

# Coordination of Neutrophil and Apoptosis-Inducing Ligand in Inflammatory Diseases

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**Abstract:** As the most abundant innate immune cells, neutrophils play a key role in host's anti-infective activity and tissue damage/repair process of sterile inflammation. Due to the restriction of apoptosis and other regulatory mechanisms, neutrophils have a short survival time in vivo. Because of the death domain of cytoplasmic regions, some members of tumor necrosis factor receptor superfamily (TNFRSF) are defined as death receptors, such as TNFR-I, Fas and DR4/DR5. TNF- $\alpha$ , FasL and TRAIL, which are known as apoptosis-inducing ligand, can bind to death receptors and activate intracellular apoptosis pathways to induce apoptosis. Accumulating studies found that these three apoptosis-inducing ligands play an important role in the immune system by coordinating with neutrophil, which including neutrophil recruitment/infiltration and function performing. In this review, we summarize existing studies targeting neutrophils as diagnosis and treatment for diseases, and focus on the involvement of neutrophils which regulated by apoptosis-inducing ligands in inflammatory diseases under current cognition.

**Keywords:** neutrophil, apoptosis-inducing ligand, infectious inflammation, sterile inflammation

## Background

Neutrophils, also known as polymorphonuclear leukocytes (PMNs), are the most abundant leukocytes in humans and are critical for innate immunity and inflammation. As an important part of the innate immune system, neutrophils have chemotactic, phagocytic and bactericidal functions. In addition to resisting pathogenic microorganisms, neutrophils are also involved in many pathophysiological processes, including tumors, autoimmune diseases and cardiovascular diseases.<sup>1</sup> Disease-related neutrophils can accelerate its regeneration in bone marrow by releasing pathological proteases, matrix metalloproteinases, chemokines and other cytokines, and maintain the number of neutrophils in blood at a high level, aggravating the pathological damage of chronic inflammation.<sup>2</sup> Consequently, the relationship between neutrophils and their mediated inflammatory response and diseases has attracted more and more attention, and has gradually become the focus of mechanism research and drug development.

Apoptosis-inducing ligand (TNF- $\alpha$ , FasL, TRAIL) is a signal protein that mediates apoptosis in pathological injury. These proteins bind to their homologous cell surface receptors (TNFR1/2, Fas, DR5/DR4) to mediate cell death or inflammatory responses.<sup>3</sup> Studies have shown that they also play an important role in regulating the migration of

neutrophils and promoting the secretion of inflammatory cytokines.<sup>4</sup> They are key molecules involved in the development of various types of diseases and important targets for drug development.

## Overview of Neutrophil

Mature neutrophil is a kind of specialized phagocytes that differentiate from granulocyte-monocyte progenitors in bone marrow. Circulating neutrophils maintain a dynamic balance with a basically constant number and short life span. Neutrophils, as the most abundant immune cells in human body, play an important role in both infectious and non-infectious inflammation.<sup>4</sup>

