NEUTROPHILS: WARRIORS AND COMMANDERS IN IMMUNE MEDIATED INFLAMMATORY DISEASES

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Abstract

Neutrophils are critical effector cells in the immune system. They not only play crucial roles in pathogenic defense but are also able to modulate the function of other immune cells and consequently contribute to the immune response fate. The herein review is focused in neutrophil biology in a general perspective and its contribution to the course of immune mediated inflammatory diseases.

Keywords: Neutrophil; Inflammation; Trafficking; Activation; Apoptosis; Rheumatoid arthritis.

Resumo

Os neutrófilos são células efectoras centrais no sistema imune. Estas células além de serem cruciais para a defesa do organismo contra agentes patogénicos, são também capazes de controlar as funções de outras células do sistema imune e, consequentemente, contribuir para a resolução da resposta imune. Este artigo de revisão apresenta a biologia dos neutrófilos sob uma perspectiva geral e a sua contribuição para o desenvolvimento de doenças inflamatórias autoimunes.

Palavras-chave: Neutrófilos; Inflamação; Recrutamento; Activação; Apoptose; Artrite reumatóide.

Introduction

Neutrophils are key cells in the immune response due to their dual role as anti-infectious and pro-inflammatory cells, being critical effectors in both innate and humoral immunity. Neutrophils generate chemotatic signals and cytokines that recruit, differentiate and activate B and T lymphocytes and program antigen presenting cells (APCs), thus establishing a "bridge" between the innate and adaptive immune system. Neutrophils are present in high numbers in areas of inflammation, where they constitute an important source of cytokines and other immune mediators, and can therefore participate in immune decision making.[1] Neutrophils' rate of production and retention in the bone marrow are in turn controlled through a reciprocal feedback mechanism which involves different molecules produced by the adaptive immune system.[1] This complex network of communication exists between innate and adaptive effectors cells throughout the immunological response and evolves until its resolution.[1] Neutrophils are important decision-shapers in this complex system and further understanding of the specific roles of these cells may well help to answer one of the main questions in the immune system domain: "What triggers an immune response?".[1]

With this question in mind this review will focus on the characteristics, functions and pathogenic properties of neutrophils and their contribution to the development of immune mediated inflammatory diseases such as Rheumatoid arthritis (RA).

Neutrophil Biology

Neutrophils have two important roles in the immune system: immune surveillance and elimination of microorganisms. These functions require a quick transition from a circulating/inactive to an adhesive/active phenotype to allow migration towards inflamed tissues. In order to achieve their main goals, neutrophils must pass through different and complex phases, as described below.

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Granulopoiesis

Neutrophils differentiate from myeloid precursors through a process named granulopoiesis, which comprises the promyelocyte, myelocyte and metamyelocyte stages. This is a multistage process which results in the continuous production of high numbers of mature neutrophils from a small number of hematopoietic stem cells.[2] Granulopoiesis is closely regulated by several cytokines and intrinsic myeloid transcription factors. Recently, it has been shown that lymphoid enhancer-binding factor (LEF)-1 is an important transcription factor in the regulation of proliferation and differentiation of granulocytes,[3] specifically in the differentiation of myeloid progenitors to mature neutrophils under granulocyte-colony stimulating factor (G-CSF) control.[2] Expression of G-CSF by macrophages, fibroblasts and endothelial cells is stimulated by inflammatory cytokines, such as interleukin (IL)-1, IL-6, IL-17 and tumor necrosis factor (TNF).[4] Also important for granulopoiesis are β-integrins, particularly β_2-integrin that supports cell retention and β_1-integrin which is necessary for neutrophils release.[5]

In normal conditions, only a small percentage of neutrophils are released from the bone marrow into circulation, where they have a very short half-life and are rapidly cleared by constitutive apoptosis. This process is crucial to keep neutrophil production balanced.[6] In a situation of inflammation the production of neutrophils increases and these cells are mobilized both in mature and immature stages. This response is designated as "emergency or stress granulopoiesis".[7] Once activated, neutrophils contribute to the recruitment of immune cells, amplification of inflammation and tissue damage by generation of reactive oxygen species (ROS) and secretion of proteases, chemokines and cytokines. Upon resolution of inflammation, neutrophils die by apoptosis and are phagocyted by macrophages, downregulating IL-23. Consequently, a suppression of IL-17 expression occurs, which results in a decreased G-CSF level and therefore reduces neutrophil production and release. Another relevant molecule is SDF-1 (CXCL12), which is a chemoattractant for neutrophils[8] that binds to CXCR4 receptor and is present constitutively at high concentrations in the bone marrow, where it provides a signal for cell retention.[5] G-CSF can disrupt SDF-1/CXCR4 signaling, thus contributing to neutrophil mobilization.[5] On the other hand, CXCR4 expression increases during neutrophil ageing leading to the preferential homing of senescent cells into the bone marrow.[8,9] Therefore, SDF-1/CXCR4 signaling has a dual role in the regulation of cell retention and homing, being an important player in neutrophil homeostasis.[5]

