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*Correspondence to
Jae Man Lee
Department of Biochemistry and Cell Biology,
Cell and Matrix Research Institute, School of
Medicine, Kyungpook National University, 680
Gukchaebosang-ro, Jung-gu, Daegu 41944,
Korea.
E-mail: jaemanlee@knu.ac.kr

†These authors contributed equally to this
work.

ORCID iDs
Jae-Han Jeon
https://orcid.org/0000-0002-9217-968X
Chang-Won Hong
https://orcid.org/0000-0001-7745-9205
Eun Young Kim
https://orcid.org/0000-0003-2375-5580
Jae Man Lee
https://orcid.org/0000-0003-2707-5136

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Current Understanding on the Metabolism of Neutrophils

Jae-Han Jeon [1,2,†], **Chang-Won Hong** [2,3,†], **Eun Young Kim** [4], **Jae Man Lee** [4,*]

[1]Department of Internal Medicine, School of Medicine, Kyungpook National University, Daegu 41944, Korea
[2]Kyungpook National University Hospital, Bio-Medical Research Institute, Daegu 41940, Korea
[3]Department of Physiology, School of Medicine, Kyungpook National University, Daegu 41944, Korea
[4]Department of Biochemistry and Cell Biology, Cell and Matrix Research Institute, School of Medicine, Kyungpook National University, Daegu 41944, Korea

ABSTRACT

Neutrophils are innate immune cells that constitute the first line of defense against invading pathogens. Due to this characteristic, they are exposed to diverse immunological environments wherein sources for nutrients are often limited. Recent advances in the field of immunometabolism revealed that neutrophils utilize diverse metabolic pathways in response to immunological challenges. In particular, neutrophils adopt specific metabolic pathways for modulating their effector functions in contrast to other immune cells, which undergo metabolic reprogramming to ensure differentiation into distinct cell subtypes. Therefore, neutrophils utilize different metabolic pathways not only to fulfill their energy requirements, but also to support specialized effector functions, such as neutrophil extracellular trap formation, ROS generation, chemotaxis, and degranulation. In this review, we discuss the basic metabolic pathways used by neutrophils and how these metabolic alterations play a critical role in their effector functions.

Keywords: Immunology; Innate immunity; Neutrophils; Metabolism; Immunometabolism

INTRODUCTION

Cells utilize diverse metabolic pathways for their growth, proliferation, survival, and even cell death. Catabolic reactions in metabolic pathways not only generate ATP, the major cellular energy source, but also fundamental metabolic intermediates. To achieve this, cells convert glucose into pyruvate through glycolysis, and pyruvate is further converted into acetyl-CoA. Acetyl-CoA is then oxidized through the tricarboxylic acid (TCA) cycle, generating GTP, NADH, and flavin adenine dinucleotide ($FADH_2$). Acetyl-CoA is also generated either from fatty acid oxidation (FAO) or from carbohydrate metabolism. The pentose phosphate pathway (PPP), a diversion pathway of glycolysis, generates NADPH and precursors for nucleotide synthesis. Thus, cells are able to utilize anabolic reactions in metabolic pathways to generate diverse intermediates for polypeptides, nucleic acids, proteins, polysaccharides, and lipids that they require for survival.

Immune cells are highly mobile cells; hence, they are exposed to various immunological environments wherein the availability of nutrients varies. Therefore, immune cells have a

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high functional plasticity, since they can reprogram diverse metabolic pathways according to the immunological environments they are exposed to. For example, proinflammatory M1 macrophages preferentially utilize the glycolytic pathway with broken TCA cycle to generate inflammatory mediators, whereas anti-inflammatory M2 macrophages utilize β-oxidation with intact TCA cycle to generate anti-inflammatory mediators [1]. T cells also often reprogram their metabolic pathways to achieve differentiation into diverse subtypes, and each resulting subtype has a distinct metabolic signature [1,2].

