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Review

The Critical Role of Cell Metabolism for Essential Neutrophil Functions

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Abstract
Neutrophils were traditionally considered as short-lived cells with abundant secretory and protein synthetic activity. Recent studies, however, indicate neutrophils are in reality a heterogeneous population of cells. Neutrophils differentiate from pluripotent stem cells in the bone marrow, and can further mature in the blood stream and can have different phenotypes in health and disease conditions. Neutrophils undergo primary functions such as phagocytosis, production of reactive oxygen species (ROS), release of lipid mediators and inflammatory proteins (mainly cytokines), and apoptosis. Neutrophils stimulate other neutrophils and trigger a cascade of immune and inflammatory responses. The underpinning intracellular metabolisms that support these neutrophil functions are herein reported. It has been known for many decades that neutrophils utilize glucose as a primary fuel and produce lactate as an end product of glycolysis. Neutrophils metabolize glucose through glycolysis and the pentose-phosphate pathway (PPP). Mitochondrial glucose oxidation is very low. The PPP provides the reduced nicotinamide adenine dinucleotide phosphate (NADPH) for the NADPH-oxidase (NOX) complex activity to produce superoxide from oxygen. These cells also utilize glutamine and fatty acids to produce the required adenosine triphosphate (ATP) and precursors for the synthesis of molecules that trigger functional outcomes. Neutrophils obtained from rat intraperitoneal cavity and incubate for 1 hour at 37°C metabolize glutamine at higher rate than that of glucose. Glutamine delays neutrophil apoptosis and maintains optimal NOX activity for superoxide production. Under limited glucose provision, neutrophils move to fatty acid oxidation (FAO) to obtain the required energy for the cell function. FAO is mainly associated

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with neutrophil differentiation and maturation. Hypoxia, hormonal dysfunction, and physical exercise markedly change neutrophil metabolism. It is now become clear that neutrophil metabolism underlies the heterogeneity of neutrophil phenotypes and should be intense focus of investigation.

Neutrophil identity and biology

The most abundant leukocyte in human peripheral blood is neutrophil with an estimated daily turnover of 1010 to 1011 cells [1, 2]. Bone marrow myeloid cells differentiate to myeloblasts through granulocytopoiesis to generate neutrophils [3]. The estimated neutrophil half-life in human blood is of only 19 h [4, 5]. The life span of neutrophils, however, can be extended up to 72 hours when activated [6, 7]. Due to the short lifespan, blood neutrophils have to be constantly replenished from bone marrow precursor cells. Evrard, et al. [8] described three bone marrow neutrophil subsets. The committed proliferative neutrophil precursor (preNeu) differentiates and generates two other subsets: non-proliferating immature neutrophils and mature neutrophils either in mice [9] and humans [10]. Mice pre-neutrophils express cluster of differentiation (CD) 117 but no markers for other leukocyte lineages. The expression profiles of chemokine C-X-C motif ligand receptor 4 (CXCR4), CXCR2, and CD101 discriminate immature neutrophils (CXCR2−CD101−) and mature neutrophils (CXCR2+CD101+). In the human bone marrow, the discriminations of the three primary neutrophil subsets associate to the absence of other leukocyte lineage markers but expression of CD101 and other cell specific surface proteins for neutrophils such as CD16 [11].

The number of granules in the cytoplasm in the promyelocyte phase during neutrophil maturation increases. For instance, azurophils granules produced in the Golgi complex. Neutrophils then develop to reach the myelocyte stage, characterized by the production of specific granules [12]. The granules contained in the cytoplasm of neutrophils are primary lysosomes. These latter granules fuse with the phagocytic vacuoles. The released hydrolytic enzymes then act as antibacterial agents [13]. There are three main types of granules in neutrophils. Azurophilic or primary are large and dense granules formed during promyelocyte stage and contain myeloperoxidase [14]. Specific or secondary granules formed during the myelocyte-metamyelocyte phase contain lactoferrin [15]. The tertiary granules formed at the band-stage of cell development contain gelatinase [16]. These cells are also known as polymorphonuclear (PMN) leukocytes since the segmented nucleus is made up of 3 to 5 lobes connected through a thin strip of nuclear material. Immature neutrophils found occasionally in the circulation are called “rods” because they do not have nuclei segmented into lobes, being identified by their horseshoe-shaped nuclei [17].

Neutrophil functions

Neutrophils are the first leukocytes to reach the inflammation or infection site to combat the invading microorganisms [18]. The activation of neutrophils involves: endothelial cell adhesion, migration to inflamed tissue (chemotaxis), phagocytosis, degranulation, ROS generation, and cytokine production. The cytokines produced participates of the inflammation process, as they attract other leukocytes to the inflammation area [19]. When activated, neutrophils migrate following the gradient of cytokines or other compounds, adhere to the endothelium of the vessels, and pass then to the tissues. Neutrophils move towards the inflammatory focus, through chemotaxis, where they perform phagocytosis of particulate material and produce ROS [20]. These cells also release various cytokines, such as IL-6, IL-1, and tumor necrosis factor alfa (TNF-α), fever-causing pyrogens and inflammation chemical mediators [21]. Neutrophils act in efferent (phagocytosis and degranulation) and afferent (release of immunomodulatory molecules) processes associated with inflammatory and immune responses [22]. Following migration to damaged/infected tissue, neutrophils rapidly

die after completing specific tasks [23], releasing toxic granular proteins and DNA genomic strands to catch and kill bacteria [24].