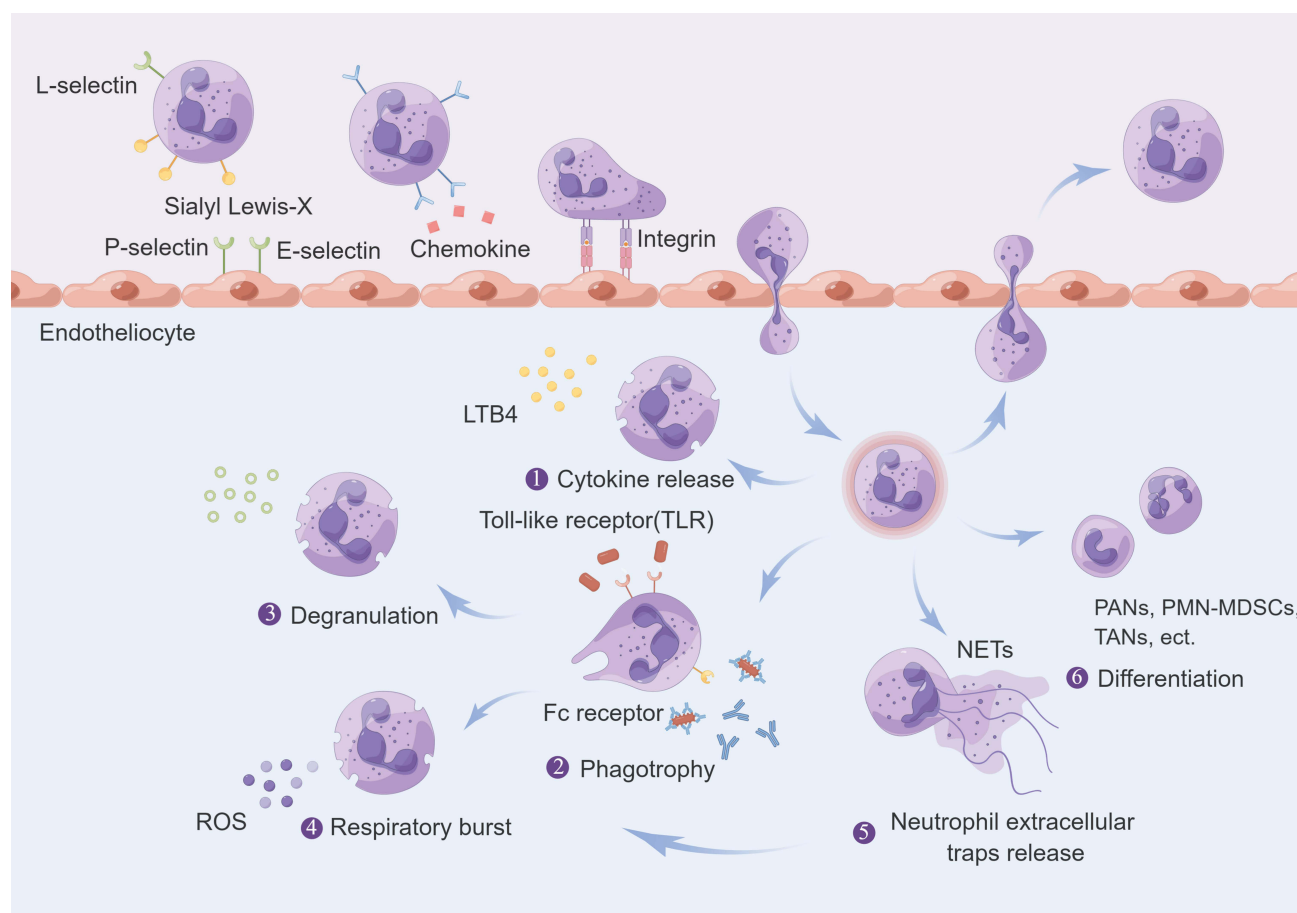
In infectious diseases, neutrophils are involved in the regulation of the inflammatory response, promoting phagocytes to eliminate harmful bacteria to resist infection, and clearing necrotic tissue to promote wound healing. They are regarded as important participants in tissue repair. However, when inflammation recurs or irritants persist, neutrophils recruitment to infectious lesions can promote the spread of pathogen to bloodstream and vital organs, resulting in systemic infection and eventually death.<sup>5</sup> In addition, Neutrophils are also involved in removing cell debris and restoring tissue homeostasis during sterile tissue injury. On the other hand, tissue damage will lead to neutrophil infiltration and prolong their survival time, which will promote the release of inflammatory mediators, resulting in adjacent tissue damage.<sup>4</sup> In conclusion, neutrophils are critical to the maintenance of host health through regulating immune system, and their deficiency or dysfunction is detrimental to human health. Therefore, moderate regulation of neutrophil is essential for effective treatment of diseases.

## Neutrophil Activation and Migration

The migration of neutrophils is a key response after acute infection.<sup>6,7</sup> Mature neutrophils circulate in the body and monitor abnormal changes in real time, including pathogen-related molecular patterns (PAMPs), damp-related molecular patterns (DAMPs), or specific cytokines and chemokines (TNF- $\alpha$ , IL-6, IL-8, and chemokines of the CXCL family) that promote neutrophil secretion.<sup>8,9</sup> Once these pathogen-derived factors bind to G-protein-coupled receptors (GPCR) on neutrophils, neutrophils will be activated to migrate to the site of injury.