Neutrophils are the most abundant leukocytes in humans and participate in the innate immune response against invading pathogens. They are the first responders during inflammation and eliminate pathogens at infection sites. Because neutrophils are terminal differentiated cells with short life spans, earlier studies suggested that neutrophils were committed into simple metabolic pathways, such as glycolysis. However, recent advances in the immunometabolism field revealed that neutrophils use diverse metabolic pathways; reviewed in Kumar and Dikshit (**Fig. 1**) [3]. Compared to other immune cells where metabolic reprogramming generally ensures differentiation into distinct subtypes, neutrophils modulate their metabolic pathways to execute different effector functions such as chemotaxis, generation of ROS, neutrophil extracellular trap (NET) formation, and degranulation. In this review, we review the basic metabolic pathways used by neutrophils and further discuss the metabolic changes required for their effector functions.

BASIC METABOLIC PATHWAYS IN NEUTROPHILS

Glycolysis

Glycolysis is a fundamental metabolic pathway for most immune cells. Most cells uptake extracellular glucose through glucose transporters (GLUTs) and the subsequent glycolytic process coverts glucose into pyruvate, generating a small amount of ATP and NADH. In normoxia, pyruvate is converted to acetyl coenzyme A (acetyl-CoA), which is further oxidized through the TCA cycle. Alternatively, in anaerobic conditions, pyruvate is converted to lactate by LDH [4]. Glycolysis also generates diverse intermediates that are processed by the PPP for nucleotide synthesis, by the serine biosynthetic pathway for amino acids, or by the TCA cycle for [1]. Therefore, glycolysis is a dominant metabolism in most immune cells, including neutrophils, which also depend on glycolysis as source of ATP. Resting neutrophils express various GLUTs, such as GLUT1, GLUT3, and GLUT4. Activated neutrophils show increased surface expression of GLUTs [5,6], which is accompanied by an increase in the uptake of glucose [6,7]. In fact, depletion of glucose totally shuts most effector functions of neutrophils [4,6,8,9]. Moreover, neutrophils have relatively lower numbers of mitochondria than other immune cells [10] and oxidative phosphorylation (OXPHOS) is considered to be dispensable for their energy production [11,12]. Under glucose-deprived conditions, neutrophils rely on the breakdown of stored glycogen (glycogenolysis) as an alternative source of glucose [9] and phagocytosis depends on stored glycogen in neutrophils [7]. Interestingly, in inflammatory conditions, neutrophils show a larger accumulation of glycogen than peripheral neutrophils [13]. Therefore, glycolysis seems to be a dominant and essential metabolic pathway for neutrophils.