Trafficking

Neutrophils in the peripheral blood are present in two pools: a circulating pool in large blood vessels and axial stream of small vessels, and a marginating pool.[10] In the absence of inflammation the marginating pool comprises granulocytes transiently arrested in narrow capillaries, mainly in the lungs.[10] Circulating neutrophils contact and transiently interact with endothelial cell surface molecules in a roll-and-release tumbleweed-like motion.[11]

Rolling

In case of inflammation, rolling constitutes the first step of neutrophil recruitment and allows for tight interactions with endothelial cells that consequently lead to the migration to tissues and sites of inflammation.[11] The main participants in the rolling process are selectins.[11] These molecules are type 1 membrane-glycoproteins characterized by a NH_2-terminal C-type lectin and an EGF-like domain. L-selectin, present on the surface of neutrophils, interacts with endothelial cells and other neutrophils via P-selectin glycoprotein ligand (PSGL)-1.[12] Endothelial cells express P-selectin (present in the Weibel-Palade granules) just a few minutes after stimulation by thrombin, histamine or ROS and E-selectin one to two hours following cell stimulation with IL-1, TNF or lipopolysaccharide (LPS) which cause increased gene transcription.[13] E-selectin counter-receptors include PSGL-1 and E-selectin ligand (ESL)-1, both located on neutrophil microvilli.[14] In opposite to P- and E-selectins receptors, which are only expressed on endothelial cell after activation, L-selectin is constitutively presented on the leukocyte surface and its binding capacity is increased upon cell activation, possibly through receptor oligomerization.[15] PSGL-1, which binds L-selectin, P-selectin and E-selectin, is also important for the triggering of intracellular signaling pathways upon ligand engagement, leading to neutrophil activation, as well as activation of β_2-integrins,[16] tyrosine phosphorilation, secretion of cytokines,[17] transcriptional activation,[18] and cytoskeleton rearrangement.[19] Furthermore, as PSGL-1

is a counter receptor for leukocyte L-selectin, some studies revealed that neutrophil roll on previously adherent cells via L-selectin,[20] and this adhesion could synergistically enhance leukocyte accumulation on inflamed endothelium.[10]

Adhesion

After rolling on activated endothelium in areas of inflammation, the response of neutrophils to a chemoattractant gradient is tight stationary adhesion.[11] Neutrophil adhesion to endothelial cells or extracellular matrix is performed via the β_2-integrin family. These receptors are composed by variable α subunits, such as CD11a, CD11b, CD11c, and a constant β subunit, named CD18, which has cytosolic domains that interact with the cytoskeleton, allowing for cell adhesion stabilization and providing a framework for signaling proteins.[11] The most important β_2-integrins on neutrophil surface are CD11a/CD18 (LFA-1), which has intercellular adhesion molecule (ICAM)-1 and ICAM-2 as counter-ligands, and CD11b/CD18 (MAC-1 or CR3), that binds to ICAM-1, ICAM-2, fibrinogen, complement fragment iC3b and heparin, among others. Chemoattractants displayed on the endothelial membrane rapidly activate a complex network of intracellular events in neutrophils, leading to β_2-mediated adhesion,[11] thus providing a mechanism that controls acute and chronic inflammation.[10] MAC-1 activation can be induced by chemoattractants such as IL-8, platelet-activating factor (PAF) and complement C5a, cytokines (e.g. TNF), growth factors such as granulocyte macrophage-colony stimulating factor (GM-CSF) and microbial molecules like formylated peptides and LPS.[10] Adhesion participants constitute targets for a number of therapeutic agents with anti-inflammatory actions, such as corticosteroids, which diminish the expression of adhesion molecules on the endothelium[21] and neutrophil surface,[22] or salicylates, which block the activation of MAC-1 leading to the inhibition of neutrophil adhesion.[23]

Diapedesis

Diapedesis of neutrophils occurs on tight junction's discontinuities of endothelial cell borders, with necessary modifications of these adherent junctions. In fact, VE-cadherin, β-catenin, and plakoglobin become disorganized in regions of firm adhesion between neutrophil and the endothelial layer.[24] In leukocytes the process of diapedesis involves two adhesion molecules of the Ig-superfamily (CAMs): platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31) and junctional adhesion molecule (JAM).[25,26] JAM is not present on neutrophils cell surface, being instead concentrated at inter-endothelial tight junctions.[10] Contrarily, PECAM-1 forms homophilic interactions (PECAM-1/PECAM-1) and is expressed on neutrophils membrane and at endothelial cell junctions.[10] This molecule also transduces intracellular signals and its dimerization increases CD11b/CD18 binding capacity.[27]

