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PFKL, a novel regulatory node for NOX2-dependent oxidative burst and NETosis

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Neutrophils are predominant leukocytes in the circulation, which are essential for killing invading pathogens via the activation of effector responses and the production of reactive oxygen species (ROS), also named as "oxidative burst." When infected, activated neutrophils fight bacteria, fungi, and viruses through oxidative burst, phagocytosis, degranulation, and the production of neutrophil extracellular traps (NETs) in a neutrophil death process named as "NETosis" (Mutua and Gershwin, 2021). NETs, consisting of DNA fibers decorated with modified histones and numerous antimicrobial proteins from cytoplasmic granules and the nucleus, can either be beneficial or detrimental (Mutua and Gershwin, 2021). Several pathways can lead to this death process. In response to various stimuli, NETosis traps and clears pathogens, facilitating phagocytosis by other neutrophils and phagocytes. However, excessive NETosis often results in disease due to increasing the pro-inflammatory response and perpetuating the inflammatory condition (Hellebrekers et al., 2018; Hidalgo et al., 2019; Klopf et al., 2021). Accordingly, inhibiting aberrant NETosis may alleviate the severity of various autoimmune and inflammatory diseases.

Thus far, two major forms of NETosis have been recognized, namely the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX)-dependent pathway and the NOX-independent pathway (Romero et al., 2020). In the NOX-dependent pathway, ROS produced by NOX promote the translocation of granular neutrophil elastase (NE) and myeloperoxidase (MPO) to the nucleus, which cleaves histones and initiates nuclear DNA (nDNA) decondensation (Papayannopoulos et al., 2010). Meanwhile, in the NOX-independent pathway, calcium ionophores A23187 and ionomycin activate peptidyl arginine deiminase 4 (PAD4) and promote nDNA decondensation (Gupta et al., 2014). Douda et al. (2015) further demonstrated that calcium-activated NOX-independent NETosis is mediated by mitochondrial ROS (mtROS).

Besides the above-mentioned classification, NETosis can also be divided into suicidal NETosis and vital NETosis (Delgado-Rizo et al., 2017). The better-studied pathway is conventional suicidal NETosis, which initiates from 2 to 4 h after stimulation. In this process, neutrophils, responding to various stimuli, activate the NOX complex to produce ROS and increase the cytosolic Ca^{2+} level to activate PAD4. Subsequently, ROS stimulate the breakdown of nuclear membrane and PAD4 promotes the deamination of histones. Then, granular NE and MPO translocate to the nucleus. Finally, chromatin decondenses and spreads throughout the cytoplasm, leading to NET formation and cellular lysis (Bonaventura et al., 2018). In vital NETosis, neutrophils form NETs without damaging the nuclear or plasma membrane independently of ROS production. Mouse neutrophils retain not only their viability, but also many of their effector functions after nDNA release (Yipp and Kubes, 2013). In addition, mitochondrial DNA (mtDNA) release induces an alternative path of vital NETosis, since only mtDNA

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extrudes without interfering with the cell viability of human neutrophils. This form of vital NETosis is primed with granulocyte/macrophage-colony stimulatory factor (GM-CSF) and stimulated by lipopolysaccharide (LPS), which is dependent on ROS production (Yousefi et al., 2009).

The presence of two opposing strategies of neutrophils to combat bacteria, with one causing cell death and the other requiring living cells, seems controversial. Thus, it is important to explain the exact mechanisms of NET formation in different physiological contexts. For instance, isolated human neutrophils were shown to discriminate the LPS of different bacterial sources and selectively release NETs under intravascular or tissue conditions. It was demonstrated that LPS derived from the *Escherichia coli* serotypes O55:B5, O127:B8, O128:B12, O111:B4, O26:B6, *Salmonella enterica* (serotype *enteritidis*), and *Pseudomonas aeruginosa* (serotype 10) could all induce vital NETosis, independent from ROS formation in the bloodstream, while only O128:B12 and *P. aeruginosa* 10 could trigger a suicidal, ROS-dependent NETosis under tissue conditions (Pieterse et al., 2016), suggesting that neutrophils selectively respond to various stimuli depending on the context.