PPP

The PPP allows diversion of intermediates from the glycolytic pathway to generate NADPH for redox signaling, and nucleotides for DNA and RNA synthesis [1]. PPP potentiates the generation of ROS in neutrophils by NADPH oxidase (NOX) through providing NADPH [8].
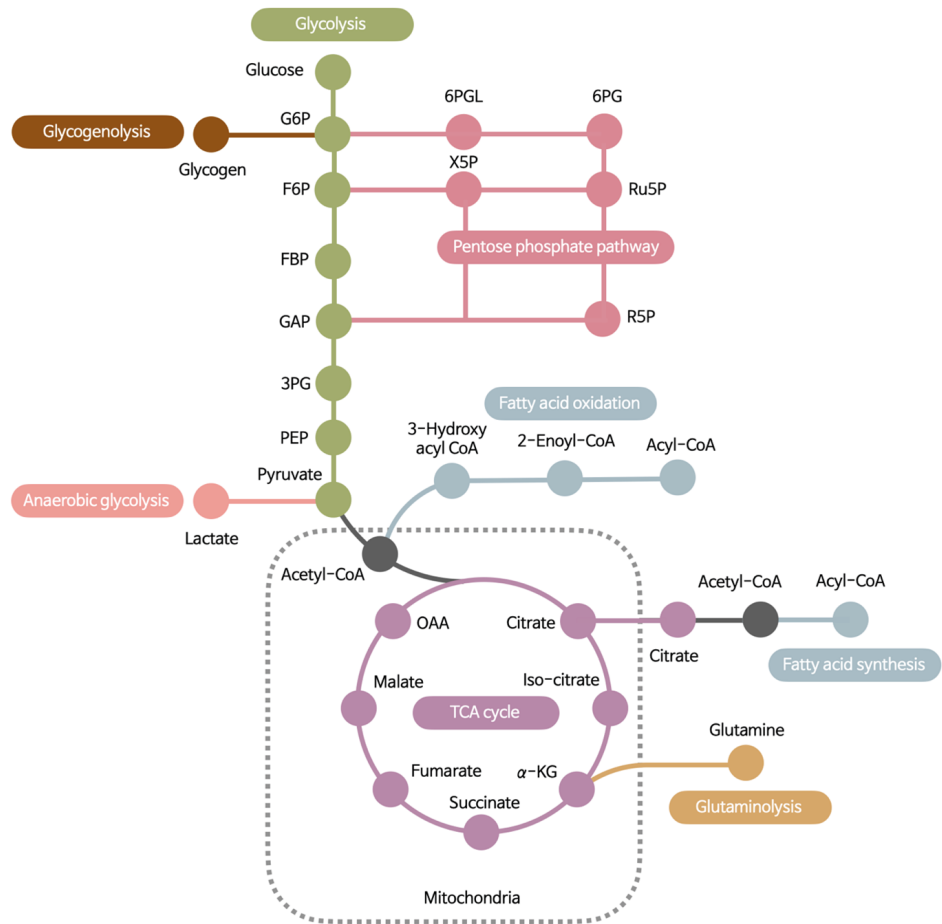
Figure 1. Basic metabolic pathways in neutrophils. Glycolysis is the dominant metabolic pathway for basal energy production in the form of ATP in neutrophils. The PPP diverts intermediates of the glycolytic pathway to produce NADPH, which is oxidated during ROS generation. The FAO pathway is an important source of acetyl-CoA through breakdown of fatty acids. FAO is used by immature neutrophils such as differentiating neutrophils and c-kit+ tumor-infiltrating neutrophils. Although neutrophils are equipped with intact mitochondrial and TCA cycle, they hardly depend on mitochondrial respiration for energy production. Mitochondrial purinergic signaling is important for chemotaxis in neutrophils. Migrating neutrophils show condensed mitochondrial in the head and tail positions and the local release of ATP drives their chemotaxis. On the other hand, an intact TCA cycle ensures the generation of various inflammatory intermediates. FAS mediates *de novo* lipogenesis, including the production of membrane phospholipids that replenish decayed membranes during inflammation. Glutaminolysis convert glutamine into α-KG, supporting the TCA cycle. Although neutrophils utilize glutaminolysis during glucose-limiting conditions, the fate of metabolized glutamine has not been fully understood. Neutrophils also use stored glycogen for glycogenolysis under glucose-limiting condition and anaerobic glycolysis under low oxygen conditions.

α-KG, α-ketoglutarate; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; FBP, fructose 1,6-bisphosphate; GAP, glyceraldehyde-3-phosphate; 3PG, 3-phosphoglycerate; PEP, phosphoenolpyruvate; 6PGL, 6-phosphogluconolactonase; 6PG, 6-phosphogluconate; X5P, xylulose-5-phosphate; Ru5P, ribulose-5-phosphate; R5P, ribose-5-phosphate; OAA, oxaloacetate.

On the other hand, the nonoxidative branch of the PPP supplies nucleotides for nucleic acid synthesis. Since neutrophils are short-lived cells, the requirements of additional production of nucleotides for DNA synthesis is questionable. Instead, neutrophils are equipped with high amounts of microRNAs that are needed for the fine tuning of gene expression through post-transcriptional regulation (14). Therefore, a detailed study on the role of the nonoxidative branch of PPP in neutrophils is still needed.