Neutrophils also participate in sterile (in the absence of microorganism) inflammation as occurs in trauma, ischemia-reperfusion injury, chemically-induced injury. Ng et al. [25] investigated mice dermis neutrophil migration to injured tissue using microscopic analysis. The authors reported three subsequent phases: scouting neutrophil migration, waves of neutrophils, and neutrophil migration stabilization. Neutrophils are also involved in pathological processes (e.g. gout and pseudogout, myocardial infarction, stroke, and infiltration of leukocytes into the tumor surrounding [26]. Release of molecules associated with endogenous damaged tissues known as DAMPs (damage-associated molecular pattern) then takes place [26, 27]. DAMPs lead to production of CXC chemokines required for the neutrophils recruitment in sterile inflammation [26, 28]. In 2004, Brinkmann et al. [24] described the neutrophil extracellular traps (NETs). Neutrophil NETs contain DNA, histones, and peptides with anti-microbial properties to trap and kill microorganisms. Formation of NETs occurs by microbes, bacterial products, and phorbol 12-myristate 13-acetate (PMA) stimuli. Azevedo, et al. [29] described that glucose metabolism has deviated from glycolysis to the PPP during NETs formation.

Biological cell death types include necrosis, apical necrosis, pyroptosis, necroptosis, and Neutrophils Extracellular Traps-induced cell death (NETosis) [24, 30]. Following completion of their biological function, neutrophils die by necrosis or apoptosis [31]. The constitutive death process of neutrophils is apoptosis. Galluzzi, et al. [32] reported an updated cell death classification based on the mechanisms and the central aspects associated. The authors listed and defined different types of regulated cell death processes and the programmed cell death. The authors pointed out that cellular senescence is not a cell death type but instead is a non-lethal process. Biochemical and cellular characteristics allow the recognition of neutrophils by resident macrophages in the tissues, they perform phagocytosis of neutrophils and their removal before the rupture of the cell membrane occurs. This process is vital to maintain tissue homeostasis [6, 7, 33, 34].

There are differences reported on neutrophils from mice and humans. Neutrophils represent the majority of white blood cells in humans (50 to 70%), but are less common in mice (10 to 30%), whereas mice blood has a preponderance of lymphocytes (75–90%) [35]. In mice, granulocyte antigen-1 (Gr-1) and lymphocyte antigen 6 complex locus G6D (Ly-6G) are well-defined markers for identifying granulocytes, whereas human granulocytes do not express these proteins. Mice also do not express the human Fc receptor for Immunoglobulin A - IgA – (FcαRI or CD89) - one of the antibody fragment crystallizable region (Fc) receptors that trigger effector functions, such as cytokine production, NETose, and phagocytosis [36, 37]. The granule content neutrophils from humans are very different from that in mice, which can alter the effector functions of neutrophils in mice as compared to humans. Mice neutrophils do not produce defensins, whereas human neutrophils produce this proteins [38]. The binding immunoglobulin (BIP), myeloperoxidase, β-glucuronidase, lysozyme, alkaline phosphatase, and arginase-1 expressions are much higher in humans than in mice neutrophils [39]. The production of pro and anti-inflammatory cytokines also differs between humans and mice.

Neutrophil plasticity

Neutrophil was considered for long time a homogenous cell type with low transcription activity and short-life time. There is now unquestionable evidence on the heterogeneity of neutrophil phenotype and function in different tissues [40]. A great variety of neutrophil phenotypes was identified in health and diseased conditions [41]. Neutrophil phenotypes are differentiated based mainly on cell-surface markers, maturity state, and functions. Neutrophils are not only microbe-killing in diseases but have a role in innate immunity. The reviews of Yang, et al. [40] and Silvestre-Roig, et al. [41] describe the neutrophil phenotypes

and functions in several diseased conditions such as inflammation, rheumatoid arthritis, systemic lupus erythematosus, autoimmune disease, cancer, and diabetes. Mortaz, et al. [42] reviewed the studies on neutrophil phenotypes in trauma. The authors present evidence that neutrophil phenotypes associated with secondary complications may be used as prognosis of trauma injured patients.

Neutrophil displays different phenotypes according to the inflammatory condition [43, 44]. Description of N1 and N2 phenotypes illustrate the plasticity of neutrophils. N1 neutrophils associate with acute inflammation to eliminate pathogens. N1 neutrophils have a hypersegmented and lobulated nuclear morphology and anti-tumor properties, and increased activity of NADPH oxidase leading to high production of ROS, and have anti-tumor properties [45, 46]. N1 highly expresses pro-inflammatory mediators – TNF-α, chemokine C-C motif ligand 3 (CCL3), and intercellular adhesion molecule-1 (ICAM-1) [47]. N2 neutrophils induce angiogenesis and tissue remodeling in chronic inflammation. N2 phenotypes have pro-tumor actions [48], causes tumor growth, and tumor cell metastasis. In chronic inflammation, N2 phenotype formation occurs in the bone marrow and tumor microenvironment [49, 50]. Priming with interferon- γ (IFN-γ) and TNF-α convert N2 to N1 [51]. The transformation of growth factor β (TGF-β), an immunosuppressive cytokine overexpressed by tumor, polarizes neutrophils into N2, whereas TGF-β blockade leads to an accumulation of N1 [43]. The plasticity of neutrophils under different stimuli and the differentiating neutrophil markers repertoire indicate that other phenotypes might exist between the extremes N1 and N2 neutrophils.

Neutrophils have antitumor or pro-tumor properties depending on the inflammatory cytokine effects [52]. Zhu, et al. [10] described neutrophil progenitor cells with pro-tumoral properties within mouse and human bone marrow. So, these cells are programmed to exhibit pro-tumoral activities even before reaching the blood stream. Neutrophils also have either pro- or anti-metastatic properties. Hsu, et al. [53] reported the involvement of immature low-density neutrophils in the induction of liver metastasis in cancer conditions. Contrary, mature high-density neutrophils impede liver metastases from occurring.

