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Glycolysis dependent lactate formation in neutrophils: A metabolic link between NOX-dependent and independent NETosis

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ABSTRACT

Neutrophil extracellular traps (NETs) play a pivotal role in the innate immune defense, as well as in the pathophysiology of various inflammatory disease conditions. Two major types of NETosis have been described - NOX-dependent and independent. The present study was undertaken to assess metabolic requirements of NETs formation by using PMA and A23187 as the inducers of NOX-dependent and NOX-independent NETosis respectively. Both these inducers caused an increase in ECAR, lactate dehydrogenase (LDH) activity, PKM2 dimerization and reduction in pyruvate kinase M2 (PKM2) activity, promoting lactate formation through Warburg effect. Interestingly exogenous treatment with lactate also induced NETs formation in human neutrophils, while inhibition of LDH activity significantly reduced NETosis by both the pathways. Moreover, NETosis and lactate accumulation during LPS induced sepsis in mice was inhibited by sodium oxamate, LDH inhibitor, demonstrating the importance of lactate in an experimental model of NETosis. Present study thus confirms importance of glycolysis in NETosis and also reveals role of lactate in NETs formation. It also reports sharing of the common metabolic pathway by NOX-dependent and independent NETosis.

1. Introduction

Neutrophils are the first cells to be recruited at the site of infection and participate in anti-microbial host defense as regulators of both innate and adaptive immunity. Conventionally, to eliminate microbes neutrophils were thought to employ three major strategies which include phagocytosis [1], reactive oxygen species (ROS) generation [2], and the release of microbicidal molecules from granules (degranulation) [3,4]. However in the last decade, NETosis was identified as another distinct and unique defense mechanism [5,6]. During NETosis, neutrophils extrude a meshwork of chromatin fibers decorated with granule proteins, antimicrobial peptides and enzymes. These trap like structures are called neutrophil extracellular traps (NETs) and this phenomenon is considered as beneficial suicidal death by neutrophils [6].

The molecular basis of NETosis is still minimally defined. However, accumulated evidences on the type of inducers have grouped it into two types: 1) NOX dependent 2) NOX-independent. The inducers like phorbol 12-myristate 13-acetate (PMA) [6,7], pro-inflammatory cytokines [8], nitric oxide [9], and oxLDL [10] which stimulate NETs via involvement of NADPH oxidase (NOX2)-mediated oxidative burst are termed as NOX dependent NETs inducers. While the other type of inducers including calcium ionophore [11], uric acid [12], soluble immune complexes [13] and diverse microorganisms [14] do not require NOX2 activation [15] and are termed as NOX- independent NETs inducers [11]. An essential role is attributed to NOX2 in the regulation of NETosis, as PMA induced NETosis is prevented either by using diphenylene iodonium (DPI), an inhibitor of NOX activity, or is diminished in chronic granulomatous disease (CGD) patients which have congenital defects in NOX2 subunits [16]. Nonetheless, final outcome in both types of NETosis is characterized by its characteristic cell swelling and subsequent DNA release by various type of stimuli and infections. Thus, both of these pathways might have a common metabolic mechanism for the efficient clearance of pathogens, which however remains elusive so far.

During the last decade, the burgeoning interest on metabolic switching or reprogramming to modulate immune function has brought immunometabolism to the forefront. Recent studies on macrophages

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and dendritic cells emphasize on metabolic adaptation and identify the decisive role of metabolic pathways in immunoregulation [17]. In this regard, PMNs are pondered to utilize glycolytic metabolism and have few mitochondria which produce little energy from respiration [18,85]. Recent reports suggest that neutrophil glycolytic metabolism contributes to NETs formation, and metabolic shift towards the pentose phosphate pathway (PPP) is important for NOX dependent NETs release [19,20]. The activation of PPP fuels NOX with NADPH to generate the majority of the cellular ROS and induce NOX dependent NETs formation. Although mitochondrial respiration is dispensable for oxidative burst or energetics in neutrophils, but maintenance of mitochondrial integrity and potential ($\Delta\psi m$) are needed for neutrophil survival [21,22].

Interestingly, glycolysis generates pyruvate that under aerobic conditions enters the Krebs cycle through acetyl-Co-A. While under anaerobic conditions pyruvate gets converted to lactate, an end product of glycolysis. Conversely, increase in glycolytic flux and preferential production of lactate even in the presence of oxygen is defined as Warburg effect which is evident in cancer cells for growth and proliferation [23]. Phosphorylation of pyruvate kinase type M2 (PKM2) on Tyrosine-105 promotes monomer/dimer form which is enzymatically less active and supports the Warburg effect [23–25]. This role of PKM2 and its association with Warburg effect in cancer cells has been well demonstrated by numerous investigators [25–27]. Though in general lactate is considered as metabolic waste, several novel function of lactate are identified recently in metabolism [28–30]. Elevated lactate is associated with pathological conditions including shock, sepsis, cardiac arrest, ischemia, diabetic ketoacidosis and malignancy [31]. Lactate exists in two isomers: L-lactate and D-lactate. The primary isomer produced in humans is L-lactate. Surprisingly, a recent study found role of D-lactate in induction of NETosis in bovine PMNs [32], suggesting functional role of lactate in neutrophil biology.

The aim of this study was to determine the cellular metabolism during both forms of NETosis in human neutrophils and to further identify decisive role of metabolic cues in NETosis. Our data reveal requirement of glycolysis specifically during initiation of NETosis by both NOX dependent and independent process. We observed a marked increase in the glycolysis rate and decreased levels of pyruvate during NETosis. Interestingly, lactate formation was significantly increased during both NOX dependent and independent NETosis inhibition of lactate dehydrogenase significantly prevented NETs formation. Further investigations identified increase in PKM2 dimer formation that represents reduced PKM2 activity suggesting involvement of Warburg effect in NETs formation.