Mitochondria

Mitochondria are dispensable for energy production in mature neutrophils. Bioenergetic analysis showed that the basal oxygen consumption rate (OCR) is relatively lower in neutrophils than in monocytes or lymphocytes and is unresponsive to mitochondrial respiratory inhibitors (11). Moreover, the inhibition of mitochondrial respiration did not affect the ATP generation in mature neutrophils (15). However, neutrophils express components of the OXPHOS complex (16) and exhibit a complex mitochondrial network (17). Moreover, mitochondrial membrane potential (ΔΨm) is detected in mature neutrophils (16,17) and metabolization studies using exogenous amino acids have shown the functional mitochondria in neutrophils (18). Overall, these studies suggest that neutrophils are equipped with intact mitochondria but do not utilize them for energy production. Interestingly, immature neutrophils depend on OXPHOS and FAO, have relatively higher numbers of mitochondria than mature neutrophils (10), and show an intact OCR activity (12). In contrast to mature neutrophils, immature neutrophils produce energy by degrading lipid droplets through lipophagy and by directing the resultant fatty acids to the TCA cycle and OXPHOS (19).

FAO

During differentiation, neutrophils are critically dependent on lipophagy-mediated FAO (19). Since mature neutrophils mainly utilize glycolysis for energy production, FAO is considered to exclusively mediate their effector functions. For example, oxidized lipopoprotein induce NETs formation (20) and tumor infiltrating c-kit+ neutrophils utilize mitochondrial FAO to support ROS generation (12). However, it is unclear whether FAO mediates the effects of short chain fatty acids (SCFAs) during chemotaxis (21) and apoptosis (22) of neutrophils. Moreover, neutrophils with defective glucose and glutamine metabolism during hyperglycemia are suggested to activate compensatory FAO (23). Therefore, FAO is dispensable for energy production in mature neutrophils, but seems to mediate their effector functions.

Fatty acid synthesis (FAS)

Endogenous FAS mediates the *de novo* lipogenesis from precursors such as acetyl-CoA. A recent study showed that neutrophils utilize FAS to convert malonyl-CoA into fatty acids that are finally converted into membrane phospholipids (24). This study demonstrated that neutrophils utilize FAS to build membranes during differentiation or to replenish decayed membranes during inflammatory conditions. Previous studies also reported the influence of SCFAs on effector functions of neutrophils such as apoptosis (22), ROS generation (25), chemotaxis (26-28), and NETs formation (29,30). However, this influence depends on histone deacetylase inhibition (31), extracellular acidification (30,32), and specific receptors for SCFAs (25), rather than on the fatty acid metabolism.

METABOLISM OF ACTIVATED NEUTROPHILS

Energetics of neutrophils during differentiation

Hematopoietic stem cells preferentially use glycolysis for energy production, whereas committed progenitors utilize mitochondrial respiration (33,34). Neutrophils are generated from myeloid committed progenitors, the granulocyte-monocyte progenitor cells (GMPs), through increasingly more mature differentiation stages: myeloblasts (MBs), promyelocytes (PMs), myelocytes (MCs), metamyelocytes (MMs), and band cells (BCs) (35). Abundant mitochondria are observed in immature neutrophils such as MBs and PMs, but their number is reduced in mature neutrophils (10). Immature neutrophils utilize OXPHOS for energetics