To date, the best-known pathway of massive ROS generation is one dependent on the NOX enzyme. The NOX enzyme family members NOX1–NOX5, and the dual oxidase (DUOX) enzymes DUOX1 and DUOX2 are membrane-associated hetero-oligomeric complexes (Brandes et al., 2014). The assembled NOX complex generates ROS by transferring one electron from NADPH to the cytosol O_2. NOX enzymes are widely distributed across different cells and tissues, while NOX2 is highly expressed in neutrophils and is rapidly activated to generate large quantities of ROS in response to external stressors (Leung et al., 2021).

Neutrophil hyperactivation and ROS generation can be a double-edged sword. Although the exact mechanism of modulation from physiological NETosis to pathological NETosis remains unknown, Maueröder et al. (2016) indicated that NET formation is promoted by alkaline pH, while it is suppressed by acidic pH. At the same time, cytokines and inflammatory factors also affect the activation of NETosis. For instance, Shishikura et al. (2016) found that prostaglandin E_2 (PE_2) inhibits NET formation through the production of cyclic adenosine monophosphate (cAMP). At low regulated levels, ROS are essential for redox homeostasis, immune effector function, and anti-pathogen defense. ROS deficiency results in recurrent and severe bacterial infections, as well as the development of chronic granulomatous disease (CGD) (Matute et al., 2009). However, excessive ROS and NET releases by neutrophils lead to oxidative damage and pathologic alterations, such as autoimmune and inflammatory disorders. For instance, mounting evidence has demonstrated that activated neutrophils release cytokines and influence other immune cells, contributing to the pathogenesis of rheumatoid arthritis (RA). Furthermore, NETs promote the production of autoantibodies initiating the immune reaction in RA (Chen et al., 2018). Similarly, the pathogenesis of acute respiratory distress syndrome (ARDS) involves inflammatory reactions associated with the massive infiltration of neutrophils in the lungs. Clinical studies have shown the significant accumulation of neutrophils in damaged alveoli and interstitial tissues (Kambas et al., 2008). In addition, gout is an autoinflammatory disease associated with the deposition of monosodium urate (MSU) crystals in the joints. MSU promotes neutrophils to secrete cytokines and induce NET formation, which participates in tophi formation and inflammation (Matosinhos et al., 2022). Interestingly, extracts of *Coffea arabica* reduced hypernociception in an MSU-induced C57BL/6 mice gout model, which could be attributed to inhibiting neutrophil migration and pro-inflammatory cytokine release (Matosinhos et al., 2022).

As mentioned above, aberrant ROS and NET constituents can injure host tissue, and therefore it has been suggested that these pathways must be tightly regulated and triggered. NOX2 has been considered as a potential target for treating neutrophil-driven disorders. In this context, several small molecules have been postulated as direct NOX2 inhibitors, including well-known diphenylene iodonium (DPI) and apocynin, which have been found to be unspecific due to formidable off-target effects (Barbieri et al., 2004; Nagel et al., 2012; Altenhöfer et al., 2015). Briefly, DPI acts as an uncompetitive inhibitor of flavoenzymes. Besides NOX2, DPI also inhibits inducible nitric oxide synthase (iNOS), xanthine oxidase, and proteins of the mitochondrial electron transport chain. For instance, nitric oxide (NO) synthesis was irreversibly inhibited after 10 μmol/L DPI incubation