Neutrophil migration involves forward migration and reverse migration. Positive migration refers to the migration of neutrophils from the peripheral circulation to the inflammation site. The chemokines that cause neutrophil migration can be derived from both immune cells (neutrophils, macrophages, and T cells) and non-immune cells (epithelial and endothelial cells), mainly including the CXCL family (CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8), IL-6, IL-8, TNF- $\alpha$ , etc. They bind to receptors such as GPCR, CXCR1 and CXCR2,<sup>10,11</sup> and transmit signals to vasodilatation-stimulating phosphoproteins (VASP, PI3K and SFK) to mediate neutrophil migration.<sup>11,12</sup> In addition, lipids are also strong inducers of neutrophil chemotaxis. Leukotriene B4 (LTB4), a multi-enzyme pathway metabolite of arachidonic acid multienzyme pathway, plays an important role in the exponential growth of neutrophil recruitment. LTB4 can activate neutrophils to produce chemokines, such as CXCL2 and IL-1 $\beta$ , to amplify neutrophil recruitment<sup>13</sup> (Figure 1). N-formyl peptide can also stimulate neutrophils to produce more LTB4 and then recruit more neutrophils by activating ERK1, ERK2 and p38 mitogen-activated protein kinase signaling pathways.<sup>10</sup>

In contrast to forward migration, reverse migration accelerates the local regression of inflammation and alleviates tissue damage by removing neutrophils from the injured site.<sup>14</sup> Buckley et al demonstrated the reverse migrate ability of human neutrophils through the endothelial single-cell layer in vitro and identified the characteristic markers of these migrating neutrophils (ICAM1<sup>hi</sup>CXCR1<sup>low</sup>). Notably, neutrophils with this phenotype were found in the peripheral blood of patients with systemic inflammation,<sup>15</sup> suggesting that reverse migration may be a possible mechanism to solve local inflammation, and may also become a potential new target for the treatment of diseases characterized by excessive neutrophil infiltration. However, reverse neutrophil migration may lead to the redistribution of activated neutrophils to other parts of the body, leading to inflammation in other parts. Therefore, the exact mechanism of neutrophil reverse migration and its role in the treatment of human diseases still need further exploration.



**Figure 1** The recruitment of neutrophils to inflammatory sites and their functions in inflammation (By Figdraw). In the process of inflammation, vascular permeability increases, plasma exudation, blood flow viscosity increases, and blood flow velocity slows down. The larger and slower moving neutrophils are pushed away from the vascular axis to the edge of the blood vessel to achieve the edge set of neutrophils. Subsequently, under the action of cytokines released from the local infection site, neutrophils expressed L-selectin and sialylated Lewis X, which combined with a small amount of P-selectin and E-selectin expressed on the surface of endothelial cells with low affinity, mediated the rolling of neutrophils on the endothelial surface. During the rolling process, neutrophils are constantly stimulated by chemokines released from the inflammatory site, and the expression of integrins on their surface increases and gradually transforms into a high-affinity form. When it binds to the corresponding receptors on the endothelium, neutrophils are tightly adhered to the surface of endothelial cells and begin to change their cytoskeleton. Then, in the form of amoeba, it overflows from the endothelial gap to the inflammatory site, and begins to perform corresponding functions after activation. 1) Release of cytokines, in which leukotriene B4 released by neutrophils can amplify the recruitment and aggregation of neutrophils. 2) Phagocytosis, Neutrophils phagocytize microorganisms such as bacteria through Toll-like receptors on their cell surface, and phagocytize antigen-antibody complexes or antibodies through Fc receptors. 3) Degranulation. Under the stimulation of bacteria and antigen-antibody complexes, neutrophils can release particles containing various antibacterial proteolytic enzymes. 4) Respiratory burst, secondary to phagocytosis and release of neutrophil extracellular traps, neutrophils release a large amount of reactive oxygen species. 5) The release of neutrophil extracellular traps, activated neutrophils, can release a network structure with DNA as the skeleton, embedded with histones, myeloperoxidase (MPO), neutrophil elastase (NE), cathepsin G, calreticulin, protease 3 and other proteins with bactericidal and increased permeability. 6) Differentiation, there are different functions of neutrophils in different pathological and physiological conditions, called different subsets, there are differences between the karyotype.

