Chapter

Neutrophil Function Impairment Is a Host Susceptibility Factor to Bacterial Infection in Diabetes

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Abstract

Diabetes mellitus is a highly prevalent noncommunicable disease globally. One of the main complications of diabetes is the increased susceptibility to bacterial infection. Neutrophils play a crucial role in inflammatory response against bacterial infections, once they are the first cells recruited to the sites of injury. In diabetes, there is a failure in the neutrophil functions, including migration, ROS production, phagocytosis, and bacterial killing, which are associated with the high incidence of bacterial infections. Herein, we point out pieces of evidence revealing the primary molecular mechanisms involved with impairment of neutrophil functions in diabetes, with relationship with high susceptibility to bacterial infections.

Keywords: diabetes, bacterial infection, neutrophils, inflammation, chemotaxis

1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by a hyperglycemic condition that results in several complications, such as neuropathy, nephropathy, retinopathy, and increased risk of cardiovascular disease [1]. DM can be classified into type 1 (T1DM) and type 2 (T2DM). T1DM is common in childhood or young adulthood and is a result of autoimmune destruction of beta-cells in pancreatic islets mediated by T cells, leading to defect in insulin synthesis [2, 3]. The T2DM appears mainly in adulthood, affecting people with the most productive age. This type of diabetes is associated with insulin resistance and inadequate compensation by beta-cells, leading to a relative insulin deficiency [1, 4]. Currently, it is known that there are over 425 million people with DM globally. Worryingly, it is estimated that in 2045, this number will grow to over 600 million [5].

Hyperglycemia, a hallmark of DM, is associated with patient vulnerability to bacterial infections, such as tuberculosis and pneumonia, besides more severe sepsis of bacterial origin [5]. In fact, diabetic patients generally present microbial persistence, greater susceptibility to new infections, recurrences, and an increase in the risk of mortality if compared to nondiabetic individuals [5, 6]. This is due to the compromised immune response presented by diabetic patients, which leads to failure in leukocytes protective effects. Cyclically, infection profile in these patients can worsen glycemic control [5]. Neutrophils present an important role in host immune response to bacterial infection, once they are one of the first leukocytes

that arrive in the infected area [7]. In normal conditions, these cells act by different manners against microorganism, leading to infection control and resolution of the inflammatory process. However, the immune response in diabetic patients is characterized by impairment in neutrophil function [7, 8]. Here, we revised the mechanisms involved with the failure of neutrophil functions noted in DM and its relationship with the high susceptibility to bacterial infections.

2. Role of neutrophils in bacterial infections

2.1 Migration of neutrophils to infected sites

Neutrophils are polymorphonuclear (PMN) versatile innate effector cells essential for immune defense, which arise from hematopoietic stem cells (HSCs) in bone marrow [9]. Under normal conditions, about 5×10^{10}–10×10^{10} new neutrophils are produced in the bone marrow daily [10, 11]. Chemokine gradients and adhesion molecules are central players that regulate neutrophil release from the bone marrow [11]. Neutrophils express CXC receptors (CXCR)-1 and CXCR2 that interact with CXC chemokines (CXCL1/KC, CXCL2/MIP-2, and CXCL8/IL-8) and result in neutrophil migration from bone marrow into the bloodstream. Neutrophils also express CXCR4, which interacts with CXCL12/SDF-1 produced by osteoblasts and other stromal cells to mediate neutrophil maintenance in the bone marrow [10, 12]. Thereby, only a small fraction of mature neutrophils is released into the blood. However, after a bacterial invasion, the host defense activates strong neutrophil release from bone marrow and migration toward infected sites [11, 12].

Under bacterial infection, sentinel cells detect the microorganisms via pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs). These receptors identify highly conserved pathogen-associated molecular patterns (PAMPs), including peptidoglycan (PGN) and lipopolysaccharide (LPS) expressed in the cell membrane surface of bacteria. They can also recognize danger-associated molecular patterns (DAMPs), such as high mobility group protein B1 (HMGB1), ATP, and uric acid, released from damaged and necrotic cells after tissue injury. Then, sentinel cells release mediators such as granulocyte colony-stimulating factor (G-CSF), which leads to neutrophil production and release from bone marrow via upregulation of CXCR2 and its ligands, and reduces expression of CXCL12/SDF-1 and CXCR4. After this event, neutrophils can be mobilized to sites of infection and combat microorganism [13–15].

Correct leukocyte recruitment requires the adhesive interactions between P-selectin glycoprotein ligand-1 (PSGL-1), E-selectin ligand-1 (ESL-1), and CD44 expressed on the neutrophil membrane surface to the P- and E-selectin which are upregulated in endothelial cells of inflamed tissue. These processes will lead to neutrophil capture and fast rolling [16, 17]. Rolling event exposes neutrophils to chemokines that are arrested on the glycocalyx of endothelial cells, such as CXCL8/IL-8. Then occurs the activation of integrin molecules such as VLA-4 (CD49D/CD29), macrophage-1 antigen (MAC-1 or CD11b/CD18), and lymphocyte function-associated antigen-1 (LFA-1 or CD11a/CD18) on neutrophils [10, 18]. The integrin binds to their ligands such as intercellular adhesion molecule (ICAM)-1, ICAM-2, and platelet endothelial cell adhesion molecule-1 (PECAM-1) on endothelial cells, resulting in slow rolling and firm adhesion of the neutrophil to endothelial cells [17]. Thence, the neutrophils perform diapedesis toward the tissue and migrate along a chemokine gradient until they arrive in the infected site. The long-distance recruitment is mediated by chemoattractants, including leukotriene B4 and CXCL8/IL-8, while near chemoattractants are peptides and C5a [17].