(19). MBs have a relatively higher autophagic flux than other differentiating neutrophil subsets, such as MCs, MMs, and BCs, and induce lipophagy-mediated degradation of lipid droplets to generate free fatty acids. FAO further supports mitochondrial respiration during neutrophil differentiation. Indeed, defective autophagy arrests neutrophil differentiation at or after the GMP stage. Interestingly, subsets of immature c-kit+ neutrophils also rely on mitochondrial respiration for energy production (12). In particular, c-kit+ neutrophils residing in tumor environments have a higher OCR compared to peripheral c-kit− neutrophils and are sensitive to treatment with etomoxir, an inhibitor of FAO. Moreover, acute myeloblastic leukemias exhibit accumulation of granulocyte precursors, which engage into aerobic glycolysis (a phenomenon known as the Warburg effect) (36). In summary, committed neutrophil precursors shift their metabolic pathway from glycolysis to FAO-mediated mitochondrial respiration. Then, as neutrophils differentiate, mitochondrial respiration diminishes (**Fig. 2A**). Finally, mature neutrophils shift their metabolic pathway back to glycolysis (**Fig. 2B**).
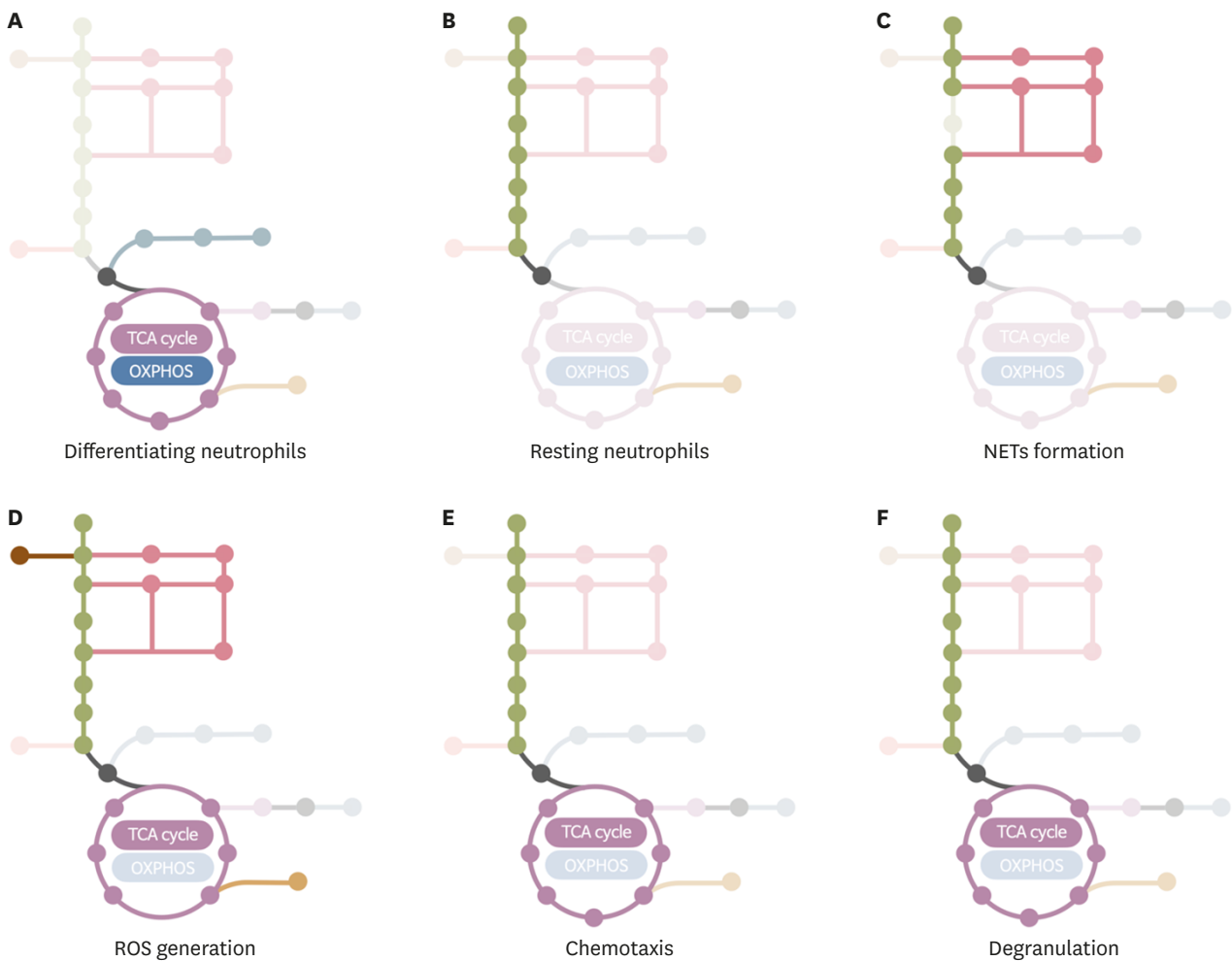