## Neutrophil Subsets

Neutrophils exhibit substantial phenotypic and functional diversity under certain physiological and pathological conditions. This heterogeneity manifests through distinct subsets of neutrophils with unique phenotypic markers (Table 1). They can be divided into angiogenic neutrophils, polymorphonuclear myeloid derived suppressor cells (PMN-MDSCs), tumor-associated neutrophils (TANs), etc.<sup>16–18</sup> The naming of neutrophil subsets is mainly determined by their functional phenotype. Few specific surface markers can clearly distinguish neutrophils from different subsets. Previously, morphology of the nucleus was used as a marker to distinguish different functional granulocytes. Because the nucleus of the progenitor cells is horseshoe-shaped, and then the morphology of the nucleus begins to segment as the neutrophils continue to mature. For example, PMN-MDSCs have banded nuclei or circular nuclear structures.<sup>19</sup> Anti-tumor neutrophils (N1) have hypersegmented nuclei compared with tumor-promoting neutrophils (N2), which may be a feature to distinguish neutrophil subsets. But there is still no more evidence, and no transcription factors that polarize

**Table 1** Phenotypic Markers of Distinct Neutrophil Subsets

|           |    |  |
|-----------|----|--|
| PANs      |    | CD11b, CD16, Ly6G, VEGFA, BV8, CXCR2 <sup>21</sup>   |
| PMN-MDSCs |    | CCR2, CCR5, CD11b, CD38, CD39, CD40, CD43, CD45, CD54 <sup>lo</sup> , CD62L, CD73 <sup>hi</sup> , CD80 <sup>lo</sup> , CD86, CD98, CD115, CD120b <sup>lo</sup> , CD124, CD162, CD279, CX3CR1, PD-L1, Gr-1 <sup>hi</sup> , Ly6C <sup>lo</sup> , Ly6G <sup>hi</sup> , Mac-2 <sup>lo</sup> , Sca-19, VEGFR, CD13 <sup>hi</sup> , CD14, CD15, CD16 <sup>lo</sup> , CD33 <sup>lo</sup> , CD34 <sup>lo</sup> , CD38, CD39, CD45, CD66b, CD117, CXCR1, CXCR2, CXCR4, Lin <sup>lo</sup> , CD300ld <sup>16,22</sup> |
| TANs      | N1 | CCL3 <sup>hi</sup> , IL-1β <sup>hi</sup> , IL-12a <sup>hi</sup> , CD66b, CD11b, CD101, CD177, CD 54, CD170 <sup>lo</sup> , HLA-DR, CD86, CD15hi <sup>21,23</sup>   |
|           | N2 | Ly6G, CD206, IL-10, CD66b, CD11b, CD170 <sup>hi</sup> , PD-L1, <sup>21,24</sup>  |

them to perform specific functions have been identified in activated neutrophils. Therefore, it is not a consensus for identifying neutrophil phenotype with nuclear morphology.<sup>20</sup>

## Function of Neutrophils

Neutrophils are innate immune cells that play a central role in immune defense. In the early stages of infection, locally released chemotactic molecules attract neutrophils to migrate from the blood to the site of infection/injury and execute functions. Generally, neutrophils function by phagocytosis, degranulation, release of ROS and NETs<sup>24–26</sup> (Figure 1).

## Phagocytosis

The phagocytosis function of neutrophils can be classified into specific phagocytosis and non-specific phagocytosis. Specific phagocytosis mainly mediates endocytosis through toll-like receptors, scavenger receptor, Fc receptors, etc. by combining with ligands. Nonspecific phagocytosis of neutrophils is immediate and independent of receptor contact.<sup>27</sup>

The formation of phagosome is the key step of neutrophil phagocytosis. It is a complex process of structural changes of actin and skeleton network regulated by Ca<sup>2+</sup> and a variety of special functional proteins. When the pseudopodia of neutrophils capture bacteria and form phagosomes, part of the plasma membrane is invaginated, so that cytochromes b558, one of the NADPH oxidase subunits on the plasma membrane, can contact with another oxidase subunit in the cytoplasm and assemble into activated NADPH oxidase to start the aerobic sterilization process. In addition, after the formation of phagosomes, the phagosomes break away from the plasma membrane and enter the cytoplasm, then contact and fuse with neutrophil lysosome particles, triggering degranulation and initiating the non-oxygen sterilization process.<sup>28,29</sup> Phagosome formation is almost synchronized with the process of non-aerobic and aerobic sterilization, which cooperate with each other to complete the sterilization and digestion process.