The different features of low- and high-density neutrophils correlate with metabolic activity. Low-density neutrophils exhibit an augmented capacity for ATP production even in mitochondria. This augmented energetic generating feature enables this neutrophil phenotype to carry out pro-metastatic functions, including NETosis, even under nutrient-deprived conditions. NETosis plays a role in the neutrophil promotion of breast cancer liver metastasis. The authors described that proline and glutamate metabolism generates ATP for low-density neutrophils to carry out NETosis even in the absence of glucose. The function of the pro-metastatic neutrophils then associates with the plasticity of this cell type to move the substrate flux from one metabolic pathway to another.

Neutrophil metabolism

The main steps catabolic pathways of glucose, glutamine and fatty acid metabolism are presented in the Fig. 1. Neutrophils eliminate and destroy microorganisms or cell debris through phagocytosis, production of ROS, extrusion of genomic DNA, such as NETs, and release of cytotoxic granules [18, 54]. Neutrophils in general use energy derived primarily from glucose [55-57]. In 1912, Levene and Meyer [58] reported that neutrophils use glucose and convert it to lactic acid. Sbarra and Karnovsky [59] described the neutrophil main ATP generation pathway is glycolysis initiated by glucose phosphorylation to glucose-6-phosphate (G6P) through hexokinase.

Glucose forms lactic acid through anaerobic glycolysis in neutrophils [60]. Neutrophils have few functional mitochondrial and so very low Krebs cycle activity and rates of oxidative phosphorylation [61]; around 3% of total ATP used by these cells [62] and oxidative phosphorylation. Mitochondria participate of apoptosis through mitochondrial membrane potential changes and release of specific signaling factors [34]. We reported that rat neu-

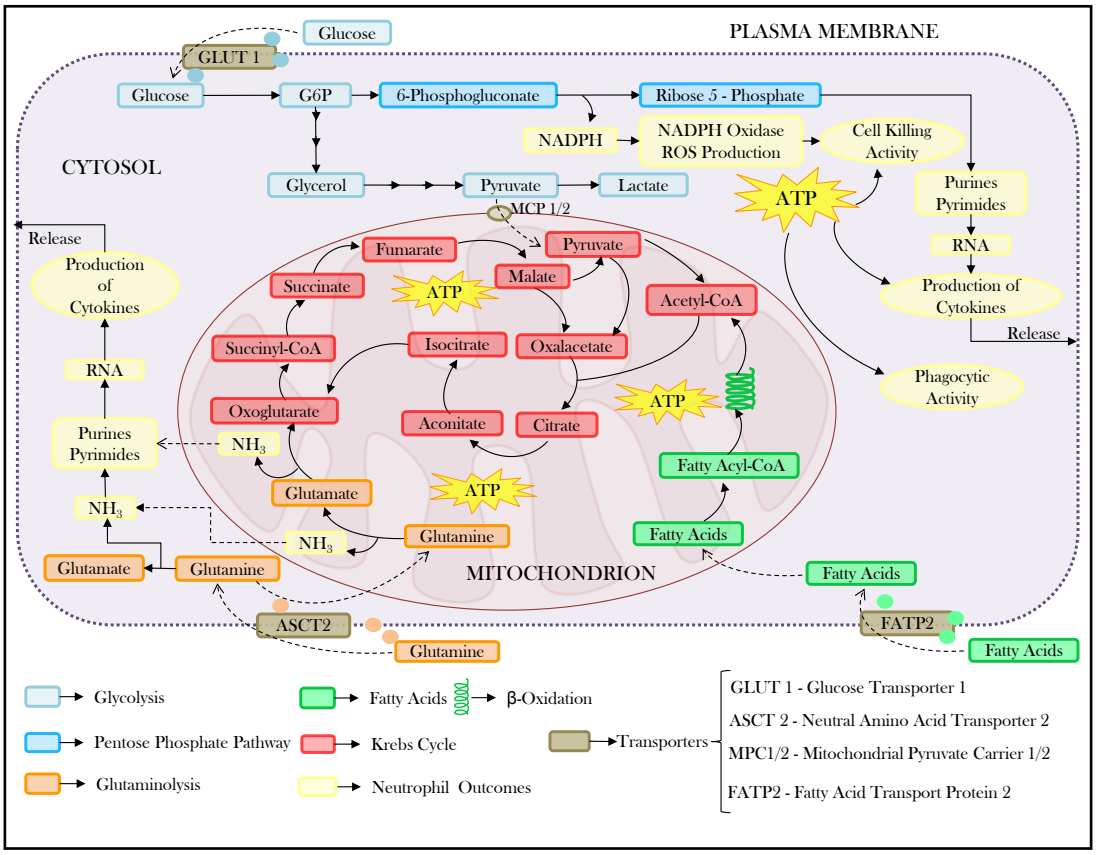

Fig. 1. Neutrophil metabolism and outcomes.

trophils have substantial phosphate-dependent glutaminase (PDG) activity, a key enzyme for glutamine metabolism by the cells, converting glutamine to glutamate. Neutrophils were obtained from adult rats by intraperitoneal cavity lavage using 40 mL sterile phosphate-buffered saline, 4 h after induction of neutrophil migration by intraperitoneal injection of 20 mL sterile oyster glycogen solution (at 1%). These cells (107 per flask) were incubated in 1 mL Krebs-Ringer medium containing 2% defatted albumin, 5 mM glucose or 2 mM glutamine, for 1 hour, at 37°C. Rat neutrophils utilize glutamine at higher rates than glucose in the experimental conditions described [63-65].