However, not only protein-protein interactions regulate leukocyte trafficking, but also locally generated lipid mediators are involved.[28] For example, recently it has been shown that lipoxins (LX), in particular LXA_4, function as an innate "stop signal", controlling local inflammatory mechanisms.[29] In addition, the synthesis of "resolvins" by macrophages upon apoptotic cell ingestion blocks neutrophil recruitment, controlling the initiation of inflammation resolution.[30]

Neutrophil transepithelial migration is driven by chemokines such as IL-8, which is secreted by infected epithelial cells on their basolateral face[31] and formyl peptides secreted by bacteria which are transported across epithelial cells.[32] These chemotactic factors trigger the complex adhesive and de-adhesive mechanisms that make crossing through the epithelium layer possible.[10]

Migration

The third step in neutrophil response is the migration to inflamed tissues due to immobilized chemoattractant gradients.[10] Chemoattractants can be released by microorganisms, necrotic, stromal and epithelial cells present in locals of inflammation, and they tend, upon release, to bind to the extracellular matrix elements due to their negative charge.[10] Crosstalk between chemoattractant receptors and the activated signaling pathways might cause desensitization to one attractant by another, allowing for leukocyte recruitment through their final target within a tissue.[10] Migration is mediated by β_2-integrin in concert with β_1- and β_3-integrins that are mainly packed in neutrophil granules and present on their plasmatic membrane upon chemoattractant activation and during migration.[33,34] The formation of new adhesion interactions at the front of the cell and cell rear detachment from the adhesive substrate are also required for migration.[35] De-adhesion is facilitated by anti-adhesive membrane molecules (for example, CD43) that cluster on the cell rear.[36]

Chemokine signaling

Neutrophils produce several chemokines, like chemokine ligand (CXCL)1, CCL3, CCL4, CXCL8 (IL-8), CXCL9 and CXCL10 in response to stimulation with LPS, TNF, interferon (IFN)-γ and G-CSF.[37] However, the production of cytokines such as IL-1β, IL-6 and TNF is still a contradictory issue.[38,39] In order to control the production of chemoattractants, neutrophils require a relatively selective combination of stimuli.[37] In addition, neutrophils are able to regulate the production of both chemokines and cytokines by other immune cells.[37] One example is that IL-8 can release TNF-related apoptosis-inducing ligand (TRAIL) from interferon-activated neutrophils,[40] which plays an immunoregulatory function on activated T cells.[37] At sites of inflammation there are several chemokines (such as IL-8) that are relevant players in the modulation of neutrophil function.[11] In fact, neutrophils express various structurally related receptors for these molecules allowing for the triggering of functional responses such as adhesion, migration, degranulation and oxidative burst. The chemokine receptors, which are G-protein-coupled seven-transmembrane glycoproteins ("serpentines"), expressed by these cells are CCR1, CCR2, CXCR2, CXCR4 and CCR6.[37]

Cytokine production and receptors expression

Neutrophils express several cytokine receptors (e.g. for IL-1 and TNF) which lead to the amplification of many of their functions, like adhesion and ROS production. On the other hand, neutrophils are able to synthesize (constitutively or inducibly), and secrete pro- or anti-inflammatory cytokines, as well as other cytokine types and growth factors.[10] These cells do not produce a wide range of cytokines, nevertheless they are an important cytokine source once present in a higher number than other leukocytes. The production of cytokines is regulated by different molecules, such as other cytokines as well as bacterial endotoxins (LPS). It is a highly controlled process dependent on the agonist and, in some cases, necessary co-stimulation by at least two agonists (e.g. the secretion of IL-12 requires both IFN-γ and LPS).[41] The secretion of cytokines is also regulated by previous accumulation of mRNA. Although neutrophils have low transcriptional activity, data have pointed out that regulation of cytokine expression can be performed at the level of mRNA stability in addition to a post-transcriptional mechanism of regulation. For example, IL-10 inhibits IL-8 gene transcription and activates IL-8 mRNA degradation while IL-1Ra under specific experimental conditions is controlled at the translational level.[10]