## Degranulation

With the occurrence of phagocytosis, the degranulation of neutrophils is also initiated. Under the stimulation of pathogenic microbial products, bacteria, antigen and antibody complexes, neutrophils were activated and then triggering particle release, particle displacement, and fusion with phagosomes formed by plasma membrane invagination, and release various antibacterial proteolytic enzymes. This process is called degranulation.

Neutrophils contain many small scattered light red or light purple particles, which can be divided into azurophil particles, specific particles, gelatinase particles and secreting bubbles. These particles are rich in receptors, proteases, antimicrobial peptides and proteins, all of which have strong killing effect and can effectively exert antibacterial function. However, toxic particles can also lead to local tissue damage and serious systemic complications.<sup>26</sup> Studies have shown that a variety of pathways, such as camp-camp-dependent PKA, phospholipase -PKC, can regulate degranulation of neutrophils. And, there are complex interactions between multiple signaling pathways. For example, Bmal1 and CXCL2-CXCR2 signaling pathways can regulate degranulation, reduce particle content, self-killing ability and prevent excessive inflammation from causing vascular and tissue damage.<sup>26</sup> In addition, accumulating evidence indicates that degranulation also shows regularity and specificity in time, and the occurrence of degranulation marks the beginning of “respiratory outbreak”.<sup>30</sup>

## Production and Release of Reactive Oxygen Species (ROS)

The production of reactive oxygen species (ROS) in neutrophils plays an important role in host defense and inflammatory response. ROS are highly reactive chemicals formed by oxygen metabolism. Common ROS include peroxides, superoxides, hydroxyl radicals, and singlet oxygen. The production of ROS in neutrophils mainly begins with the activation of NADPH oxidase on the surface of phagocytic bodies, which deoxidize  $O_2$  to  $O_2^-$ , and then  $O_2^-$  rapidly is rapidly converted to  $H_2O_2$  through superoxide dismutase. These molecules can react with and oxidize many cellular components including lipids, proteins and nucleic acid and therefore can directly damage and kill bacteria.<sup>31</sup> In the presence of  $CL^-$ , MPO enzyme can catalyze  $H_2O_2$  to produce HOCL. HOCL is an effective fungicide, which exerts its killing toxicity by reacting with adjacent sulfhydryl groups and amino groups.<sup>32</sup> In conclusion,  $H_2O_2$ -MPO-HOCL system is a powerful tool for the host to resist microbial invasion, and the release of reactive oxygen species (ROS) plays an important role in inflammatory response. However, activated neutrophils also damage the surrounding normal tissues by releasing a large amount of superoxide while eliminating pathogens, leading to a variety of diseases related to reactive oxygen species metabolism.<sup>33</sup> Release of ROS during the human neutrophil respiratory burst is thought to be mandatory for effective defense against bacterial infections and may play a vital role in damage to host tissues. Part of the critical bacterial and host tissue damage has been attributed to hydroxyl radicals produced from superoxide and hydrogen peroxide.<sup>34</sup>

In the absence of NADPH oxidase, the mitochondria are an important alternate source of cellular ROS. Mitochondrial ROS (MitROS) are produced when electrons escaping from the electron transport chain are picked up by oxygen.<sup>35</sup> Neutrophils have an extensive mitochondrial network.<sup>36</sup> MitROS production in the context of infection has been mostly characterized in macrophages.<sup>37</sup> Recent studies have revealed that mitochondrial ROS production by neutrophils is required for host antimicrobial function against *Streptococcus pneumoniae* given that the NADPH oxidase is not required for host defense against these bacteria.<sup>38–40</sup>