Glycolysis

Cells require glucose for survival, proliferation, and function. Rui Curi, Tania Pithon-Curi and others measured the maximal enzyme activities of glucose and glutamine metabolism in rat neutrophils obtained from intraperitoneal cavity. As pathway markers, they determined the activities of hexokinase (glycolytic pathway), glutaminase (glutaminolysis), and citrate synthase (Krebs cycle) [63-66]. The rates of metabolite utilization and production in incubated rat neutrophils were also determined [63, 66]. Glycolysis generated the majority of ATP required for neutrophil function [67] so generating lactate [68]. The glycolysis rate remains unchanged during phagocytosis [56], whereas ATP levels, which usually are approximately 1.9 nmol/10^6 cells, fall to 0.8 nmol/10^6 cells [55], suggesting high rates of ATP consumption during phagocytosis. The neutrophil is particularly rich in glycogen. The concentration of this complex polysaccharide is 7.36 mg/10^9 cells [69, 70].

Neutrophils use glucose from intracellular glycogen breakdown [55] and from the circulation through the glucose transporters (GLUTs) uptake system [57]. Expressions of GLUT1, GLUT3, and GLUT4 vary according to neutrophil biological conditions [71]. In resting conditions, neutrophils express GLUT1 and GLUT3 on cells surface that do not require insulin

to promote glucose uptake. However, in the presence of PMA stimulation, insulin induced GLUT4 transfer to plasma membrane. Under physiological circumstances, glucose is mainly transported through GLUT1 in neutrophils [72].

Intracellular G6P is the precursor of glycogen [69]. Glucose-6-phosphate transporter (G6PT) mediated endoplasmic reticulum (ER) uptake [73] or metabolization through glycolysis [56] or the PPP are other G6P destinations [74]. Glycogen breakdown in neutrophils is considered primarily restricted to phagocytic activity [56, 75]. The primary competing glucose/G6P pathways in neutrophils are glycolysis and PPP. Glycolysis supplies the necessary ATP for neutrophil locomotion and chemotaxis whereas PPP generates NADPH.

Pentose-phosphate pathway (PPP)

Glucose utilization through the PPP is required for several neutrophil functions [59, 76, 77]. In resting neutrophils, the amount of glucose metabolized via this route is only 2 to 3 percent of the total glucose consumed by the cells [77, 78]. The PPP is of particular importance for neutrophils because it provides the NADPH needed for *de novo* fatty acid synthesis and for NOX activity. NOX uses NADPH to reduce oxygen and generate superoxide (O_2) [73, 79-81].

Superoxide generated by this enzyme complex serves as the starting point for the generation of a wide variety of reactive oxidants such as oxidized halogens, free radicals, and singlet oxygen. Neutrophils use these oxidant compounds to kill invading microorganisms [82]. Glucose-6-phosphate dehydrogenase (G6PD) and gluconate-phosphate dehydrogenase enzymes are steps of NADPH generation in the PPP [83]. As mentioned, NADPH donates electrons for the production of superoxide and hydrogen peroxide that induce the release of toxic granular proteins. Elastase, myeloperoxidase, and chromatin traps (DNA genomic strands) kill a large quantity of bacteria at the same time [84].

Glycogen metabolism

Glycogen content increases with neutrophil maturation [85]. Glycogen breakdown increases when neutrophils are exposed to limited extracellular glucose. Glycogen synthesis occurs when glucose supply is adequate [56, 69, 86]. The re-establishment of normal intracellular glucose levels promotes the re-synthesis of glycogen [59]. Glucose-starved neutrophils exhibit increased glycogen phosphorylase activity during phagocytosis to form glucose-6-phosphate [86]. Phagocytosis promotes glycogen breakdown to generate ATP via glycolysis [55-57].

The neutrophil glycogen level is also regulated by glucose recycling between ER and cytoplasm. Jun, et al. [73] reported the ER and cytoplasm glucose cycling plays a key role to regulate neutrophil function and apoptosis. Deficiency of the ER glucose-6-phosphatase-β (G6Pase-β also known as G6PC3) underlies the G6PC3–deficient congenital neutropenia syndrome. It is reported enhanced ER stress and apoptosis in neutrophils from patients with this disease. G6PC3 generates glucose and phosphate from the G6P that enters the ER through the G6P transporter (G6PT). The decreased cytoplasmic concentrations of glucose, G6P, lactate, and ATP due to the deficiency of ER G6PC3 impairs neutrophil function and causes apoptosis leading to neutropenia.

Fatty acids

Fatty acids have different chain lengths varying from 3 to 30 carbon atoms. Short-chain fatty acids have less than six carbon atoms, whereas medium-chain fatty acids have six to ten carbon atoms, and long-chain fatty acids have over twelve carbon atoms. Monounsaturated fatty acids have one carbon - carbon bond whereas the polyunsaturated contain two or more double bonds. Saturated fatty acids have no double bonds in the molecule [87].

Neutrophil activation and function also rely on fatty acid utilization and oxidation. Several free fatty acid receptors exist in this leukocyte. Free fatty acid receptor-1 (FFAR1/GPR40) and 2 (FFAR2/GPR43), and GPR84 transport long-chain fatty acids (>C12), short-chain fatty acids (C2-C6), and medium-chain fatty acids (C7-C12), and fatty acid transporter

proteins (FATP) also transport long-chain fatty acids [88, 89]. Fatty acids play an essential role in leukocyte metabolism [90]. Neutrophils release arachidonic acid derivatives such as leukotriene B4 (LTB4) and hydroxyeicosatetraenoic acids (HETE) [91, 92]. In conditions of limited glucose availability or under fasting conditions, cells rely on fatty acid metabolism.