Despite the canonical neutrophil migration during infections, in sepsis occurs an inadequate migration of neutrophils even with high levels of chemokines at the infection site [12]. The decrease of CXCR2 expression on the cell surface of neutrophils is among the mechanisms leading to this failure. The prolonged exposure to CXCR2 agonists, which leads to phosphorylation of G protein-coupled receptors (GPCRs) by GPCR kinases (GRKs) and induces the desensitization and internalization of CXCR2, can explain the down-regulation of this receptor [16]. In addition, the activation of lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) by inflammatory products, such as C-reactive protein (CRP), bacterial products, apoptotic cells, or activated platelets, can also account for CXCR2 neutrophil endocytosis [12, 19].

2.2 Actions of neutrophils in infected sites

At the sites of infection, neutrophils can combat pathogenic microorganisms and clear infections by different ways including phagocytosis, degranulation of microbicidal molecules, production and secretion of reactive oxygen species (ROS), and release of neutrophil extracellular traps (NETs) [20]. For efficient bacterial phagocytosis, the microorganism needs to be covered with opsonins, such as immunoglobulins (Igs) and components of the complement system, which are recognized by neutrophil specific surface receptors. After phagocytosis of an opsonized pathogen by neutrophils, there is a mobilization of intracellular granules or lysosomes, leading to the killing of the ingested bacteria [21].

Neutrophil activation can induce the production of ROS to combat infection. It happens mainly due to the action of NADPH oxidase complex (NOX), but can also be generated by mitochondria. After neutrophil activation, NOX acts converting molecular oxygen (O_2) into superoxide anion ($O_2^{\cdot -}$) which suffers dismutation, spontaneously or catalyzed by myeloperoxidase (MPO), generating hydrogen peroxide (H_2O_2) [22, 23]. In addition to ROS, neutrophils can also enhance the inducible nitric oxide synthase (NOS2) expression, which will convert O_2 to nitric oxide (NO), resulting in reactive nitrogen species (RNS). Both ROS and RNS contribute to microbicide activity and are crucial for the defense against intracellular microorganisms [22]. Curiously, NO is supposed to be involved in the failure of neutrophil migration during sepsis, once it stimulates the internalization of CXCR2 on the neutrophil surface and reduces expression of adhesion molecules, leading to diminished leucocyte rolling and adhesion to the endothelium [12]. After neutrophil migration, degranulation occurs, which is the process mediated by microbial or inflammatory stimuli in which neutrophils release the granule contents, such as MPO, defensins, cathepsin G, neutrophil elastase, and collagenase. These granule contents are released by exocytosis or into the phagosome to kill microorganisms [24].

Neutrophils may also perform the antimicrobial activity directly attacking and restraining microorganisms by releasing NETs (NETosis) [25, 26]. NETs are extracellular fibrous structures composed by a network of extracellular chromatin fibers, histones, antimicrobial peptides, and enzymes, including MPO, α-defensins, cathepsin G, elastase, and lactoferrin, to capture and kill microorganisms [12, 20, 26]. NETosis occurs after neutrophil exposure to bacteria or stimulation with mediators such as interleukin CXCL8/IL-8. This neutrophil stimulation will result in activation of intracellular pro-inflammatory kinases, such as Akt, p38 MAPK, or MEK/ERK, a release of neutrophil elastase, oxidative burst, and actin polymerization [20, 26, 27]. This mechanism will result in microorganism destruction and neutrophil death [25]. NETs also limit the microorganism growth and dissemination; however, excessive formation of NETs in association with the

uncontrolled inflammatory response that occurs in sepsis can result in multiple organ damage to the host [12, 20].

2.3 Resolution of neutrophilic inflammation

Resolution phase is an essential process to interrupt the inflammatory response after the danger signal or when microorganism has been eliminated, preventing the development of chronic inflammation and fibrosis [28]. Resolution of inflammation was previously considered a passive response, associated with clearance of inflammatory stimulus, reduction of pro-inflammatory mediators, and prevention of leukocyte recruitment. Currently, it is known that resolution is an active and tightly controlled process, carried out by specialized pro-resolving mediators (SPM) such as resolvins, lipoxins, maresins, and protectins, which are produced locally from polyunsaturated fatty acids and act orchestrating the end of inflammation, but do not evoke unwanted immunosuppression [29, 30]. For a correct resolution of inflammation, the neutrophil reverse migration, lymphatic drainage, exudation to the external environment, apoptosis of activated neutrophils followed by efferocytosis, and autophagic clearance of intracellular inflammatory signals are necessary [31, 32].