2. Materials and methods

2.1. Reagents and antibodies

Percoll was obtained from Amersham Biosciences Corp. (Uppsala, Sweden). PKM1, PKM2, pPKM2 antibodies were purchased from Cell Signaling Technology (Danvers, MA). DASA-58, Lactate and Pyruvate estimation kit were obtained from Cayman Chemical (Ann Arbor, USA). RPMI 1640, FBS, Quant-iT™ PicoGreen dsDNA Assay Kit and sytox green was obtained from Invitrogen (Carlsbad, CA, USA). l-Sodium lactate, sodium oxamate and other chemical unless otherwise specified were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2. Isolation of PMNs from human blood

Peripheral blood was obtained from Blood Centre, King George Medical University (KGMU), Lucknow after prior consent from healthy volunteers. All the procedures were performed in accordance with the guidelines of the ethical committees of KGMU, and CSIR-CDRI (Lucknow, India). Human PMNs were isolated by the percoll density gradient centrifugation method as previously described [33]. Purity of the isolated PMNs from human was assessed by CD15 using flow cytometer (FACS Calibur, Becton Dickinson, USA). Cell viability was found to be > 95% as assessed by trypan blue exclusion assay.

2.3. In vivo experiments

For LPS-Induced sepsis Model, LPS at a dose of 10 mg/kg was administered intra-peritoneally to wild-type C57/BL6 mice for 12 h. To establish the involvement of lactate during NETosis sodium oxamate, a lactate dehydrogenase (LDH) inhibitor, the mice were divided into 4 groups with 6–8 mice in each group and administered a single intraperitoneal (i.p.) injection of PBS, LPS alone, sodium oxamate (300 mg/kg), LPS + sodium oxamate in 300 µl PBS. After 12 h of injection, peritoneal exudate cells and blood was collected and further used for NETs incidence. In another set of experiment, mice (6–7 mice/group) injected with 30 mM sodium L-lactate in 300 µl PBS. After 12 h of injection, peritoneal exudate cells and blood was collected and further used for NETs incidence [34,35]. Two independent experiments were performed with 6–8 mice per group in each experiment.

2.4. Quantification of plasma DNA

Blood plasma was separated by centrifugation from control (PBS-treated) and treated mice from in vivo experiments and stored at −80 °C until analysis. Plasma DNA was quantified using a Quant-iT™ PicoGreen dsDNA Assay Kit according to the manufacturer' instructions [34].

2.5. NETs formation

5×10^4 PMNs seeded in coverslips pre-coated with 0.001% poly-L-lysine were treated with PMA (50 nM) or A23187 (5 µM) or L-sodium lactate (20 mM in 0.9% saline) for 1–3 h in a CO_2 incubator (RS Biotech, UK) at 37 °C. After treatment cells were fixed with 4% PFA for 30 min. NETs incidence were further analyzed by DAPI (1 µg/ml) staining. After mounting, images were acquired using fluorescence microscope. The percentage of NETs formations was assessed by counting the number of NETs forming neutrophils out of the total number of neutrophils as observed under 40× objective lens. A NET has been defined as a discrete area of bright fluorescence larger in size than neutrophils [9].

2.6. Measurement of DNA release

DNA release was measured by plate reader assay as described earlier with some modifications [11]. Briefly, PMNs were resuspended in RPMI (phenol red free) with 0.5% FBS. 5×10^4 PMNs were seeded per well in a 96-well plate pre-coated with 0.001% poly-L-lysine in the presence of 5 µM Sytox Green cell-impermeable nucleic acid stain and subsequently stimulated with 50 nM PMA or 5 µM A23187 or L-sodium lactate (20 mM in 0.9% saline). The fluorescence was measured using POLARstar OMEGA fluorescence microplate reader (BMG Labtech) at specific time intervals for up to 180 min after the activation of cells. In each figure, DNA release mentioned as florescence of Sytox green for vehicle, PMA or A23187 treated PMNs have been pooled and reported as mean values of ten independent experiments.

2.7. Real-time cell metabolism assay

XFp Extracellular Flux Analyzer (Seahorse Bioscience) was used for real-time analysis of the extracellular acidification rate (ECAR) and the oxygen consumption rate (OCR). Briefly, (5×10^4 cells/well) human neutrophils or mice BMDN (bone marrow derived neutrophils) were resuspended in sterile XF base media (supplemented with 10 mM D-glucose, pH 7.4) and plated in XFp cell culture plates pre-coated with 0.001% poly-L-lysine and allowed to settle for 30 min at 37 °C. Manufacturer's instructions were followed to obtain real-time