Neutrophil Granules

The majority of neutrophil functions, such as adhesion and phagocytosis, require the mobilization of cytoplasm granules and secretory vesicles, which contain antimicrobial proteins, enzymes, components of the respiratory burst oxidase and diverse membrane-bound receptors[42] (see Table I.). Granules are formed in a process named granulopoiesis that follows myeloid cell differentiation, beginning at early promyelocyte state when immature transport vesicles arise from the Golgi apparatus and fuse together.[43] Specifically, neutrophils harbor four types of granules, named azurophilic, specific and gelatinase granules, and secretory vesicles that appear sequentially during different granulopoiesis stages. Although granules share common structural features, such as a phospholipidic bilayer membrane and an intra-granular matrix containing proteins for exocytosis or phagosome delivery, their protein content is quite different.[42] This difference can be explained by the "targeting-by-timing" hypothesis, which proposes that the targeting of proteins into granules is determined by the time of their biosynthesis[44,45] and their targeting efficiency.[46] Additionally, gene expression is highly regulated by combination of myeloid transcription factors present at specific stages of cell development.[45,47]

The ability for exocytosis is different among granules. Secretory vesicles have the highest propensity for exocytosis followed by gelatinase, specific and azurophilic granules.[48] A higher ability for exocytosis is related to a higher density of vesicle-associated membrane protein (VAMP)-2, a fusogenic protein.[49] Besides the functions mentioned in the table above, some granule components, such as defensins, azuricidin and human cathelicidin hCAP-18, also have the ability to induce $CD4^+$ and $CD8^+$ T cells chemotaxis,[50] revealing the capacity of neutrophils to participate in the amplification of the inflammatory response and to communicate with adaptive immune cells.

Phagocytosis

Neutrophils, as well as macrophages, are pha-

Table I. Neutrophil granular content

Granule	Granular components	Function
Azurophilic	Myeloperoxidase (MPO); serine proteases; antimicrobial proteins; granulophysin (CD63); CD68; presentin-1; stomatin; vacuole-type H+-ATPase	Phagocytosis
Specific	Cathelicidins	Antimicrobial activities (fusion with phagosome or exocytosis)
Gelatinase	Matrix-degrading enzymes and membrane receptors	Transmigration
Secretory vesicles	CD11b/CD18; complement receptor 1 (CR1); fMLP receptors; LPS/lipoteichoic acid (LTA); receptor CD14; FcγIIIR CD16; Leukolysin	Early neutrophil-mediated inflammatory response

gocytes that eliminate pathogens and cellular debris. The phagocytosis of opsonized particles has two different pathways: through Fcγ receptors (FcγRs) for immunoglobulin (IgG)-coated particles and complement receptors for complement-coated particles. Human neutrophils constitutively express low-affinity FcγRIIA (CD32) and FcγRIIIB (CD16b) receptors. Additionally, interferon-primed neutrophils express FcγRI (CD64), a high-affinity receptor.[51] After Fcγ receptor binding, pseudopods are formed to surround and engulf the particle.[52] Concomitantly, particles coated with complement fragment C3bi bind to activated CD11b/CD18 with ingestion occurring by "sinking" into the cell.[52]

Binding of IgG-opsonized particles to FcγRs triggers downstream activation of signaling pathways, which contribute for membrane extension over the particle, fusion and final closure of the "phagocytic cup". After formation of the phagosome microbicidal functions must be acquired, such as enzymes, vacular (V)-ATPases and NADPH oxidase complex. This maturation process occurs by the fusion with granules and secretory vesicles, as well as removal of components by vesicular fission, processes that require cell signaling. Contrarily to macrophages, phagosomes of neutrophils are not very acidic even with fusion of acidic granules and, consequently, of V-ATPases insertion (which pump H^+ into the lumen of the phagosome). This is due to NADPH oxidase action that alters the pH of phagosome by consuming luminal H^+ and producing ROS, thereby diminishing the efficiency of granule fusion and decreasing its permeability to H^+.[53] Altogether, NADPH oxidase shows a more relevant role in phagosome acidification that is thought to be required for optimal phagosome maturation.[54]

The phagocytosis pathway via C3bi-opsonized targets, which is performed by complement receptor 3 (CR3), is different from FcγR via. One difference is the fact that ingestion is independent of a rise of cytosolic-free Ca^{2+} and of increased inositol phosphate production.[55] Moreover, phagocytosis is not accompanied by respiratory burst activation and arachidonic metabolites and cytokine production.[56] Finally, it also does not involve Rac or Cdc42;[57] instead it is Rho activation, which follows complement receptor stimulation, that leads to membrane protrusions extending over the surface of the opsonized particle, forming the "phagocytic cup".[58,59]

Oxidative burst

Neutrophils have oxygen-dependent and oxygen--independent microbicidal weapons.[10] Oxygen-dependent pathways lead to the production of ROS by NADPH oxidase complex, in a process named oxidative or respiratory burst.