Figure 2. Representation of the current knowledge on metabolic pathways in working neutrophils. (A) Immature neutrophils utilize FAO-mediated OXPHOS for energy production. (B) Mature neutrophils preferentially utilize glycolysis as source for energy production. (C) Neutrophils depend on glycolysis bypassing the PPP for NET formation. (D) Neutrophils utilize diverse metabolic pathways for ROS generation. Glycolysis provides ATP and the PPP pathway provides NAPDH, which are required for ROS generation. NADPH generated through mitochondrial glutaminolysis is involved in ROS production. In contrast, immature neutrophils utilize mitochondria for ROS generation. (E) Mitochondrial purinergic signaling in migrating neutrophils mediates the chemotaxis of neutrophils, whereas glycolysis provides ATP for chemotaxis. (F) Both glycolysis and mitochondrial ATP production are involved in degranulation of neutrophils.

Basal energetics of neutrophils

As previously mentioned, mature neutrophils preferentially utilize glycolysis for energetics (**Fig. 2B**) and the inhibition of mitochondrial respiration does not affect their effector functions (4,37). However, the inhibition of glycolysis markedly attenuates ATP production in resting neutrophils and the production of lactate increases according to available glucose concentration (4,9). Neutrophils often encounter harsh environments where glucose and oxygen are lowly available. In glucose-deprived environments, neutrophils use the glycogenolysis pathway (4), whereas under low oxygen conditions neutrophils might adopt glycolysis for energy production rather than oxygen-requiring mitochondrial respiration (**Fig. 2**).

NETs formation

NETs are extracellular structures composed of DNA, histones, and antimicrobial granules (38). Neutrophils release NETs in response to immunological stimulations such as bacteria, fungi, viruses, parasites, and damage-associated molecular patterns to limit the dissemination of pathogens (39). Neutrophils mainly use glycolysis as energy source for NET formation. This is suggested by experiments showing that 2-deoxyglucose (2-DG, a hexokinase inhibitor) inhibits NET formation induced by phorbol 12-myristate 13-acetate (PMA) and amyloid fibrils (AF) (6,8). Moreover, the depletion of glucose totally abolishes NET release (6,8) and, conversely, the addition of glucose is sufficient to sustain NET formation (8). PMA stimulation enhances GLUT-1 expression, leading to an increase in glucose uptake and in glycolysis (6). The stimulation of neutrophils with either PMA or ionomycin enhances the extracellular acidification rate and the LDH activity (40). Interestingly, the inhibition of PPP by 6-aminonicotinamide (6-AN, a glucose-6-phosphate dehydrogenase [G6PDH] inhibitor) also inhibits PMA- and AF-induced NETs formation (8). Additionally, the exposure of bovine neutrophils to lactate enhances NET formation (41) and the inhibition of either monocarboxylate transporter 1 or LDH affects the lactate-induced NET formation (41). Because LDH mediates the bidirectional conversion of pyruvate and lactate, this result suggests that neutrophils utilize lactate during NETs formation. Therefore, glycolysis bypassing the PPP comprises the major metabolic pathway underlying NET formation (**Fig. 2C**).

ROS generation

ROS generation is one of the most important mechanism for the bactericidal activity of neutrophils. The generation of superoxide by NOX is the rate-limiting step for ROS generation. NOX transports electrons from NADPH into the phagosome to generate superoxide and these superoxide radicals are further converted into various ROS. Indeed, patients with chronic granulomatous disease (CGD), a genetic defect in NOX, show a predisposition to bacterial and fungal infections. ROS generation results in an increase in the OCR, which masks the mitochondrial respiration-induced OCR (11).