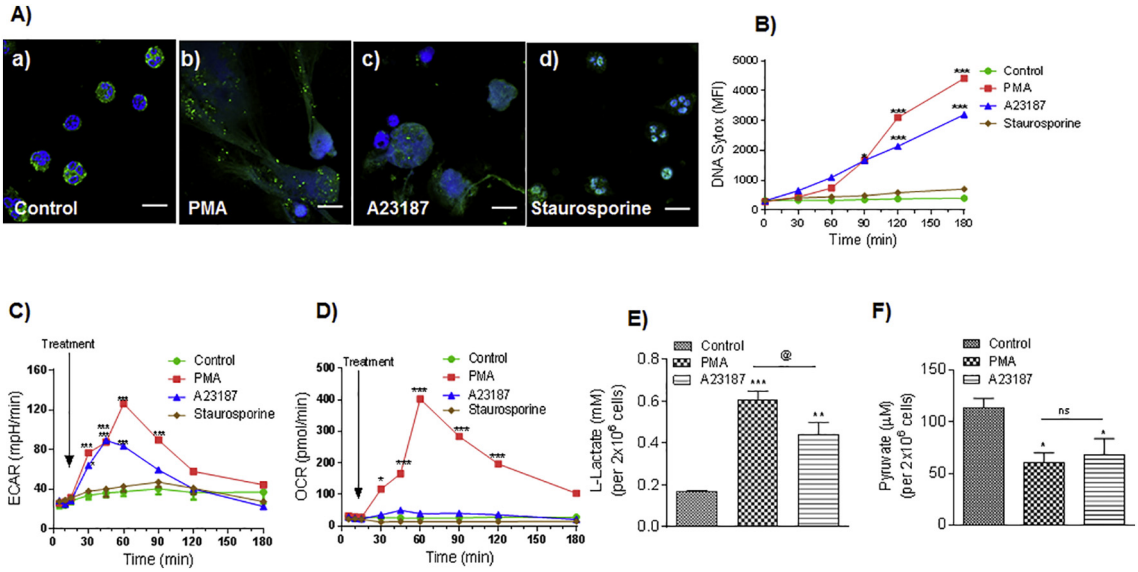

Fig. 1. Both PMA and A23187 induced NETosis are committed to glycolytic metabolism for its initiation, but not staurosporine induced apoptosis. (A) Unstimulated control neutrophils or treated with various inducers for 3 h in a CO_2 incubator were fixed and analyzed after labelling with antibody against MPO and DAPI (1 μg/ml).stained with DAPI. Representative confocal images show, a: Resting neutrophils b: PMA (50 nM) induced NETs c: A23187 (5 μM) induced NETs d: Staurosporine (5 μM) treated neutrophils with overlay labelling of MPO and Alexa 488 (green) with DAPI (blue). (B) NET release in response to PMA or A23187 or staurosporine or vehicle control was measured using a plate reader assay and real time kinetics of NET release in terms DNA sytox fluorescence was recorded. DNA release in untreated, PMA or A23187 by sytox green is the common mean values of ten independent experiments. Neutrophils were seeded in a Seahorse XFp analyzer culture plates and real-time rates of (C) ECAR as a readout for lactate production and (D) OCR of control or when treated with various agonist. ECAR values in untreated, PMA or A23187 are the common mean values of twelve independent experiments. Bar diagram represents (E) Lactate levels (F) Pyruvate levels was determined by using their respective assay kit. (Data represent mean ± SEM of three independent experiments). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. vehicle control neutrophils. (Scale bar: 50 μm).

measurements of ECAR and OCR in vehicle control PMNs, and where indicated 50 nM PMA or 5 μM A23187 or staurorosprin was added and kinetics was recorded for upto 180 min. ECAR values reported in each figure for vehicle, PMA or A23187 treated cells have been pooled and are common mean value of twelve independent experiments [36].

2.8. Pyruvate and lactate quantification assay

Lactate and pyruvate accumulation were assessed using a commercially obtained lactate and pyruvate estimation kit according to manufacturer's instructions (Cayman). In each figure, lactate levels in the vehicle, PMA or A23187 treated cells have been pooled from six independent experiments.

2.9. Enzymatic assays

2.9.1. LDH activity

Lactate dehydrogenase enzymatic activity was assessed using a commercially available LDH Assay Kit according to manufacturer's instructions (Abcam).

2.9.2. PKM2 activity

PKM2 activity was measured by protocol described previously [37]. Neutrophils were harvested after treatment and resuspended in 200 μl of lysis buffer (10 mM Tris–HCl (pH 7.5), 1.5 mM $MgCl_2$, 20 mM NaCl, 1 mM DTT, 1 mM PMSF and a protease inhibitor cocktail), followed by incubation 10 min in ice. Cells were further lysed using Dounce homogenizer. The lysate was further centrifuged at 15,000 rpm for 30 min at 4 °C. The supernatant was collected to measure PKM2 activity by a lactate dehydrogenase (LDH) coupled assay. The 200 μl reaction mixture was prepared in assay buffer (100 mM Tris– HCl (pH 8.0), 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 0.2 mM β-NADH, 1.5 mM ADP, 5 mm phosphoenolpyruvate (PEP) and 8 units/mL LDH). The reaction was initiated by the addition of 5 μg of total cell extract. Activity was measured as the decrease in absorbance recorded at 340 nm at 37 °C using commercial LDH, a blank (without cell lysate).

2.10. Western blotting

Human PMNs (2×10^6) were harvested after treatment and lysed in ice-cold neutrophil lysis buffer for 30 min on ice. Sample were prepared in reducing or non-reducing laemmli dye and boiled for 5 min at 100 °C. After centrifugation, supernatant (30 μl) was run on the 10% SDS polyacrylamide gel, and transferred to PVDF membranes. After blocking (5% BSA in TBST), the membrane was incubated overnight at 4 °C with primary antibody against various candidate proteins (1:1000). This was followed by incubation with specific HRP-conjugated secondary Ab. The specific bands were detected by ECL as described earlier.

2.11. Statistical analysis

Data have been represented as mean ± SEM from atleast 3–5 independent experiments and were analyzed by one-way ANOVA test followed by Newman–Keul's post analysis. A p value equal or < 0.05 was considered as statistically significant. All statistical analyses were performed with the GraphPad Prism 5.0 program (GraphPad Inc., San Diego, CA).