for 30 min in activated macrophages (Stuehr et al., 1991). Likewise, apocynin has been shown to be an intrinsic antioxidant acting as a radical ROS scavenger. Apocynin at 0.1–1.0 mmol/L directly interfered with peroxides generated by xanthine oxidase. However, at 10–600 μmol/L, it failed to inhibit superoxide anion generated by NOX in HEK-293 cells (Heumüller et al., 2008). Obviously, more specific compounds are needed for better efficiency. Recently, 4-[(4-(dimethylamino)butoxy)imino]-1-methyl-1H-benzo[f]indol9(4H)-one (CYR5099), as a new specific NOX2 inhibitor, reduced ROS generation, neutrophil infiltration, and edema in complete Freund's adjuvant (CFA)-induced inflammatory arthritis in C57BL/6 mice. Mechanically, CYR5099 (3–15 μmol/L) effectively inhibited NOX2 activity and respiratory burst in activated neutrophils, which was associated with selectively binding to $gp91^{phox}$, a catalytic subunit of NOX2 (Liu et al., 2019). Similarly, two small NOX2 inhibitors named CPP11G and CPP11H were evaluated for their efficacy in in vitro and in vivo models of inflammation (Li et al., 2019). Li et al. (2019) reported that CPP11G and CPP11H disrupted $p47^{phox}$–$p22^{phox}$ interaction, and significantly abolished ROS generation in human aortic endothelial cells. Consistently, these two inhibitors (dose of 15 mg/kg, intravenously (i.v.), once) decreased ROS production and ameliorated endothelial dysfunction in acute inflammatory mouse models. However, $p47^{phox}$ is also a cytosolic organizer in NOX1, and therefore, whether CPP11G and CPP11H inhibit the NOX1 system needs further investigation. Recently, a smaller compound named LMH001 (molecular weight (MW)=290.079) was observed to effectively inhibit NOX2 activation and superoxide production (half maximal inhibitory concentration (IC_{50})=0.25 μmol/L) without compromising peripheral leucocyte oxidative response to pathogens. In addition, LMH001 (dose of 2.5 mg/kg, intraperitoneally (i.p.), once) inhibited ROS production and hypertension, and reduced inflammatory damage and incidences of aortic aneurysms in an angiotensin II (AngII)-induced mouse model (Fan et al., 2022). Nonetheless, there are concerns in these clinical applications, since NOX2 plays a critical role in controlling the process of neutrophil activation and phagocytosis, and hence the inhibition of NOX2 for therapeutic purpose should be purposefully considered.

Glucose-6-phosphate dehydrogenase (G6PD), as a key enzyme in the pentose phosphate pathway, is involved in the production of NADPH and reduction of ROS. Consequently, an alternative approach would be the use of G6PD inhibitor to fight oxidative stress. It was reported that 6-aminonicotinamide (6-AN; 200 μg each mouse, intranasally (i.n.)), inhibited G6PD activity, reduced ROS production, limited oxidative damage, and ameliorated airway inflammation in acute lung injury (ALI) mice induced by LPS (Nadeem et al., 2018). In addition, Ghergurovich et al. (2020) identified a small molecule, named G6PD inhibitor-1 (G6PDi-1), which effectively inhibits G6PD. Employed on many mammalian cells, G6PDi-1 was demonstrated to markedly deplete NADPH production in lymphocytes and decrease inflammatory cytokine release from T cells. Moreover, G6PDi-1 (50 μmol/L) suppressed the respiratory burst in neutrophils. However, G6PD expression occurring under various physiopathologic conditions might be either beneficial or harmful, depending on whether ROS are produced or scavenged. Also, the complete abrogation of G6PD activity might be unsuitable in humans.

It is well-known that glucose is metabolized by two pathways, glycolysis to make adenosine triphosphate (ATP) and the pentose phosphate pathway to form NADPH. It is tempting to strengthen glycolysis and selectively dampen flux through the pentose phosphate pathway in neutrophils. Recently, Sollberger et al. (2018) screened a chemical library and found a small molecule, named LDC7559 (10 μmol/L), which efficiently inhibited NETosis in human cells. However, the mechanism and reason for LDC7559 to inhibit NOX2-dependent NETosis remain elusive.

Strikingly, Amara et al. (2021) revealed that LDC7559 and its more effective analog NA-11 selectively activated phosphofructokinase-1 liver type (PFKL), a key enzyme of glycolysis, thus dampening the flux through the pentose phosphate pathway and consequently limiting NADPH generation. They reported that NA-11 rescued PFKL inhibited by ATP with a concentration for 50% of maximal effect (EC_{50}) of (14.00±2.91) nmol/L. Vertebrates have three phosphofructokinase-1 (PFK1) isoforms (PFKL, PFK1 platelet type (PFKP), and PFK1 muscle type (PFKM)), while PFKL is the dominant *PFK1* gene highly expressed in neutrophils (Fernandes et al., 2020). Notably, NA-11 was selectively bound at the AMP/adenosine diphosphate (ADP) allosteric pocket of PFKL and activated PFKL in neutrophils. Three residues (K315, V545, and V582) at the NA-11-binding site are specific