Neutrophils mainly use the PPP for ROS generation. In fact, the inhibition of PPP by 6-AN attenuates high glucose-induced ROS generation in neutrophils in pregnant women (42). The oxidative phase of PPP reduces 2 molecules of NADP+ to NADPH using energy obtained from the conversion of glucose-6-phosphate to ribulose 5-phosphate. Although the PPP provides the majority of NADPH, a substantial amount of NADPH is also obtained by glutaminolysis. Of note, the PMA treatment increases the OCR in human neutrophils and the inhibition of mitochondrial respiration does not affect PMA-induced OCR (11). PMA-stimulated neutrophils show an increase in the phosphorylation of 6-phosphofructo-2-kinase, the limiting enzyme in glycolysis, and the inhibition of this enzyme decreases both the glycolysis rate and the NOX activity in neutrophils (43). Additionally, neutrophils isolated from patients

with glycogen storage disease type Ib (GSD-Ib), a deficiency in glucose-6-phosphatase-β, showed impaired ROS generation (44).

In contrast, immature neutrophils were suggested to use mitochondrial respiration for ROS generation. As described earlier, immature neutrophils heavily rely on FAO-supported mitochondrial respiration to fulfill their energetic requirements. The inhibition of glycolysis with 2-DG attenuates the early phase of the OCR, whereas the inhibition of mitochondrial respiration with rotenone and antimycin A inhibits the later phase of the OCR in tumor-infiltrating immature neutrophils stimulated with PMA (12). The combined treatment of 2-DG and etomoxir (a carnitine palmitoyltransferase 1 inhibitor) completely inhibits the PMA-induced OCR in immature neutrophils (12). In contrast, in mature neutrophils, PMA-induced ROS generation is not affected by carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (11,17), suggesting a negligible role of mitochondria for ROS generation in mature neutrophils. Interestingly, exogenous α-ketoglutarate and pyruvate enhance ROS generation in neutrophils containing increased amounts of cellular intermediates such as glutamate (45,46).

Moreover, supplemental glutamine enhances ROS generation in human neutrophils (47). Glutaminolysis is an alternative pathway for provision of NADPH (48) and mitochondrial glutaminase is pivotal for glutamine metabolism (49). Because mitochondria in neutrophils are functionally intact (despite being dispensable for energy production) (17,50), neutrophils may alternatively use the mitochondrial glutaminolysis pathway for supplementing NADPH during ROS generation (**Fig. 2D**).

In summary, the PPP is the major metabolic pathway used by neutrophils for the generation of ROS but mitochondrial glutaminolysis seems to play an alternative role as an important NADPH supplementing pathway (**Fig. 2D**).

Chemotaxis

Neutrophils actively migrate into inflammatory foci to eliminate invading pathogens. Neutrophils recognize a gradient of chemoattractants, reorganize their cytoskeletons, and migrate toward chemoattractants. Various signaling molecules, such as small GTPases and PI3K, coordinate the migration of neutrophils through spatial control of the cytoskeleton (51). Mitochondria-derived purinergic signaling is important for neutrophil chemotaxis. For example, N-formyl-methyl-leucyl-phenylalanin (fMLP), a major chemoattractant for neutrophils, enhances the mitochondrial activity and ATP production at the front side of the migrating neutrophils (50,52). This release of ATP triggers the P2Y2 receptor resulting in pseudopod protrusion supported by actin polymerization. Mitochondria respiration is hardly involved in ATP generation in response to chemoattractants due to the inhibition of chemotaxis by the disruption of the mitochondrial potential but not due to the inhibition of ATP synthase (17). Moreover, the inhibition of mitochondrial complex I by metformin inhibits neutrophil chemotaxis (53) and iso-citrate dehydrogenase-deficient neutrophils show impaired chemotaxis (54).

Additionally, neutrophils use glycolysis as source of ATP for chemotaxis. Migrating neutrophils show increased uptake of glucose (7) and, in accordance, impaired chemotaxis is observed in G6PD-deficient neutrophils (44). Furthermore, the maximal dose of 2-DG completely inhibits neutrophil chemotaxis, whereas the half-maximal dose of 2-DG paradoxically enhances neutrophil chemotaxis (55).