3. Results

3.1. Increase in metabolic ECAR during NOX-dependent and independent NETosis

NETosis has been known to occur via both NOX-dependent and NOX-independent pathways. To elucidate the metabolic requirement for both these forms of NETosis, human neutrophils were treated with PMA, the most widely used NOX-dependent inducer of NETs, while for

NOX-independent NETosis A23187 was used [38]. Neutrophils incubated with either PMA or A23187 exhibited its characteristic cell swelling accompanied by the loss of the multilobed nuclear morphology, chromatin decondensation, and intermixing of DNA and granular proteins that together released as complex fibrous or cloud-like NETs structures (Fig. 1A). Consistently, real-time kinetics measurement of extracellular DNA release i.e. typical feature of NETosis, confirmed NET-inducing effect of both types of inducers (Fig. 1B).

To understand metabolic changes/requirement in NETosis, we analyzed the real-time changes in oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), a measure of lactate production, following stimulation with PMA or A23187 using Seahorse system. As expected, PMA induced a sharp increase in OCR due to rapid activation of NOX; in contrast A23187 did not show much increase in OCR (Fig. 1D) confirming its NOX independence. Surprisingly, both NETosis inducing agents PMA as well as A23187 caused a rapid increase in ECAR at an early time point (Fig. 1C), suggesting a common link to NETosis, while staurosporine, an apoptosis inducer, did not show significant increase in OCR, ECAR and DNA release (Fig. 1A, B, C, D). Thus both forms of NETosis seem to be associated with glycolysis dependent lactate formation. Consistent to ECAR increase in NETosis (Fig. 1C), lactate levels were significantly increased in PMA and A23187 stimulated cells, while pyruvate levels were decreased (Fig. 1E, F). The data further confirmed that increase in glycolysis/ECAR/lactate production were specific to NETosis and not to apoptosis.

3.2. Glycolysis dependent lactate formation following NETs formation

Glycolysis produces pyruvate that can be converted to either acetyl CoA to enter the Kreb's cycle or lactic acid by lactate dehydrogenase (LDH) to generate lactate. The results obtained so far suggest that glycolysis dependent lactate formation correspond with NETosis. Therefore, we next investigated the enzymatic activities of glycolytic enzymes that are associated with lactate production, like LDH and PKM2. In contrast to LDH activity which was significantly increased in a time dependent manner (Fig. 2A), PKM2 activity was decreased within 15 min and this low activity was maintained until 3 h after stimulation with either PMA or A23187 (Fig. 2B). To further validate dependence of lactate production on glycolysis in our experimental conditions we used 2DG (2-deoxy glucose), a glycolysis inhibitor. As expected, 2DG completely abrogated both ECAR and lactate production, and also the subsequent NETs formation from both types of inducers (Fig. 2C, D, E, F). To further explore the selective role of lactate in NETs formation, human neutrophils were treated with L-sodium lactate (20 mM). Interestingly, exogenous treatment of lactate resulted in a time dependent increase in NETs formation from human neutrophils (Fig. 3A, B, C) contrary to earlier findings that pyruvate do not induce NETs formation [19]. To further evaluate the importance of LDH and lactate formation during NETosis, we used sodium oxamate, a lactate dehydrogenase inhibitor. Interestingly, sodium oxamate reduced ECAR and lactate production following PMA or A23187 stimulation (Fig. 3D, E). Furthermore, pre-treatment with sodium oxamate also reduced PMA and A23187 induced NETs formation (Fig. 3F, G, H) suggesting a crucial role of LDH dependent intracellular ECAR-lactate in NETosis.

3.3. PKM2 oligomerization and phosphorylation during NETosis

Since pyruvate kinase (PK) catalyzes the final step of glycolysis to produce pyruvate, a substrate of LDH, we further investigated regulation of PK in NETosis. PK can be allosterically activated by the upstream glycolytic metabolite fructose 1,6-bisphosphate (FBP), in addition to the modulation of its enzymatic activity by its the terameric, dimeric, or monomeric state. PK exists as two isoforms- PKM1 and PKM2. PKM1 has high constitutive enzymatic activity as it naturally exists as a stable tetramer and optimally binds to its substrate. While, PKM2 shuttles between active tetrameric or less active dimeric state depending upon its activator binding and its phosphorylation status [39], PKM2 is a tetrameric rate limiting glycolytic enzyme which modulates the ratio of lactate/pyruvate production and is more active [40,41]. Interestingly, we found that there was a modest statistically insignificant increase in expressions of PKM1 and PKM2, during both PMA and A23187 induced NETosis (Fig. 4A, B). Tyrosine phosphorylation of PKM2 disrupts its tetramer formation, decreases its enzymatic activity by releasing fructose 1,6-bisphosphate, and promotes the Warburg effect with the high rate of glucose to lactate conversion [23]. We found a significant increase in tyrosine-105 phosphorylation with reduced PKM2 activity in both PMA and A23187 induced NETosis (Fig. 4C, D), while staurosporine induced apoptotic cells did not exhibit these changes (Data not shown). Moreover, PKM2 can change its oligomeric status, ranging from metabolically less active monomeric and dimeric forms to metabolically more active tetrameric forms. Importantly, resting neutrophils exhibited predominant tetrameric isoform while NETotic neutrophils exhibit increase in monomeric/dimeric form of PKM2 (Fig. 4E, F, G, H). DASA 58, is a highly specific activator of PKM2 [42] binding at a site different from the FBP binding site, consequently, leading to a tight tetramer of PKM2 thus making it resistant to inhibition by tyrosine phosphorylation. Serine is also shown to act as a natural ligand and allosteric activator of PKM2 [43]. To examine the impact of oligomeric status of PKM2 on NETs, we enforced PKM2 to its constitutively active tetrameric assembly by using DASA-58 and serine. DASA-58 and serine treatment led to an increase in PKM2 activity and decrease in lactate to pyruvate ratio (Fig. S1A, B) that was well supported by decrease of NETs release (Fig. S1C, D, E). Thus, these results identify crucial role of PKM1/2 regulation and lactate accumulation in NETs formation.