Autophagy is involved in cell proliferation, death, and differentiation. Autophagy plays a critical role in cell fate decisions [93]. Riffelmacher, et al. [94] reported that autophagy-dependent release of free fatty acids participates in the bone marrow neutrophil maturation.

Free fatty acids enter the FAO pathway to produce ATP through oxidative phosphorylation (OXPHOS) for neutrophil differentiation, maturation, and function [95]. FA can block glycolysis through the glucose-fatty acid cycle. Randle, et al. [96] described that fatty acids reduce the uptake and glucose metabolism in cardiac muscle cells. The oxidation of fatty acids generates acetyl-coenzyme A (acetyl-CoA) that in turn forms citrate via the citrate synthase activity. High acetyl-CoA/CoA and NADH/NAD ratios promote pyruvate dehydrogenase kinase (PDK) activation that inactivates pyruvate dehydrogenase (PDH). ATP and citrate cause inhibition of phosphofructokinase (PFK), and so glucose-6-phosphate accumulation and hexokinase inhibition. As a consequence, glycolysis is inhibited.

Fatty acyl-CoAs are transported to the mitochondrial matrix where acetyl-CoA is produced which can enter the tricarboxylic acid (TCA) cycle for oxidation [64]. Carnitine is necessary for energy production due to its role in FAO. Carnitine palmitoyltransferase-1 (CPT-1) catalyses an ester bond of carnitine with long-chain fatty acids and generates acylcarnitines. This enzyme is on the outer mitochondrial membrane. Acylcarnitines are then translocated across the inner mitochondrial membrane by carnitine acylcarnitine translocase [97]. Acylcarnitines regenerate acyl-CoAs inside the mitochondrion through the carnitine palmitoyltransferase-2 (CPT-2) activity that is placed in the inner mitochondrial membrane [98]. Carnitine returns to the cytoplasm for another cycle (using carnitine-acylcarnitine translocase - CACT), while acyl-CoAs enter the pathway of β-oxidation generating acetyl-CoA (under aerobic conditions and low ATP levels) [97].

Glutamine metabolism

Glutamine concentration is relatively high in the human blood and intracellular pools as compared to all other amino acids. Mammals synthesize glutamine in skeletal muscle, liver, and lung [99]. In high catabolic states (e.g. injury, trauma, burns, and sepsis), the cellular requirement for glutamine is increased. Glutamine then becomes an essential metabolite; it has been referred to as a "conditionally" essential amino acid. In the 1980s, Eric Newsholme's laboratory performed pioneering studies regarding glutamine metabolism in leukocytes. His group was the first to establish that lymphocytes and macrophages utilize glutamine at high rates. Up to then, glucose metabolism was the solely well studied in these cells. Glutamine is of critical importance for proliferation of lymphocytes and the inflammatory response of macrophages. We reported that glutamine is utilized by rat neutrophils and it participates in several neutrophil functions [63, 100]. Castell, et al. [101] described on the glutamine metabolism in human neutrophils. Glutamine is involved in production of ROS and apoptosis of neutrophils. Due to the high utilization of glutamine by leukocytes and its importance for these cells functions, Eric Newsholme and members of his laboratory postulated skeletal muscle provides this amino acid for leukocyte functions. Indeed, marked skeletal muscle mass wasting occurs in conditions of increased leukocyte function and glutamine requirement [102].

Krebs [103] first described the reactions of glutamine synthesis and hydrolysis in mammalian tissues. The most significant proportion of circulating glutamine (concentration of approximately 0.6 mmol/L in humans) derives from skeletal muscle, which synthesizes and exports glutamine and alanine to the circulation. The skeletal muscle glutamine synthesis substantially increases during situations of intense catabolism, such as fasting and prolonged exercise. In patients with acquired immune deficiency syndrome (AIDS), sepsis, severe injury, burns, or after surgery, plasma glutamine concentration decreases to levels less

than 50% of the standard values. Low plasma glutamine level is associated with impaired immune defense functions [104, 105].

Glutamine is the free amino acid with the highest concentration in the skeletal muscles, reaching a concentration of 20 mmol/L [106]. In addition to skeletal muscle, glutamine synthesis also occurs in the liver, adipose tissue, and lung. In contrast, hydrolysis occurs in a variety of tissues and organs, including kidneys, lymph nodes, macrophages, gastrointestinal tract, liver, brain, and adipose tissue [107, 108].

The first step of glutaminolysis is the conversion of glutamine to glutamate through PDG. Glutamate is converted into α-ketoglutarate via transamination or under some conditions, via glutamate dehydrogenase (releasing NH_4^+), that enters the TCA cycle. α-Ketoglutarate forms succinyl-coenzyme A, succinate, fumarate, and then malate. Malic enzyme converts malate and nicotinamide adenine dinucleotide phosphate ($NADP^+$) into pyruvate and NADPH. Pyruvate can be converted into lactate or to a lesser extent enters the TCA cycle via generation of acetyl-CoA by PDH. Intermediates and products of glutaminolysis undergo several chemical reactions and have different functions in various cell types [102].

Glutamine is metabolized in neutrophils and produces glutamate, but also aspartate, alanine, and lactate [63]. Pyruvate is a then a common product of glycolysis and glutaminolysis. Glutamate is the precursor of several other metabolites including malate, and therefore NADPH generation through malate dehydrogenase and malic enzyme [109]. Glutamine and glutamate provide nitrogen for nucleotides and nucleic acids synthesis [99] . Glutamine transfers an amino group to a fructose-6-phosphate molecule. Fructose-6-phosphate transaminase catalyzes this reaction and generates glucosamine. The latter is an amino sugar involved in the glycosylation of proteins and lipids [110] with specific roles in cell functions.