NADPH oxidase is an enzymatic complex composed of membrane and cytosolic components (see Figure 1.). After neutrophil activation, cytosolic components present in a heterotrimeric complex $p47^{phox}$-$p67^{phox}$-$p40^{phox}$ are phosphorylated by kinases, such as p38 MAPK and phosphatidylinositol--3-OH-kinase (PI3K),[60] and are translocated to the plasma membrane, allowing its interaction with the membrane and with other oxidase proteins.[61] Additionally, specific and azurophilic granules and secretory vesicles fuse with the plasma membrane to form the phagosome, thus allowing for $gp91^{phox}$ and $p22^{phox}$ interaction with the membrane.[62] In addition, phorbol 12-myristate 13-acetate (PMA)

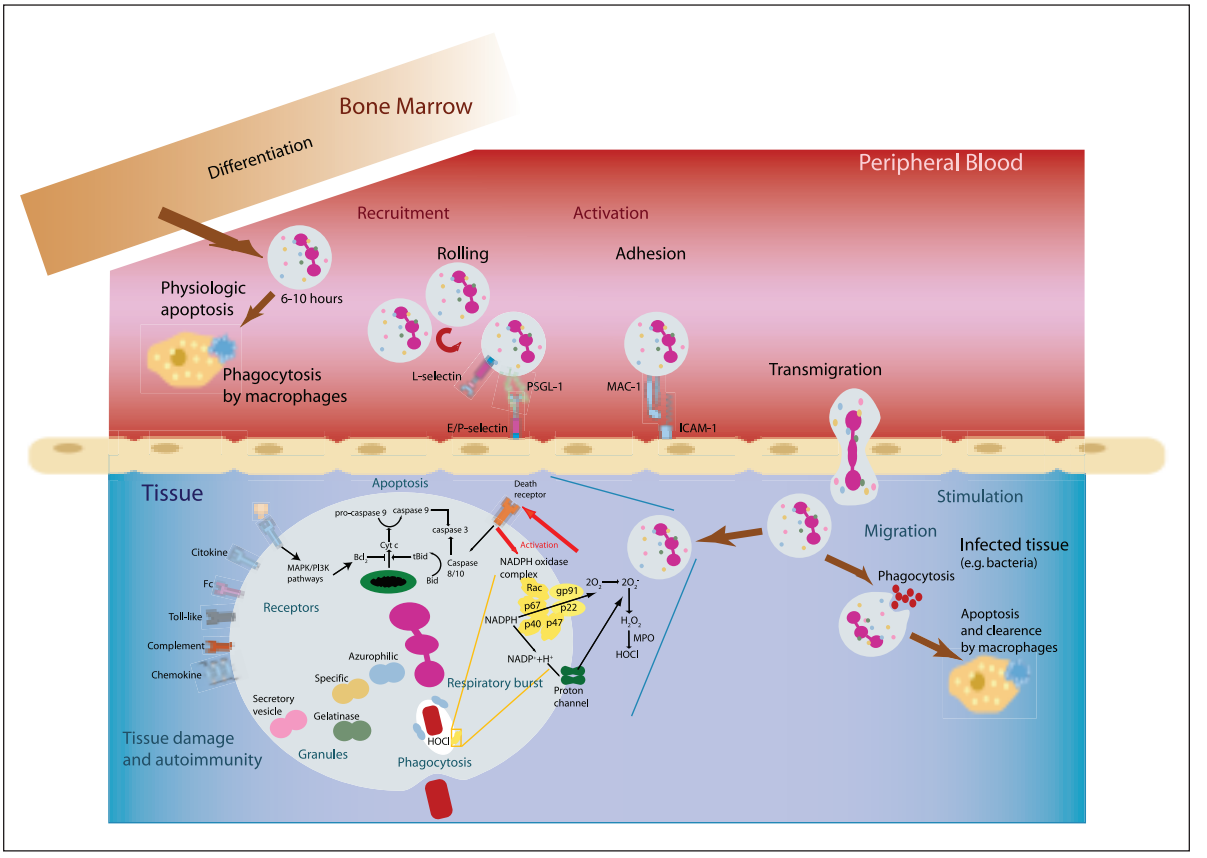

Figure 1. Neutrophil Biology (see text for details).

activation leads to $p47^{phox}$, $p67^{phox}$ and Rac2 translocation to the plasma membrane as well as to specific granules where, in complex with membrane components, they can produce O_2^- for a short period of time.[63] In granules NADPH oxidase activity is dependent on protein kinase C (PKC)δ and PI3K for proper assembly.[64,65] NADPH oxidase complex accepts electrons from reduced NADPH at the cytosolic surface of the membrane and transfers them to O_2 on the extracellular surface of the membrane leading to the downstream production of O_2 and H_2O or hypochlorous acid (HOCl). HOCl constitutes a strong microbicidal agent as it oxidates several bacterial molecules.[66] However, this molecule also damages most tissues.[11] Indeed, HOCl mediates the activation of pro-collagenases and pro-gelatinases[67] and the production of cholesterol chlorohydrins,[68] leading to tissue injury. Inappropriate NADPH oxidase assembly and activation can be regulated and prevented by neutrophils through differential location of its components. Production of O_2^- by NADPH oxidase is only possible after certain events, such as phosphorylation, translocation, and multiple conformational changes.[69] Neutrophils can also regulate gene expression of NADPH oxidase proteins at the transcriptional level by several transcription factors depending on cytokines and other inflammatory mediators.[70]