3.4. Involvement of lactate in the NETs release in a mice model of LPS induced sepsis

To further corroborate our findings and their clinical relevance, we used LPS induced sepsis mice model that is commonly associated with NETosis and lactate accumulation [34]. Indeed, LPS treated mice showed increased lactate accumulation in the cells present in peritoneal exudates along with significantly elevated levels of TNF-α, and DNA in plasma implying NETosis (Fig. 5C, D). Treatment of sodium oxamate along with LPS in these mice significantly reduced DNA in both plasma and peritoneal exudates (Fig. 5A, B). Consistently, administration of lactate to mice led to a modest increase in DNA content in both plasma and peritoneal exudates (Fig. 5E). These results thus reveal a crucial role of glycolytic product, lactate in NETs formation in this experimental model of sepsis.

4. Discussion

Recent researches on the roles of diverse metabolic pathways and metabolites in immune cell function have demonstrated metabolic reprogramming as a key immuno-regulatory phenomenon that govern the nature of the immune response and also influence their differentiation, function and even its fate [44]. The energy required for neutrophil functions like chemotaxis and phagocytic activity is derived from glycolysis [18,45,85]. Recently, requirement of glycolysis has also been shown for NOX-dependent NETosis [19]. The present study investigated the metabolic changes and bioenergetics requirements of PMNs during NOX-dependent and independent NETs formation with an interest to identify specific and common metabolic link. Here, we observed that glycolysis activation during both NOX dependent and independent NETosis significantly increased lactate accumulation.

Moreover, enhanced glycolysis was associated with NETs release as demonstrated by the real time changes in glycolytic rate during both NOX- dependent and independent NETs formation by analysis of ECAR and OCR. Consistent to our finding, Nunn and colleagues has reported a drop in lactate, the end product of non-oxidative glycolysis in apoptotic