The reduction in neutrophil recruitment is regulated by a class-switch from the production of pro-inflammatory to pro-resolving mediators, resulting in down-regulation of CXCR2 on neutrophils [28]. Pro-resolving lipid mediators also resolve inflammation by promoting neutrophil apoptosis [28, 32]. Apoptotic neutrophils or cell bodies are phagocytosed by professional phagocytes, mainly macrophages, in a process known as efferocytosis. This event is mediated by an interaction between phosphatidylserine expressed on the neutrophil surface and macrophage receptors, such as TIM1 and TIM4 [32, 33]. During resolution, macrophages change their profile decreasing the pro-inflammatory feature and acquiring anti-inflammatory and pro-resolving functions, acting in apoptotic cell clearance, and producing immune regulatory intracellular messengers, including cyclic adenosine monophosphate (cAMP) [28, 34]. Macrophage phagocytosis allows the complete elimination of dead neutrophils and tissue debris of the infected and inflamed area. Generally, this process is followed by macrophage autophagy [35, 36]. Together, all these processes contribute to the resolution of neutrophil inflammation and tissue homeostasis [31].

3. Impaired neutrophil migration in diabetes

The causes of increase in susceptibility to infections in DM are not yet fully known, but one of the possible and well-established explanations is that diabetics present an impairment in defense mechanisms of innate immunity, including neutrophil migration to the site of inflammation, phagocytosis, ROS production, and bactericidal activity [37].

The number of neutrophils in the circulation is also altered in DM. Older studies have shown that in T1DM patients, there is an increase in neutrophil counts compared to healthy individuals [38, 39]. Recent researches described a decrease in circulating neutrophil numbers in T1DM patients in comparison with nondiabetics [40, 41]. Impairment in neutrophil yield and maturation in bone marrow, increase in peripheral neutrophil consumption, and/or tissue sequestration could explain this reduction in blood neutrophil counts observed in T1DM [42]. This divergence between studies can be attributed to differences between ethnic groups and the discovery of the existence of various stages of DM [43]. While in T1DM, the data about circulating neutrophil counts are still controversial, most of the studies

described that in T2DM patients, there is an increase in the number of neutrophils in circulation in comparison to healthy individuals [40, 44]. This neutrophilia was related to elevation in the circulation levels of inflammatory cytokines, including TNF-α, IL-1β, and IL-6, and CRP, a known marker of inflammation [45].

Regarding migration, *in vitro* studies described a reduction in CXCL8/IL-8, platelet-activating factor (PAF), or N-formyl-methionyl-leucyl-phenylalanine (fMLP)-induced chemotaxis of neutrophils from T1DM or T2DM patients compared to cells from healthy subjects [40, 46]. *In situ* evaluation of chemotaxis toward fMLP using T1DM rat neutrophils also revealed a deficiency in migration. This impairment in neutrophil chemotaxis was positively related to DM severity which was characterized by glycaemia values greater than 400 mg/dL [47]. In addition, blood neutrophils of diabetic animals presented a decreased migratory response to CXCL2/MIP-2 *in vitro* and *in vivo* compared to nondiabetic animals. Despite the deficiency in CXCL2/MIP-2 induced-neutrophil migration, there was no difference between the expression of CXCR-2, a CXCL2/MIP-2 receptor, on neutrophils from diabetic animals [48, 49]. Similarity in CXCR-2 mRNA levels was also found among bone marrow neutrophils obtained from NOD mice (a strain that spontaneously develops T1DM), NOR mice (a strain that is resistant to diabetes), and control mice strain. However, CXCR-1 mRNA levels were reduced in neutrophils isolated from NOD mice in comparison to neutrophils from NOR and control mice [50]. Then, it is possible to consider that alterations in CXCR-1 expression and activity may also contribute to the impairment of neutrophil migratory activity in diabetics.

A feature well described in DM is the increase in oxidative stress which may also be related with impairment of neutrophil migration. Oxidative stress can induce glutathionylation (S-thiolation) of several proteins, including L-plastin (LPL) [51] that is expressed exclusively in leucocytes and controls polarization and migration of neutrophils through bundling of β-actin filaments [52]. Neutrophils from diabetic patients and from T2DM mice showed enhanced S-thiolation of LPL in comparison to neutrophils from nondiabetic subjects, which culminate with impaired fMLP-chemotaxis of neutrophils from diabetics. S-thiolation of LPL reduces its interaction with β-actin and this may be another mechanism involved in defective migration of neutrophils in DM [51].