Some studies suggest that ROS and MPO activity are not enough for microbicidal capacity; instead, the proteases activated by the respiratory burst process are actually responsible for destroying invading agents.[71] Additionally, ROS is implicated in the regulation of cellular signaling pathways related with homeostasis, proliferation, differentiation, inflammatory and immune responses.[72] It is also interesting to refer that ROS have characteristics of intracellular messenger such as diffusibility and rapid turnover, allowing for spatial and temporal signaling specificity[73,74] in a nontoxic concentration.[75] Intracellularly, ROS can alter redox state and oxidize proteins.[76] Alteration of redox state can regulate signaling pathways at many levels including receptor functions, enzymatic activity, transcription fac-

tors and gene expression patterns.[73,77] ROS can also participate in pathways triggered by pro-inflammatory cytokines and chemokines.[75] For example, neutrophil apoptosis can be increased by activation of inositol phosphatase (SHIP) by the tyrosine kinase Lyn (of the Srk-family) via NADPH oxidase-derived ROS.[78] Another example is that ROS can activate transcription factor nuclear factor (NF)-kB that is involved in cytokine and chemokine expression by neutrophils in an inflammatory milieu.[79] Additionally, ROS can also modulate signaling pathways in adjacent cells such as macrophages and endothelial cells.[75] In the case of inflammation, ROS can be also released extracellularly leading to tissue damage.

Apoptosis

In the absence of activating stimulus, neutrophil stay in circulation approximately 6 to 18 hours and undergo constitutive apoptosis,[6] a systematic and stereotyped programmed cell death.[80] Therefore, neutrophils are cells characterized for having a very short half-life.

Apoptosis has two different pathways: extrinsic and intrinsic. The extrinsic pathway is triggered by the ligation of external pro-apoptotic molecules to neutrophil surface death receptors, such as Fas (Apo-1/CD95), TNFRs, TRAIL-R2 and TRAIL-R3.[81] Death receptors are cell surface receptors which contain cysteine-rich extracellular domains and a cytoplasmic motif named "death domain" (DD).[82,83] These domains allow for the interaction of receptors and intracellular molecules of the apoptotic process,[81] like pro-caspases (cysteine-dependent aspartate-specific proteases) that become activated. The intrinsic pathway is triggered by the release of cytochrome (cyt) c from the mitochondria leading to caspase activation. Neutrophil possesses caspases-1, -3, -4, -6, -7, -8, -9, -10 and -14.[84] In the intrinsic pathway pro-apoptotic Bcl_2 proteins are able to localize in the outer mitochondrial membrane, altering its permeability. Then cyt c is released into the cytoplasm, where it forms a complex with apoptosis protease-activating factor (APAF)-1, present in high levels, and caspase-9. Ultimately, caspase-9 cleaves downstream caspases and initiates apoptosis.[85]

In the setting of inflammation the apoptotic delay is an important factor for the accumulation of neutrophils in the place of injury. Actually, host and bacterial anti-apoptotic mediators are also able to delay neutrophil apoptosis, converging on common intracellular molecular pathways. The process of extravasation itself can mediate cell survival,[86] by cell contact with activated endothelium[87] and exposure to cytokines. In fact, neutrophil apoptosis can be delayed by IL-1β, IL-2, IL-6, IL-15, TNF, INF-γ, G-CSF, GM-CSF, LPS[88] and IL-8.[89] It is interesting to refer that IL-1, IL-6 and TNF can be produced by activated neutrophils to regulate themselves.[90] Antimicrobial human β-defensins (hBDs), particularly hBD-3, also prolong the lifespan of neutrophils through down-regulation of truncated Bid (tBid) and up-regulation of Bcl-x_L.[91] Most interesting are the recent data which reveal that cathepsin D (stored in azurophilic granules) activates caspase-8 in a caspase-independent but ROS-dependent manner.[92] However, under inflammatory conditions cathepsin D is kept in granules and neutrophil apoptosis became reduced.[92] On the other hand, bacterial molecules, such as LPS and LTA, can delay constitutive apoptosis via engagement of Toll-like receptors (TLR) 4 and TLR 2, respectively.[93,94] Also, TLRs 7, 8 and 9 affect neutrophils life span. Contrarily, after neutrophil phagocyte bacteria, apoptosis is accelerated[95] by a process called phagocytosis-induced cell death (PICD). However, bacterial ingestion has also been shown to delay apoptosis.[80] Regarding this matter, it has been shown that engagement of β_2-integrins, which are involved in PICD, can both accelerate and delay constitutive apoptosis depending on their activation state and the balance between death and survival signals, some of which appear to be cell lineage specific.[96]