## Neutrophil Extracellular Traps (NETs)

In 2004, Brinkmann et al firstly reported that neutrophils can also capture and kill pathogenic microorganisms by forming neutrophil extracellular traps (NETs).<sup>41</sup> This is a new unique feature besides to phagocytosis, degranulation and ROS.<sup>42</sup> NETs are a reticular structure released by activated neutrophils. By analyzing its structure, it is found that NETs are DNA-based skeletons, embedded with histones, myeloperoxidase (MPO), neutrophil elastase (NE), cathepsin G, calreticulin, proteinase 3 and other proteins with bactericidal and permeability increasing effects.<sup>41</sup>

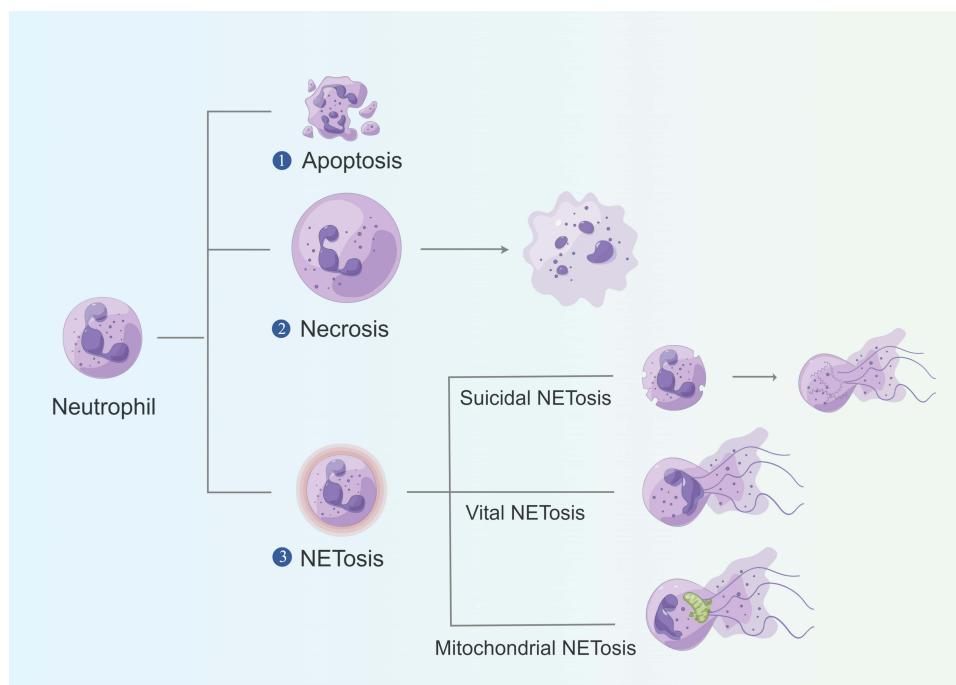
The production of NETs is affected by a variety of inducible factors. ROS, IL-8, LPS, complementary 5a (C5a), carbonic anhydrase inhibitor (CAI),  $\beta$ -glucan (BG), phorbol-12-myristate-13-acetate (PMA), glucose oxidase (GO) bacteria, DAMPs, and cytokines can significantly activate neutrophils to induce NETs formation through PRR (such as TLR2 or TLR4).<sup>43</sup> The formation of NETs was initially thought to require neutrophil death, and subsequently, it was found that NETs may also be released from living cells.<sup>44</sup> The released NETs capture and kill various pathogens by their unique three-dimensional network structure, and provide high -concentration antibacterial molecules locally, which can quickly control the growth of bacteria in the body and play an immune antibacterial role. The mechanism of NETs capturing pathogens remains unclear. It is currently believed that the charge attraction between positively charged NETs components and the negatively charged pathogen components is a possible mechanism. In addition to its antibacterial function, NETs can also play an immunomodulatory role through regulating the activation and differentiation of macrophages, dendritic cells and T cells.<sup>45</sup>

At present, the cellular signals that regulate the release of NETs are not yet fully understood. It has been reported that the release of NETs depends on the activation of PKC and RAF-MEK-ERK signaling pathways, increased intracellular calcium levels (for example, initiated by calcium ion vectors such as A23187 or ionomycin) and mitochondrial ROS production.<sup>1</sup>

## The Death Patterns of Neutrophil

As the first line of defense of the body's immunity, neutrophils will die after completing their biological functions. Depending on its mode of action, it may undergo different types of cell death, including apoptosis, necrosis and NETosis, involving the participation of multiple signaling pathways<sup>46</sup> (Figure 2).