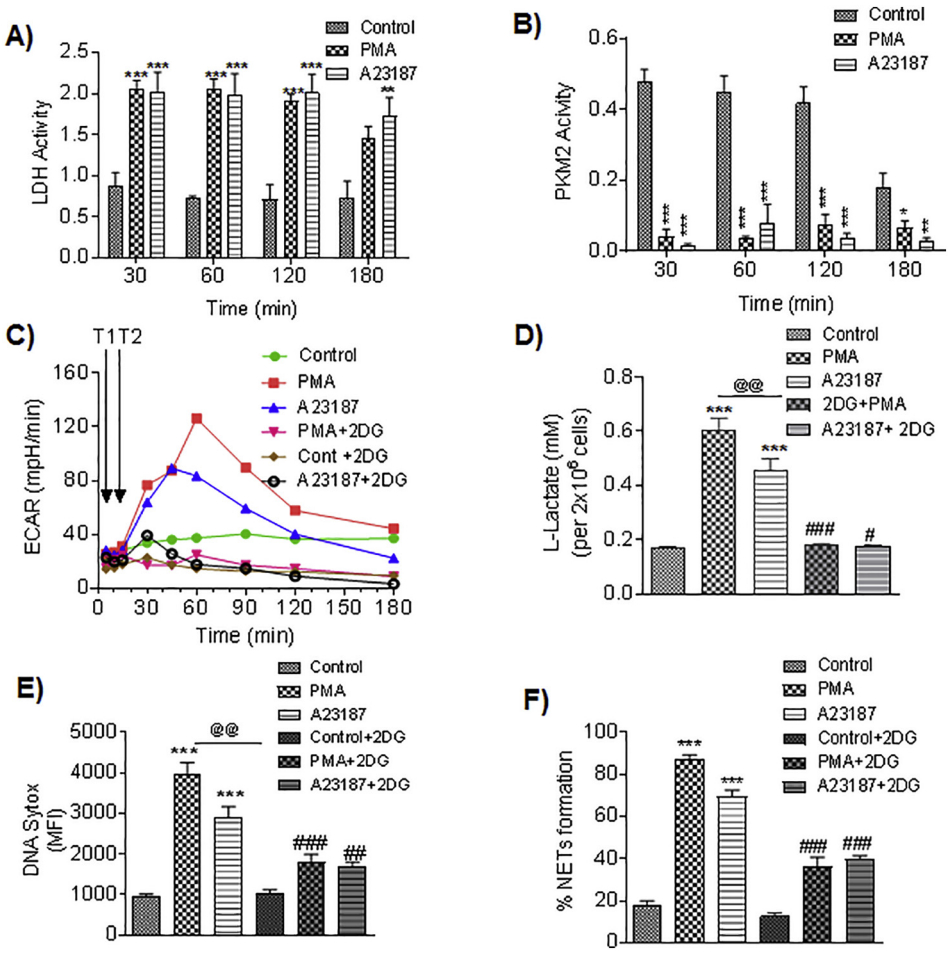

Fig. 2. Glycolysis dependent lactate accumulation modulated both NOX-dependent and independent NETosis. Human neutrophils were treated with PMA or A23187 for various time duration as indicated and enzymatic activities were measured. Bar graph represents (A) relative LDH activity (B) relative PKM2 activity in vehicle control and PMA or A23187 treated neutrophils. (C) Human neutrophils were seeded in a Seahorse XFp analyzer culture plates and real-time rates of ECAR as readout for lactate production with or without 2-DG (2 mM) as first injection (T1) followed by vehicle or PMA or A23187 as second injection (T2) in the appropriate port, was measured (Data represent mean values of six independent experiments). ECAR values in vehicle, PMA or A23187 treated PMNs are pooled from twelve independent experiments. (D) Bar diagram represents lactate level in presence or absence of neutrophils pre-treated with 2 mM 2-DG for 30 min followed by PMA (50 nM) or A23187 (5 μM) or vehicle control. Lactate levels in vehicle, PMA or A23187 treated cells are the common mean values of six independent experiments. (E) DNA release by DNA sytox fluorescence intensity in plate reader assay. DNA release after vehicle, PMA or A23187 treatment are the common mean values from ten independent experiments. (F) Percent NETs forming cells. (Data represent mean ± SEM of three independent experiments. Data presented as % release, are mean count ± SEM of five transect from three individual experiment) $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. vehicle control neutrophils, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs.PMA or A23187 treated neutrophils.

neutrophils [46]. Furthermore changes in various metabolically important proteins like pyruvate kinase and lactate dehydrogenase suggest metabolic requirements of NETs release. Importantly, during both NOX dependent and independent NETosis we observed activation of glycolysis, along with reduced pyruvate kinase M2 (PKM2) activity and a significant increase in lactate as well as LDH activity. Moreover, enforced PKM2 tetramerization/activation and inhibition of lactate dehydrogenase reduced NETosis. This study thus explores and delineates a common metabolic link for NOX dependent and independent NETosis.

Accumulated evidences demonstrated association of NETosis with inflammation and identified enhanced NETs in various disease pathologies [47–49] such as sepsis [50], chronic lung disease [51], cystic fibrosis [52], glomerulonephritis [53], SIRS [8], pneumonitis [54], systemic lupus erythematosus (SLE) [55] and pre-eclampsia [56]. We further noticed that a time dependent increase in LDH activity which resulted in accumulation of lactate, is a hallmark of the inflammatory milieu. Importantly, Sepsis a potentially fatal disease with elevated lactate has been shown to present with detrimental NETs [50,57] and the role of lactate in disease severity and patient survival was identified by Noritomi et al., in ICU patients with severe sepsis or septic shock, where survivors showed lesser lactate than non-survivals [58]. Similarly, Podaza et al., reported that neutrophils from chronic lymphocytic leukemia (CLL) patients were more prone to release NETs. Interestingly, numerous studies reported increased levels of lactate in these patients [59–61] suggesting that the chronic inflammatory environment possibly through lactate promotes neutrophil priming and NETosis [49]. In the present study, we explored this lactate-NETosis link using LPS induced sepsis model (*in-vivo*) and exogenous treatment of lactic acid (in vitro) that confirmed the connection between lactate and NETosis. Moreover, recent studies have demonstrated that ERK1/2 activation phosphorylates PKM2 and consequently its nuclear translocation which promotes the expression of glycolytic enzyme genes, while inhibition of ERK1/2 activity has been shown abrogate glucose consumption and lactate production [26,62–64]. Interestingly, others and our lab have confirmed that both NOX-dependent and independent NETosis inducer involves phosphorylation and activation of ERK1/2 and p38 MAPK pathway, which subsequently leads to NETs formation [11,65–67]. Published literature has often linked elevated plasma lactate as a marker of disease severity and cellular stress [68–71]. Moreover, previous studies have demonstrated that lactate enhanced LPS-stimulated cytokine expression in macrophages by NF-κB and MAPK Pathway [72]. Moreover, pretreatment of U937 macrophage-like cells to sodium lactate increased LPS-induced matrix metalloproteinase (MMP)-1, IL-1β, and IL-6 secretion [73]. HIF-1α is a master transcription factor that is stabilized by the products of glycolysis, lactate and pyruvate, which leads to an accumulation even under normoxic conditions [74]. Interestingly, mTOR regulates NET formation by human PMNs through induction of HIF-1α protein expression and show that inhibition of mTOR and HIF-1α signaling in these immune effector cells leads to decreased bacterial killing [75]. However, how lactate could trigger NETosis still remain elusive and warrants further investigation.

Pyruvate kinase (PK) is traditionally considered as rate-limiting enzyme during glycolysis that catalyzes the production of pyruvate from phosphoenolpyruvate (PEP). Recent studies have identified non-canonical roles of PK in tumour environment as regulator of Warburg effect and other pathways. Christofk et al., demonstrated that phosphotyrosine binding inhibited the enzymatic activity of the pyruvate kinase M2 isoform (PKM2); and provides a growth advantage to tumors [24,76]. Neutrophils express PKM2 [77–79] and PK can exist in either active tetramers or inactive dimers. Importantly phosphorylation/