Neutrophil apoptosis is controlled by Bcl_2 family proteins, which include anti-apoptotic proteins such as Mcl-1, A1 and Bcl-x_L, and pro-apoptotic proteins, such as Bax-α, Bid, Bak and Bad.[6,97] The ratio established between anti- and pro-apoptotic molecules, for example, upregulation of Bcl-x_L and downregulation of Bax-α,[90] regulates cell death delay.[98] Activated neutrophils produce high amounts of ROS that can increase apoptosis.[99] Recent studies suggest that death receptor clustering and the subsequent activation of caspase-8 are ROS dependent and may occur independently of Fas ligation in spontaneous apoptosis.[100] In addition, many cell signaling pathways and cell molecules known to be important in the regulation of apoptosis are influenced by the redox environment.[85] Some studies pointed out that early mitochondrial dysregulation[101,102] is a critical step in the induction of apoptosis by oxidant stress. Due to the alteration of mi-

tochondria permeability, ROS can be released into cytoplasm,[103] promoting alternative cell death pathway.[104] Additionally, exogenous ROS can act upon mitochondrial membrane leading to its depolarization, thus constituting initial stimuli for the activation of the intrinsic pathway.[85] Therefore, in inflammation sites where activated neutrophils produce higher quantities of ROS there exists a mechanism of apoptotic induction acting as a potential negative feedback in the inflammatory response.[85]

The resolution of inflammation requires at least two different processes, neutrophil apoptosis and clearance of apoptotic cells by macrophages, preventing the host tissue damage by inappropriate release of cell enzymes and proteases.[105] Apoptotic neutrophils externalize phosphatidilserine (PS) and express CD35 and CD63 at the cell surface thus facilitating the recognition by macrophages.[106-108] These are not the only clearance signaling mechanisms. Apoptotic neutrophils have different ways to signal for macrophage ingestion and clearance, such as recruitment signals,[109] membrane changes and cell receptors (e.g. vβ3/CD36, CD14, CD31, CR3/CR4 among others).[110] On the other hand, the ingestion of apoptotic neutrophils, as well as opsonized particles, by macrophages promotes their release of soluble Fas ligand (FasL) which reacts with its receptor (FasR) present on neutrophils,[10] leading to apoptosis of the remaining neutrophils.[111] Macrophages phagocyte apoptotic cells via their αvβ3 integrin-CD36 complex that binds to neutrophils through thrombospodin and other unknown ligands present on the cell surface.[105] CD36 is also important for the recognition of PS on apoptotic cell surface.[112] Additionally, the phagocytosis of apoptotic neutrophils can inhibit IL-1β, IL-8, IL-10, GM-CSF, TNF, leukotriene C_4 and thromboxane B2 production by macrophages,[106] thus suppressing the secretion of inflammatory mediators and, consequently, leading to the resolution of inflammation.[10]

NETosis

In 2004 it was shown by Brinkmann *et al* that neutrophils were able to form extracellular structures, named neutrophil extracellular traps (NETs).[113] NETs are assembled in cells activated by different pathways and are composed by nuclear components, such as chromatin DNA, histones anchored to this molecular backbone, and cytoplasmic components, such as granular peptides and enzymes. Upon activation of several receptors, such as TLRs and FcRs, there is a triggering of a signal transducing cascade that induces the activation of NADPH oxidase and downstream leads to the assembly of NETs, suggesting that its formation is ROS-dependent.[114] Due to its composition, NETs function as a web of high concentration of antimicrobial proteins that can trap and kill Gram-positive and -negative bacteria, but also fungus.[115] Neutrophils die upon release of these structures. However, this is a form of cell death different from apoptosis and necrosis, named "NETosis".[116] NETs represent an unconventional form of immune defense, because neutrophils can trap microorganisms that had no direct contact with the cell and the structure remains active even after neutrophils' death, thus prolonging the microbicidal response. Although NETs assume a role in sites of infection, the extracellular release of proteins, such as cathepsin G and elastase, can cause tissue damage.[117] In addition, the presence of nucleic acid can contribute to the development of autoimmune diseases like systemic lupus erythematosus (SLE) in which there is an exacerbated reaction against the host DNA.[115]