Overall, these results suggest that neutrophils mainly use purinergic signaling from the mitochondrial TCA cycle as source of chemotactic signals and glycolysis as an energy source for chemotaxis (**Fig. 2E**).

Degranulation

Neutrophils contain diverse antimicrobial molecules, such as peptides and proteases, in the form of stored granules. During differentiation in the bone marrow, neutrophils acquire and store these antimicrobial molecules into at least four kinds of granules: azurophil (primary) granules, specific (secondary) granules, gelatinase (tertiary) granules, and secretory vesicles (56). Because these molecules are highly toxic to microorganisms, they comprise an important non-oxidative resource with bactericidal functions. Upon sequestration of pathogens by phagocytosis, neutrophil granules fuse resulting in the formation of phagolysosomes and release antimicrobial molecules into these vesicles (57). This degranulation process comprises membrane fusion between granules and phagosomes and is mediated by membrane fusion-associated signals such as small GTPase, Ca^{2+}, vesicle-associated membrane protein, syntaxin, and synatosomal-associated protein receptor (58).

A detailed understanding of how neutrophils produce energy to use during the degranulation process has not been attempted. However, 2-DG was shown to inhibit degranulation of azurophil (59), specific (60), and tertiary granules (55,60), in accordance with a role of glycolysis in the process. Furthermore, neutrophils isolated from CGD patients exhibit exacerbated degranulation in response to stimulation with fMLP and PMA (61), suggesting that neutrophils rarely use the PPP for degranulation. Interestingly, mitochondrial functions are also involved in degranulation of neutrophils. In particular, the inhibition of mitochondrial ATP production prevents the degranulation process (62) and the purinergic signaling can mediate degranulation, among other effector functions (63). In summary, neutrophils utilize both glycolysis and mitochondrial TCA cycle to fuel degranulation (**Fig. 2F**).

Metabolism of neutrophils during metabolic disorders

Metabolic adaptation of neutrophils during pathological conditions is reviewed in an excellent article (3). Therefore, we will briefly review neutrophil metabolism during metabolic disorders. Neutrophils exhibit defective functions in genetic disorders on metabolism. The deficiency of G6PDH, a key enzyme for diversion of glycolytic pathway into PPP, results in defective PPP with reduced production of NADPH. Neutrophils isolated from patients with G6PDH deficiency showed defective PPP with reduced respiratory burst (64) and NET formation (65). Neutrophils isolated from patients with GSD-1b, a deficiency in glucose-6-phosphate transporter, also showed defective production of ATP with impaired ROS generation and phagocytosis (44). In contrast, neutrophils exhibit metabolic reprogramming during diabetes. Most effector functions of neutrophils including ROS generation, NET formation, bactericidal activity, and chemotaxis, are dysregulated in patients with diabetes (66-70). Since neutrophils are exposed to hyperglycemia during diabetes, neutrophils isolated from diabetic rats exhibit the decreased activity of G6PDH with the defective PPP and reduced ROS generation (23). Therefore, a compensatory FAO utilization by neutrophils has been suggested. However, the detailed study regarding metabolic adaptation of neutrophils during diabetes has not been fully elucidated yet.

DISCUSSION

Despite the recent advances in the field of immunometabolism, the metabolism of neutrophils is not fully understood. Earlier studies revealed that neutrophils mainly use glucose as source for their metabolic processes; however, emerging evidence shows that neutrophils also use other nutrients such as amino-acids, carbohydrates, proteins, and lipids for energy production. Neutrophils are often exposed to various immunological environments where nutrients are limited. Therefore, they must adapt diverse metabolic pathways for diverse functions in response to the required immunological needs. Moreover, recent studies suggest that neutrophils undergo metabolic adaptation under diverse disease conditions. Further studies using dedicated techniques, such as metabolic flux assay and metabolomics, might be useful for the complete understanding of the neutrophil metabolism.

ACKNOWLEDGEMENTS

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