In addition, T1DM rats administered with LPS by intra-tracheal route exhibited a reduction in neutrophil accumulation in the bronchoalveolar fluid (BAL), which occurred in association with a decrease in TNF-α and IL-1β levels, when compared with nondiabetic rats provoked with LPS. However, no difference was observed in relation to the expression of ICAM-1 and E-selectin in lung vascular endothelium and cytokine-induced neutrophil chemoattractant-1 (CINC-1) amount in BAL [53]. A deficiency in neutrophil migration to airways after LPS intra-tracheal injection was also observed in a spontaneous rat model of T2DM, using Goto-Kakizaki (GK) rats. This reduction in neutrophil migration to the airways in GK rats stimulated with LPS occurred despite an increase in the number of neutrophils in the blood. These data showed that there was no failure in the production of these cells by the bone marrow, but impairment in the recruitment mechanisms of these leukocytes to the lungs. Indeed, GK rats exhibited a decrease in IL1-β, IL-6, and TNF-α concentration in BAL and also a reduction in the expression of adhesion molecules, such as LFA-1 and ICAM-2, on neutrophils. All these alterations were associated with a reduction in the TLR4 expression and activation in neutrophils [54].

3.1 Failure in neutrophil migration associated with hyperglycemia

Hyperglycemia can influence various components of the immune response, including activities of inflammatory cells [55]. Incubation of human neutrophils

with supraphysiological levels of glucose decreased both chemotaxis in response to zymosan and phagocytosis/killing of the intracellular bacteria *Staphylococci in vitro*. In addition, high glucose concentrations increased neutrophil adherence *in vitro*, and this also can limit neutrophil locomotion from blood vessels toward infected tissues *in vivo* [56].

It is debated which mechanisms are involved in the benefit of insulin treatment on the immune response of diabetics. While some authors argue that the beneficial effects are dependent on the correction of hyperglycemia by insulin, others believe that the insulin may have direct actions on immune system independently of glycemic control [55]. Indeed, it has been shown that insulin *in vitro* increases human neutrophil chemotaxis induced by fMLP, calcium ionophore, or phorbol-myristyl acetate (PMA) [57, 58]. Besides, insulin presents a chemokinesis effect which required activation of tyrosine kinase and phosphatidylinositol 3-kinase (PI3K), but did not depend on protein kinase C (PKC) stimulation [59, 60]. Interestingly, in a hyperglycemic medium, the chemokinetic action of insulin in neutrophils is blocked through a mechanism that involved activation of PKC [60]. These data suggest that insulin is able to exert direct effects on neutrophils, but the maintenance of glucose levels is also important for actions of this hormone on these leukocytes. In addition to acting on neutrophils, insulin can increase expression of the PECAM-1 in endothelial HUVEC cells and thus enhance transmigration of neutrophils across these cells in response to fMLP *in vitro* [61]. Finally, *in vivo* studies showed that insulin restored neutrophil migration to the lungs in T1DM rats subjected to LPS provocation. This effect of insulin occurred in parallel to a reduction of 50% glycemia; however, the glycemic levels continued to be high in these animals compared to nondiabetic rats [53]. These data suggest that the action of insulin on LPS-induced inflammatory response was not totally dependent on its effect on blood glucose.

It is well known that chronic hyperglycemia upregulates the generation of advanced glycation end-products (AGE). AGEs are produced by a nonenzymatic reaction between reducing sugars, such as glucose, and amino acids of proteins. AGEs can induce cross-link between proteins and also can bind cellular receptors; among them, the best described is the receptor for AGE (RAGE) [62]. AGE accumulation has been associated with the development of several diabetic complications, including retinopathy, nephropathy, and neuropathy [63]. RAGE is expressed on neutrophils and its activation by AGEs, like glycated albumin, induces a transient rise in intracellular free-calcium levels and actin polymerization. Nevertheless, the dimension of increase in calcium levels induced by glycated-albumin is smaller than that induced by fMLP. In addition, glycated-albumin pre-treatment in neutrophils inhibited elevation of intracellular calcium levels promoted by fMLP, causing a defective signal processing and, consequently, a reduction in fMLP-induced-transendothelial migration *in vitro* [64]. Furthermore, glycated collagen also inhibited chemotaxis in response to fMLP, and this effect was associated with the capacity of glycated collagen to increase adhesion strength of neutrophils *in vitro* [62]. In addition, the blockade of AGE formation in diabetic animals restored leucocyte rolling, adhesion, and migration in response to zymosan *in vitro* [65], and also restored neutrophil accumulation toward traumatic skin tissue induced by hot water [66]. Therefore, it is possible that in DM, AGEs promote sustained stimulation of neutrophils which decreases the responses of these cells to chemotactic stimulus.

A positive relation between hyperglycemia and serum NO levels was also described in rats [67], and some studies have reported an increase in serum or plasma NO concentrations in T1DM and T2DM patients [67, 68]. Human neutrophils treated with L-Arginine, a NO precursor, have decreased chemotaxis toward CXCL8/IL-8 *in vitro*, while treatment with NOS inhibitor increased CXCL8/

IL-8-induced-chemotaxis of neutrophils *in vitro* [69]. In addition, a NO donor inhibited human chemotaxis promoted by fMLP *in vitro* and incubation with a guanylate cyclase inhibitor did not interfere with the effect of NO donor. These data suggested that the inhibitory action of NO on neutrophil chemotaxis is independent of cGMP [51]. The NO-induced impairment of neutrophil migration was confirmed using bone marrow neutrophils isolated from $NOS2^{-/-}$ mice stimulated with fMLP *in vitro,* which showed increased chemotaxis in comparison to that isolated from $NOS2^{+/+}$ mice [51]. Furthermore, the pre-treatment with NOS inhibitor prevented impairment of neutrophil recruitment toward peritoneal cavity observed in severe sepsis [70]. Therefore, it is possible to hypothesize that deficiency on migration activity of neutrophils may be associated with increased serum levels of NO in diabetics.