**Figure 2** The way of neutrophil death (By Figdraw). 1. Apoptosis is a way of programmed cell death. It is mainly characterized by slender shape, pseudopod retraction, reduced cell volume, chromatin concentration, shrinkage and nuclear fragmentation, but no nuclear membrane rupture. 2. Necrosis is a pathological way of cell death. The first changes were cell swelling and organelle disintegration. Subsequently, the cell membrane ruptured, releasing a large amount of pro-inflammatory substances. 3. Neutrophils release extracellular traps with their own death (NETosis), which is also a way of programmed cell death. There are three different formation mechanisms, which are soluble NETosis (Suicidal NETosis), nuclear DNA release NETosis (Vital NETosis) and mitochondrial release NETosis (Mitochondrial NETosis). The DNA released in soluble NETosis comes from the nucleus and is accompanied by nuclear lysis. Vital NETosis (Vital NETosis) only loses its nuclear DNA, and some of its functions remain; the DNA released by mitochondrial-released NETosis comes from mitochondria.

## Apoptosis

Neutrophil apoptosis is a non-inflammatory cell death process of senescent neutrophil under the challenge of cytokines and growth factors, which is characterized by elongated shape, pseudopodia retraction, cell volume reduction, chromatin aggregation and nuclear fragmentation, but no rupture of nuclear membrane. This is the least damaging way of death to the surrounding tissue. Once neutrophils are differentiated and mature, they will initiate their spontaneous apoptosis process. The endocytosis of macrophages in liver, spleen and bone marrow can remove apoptotic neutrophils in the circulation to promote anti-inflammatory signal transduction, prevent neutrophil cytolysis and inhibit immune response.<sup>47</sup>

Neutrophil apoptosis is regulated by a variety of factors and associated with a variety of signaling pathways.<sup>48</sup> Here we mainly introduce the following two kinds: one is the mitochondrial pathway of apoptosis. The imbalance between pro-apoptotic factors and anti-apoptotic factors will promote cytochrome C release from mitochondria and cause the loss of mitochondrial membrane integrity, which in turn causes more cytochrome c to be released into the cytoplasm. Subsequently, cytochrome C promotes Apaf-1-containing apoptotic bodies to up-regulate caspase-9, eventually activating caspase-3 and leading to apoptosis. The binding of FasL, TRAIL, TNF- $\alpha$  to their receptors can drive caspase-8-dependent caspase-3 activation, thereby mediating apoptosis. The other is death receptor and ligand mediated apoptosis.<sup>49</sup> Spontaneous apoptosis of neutrophils is the key to the dissipation of infection or inflammation, and it is also an important way for the body to self-limit the inflammatory response, which is very important for the maintenance of homeostasis. Delaying or preventing neutrophil apoptosis can lead to neutrophil proliferation, which may be an important cause of chronic inflammatory disease.

## Necrosis

Adverse environmental conditions, such as lack of oxygen or essential nutrients, high temperature, toxic compounds, and external mechanical forces, may lead to cell necrosis. Protein denaturation and autolysis or isolyisin digestion after

necrosis,<sup>50-52</sup> can cause swelling of cytoplasmic organelles (mitochondria, endoplasmic reticulum and golgi apparatus), moderate the concentration of chromatin, release of cytochrome C and nucleosome DNA breakage in cytoplasm.<sup>50,53</sup>