The activity of NOX has been assessed in rat neutrophils cultured with glutamine [80]. The NOX activity and the production of superoxide induced by PMA is enhanced still further by up to 100% in the presence of glutamine in comparison to neutrophils cultured without glutamine [111]. α-Ketoglutarate also enhances neutrophil function. α-Ketoglutarate stimulates the generation of superoxide and hydrogen peroxide, and raises myeloperoxidase activity in cultured neutrophils [112]. Extracellular glutamine levels regulate neutrophil superoxide production and cytolytic activity [113]. Glutamine raises bactericidal activity, phagocytosis, and production of ROS in neutrophils from postoperative patients [114, 115]. The administration of glutamine improves neutrophil function in burned patients. Glutamine enhances the bactericidal activity of neutrophils in the mentioned patients [114, 116]. In immunostimulated macrophages and monocytes, glutamine enhances IL-1, IL-6, TNF-α, and IL-8 release [102, 111, 117]. These reports above support the administration of glutamine as a nutritional supplement in critically ill patients [64, 118].

Control of neutrophil metabolism

Several researchers reported findings on the changes in neutrophil metabolism induced by hormones, endocrine dysfunction, and physical exercise (Table 1). Alba-Loureiro, et al. [66] reported decreased activities of G6PD and PDG and increased of PFK in in neutrophils from streptozotocin-induced diabetic rats. The authors also found reduced decarboxylation of glucose and glutamine and increased palmitic acid oxidation. The increase in palmitic acid oxidation under this condition may be an attempt to compensate for the reduced ATP production from glucose and glutamine metabolism. These metabolic changes associate with impaired neutrophil functions, such as reduction in phagocytosis and ROS production in diabetic states. The treatment with insulin abolished the diabetic state induced changes, and this effect was not associated with changes in blood glucose levels. Insulin does exhibit a direct impact on neutrophil metabolism and function [66].

Sexual steroid hormones controlled locally chemotactic mechanisms are associated with the periodic neutrophil accumulation in the rat vagina after estrus. This data provide

Table 1. Changes in neutrophil metabolism and outcomes induced by hormones, endocrine dysfunctions, and physical exercise. HG - Hyperglycemia; HT - Hypothyroidism; HP – Hyperthyroidism; ROS - reactive oxygen species

Treatments	Hormones		Endocrine Dysfunctions			Exercise		References
Changes	Insulin	Adrenaline	HG	HT	HP	Moderate	Intense	
Cell Metabolism								
Citrate Synthase (Krebs Cycle)	Down					Up		[22, 66]
G6PDH (Pentose Phosphate Pathway)		Up	Down					[66]
Glutaminase (Glutaminolysis)	Down							[66]
Glucose Consumption			Down					[66]
Glutamine Consumption			Down					[66]
Fatty Acid Oxidation			Up					[66]
Outcomes								
Migration						Up		[22]
Production of ROS		Down		Down	Up	Up	Up	[22, 121-123, 128, 136]
NADPH Oxidase Activity						Up		[130]
Phagocytosis			Up			Up	Down	[22, 66, 116, 131, 133]
Apoptosis						Up	Up	[22, 130, 136]

evidence that hormonal steroid changes control chemotactic factors and thus indirectly cell migration [119].

Testosterone increased neutrophil phagocytosis but decreased microbicidal activity [120]. Dexamethasone, a synthetic corticosteroid drug, was tested in cultured rats neutrophils. There was an augment of glucose consumption, but glucose oxidation did not alter. Glutamine consumption and oxidation remained unchanged, but glutamate production rose. Decreases of G6PD and PDG activities and mRNA expression occurred [121].

Hyperthyroidism associates with enhanced stimulated ROS production by neutrophils compared to cells from euthyroid rats. Hypothyroidism has the opposite effect and limits rat neutrophil ROS generation [122, 123]. Consumption of glutamine and oxidation by neutrophils decreased in the presence of adrenaline, an effect that is reversed by propranolol, thus confirming the participation of β adrenergic receptors [124]. Adrenaline did not affect the phagocytic capacity of neutrophils. However, the addition of 5 nM or 50 μM adrenaline potently reduced the rate of PMA-induced ROS production in the presence of glucose. Adrenaline reduces the glucose flux through the PPP and so NADPH production for NOX activity [121].

Glutaminase activity was elevated by 50μM adrenaline treatment, suggesting that glutamine utilization is more significant in the presence of this hormone. The level of the amino acid transporter for glutamine is under translational control and is modulated by nutrient availability and hormone concentrations [121]. Weiss, et al. [125] reported that the inhibition of O_2 production by adrenaline in the presence of glucose occurs via β (beta)-adrenoceptors, and dibutyryl cyclic adenosine monophosphate (db-cAMP) treatment mimicked this condition. Adenylate cyclase activity is stimulated by various respiration-enhancing agents in neutrophils [126]. Thus, cAMP and its immediate target, protein kinase A, may mediate the effects of adrenaline. There is a report on the reduction of respiratory burst induced by adrenaline via inhibition of phospholipase A_2 activity in human neutrophils [127].

Hack, et al. [128] reported that graded exercise to exhaustion on a treadmill is associated with an increase in circulating neutrophil number, an increase in phagocytic capacity, and a marginal decrease in bacterial killing immediately after the effort. After 11 weeks of training, the exercised group had increased phagocytosis capacity (by 49%) and production of ROS (6.6-fold) when compared with neutrophils from the sedentary group [22]. One

session of ballet class increased neutrophil apoptosis as determined 18 hours afterward [129]. Regular moderate exercise increased neutrophil citrate synthase activity, phagocytosis, and chemotaxis activity [130, 131].