3.2 Failure in neutrophil migration independent of hyperglycemia

DM has altered levels of several molecules in serum that are not directly related to hyperglycemia, some of which can interfere with components of immune response, including neutrophils. Alpha-1-acid glycoprotein (AGP) is one of the main acute-phase proteins in organisms; its synthesis depends mainly on liver, and during an inflammatory response, the concentration in serum increases. [71]. AGP can bind to hormones and interfere with functions of endothelial cells, platelets, and leukocytes, and in fact, it inhibits human neutrophil chemotaxis in response to fMLP *in vitro* [72]. In addition, intravenous administration of AGP in rats prevented migration of neutrophils to peritoneal cavity, reducing rolling and adhesion of these leukocytes on endothelium of mesenteric microcirculation induced by carrageenan [73]. DM patients present high serum levels of AGP [48], so it is feasible that AGP can mediate the impairment of neutrophil locomotion described in DM.

Furthermore, AGP-mediated neutrophil dysfunction was also demonstrated in diabetic animals upon sepsis induction by cecal ligation and perforation (CLP). Neutrophils from septic T1DM mice showed impaired rolling, adhesion, and migration from mesenteric tissue toward the peritoneal cavity, while accumulated in lung tissue. These observations were associated with an altered expression of adhesion molecules (CD62L-CD11b) and a clear reduction in CXCR2 in neutrophils from diabetic animals compared to nondiabetic, after CLP. Accordingly, neutrophils from diabetic mice presented an increased expression of GRK2, a key modulator of CXCR2 receptor desensitization, upon sepsis induction compared to control septic mice. AGP administration in septic nondiabetic mice impaired neutrophil migration to peritoneal cavity, augmenting GRK2 expression, and reducing CXCR2, which reproduced the diabetic condition. On the other hand, insulin treatment reduced GKR2 and augmented CXCR2 on neutrophils obtained from diabetic mice, while decreased AGP serum concentrations. Thus, AGP increased production is involved in neutrophil impaired migration to infection during diabetes, possibly by enhancing GRK2 expression and/or augmenting NO production in these cells [48]. Notably, CXCR2 downregulation in diabetic animals seems to depend on the presence of comorbidity since several studies showed no difference in CXCR2 expression between normal and diabetic mice.

Histamine for a long time was considered as a pro-inflammatory mediator whose main role is played in allergic inflammation. However, some evidence has shown that histamine can modulate other immunological events. Neutrophils express both histamine receptors, H1 and H2 [74] and activation of H2 inhibited human neutrophil chemotaxis *in vitro* [75]. Furthermore, blood neutrophils obtained after systemic or inhalatory administration of histamine in normal volunteers showed a reduction in chemotactic response to zymosan *in vitro* [75].

After septic stimuli, T1DM mice exhibited mast cell accumulation in the peritoneal cavity and higher plasma levels of histamine than nondiabetic mice. In addition, the augmented activation of H2 receptor promoted an increase in intracellular expression of GRK2 and cAMP levels in diabetic septic mice neutrophils, favoring CXCR2 desensitization [74].

Resistin is a cysteine-rich protein that belongs to the resistin-like molecule (RELM) family that, in humans, is released mainly by macrophages but can be also produced by adipose tissue [76]. Resistin impairs glucose tolerance and insulin action and therefore has been related to obesity-induced insulin resistance and T2DM [77]. Beyond metabolic effects, resistin can act directly in immune cells, including neutrophils. Resistin decreased fMLP-induced neutrophil chemotaxis *in vitro* through inhibition of PI3K pathway activation. Resistin also decreased oxidative burst in neutrophils after stimulation with PMA and *Escherichia coli* [78]. Since resistin directly affects neutrophil function and T2DM patients present higher serum levels of this hormone [79], it can be suggested that resistin is also involved with the deficiency of neutrophil responses in DM independently of hyperglycemia.

4. Neutrophil response to bacterial infections in diabetes

It is now generally accepted that high glucose concentrations impaired several functions of neutrophils beyond their migratory capacity, including phagocytosis and bacterial killing. Hyperglycemia hinders neutrophil activity by inducing higher concentrations of intracellular calcium and thereby reducing ATP levels, which in turn leads to reduced phagocytic ability of PMN cells. Nevertheless, under glycemic control, diabetic patients restored intracellular calcium levels and increased cellular ATP content in neutrophils, which consequently improved phagocytosis. In addition, hyperglycemia was shown to affect other immune and hemostatic responses during experimental human endotoxemia. Healthy patients submitted to high blood glucose levels presented a reduction of *E. coli* endotoxin-induced neutrophil degranulation and exaggerated coagulation. A reversal of these effects was observed when glucose was controlled with insulin therapy [55].