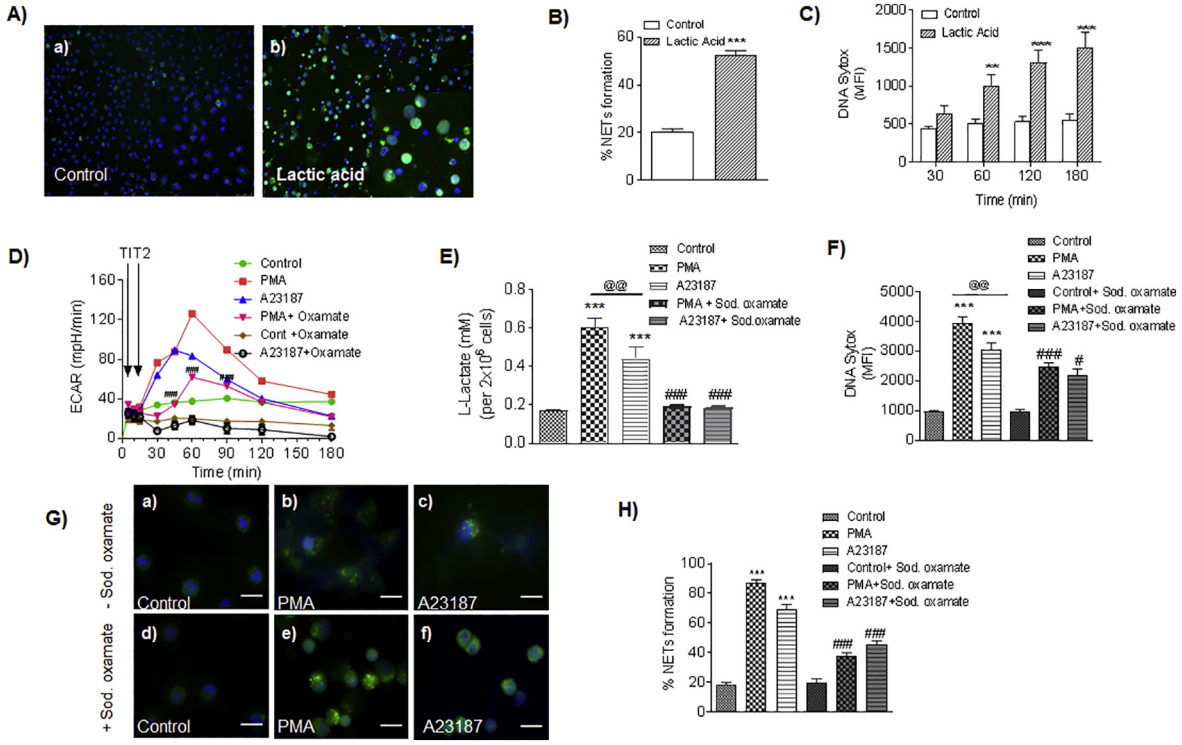

Fig. 3. Effect of lactate and its inhibitor in NOX-dependent and independent NETosis. (A) Unstimulated control neutrophils or treated with L-sodium lactate (20 mM) kept in a CO_2 incubator for 3 h in saline condition then fixed and analyzed after labelling with antibody against neutrophil Elastase and DAPI (1 μg/ml). Representative images show, a: Resting neutrophils b: treated with L-sodium lactate, neutrophils with overlay labelling of neutrophil elastase and Alexa 488 (green) with DAPI (blue). (B) Bar diagrams represents quantification of percent NETs forming cells in presence of L-sodium Lactate. (C) NET release in response to L-sodium Lactate or vehicle control was measured using a plate reader assay and real time kinetics of NET release in terms DNA sytox fluorescence was recorded. (D) Human neutrophils were seeded in a Seahorse XFp analyzer culture plates and real-time rates of ECAR as readout for lactate production with or without sodium oxamate (50 mM) as first injection (T1) followed by vehicle or PMA or A23187 as second injection (T2) in the appropriate port, was measured. ECAR values in vehicle, PMA or A23187 treated cells are common mean values of twelve independent experiments. (E) Lactate levels. Lactate levels in vehicle, PMA or A23187 treated PMNs are pooled from six independent experiments. (F) Bar diagram represents DNA sytox fluorescence in presence or absence of sodium oxamate (50 mM) for 30 min followed by vehicle, PMA (50 nM) and A23187 (5 μM) treatment for 3 h DNA release after vehicle, PMA or A23187 treatment have been pooled from ten independent experiments. (G) Representative microscopy images with overlay of DNA (DAPI, blue) and MPO (green) a: Unstimulated vehicle control b: PMA (50 nM) c: A23187 (5 μM) after 3 h without pretreatment with sodium oxamate, d, e, f neutrophils were pre-treated with sodium oxamate (50 mM) for 30 min followed by vehicle, PMA (50 nM) and A23187 (5 μM) treatment for 3 h. (H) Bar diagrams represents quantification of percent NETs forming cells. (Data represent mean ± SEM of three independent experiments. Data presented as % release, are mean count ± SEM of five transect from three individual experiments) $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. vehicle control neutrophils, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. PMA or A23187 treated neutrophils. (Scale bar: 50 μm).

dephosphorylation at tyrosine residue 105 (Y105) of PKM2 may be responsible to control this balance, thus regulating the switch between aerobic glycolysis and oxidative phosphorylation. Cancer cells under hypoxic conditions with PKM2 mutation at Y105 and substituted by phenylalanine (Y105F), have exhibited decreased cell proliferation and reduced lactate production [23]. Interestingly, apart from its important role in glycolysis, inhibition of PKM2 by oxidation at its Cys358 residue increases intracellular ROS formation [80]. Moreover, dimerization of PKM2 induces its localization to nucleus where it may form complex with Prolyl hydroxylase 3 (PHD3) and promote trans-activation of