Neutrophils and Rheumatoid arthritis: a case study

Based on their characteristics and functions it is easy to conclude that neutrophils have an enormous potential for host defense. However, they also have a non-specific action being dependent on components of the immune system to distinguish between invading agents and host antigens.[10] Therefore, if the shutting off of the recruitment of neutrophils is impaired or if the acute insult is not resolved, these cells can inflict injury to the tissues.[10] Specifically, in RA, neutrophils are involved in joint damage.[118] RA is a chronic inflammatory disease mainly characterized by synovial hyperplasia and joint destruction. Although the etiopathology of this condition is not completely understood, it is known that it is associated with misregulation of both the cellular immune system and the cytokine network.[119,120] Neutrophils are prominent participants in the joint inflammation of patients with RA. Insight from a mouse animal model of autoimmune arthritis has suggested that neutrophils are the first immune cells to infiltrate the joint at the early disease stage, with the earliest signs of ankle joint

inflammation correlating with the presence of neutrophils in the synovia.[121,122] In the same model, abrogation of the synovial inflammatory response was achieved by previous neutrophil depletion,[122] further strengthening the importance of the neutrophil as an essential component of the initial immune response in RA. In the clinical setting, we have observed that neutrophils are present in high numbers in the synovial tissue during the initial stages of RA (unpublished data),[123] as already reported in previous studies,[1] and are described to persist in the synovial fluid during the perpetuation of this disease.[124] A patient in an active disease state may have a synovial fluid cellular infiltrate containing up to 90% of neutrophils.[125] However, it is assumed that they are largely absent in joint tissues, except in cartilage and pannus-cartilage interface where in the early stages of disease cartilage damage occurs due to the action of serine and metalloproteinases (MMPs)[126,127] stored in their granules and by the production of ROS and chlorinated oxidants.[118] Despite these findings, neutrophils are generally an understudied cell in RA and are often considered simply as a terminally differentiated cell of the innate immune system, merely with cytotoxic potential and capacity to inflict tissue damage, and lacking the potential to interfere with the initiation and development of an autoimmune disease such as RA.

In RA, neutrophils are recruited into inflamed joints by chemoattractants present in synovial fluid, such as LTB_4, C5a, IL-8 and TGF-β. When these cells arrive at the joints, they become exposed to a wide spectrum of pro-inflammatory cytokines and growth factors such as IL-1β, IL-6, TNF and GM-CSF.[128] In addition, IL-17 appears to stimulate the production of cytokines that attract neutrophils to the site of inflammation, stimulate granulopoiesis and/or induce production of chemokines.[129,130] Other important activating factor appears to be IgG-containing immune complexes that trigger stimulation of the respiratory burst and degranulation.[131,132] In addition to cytokines, other stimuli, such as adhesion, transmigration and hypoxia, are also able to activate gene expression.

There are many differences in protein expression between synovial fluid and blood-derived neutrophils in RA patients. Neutrophils from synovial fluid have mobilized pre-formed molecules from intracellular stores to the cell surface and activated gene expression resulting from enhanced transcription and translation.[133,134] Consequently, several gene products are up-regulated such as IL-8, CXCR4, TNFR and MMP-9, allowing not only the up-regulation of cell functions but also the development of new cellular responses, e.g. the antigen-presentation to T cell via activated major histocompatibility complex (MHC) classe II expression.[125] In fact, synovial fluid neutrophils were described to acquire an antigen-presenting function through cytokine stimulation, allowing T cell function modulation, an important feature in RA pathology.[134] This hypothesis is conflicting with the traditional view of neutrophils as a terminally differentiated cell.[135]

Moreover, recent data from animal studies show that neutrophils play a crucial role in the initiation and progression of arthritis. For example, in the K/BxN mice model the administration of a neutrophil-depleting antibody before or simultaneously with disease induction prevent its initiation. Also, its administration performed until 3 days after arthritis induction can revert its progression.[122]

Considering RA as a T cell driven pathology, some authors assumed that the differences between rheumatoid and non-rheumatoid circulating neutrophils are a consequence rather than an initially event in RA.[10] However, therapies in use and others being tested in clinical trials for RA are commonly anti-inflammatory drugs that have profound effect on neutrophil functions, molecules (e.g. anti--adhesion) and activation (e.g. anti-cytokine antibodies),[136] reinforcing the idea that these cells are relevant players in RA. A good and simple example of this is the fact that methotrexate, the gold standard drug for the treatment of RA, abrogates the delayed neutrophil apoptosis observed in early RA patients.[137]

Conclusion

The old view of the neutrophil as a terminally differentiated cell completely focused on destroying pathogens and tissues is no longer in line with the new data related with their cellular and molecular mechanisms. In fact, neutrophils are unique cells in their ability to be immune decision-shapers and to induce damage and healing. The knowledge about the neutrophil complex biology and their role in immune-mediated inflammatory diseases is expected to reveal new promising therapeutic targets. Hopefully, time will come when specific neutrophil targeted therapies will play a role in the treatment of diseases such as RA.

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