The increase of ROS production in neutrophils induced by moderate exercise involves an increased expression of neutrophil cytosol factor 1 (p47phox), a cytosolic component of the NOX complex [132]. There is an increased expression of the ICAM adhesion molecule in neutrophils immediately after a marathon competition. Phagocytic capacity reduction occurs in triathletes and marathon runners that may result from enhanced neutrophil apoptosis [133]. Our research group postulated that augmented blood levels of fatty acids (e.g., oleic, linoleic, and stearic acids) might be involved in elevated neutrophil death though apoptosis during a triathlon competition [134].

Robson, et al. [135] reported reductions in incubated neutrophil functions (e.g., degranulation and oxidative burst) after an exhaustive physical exercise session in healthy athletes do not associate with plasma glutamine level changes. However, the increase in neutrophil apoptosis and decreased neutrophil function described above due to physical exercise may be attenuated by glutamine. Supplementation of this amino acid partially attenuated apoptosis induced by exercise in neutrophils from sexually immature and mature rats [136]. Neutrophils from rats orally treated with glutamine exhibit increased phagocytosis capacity. The same supplementation abolished the decreased neutrophil nitric oxide production and the increased production of ROS induced by exercise [6].

Impact of hypoxia on neutrophil metabolism and function

Tissue hypoxia is part of an inflammatory response, and neutrophils possess essential cellular and molecular mechanisms that enable them to function even at low oxygen concentrations [137]. Neutrophils have evolved several oxygen-sensing pathways, of which the principal regulators are the hypoxia-inducible transcription factor 1 and transcription factor 2 (HIF-1 and HIF-2) that are translocated to the nucleus and regulate gene transcription [138]. Semenza group discovered a constitutively expressed beta unit HIF-1β and three alpha subunits (HIF-1α, HIF-2α, HIF-3α) [139-141]. Hypoxia induces neutrophil survival through the HIF-1α-dependent NF-κB pathway [138, 142]. This mechanism is critical for the resolution of inflammation since both prolonged survival and excessive neutrophil activation occur in many disease settings [137].

Hypoxia reduces neutrophil production of ROS due to the shortage of available molecular oxygen [143, 144]. This latter finding plays a crucial role in neutrophil degranulation response; ROS inhibit degranulation induced by signaling mediators. Neutrophils incubated under hypoxic conditions (0.8% O_2, 3 kPa for 4 hours) compared with cells incubated under normoxic conditions (atmospheric O_2 concentration), exhibit elevated release of elastase, myeloperoxidase, lactoferrin, and matrix metalloproteinase-9. These findings indicate increased degranulation of azurophil (primary), specific (secondary), and gelatinase (tertiary) granules [145]. Despite potential benefits of hypoxia-augmented degranulation, including improved neutrophil access to sites of infection and intensified pathogen clearance, toxic granule products cause both local tissue damage and systemic complications [137, 143]. Thompson et al. reported that HIF-2 α-deficient murine inflammatory neutrophils display no impairment of chemotaxis, phagocytosis, or respiratory burst but elevated sensitivity to apoptosis, leading to reduced neutrophilic inflammation. Neutrophils carrying HIF-2 α gain-of-function mutations have lower apoptosis rates and play a role in the resolution of inflammation [146]. These isoforms exhibit distinct temporal expression profiles, with early HIF-1α upregulation and delayed HIF-2α, indicating functional divergence during different phases of an inflammatory response [147].

Hypoxia prolongs neutrophil survival by inhibiting apoptosis and represents an essential regulator of timely resolution inflammatory responses [137, 148]. Neutrophils reduce mitochondrial respiration and increase ATP production through anaerobic glycolysis,

accompanied by a time-dependent induction of key glycolytic enzymes such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and triosephosphate isomerase-1 [138, 142]. HIF up-regulates glycolytic flux and suppresses the TCA cycle and the mitochondrial oxidative phosphorylation chain [139, 149].

The HIF pathway's regulation of leukocyte metabolism provides a mechanism by which innate immune cells can adapt to the hypoxic tissue environment. The understanding of the mechanisms by which hypoxia acts to control neutrophil metabolism and function will indicate the host outcomes.

Neutrophil metabolism and outcomes

The major associations of neutrophil metabolism and outcomes are presented in summary (Fig. 1). Neutrophils play an important role in our body by triggering essential functions when activated. In different inflammatory disorders, neutrophils exhibit different phenotypes, that requires appropriate metabolic activity. The metabolism of glucose mainly produces the ATP required for neutrophil activity. Neutrophils utilize glucose for the energy generating activity of the glycolytic metabolic pathway and for activation of the PPP, which is necessary to generate large quantities of NADPH and ROS. Glycogen breakdown increases when neutrophils are restricted with respect to glucose, mainly if they are actively engaged in phagocytosis, whereas re-synthesis occurs when adequate glucose is available. In a microenvironment with decreased glucose supply, neutrophil metabolism is diverted from glycolysis to mitochondrial energy production through oxidative phosphorylation. There is evidence that glutamine metabolism plays a role for several neutrophil functions including phagocytosis, ROS and cytokine productions, and apoptosis.

Chokesuwattanaskul, et al. [150] reported changes in human neutrophil metabolome induced by treatment with PMA using nuclear magnetic resonance (NMR) ^{1}H spectroscopy. PMA induced significant changes in the contents of 43 metabolites either increase or decrease. The authors reported a redirection of glucose metabolism from glycolysis to PPP and so production of NADPH.