to PFKL, and therefore, NA-11 could not activate PFKM or PFKP even at high concentrations up to 100 mmol/L. It was reported that, as a negative regulator of the oxidative burst, PFKL deficiency shifts the glycolytic flux to the pentose phosphate pathway in neutrophils, leading to increased NADPH and ROS production (Graham et al., 2015). Importantly, as a non-natural agonist of PFKL, NA-11 maintained NADPH generation at basal levels without compromising regular cellular redox activity. Thus, it markedly blocked but not completely suppressed phorbol-12-myristate-13-acetate (PMA)-induced ROS in neutrophils. Consistent with NA-11 inhibiting the oxidative burst, NA-11 prevented PMA-induced NETosis in activated neutrophils, but it did not block spontaneous NETosis in resting neutrophils (Amara et al., 2021) (Fig. 1). Meanwhile, NA-11 protected the epithelial layer from neutrophil-mediated tissue injury. As commonly known, coronavirus disease 2019 (COVID-19) might result in ARDS, while excessive NETosis occupies a central role in ARDS pathology (Zuo et al., 2020).

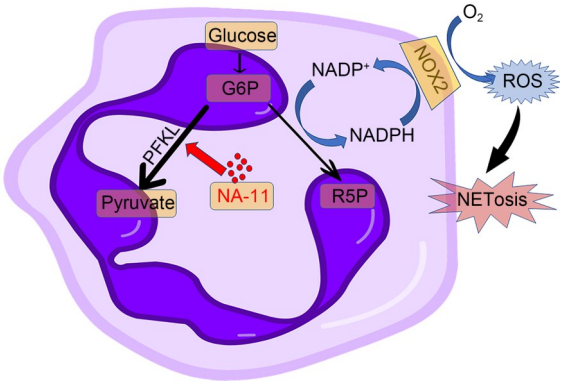

Fig. 1 NA-11 activates PFKL to suppress NETosis. In neutrophil cells, NA-11 selectively activates the glycolytic key enzyme PFKL to inhibit flux through the pentose phosphate pathway, thus limiting NADPH production. NA-11 suppresses NOX2-dependent ROS production and NETosis with the decrease of NADPH generation (Amara et al., 2021). G6P: glucose-6-phosphate; $NADP^+$/NADPH: nicotinamide adenine dinucleotide phosphate; NOX2: NADPH oxidases 2; PFKL: phosphofructokinase-1 liver type; R5P: ribose-5-phosphate; ROS: reactive oxygen species.

Targeting NETosis might have therapeutic significance in inflammatory disorders. Nevertheless, completely suppressing G6PD or NOX2 activity to inhibit ROS production is unacceptable in humans. As mentioned above, the inhibition of induced rather than basal activation of NOX2 is probably better suited for therapy. NA-11, identified as an inhibitor of the phagocyte oxidative burst via selectively activating PFKL in neutrophils, might have therapeutic benefits in neutrophil-driven diseases. However, many questions about the underlying mechanisms and its physiological role remain unanswered. Firstly, mtROS can activate NOX and are involved in the initiation of NETosis caused by various stimuli, and therefore, whether NA-11 affects mtROS production and then interferes with mtROS-dependent NETosis needs to be further investigated. Secondly, PFKL is expressed not only in neutrophils, but also in hepatocytes and macrophages, whereas the roles of NA-11 on these cells have not been determined. Lastly but most importantly, developing strategies to keep the balance of NETosis between their beneficial and harmful effects is critical in defending the host from infections by physiological NETOsis or averting damage resulting from aberrant NETosis.

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Author contributions

Xiaobo HU and Zhaohui CAO provided the theme and design, and edited the manuscript. Di HUANG, Cifei TANG, and Min ZENG participated in searching and summarizing the relevant literature as well as writing the manuscript. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Zhaohui CAO, Di HUANG, Cifei TANG, Min ZENG, and Xiaobo HU declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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