Neutrophils from diabetic patients showed increased production of inflammatory cytokines [80] and ROS without any stimulation, although neutrophil oxidative responses to certain pathogens appear to be predominantly suppressed in diabetes [64, 81, 82]. Furthermore, hyperglycemia led to decreased mRNA synthesis of different pro-inflammatory cytokines in neutrophils after LPS stimulation, compared with the euglycemic state [55]. In addition, T1DM mice showed a hyperglycemia-induced pre-activation of NOX, resulting in a significantly higher release of superoxide. Sustained hyperglycemic condition may, therefore, induce oxidative damage and the onset of diabetic complications, particularly at sites with neutrophilia [83, 84].

In DM, neutrophils increased basal ROS generation in a close-relationship to sustained hyperglycemia and the generation of AGEs [64]. On the other hand, decreased pathogen-stimulated ROS production is thought to be related to impaired glucose metabolism by the pentose-phosphate pathway, which produces NADPH that is a requirement for optimal superoxide generation by NOX [6]. Off noted, phagocytosis and NETosis were shown to depend on oxidative burst in neutrophils. Nevertheless, the relevance of the ROS production misbalance noted in neutrophils obtained from diabetics is not clear, since not all the diabetic patients with diminished ROS production presented recurrent bacterial infections [82].

4.1 Neutrophil dysfunction and sepsis

According to The Third International Consensus Definitions for Sepsis and Septic Shock, "sepsis is a life-threatening organ dysfunction secondary to a deregulated host response to an infection" [85]. During septic processes, serum inflammatory marker concentration increases in patients although innate immune response appears to be impaired. Particularly, defective neutrophil recruitment to the sites of infection was reported in animal models of sepsis [86, 87]. Clinical studies reported that the incidence of sepsis is increased in diabetic patients [5]. Accordingly, DM is associated with high severity of sepsis, likely due to compromised immune responses, such as adhesion, chemotaxis, phagocytosis, and bacterial killing by immune cells [88]. Few studies reproducing septic inflammations in the context of diabetes had been performed in animals. T1DM or T2DM animals have worse prognosis upon CLP-induced sepsis even though plasma levels of systemic pro-inflammatory cytokines, like TNF-α, CXCL2/MIP-2, and IL-6, are increased in diabetic animals compared with control animals upon sepsis induction. This situation is normally attributed to neutrophil dampened activity [48, 74, 89, 90].

On the other hand, results obtained upon CLP-induced sepsis in a mouse model of T2DM showed an increased neutrophil infiltration in the peritoneal cavity in diabetic animals compared to nondiabetic upon sepsis induction. Nevertheless, neutrophils from diabetic animals presented reduced phagocytic activity and ROS generation after sepsis induction compared to control animals in the same condition. This impairment in neutrophil functions was related to a downregulation of TAM family of receptor tyrosine kinases. The lack of an appropriated innate immune response results in deficient bacterial elimination and augmented death rate in diabetic septic animals compared to control septic animals [90].

Similar results were observed in T1DM NOD mice intraperitoneally challenged with *Staphylococcus aureus*. The augmented neutrophil presence in the peritoneum of diabetic mice was associated with a sustained TNF-α production which prevents apoptosis in these leukocytes. Despite it, diabetic mice were more susceptible to *S. aureus* infection possibly associated to neutrophil decreased oxidative burst [91]. In addition, administration of GM-CSF, a cytokine known to activate PMNs, in diabetic animals submitted to CLP was able to restore neutrophilic activity and prevent the increased mortality of the animals. These effects of GM-CSF were associated with an increased neutrophil phagocytic activity and ROS generation, which controlled bacterial proliferation in the peritoneal cavity [90].

4.2 Neutrophil counts and function in tuberculosis

Several clinical and epidemiological studies have identified DM as a risk factor for the development of pulmonary tuberculosis (TB). T2DM and TB are two of the most common co-morbid conditions in many parts of the world. In addition, DM has been associated with a greater severity of TB disease among the infected population and worse outcome in response to treatment [92]. TB-DM co-morbidity is characterized by heightened levels of bacterial loads in sputum accompanied by increased neutrophil counts in peripheral blood [93]. Neutrophilic inflammation is a central feature of TB-DM, accompanied by elevated levels of biomarkers associated with macrovascular complications.