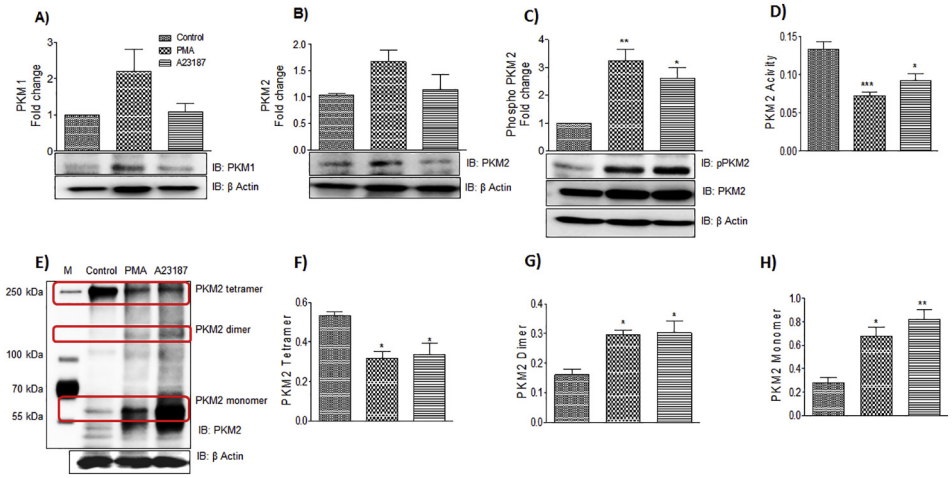

Fig. 4. PKM2 oligomerization status in both NOX-dependent and independent NETosis. Representative blots and densitometry of (A) PKM1 (B) PKM2 (C) phosphorylation and total of PKM2. Equal loading was shown by re-probing with anti-human β-actin antibody. (D) PKM2 activity in neutrophils treated with PMA or A23187 or vehicle control without stimulation for 3 h. (E) Representative blots and (F, G, H) densitometry of PKM2 oligomerization status in neutrophils treated with PMA or A23187 or vehicle control without stimulation for 3 h. Equal loading was shown by re-probing with anti-human β-actin antibody. Blots shown are representative of three independent experiments. (Data represent mean ± SEM of three independent experiments). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. vehicle control neutrophils.

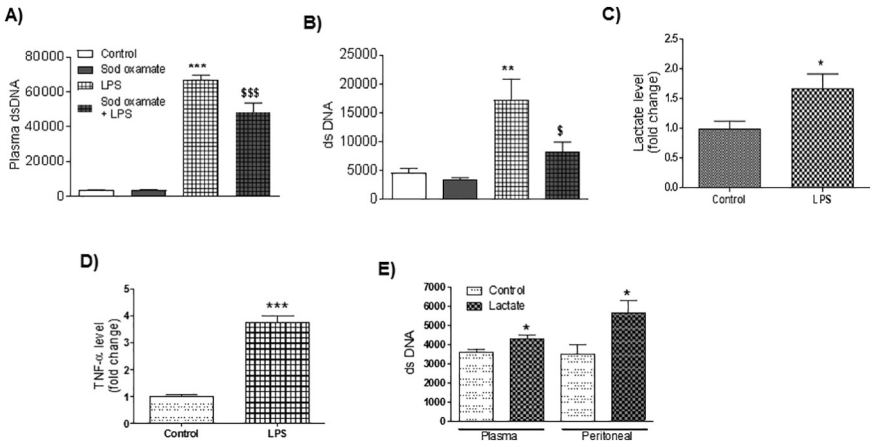

Fig. 5. Effects of sodium oxamate and lactate on NETs release in vivo. C57BL/6 mice divided in 4 groups (6–8 in each group) and injected i.p. with control PBS or sodium oxamate (300 mg/kg) alone or LPS alone or sodium oxamate along with LPS for 12 h. Bar diagram represents change ds DNA (A) in plasma (B) peritoneal exudates in terms of arbitrary units as quantified by using a Quant-iT™ PicoGreen dsDNA Assay Kit (C) Lactate (D) TNF-α. In second experiment, C57BL/6 mice (6–7 mice per group) were injected i.p. with control PBS or lactate 30 mM for 12 h. (E) Bar diagram represents change ds-DNA in plasma and peritoneal exudates in terms of arbitrary units as quantified by using a Quant-iT™ PicoGreen dsDNA Assay Kit. C57BL/6 mice divided in 2 groups and injected i.p. with control PBS or LPS (10 mg/kg for 12 h). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. PBS control, $^{\$}p < 0.05$, $^{\$\$\$}p < 0.001$ vs. LPS treated.

HIF1α and its target genes such as lactate dehydrogenase (LDH), the glucose transporter GLUT-1, and pyruvate dehydrogenase kinase-1 (PDK-1) [81]. While, DASA-58 that enforces tetramer formation of PKM2 prevent nuclear localization of PKM2 [82]. In the present study, we found PKM2 tyrosine phosphorylation in both PMA and A23187 induced NETs, which resulted in its dimeric state leading to inhibition of PKM2 enzymatic activity and subsequently increased lactate accumulation in the NETotic cells. Interestingly, Shirai et al., demonstrated PKM2 dimerization that phosphorylates the transcription factor STAT3, which enhances IL-6 and IL-1β production by monocytes and macrophages of patients with atherosclerotic coronary artery disease [83]. Corroborating to this activation of STAT3 was recently reported to be required for both PMA and A23187 induced NETosis [84].

5. Conclusion

In conclusion, we reveal requirement of glycolysis specifically during initiation of NETosis by NOX dependent and independent processes. Interestingly, lactate formation was significantly increased during NETosis and correlated with increased glycolysis which is a common feature in both NOX-dependent and independent NETs forming cells. Furthermore inhibition of lactate dehydrogenase significantly prevented NETs formation by both interventions. Further investigation identified decisive role of metabolic cues in NETosis as increased in PKM2 dimer formation with reduced PKM2 activity in the NETotic PMNs, this might be possibly causing lactate production through Warburg effect. Thus, understanding metabolic flexibility by lactate shuttles and better understanding of the metabolic checkpoints offers new perspectives to develop treatments that target the unregulated NETosis during various inflammatory conditions and overcome the limitations of glycolytic inhibitors.

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Transparency document

The Transparency document associated with this article can be found, in online version.

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Declaration of competing interest

There are no conflicting financial interests.

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