Metabolism of glucose and glutamine participates in NETs formation. 2-Deoxy-glucose (a glycolysis inhibitor) inhibits NET formation. Oligomycin, an ATP synthase inhibitor, also inhibits NET formation in a less pronounced manner. Rodríguez-Espinosa, et al. [151] divided NET formation metabolism into two phases. The first is independent of exogenous glucose (chromatin decondensation). The second (NET release) strictly depends on exogenous glucose and glycolysis. NOX-dependent and independent NETosis have been reported. Lactate formation from glycolysis closely associates with the initiation of the two mentioned NETosis types [152].

Glutamine delays the process of neutrophil apoptosis and changes mitochondrial function after only three hours in culture. Glutamine concentration positively correlates with phagocytic activity. Metabolism of glutamine, through a protective effect on mitochondrial integrity, may delay spontaneous apoptosis in neutrophils of rats and humans [153]. A product of glutamine/glutamate metabolism is glutathione, which has been reported to stabilize the mitochondrial function of neutrophils and delay apoptosis [154].

Glutamine modulates expression of cytokines and transcription factors in different cell types [155, 156]. Glutamine supplementation reduces the neutrophilia and suppresses IL-8 production by neutrophils after an exhaustive exercise in humans [157]. This amino acid decreases TNF-α production by *in vitro* LPS-treated neutrophils, indicating a possible preserving effect of this cell function in infections. Glutamine is also involved in the modulation of heat-shock proteins, a group of proteins that function as signaling proteins and as chaperones that help to fold denatured proteins caused by heat or other stressors [158]. The increase of HSP27, HSP70, and HSP72 protects against inflammatory injury or after cell exposure to heat shock or toxic contents [159, 160]. Glutamine carbon can be used to synthe-

size amino acids during periods of active synthesis and secretion of protein molecules such as cytokines [161, 162].

Rice, et al. [163] reported that immature neutrophils require mitochondrial FAO to support NOX-dependent ROS production when glucose utilisation is restricted to ensure neoplastic cell growth. Neutrophils promote tumor progression partly by generating ROS that suppress T lymphocyte functions. There is evidence that blood neutrophils from patients with cancer display features of immaturity with enhanced mitochondrial content and oxidative phosphorylation activity. Naffah de Souza, et al. [164] discovered a NOX-independent NETosis caused by high pH through increased ROS production in the mitochondria and induction of histone citrullination and cleavage.

As reported above, neutrophils exhibit a remarkable plasticity that enables the cells to survive in extremes of metabolites availability. Metabolic shifts might occur to accomplish the neutrophil population heterogeneity described in cancer-associated neutrophil and in low and high density neutrophils of auto-immune diseases, for instance [165]. Intracellular metabolism of glucose, glutamine, and fatty acids may play a critical role in the differentiation and functions of different neutrophil phenotypes. The close association of neutrophil metabolism with neutrophil plasticity requires further investigation.

Abbreviations

ATP (adenosine triphosphate); BIP (binding immunoglobulin); CACT (carnitine-acylcarnitine translocase); CCL3 (chemokine C-C motif ligand 3); CPT-1 (carnitine palmitoyltransferase 1); CPT-2 (carnitine palmitoyltransferase 2); CXCR (chemokine C-X-C motif ligand receptor); DAMPS (damage-associated molecular patter); db-cAMP (dibutyryl cyclic adenosine monophosphate); ER (endoplasmic reticulum); FAO (fatty acid oxidation); FATP (fatty acid transporter proteins); Fc (antibody fragment crystallizable region); FcαRI (human Fc receptor for IgA); FFAR1/GPR40 (free fatty acid receptor-1); FFAR2/GPR43 (free fatty acid receptor- 2); G6P (glucose-6-phosphate); G6Pase-β/G6PC3 (Glucose-6-phosphatase-β); G6PD (glucose-6-phosphate dehydrogenase); G6PT (glucose-6-phosphate transporter); GLUTs (glucose transporters); GPR84 (probable G-protein coupled receptor 84); Gr-1 (granulocyte antigen-1); HETE (hydroxyeicosatetraenoic acids); HIF (hypoxia-inducible factor); HSP (heat shock proteins); ICAM (intercellular adhesion molecule); IFN-γ (interferon gamma); IgA (immunoglobulin A); IL (interleukin); LTB4 (leukotriene B4); Ly-6G (lymphocyte antigen 6 complex locus G6D); NAD (nicotinamide adenine dinucleotide); NADH (reduced nicotinamide adenine dinucleotide); NADP (nicotinamide adenine dinucleotide phosphate); NADPH (reduced nicotinamide adenine dinucleotide phosphate); NETs/NETosis (neutrophil extracellular traps); NH_4^+ (ammonium ion); NMR (nuclear magnetic resonance); NOX (NADPH oxidase); O_2 (superoxide anion); OXPHOS (oxidative phosphorylation); p47phox (neutrophil cytosol factor 1); PDG (phosphate-dependent glutaminase); PDH (pyruvate dehydrogenase); PDK (pyruvate dehydrogenase kinase); PFK (phosphofructokinase); PMA (phorbol myristate acetate); PMN (polymorphonuclear neutrophil); PPP (pentose-phosphate pathway); ROS: reactive (oxygen species); TCA (tricarboxylic acid); TGF-β (transforming growth factor beta); TNF-α (tumor necrosis factor alfa).

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Author Contributions

Conceived and designed the Review: RC, PN, ACLP, and TCPC. Contributed to the preparation of the figures and tables: RBG, TSS, TCPC, ACLP, ESB, SOP, RFZ, MMA, PD, RG, and PN.

Wrote the paper: RC, RBG, TSS, ACLP, ESB, SOP, RFZ, MMA, PD, RG, and PN. Final approval of the version to be submitted: RC, ACLP, TCPC, RG, and PN.

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