Whole blood gene expression and plasma analyses showed that several inflammatory markers, including IL-1β, CXCL8/IL-8, IL-17A, CCL3/MIP-1, TNF-α, and VEGF, associated with neutrophilic activity and absolute neutrophil counts were highly increased in TB-DM patients compared to TB or DM patients. A higher

frequency of participants with high molecular degree of perturbation (MDP) was also noted in the TB-DM subgroup. MDP is a parameter that reflects the "distance to health," based on molecular expression scores in comparison with a healthy population. Consequently, they suggest that epigenetic reprogramming and neutrophilic inflammation determine the pattern of plasma cytokines and growth factors in TB-DM co-morbidity, highlighting neutrophilic inflammation as the main cause of susceptibility to develop TB by DM patients. Thereby, neutrophilic inflammation may be a useful target to improve TB treatment outcomes in this growing TB-DM patient population [94]. In addition, increased levels of three of the most prominent antimicrobial peptides, cathelicidin (LL37), human β-defensin 2 (HBD2), and human neutrophil peptide 1–3 (HNP1–3), principally secreted by neutrophils were found in individuals with TB-DM and TB compared with individuals with latent TB or non-TB-infected [7]. However, neutrophils isolated from T2DM patients showed a decreased capacity to phagocyte *Mycobacterium tuberculosis* or other *M. tuberculosis*-related molecules compared to control donors [95].

There are few studies using animal models of TB-DM co-morbidity focusing on neutrophil activity. Even though it is frequently observed that diabetic animals have an increased accumulation of neutrophils within lung tissue upon infection [96, 97], T2DM animals were more vulnerable to *M. tuberculosis* showing a decreased survival rate compared to control infected animals. Also, diabetic animals recruited more neutrophils and express higher levels of CXCL8/IL-8 in lung tissue than control infected animals [96]. In T1DM mice, infection with *M. tuberculosis* led to a decreased survival rate associated with an impaired bacterial control compared to nondiabetic infected mice. This high mortality of T1DM mice was accompanied by a lung neutrophilia and IL-6 overexpression. The treatment of TB-DM animals with neutralizing anti-IL-6 antibodies reduced neutrophil numbers and controlled bacterial burden in lung tissue, improving the survival rate [97].

4.3 Neutrophil counts and function in pneumonia

DM increases the risk of patients acquiring a pneumococcal disease, and besides, adversely affects the severity and outcome of this infectious illness [98]. In fact, DM has been shown to be a significant predictor of hospitalization in patients with community-acquired pneumonia (CAP) and also, a risk factor for the development of bacteremia in patients with pneumococcal pneumonia. T2DM is frequently associated with increased mortality rate from pneumonia, which appeared to be highest in the early phase of infection where neutrophilic inflammation is more important [99]. *Streptococcus pneumoniae* is the most frequent cause of CAP irrespective of age and comorbidity. The phagocytosis of *S. pneumonia* was reduced in neutrophils recovered from eight patients with poorly controlled DM, but this defect improved with insulin treatment. Notably, control neutrophils incubated with serum taken from patients with diabetes also demonstrated a defective phagocytosis, suggesting that the inefficient bacterial opsonization might be occurring in the diabetic patient's serum [100].

Once phagocytosed, bacterial killing by neutrophils depends on the generation of ROS. *Ex-vivo* studies using neutrophils from T2DM patients have demonstrated a defect in the intracellular killing of *S. pneumoniae* together with a reduced O_2 production, reduced MPO activity, and H_2O_2 generation. In addition, chronic hyperglycemia induces inactivation of the source of leukocyte ROS, which results in high prevalence of oral abscesses, progressive interstitial inflammation, and fibrosis in the lung of mice in the absence of an inflammatory stimulus, leading to cachexia and death. These data suggested that ROS generated by NOX is not only

beneficial but also essential to oral and respiratory health in DM, particularly when the glycemia is uncontrolled [84].

Klebsiella pneumoniae is emerging as an agent which induces severe CAP. DM is associated with increased susceptibility to *K. pneumoniae* and poor prognosis of infection. Streptozotocin-induced diabetic mice are more susceptible to oropharyngeal infection with *K. pneumoniae*, presenting increased mortality rate and less bacterial control. There was no difference in the antibacterial activity of neutrophils recovered from nondiabetic and diabetic mice, indicating that the higher bacterial burden in hyperglycemia is probably related to a defective inflammatory signaling and late neutrophil recruitment. In fact, *K. pneumoniae* LPS induced a fewer recruitment of neutrophils to the alveolar airspace in diabetic mice compared to nondiabetic mice. Also, diabetic mice reduced neutrophil accumulation and early production of CXCL1/KC, CXCL2/MIP-2, IL-1β, and TNF-α in lung. Additionally, TLR2 and TIRAP, a Toll receptor and adaptor protein, were under-expressed in lungs of diabetic mice following *K. pneumoniae*-LPS provocation compared to nondiabetic infected mice, while no differences were observed for TLR-4 expression. These observations suggested that the failure in neutrophil recruitment and activation during the first hours of infection with *K. pneumoniae* is a most probable mechanism for high susceptibility to pneumonia in diabetics [101].

Commonly, *K. pneumoniae* infections cause pneumonia or urinary tract infections; however, during the past two decades, a distinct invasive syndrome that causes liver abscesses (KLA) has been increasingly reported in Asia, and this syndrome is emerging as a global disease [102]. DM is the most common comorbidity in KLA patients. It was shown that DNA and MPO levels were elevated in the plasma of KLA patients compared to uninfected individuals, indicating neutrophil activation independently of diabetic status. In addition, clinical *K. pneumoniae* isolates induced phagocytosis, bacterial killing, and NETosis comparable by neutrophils from diabetic and nondiabetic patients. Notably, the IL-12-IFNγ axis and its downstream chemokines CXCL9/MIG, CXCL10/IP-10, and CCL5/RANTES were produced at lower levels by peripheral blood mononuclear cells (PBMCs) from T2DM compared to PBMCs from healthy individuals in response to *K. pneumoniae* strains. These observations indicated that although T2DM does not overtly impact on neutrophil intra- and extra-cellular killing of *K. pneumoniae*, it may influence cytokine/chemokine production and intracellular killing by PBMCs.

4.4 Neutrophil function in bacterial infection-induced deficiency in wound healing

Delayed wound healing is one of the main diabetes-related morbidities. Neutrophil inefficient activity has been pointed as one of the major responsible factors for the impaired wound healing in diabetes, since neutrophil depletion accelerates wound resolution independently of the presence of an infection [103]. Furthermore, increased serum elastase levels, a marker of neutrophilic inflammation, predicted delayed wound healing in diabetic patients. In addition, proteomic analyses of the diabetic patient's foot ulcers (DFUs) showed elevated expression of NET components, including elastase, histones, neutrophil gelatinase-associated lipocalin, and proteinase-3, in nonhealing wounds as also in circulating blood. Consistently, neutrophils isolated from blood of DFU patients showed an increase of spontaneous NETosis but an impaired inducible NETosis [104]. Isolated neutrophils from T2DM patients presented higher NETosis rate than neutrophils from healthy patients in the absence of stimulation, which was associated with elevated intracellular calcium levels. Hyperglycemia is strongly related to these effects since

neutrophils derived from healthy patients produced more NETosis after pre-incubation with high glucose medium *in vitro*. In addition, large amounts of NETs were found in excisional skin sterile-wounds of streptozotocin-induced diabetic mice. Although the role of NETosis in wounds remains elusive, it has been confirmed that the inhibition of NETosis or degrading NETs improved sterile-wound healing and reduced NET-driven chronic inflammation in diabetic mice [105].

Gram-positive bacteria cause more than half of cases of diabetes-related wound infections. Especially, *Staphylococcus aureus* is a major pathogen in these infections, and its presence correlates with significant delays in wound healing [106]. Wounds induced by *S. aureus* in T2DM mice showed delayed resolution compared to non-diabetic mice. Seven days after infection, the lesions of diabetic mice presented exacerbated NETosis, while nondiabetic mice had their inflammatory process already resolved and healing was nearly completed. Although neutrophils derived from both T1DM and T2DM patients produced greater amounts of NETs compared to healthy volunteer's neutrophils, the induction of NETosis cannot be explained just by hyperglycemia. In fact, some works showed that high glucose exposure reduced LPS- or IL-6-induced NETosis *in vitro* [105, 107, 108].

Some mechanisms that could also explain the increased neutrophil NETosis in diabetic patients are the elevated levels of zonulin and the overexpression of PAD4. Zonulin is a protein that modulates the permeability of tight junctions between cells of the digestive tract. Interestingly, the increased zonulin levels in diabetic patients revealed a strong correlation with neutrophil elastase concentration and NET formation in a glucose-independent way [109]. PAD4 is a calcium-dependent enzyme that mediates NETosis. In diabetes, PAD4 was upregulated in neutrophils from individuals with diabetes and was responsible for the unbalanced NET production by these leukocytes.

In T2DM mice, although neutrophil infiltration toward the lesion was augmented, the impaired wound healing upon surgical site infection with *S. aureus* was related to a significant reduction in phagocytic activity and bacterial killing by neutrophils. Consistently, *S. aureus*-induced phagolysosome maturation was abolished and PMA-stimulated superoxide production was decreased in neutrophils recovered from diabetic mice. In addition, treatment of neutrophils with insulin significantly restored neutrophil killing activities and increased phagocytosis. Interestingly, phagosome maturation and superoxide production restoring were dependent on glycemic control and not on a direct effect of insulin. These abnormalities in neutrophil functions were closely related with impaired wound healing in DM, once treatment with insulin restored normal wound healing in diabetic mice [110].

5. Conclusion

The increased susceptibility to bacterial infections is one of the hallmarks of diabetic complications. Under comorbidity with diabetes, the high prevalence and severity of bacterial infections, as observed in tuberculosis, pneumonia, and sepsis, is closely associated to impairment in neutrophil functions, such as migration, phagocytosis, ROS production, and NET formation. The alterations in neutrophil functions noted in diabetics occur both dependently and independently of the glycemic control. Among the mechanisms that lead to neutrophil dysfunction in diabetic conditions not related to glycemic control, some targets have been highlighted, such as AGP, H2 receptor, IL-6, PAD4, resistin, and zonulin. These potential targets should be better explored in clinical studies concerning their putative benefits to diabetic patients.

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Conflict of interest

The authors declare no conflict of interest, including with the financial support agencies.

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