The necrosis is mainly mediated by  $\text{Ca}^{2+}$  and ROS. During necrosis, increased  $\text{Ca}^{2+}$  levels in the cytoplasm lead to calcium overload in the mitochondria, which subsequently activates proteases and phospholipases to destroy the cell structure.<sup>54</sup> Meanwhile, reactive oxygen species attack intracellular lipids, proteins and DNA, resulting in mitochondrial dysfunction, ion imbalance and loss of membrane integrity, thereby further aggravating calcium overload in neutrophils. In addition, necrotic neutrophils can also activate NF- $\kappa$ B in adjacent cells (such as fibroblasts or macrophages), thereby stimulating the production of IL-6, IL-8, and TNF- $\alpha$ .<sup>54</sup> Necrosis of neutrophils will cause additional tissue damage or promote chronic inflammation, causing tissue damage.<sup>55,56</sup>

## NETosis

The process by which neutrophils release extracellular traps (NETs) accompanied by their own death is called NETosis. It is another way of programmed cell death different from apoptosis and necrosis. There are three different mechanisms responsible for NETosis formation, which are soluble NETosis, nuclear DNA- released NETosis and mitochondrial - released NETosis.

The released DNA in both soluble NETosis and nuclear DNA-released NETosis comes from the nucleus. The process of soluble NETosis is relatively slow and relies on reactive oxygen species produced by NADH oxidase. Neutrophil elastin (NE) located in nitrogenous granules is released into the cytoplasm under the action of ROS, and its proteolytic activity is obtained under the action of myeloperoxidase (MPO), and then translocated into the nucleus together with MPO to drive histone degradation and chromosome depolymerization.<sup>57</sup> Nuclear DNA-released NETosis is very rapid which is not dependent on ROS production, but through the activation of Toll-like receptor 2 and complement C3 receptors. At the same time, in nuclear DNA-released NETosis, neutrophils that lose nuclear DNA still retain some of their functions. The DNA released by mitochondrial- released NETosis comes from mitochondria, which depends on the presence of ROS.

NETosis requires activation of neutrophils. In addition to binding of plasma membrane surface receptors to corresponding ligands, positive feedback regulation also exists in excessive NETosis. The release of NETs caused by NETosis leads mucus to become more viscous, thereby promoting bacterial infection, recruiting more neutrophils and promoting further production of NETs. This is also the reason why the respiratory function of patients with lung diseases (such as ARDS) is affected and the severity of the disease is aggravated.<sup>58</sup> At present, there is no clear understanding of the inhibitors and regulating factors of NETosis.

## TNF- $\alpha$ , FasL, TRAIL and Other Ligands Regulate Neutrophils to Participate in the Development of Inflammatory Diseases

### Neutrophil and TNF- $\alpha$

TNF- $\alpha$ , also known as tumor necrosis factor  $\alpha$ , is one of the cytokines involved in acute inflammatory response. Neutrophils express both TNF receptor 1 (TNF-R1) and TNF receptor 2 (TNF-R2). TNF-R1 and TNF-R2 can trigger inflammatory signals, while only TNF-R1 can induce apoptosis.<sup>59</sup> At physiological concentration, TNF- $\alpha$  stimulation tends to induce inflammatory responses, while at high concentrations, it mainly plays a pro-apoptotic role. In addition, the stimulation time and the type of costimulatory molecules also effect the role of TNF- $\alpha$ .<sup>60,61</sup>

In addition to promoting neutrophil degranulation, phagocytosis,<sup>62</sup> phagocytosis,<sup>63</sup> releasing NETs and generating reactive oxygen species, TNF- $\alpha$  can also stimulate neutrophils to produce various cytokines, thereby amplifying inflammatory response.<sup>64</sup> The transmission of inflammatory signals is achieved by the recruitment of TRAF2, TRAF3, cIAP1 and cIAP2 by TNFR1, TRADD and RIP1 complex 1 or TNFR2, which ultimately activates NF - $\kappa$ B and JNK pathways.<sup>59</sup>

In addition, TNF- $\alpha$  is also involved in the process of programmed death of neutrophils. Its pro-apoptotic effect activates caspase by binding to TNFR1, recruiting RIP-1, TRADD, TRAF2, FADD, and procaspase-8 to form a death-inducing signal complex (DISC), thereby inducing neutrophil apoptosis.<sup>59</sup> Under normal circumstances, inhibition of caspase can

block TNF- $\alpha$ -mediated apoptosis. But, recent studies have shown that inhibition of caspase in neutrophils lacking X-linked IAP (XIAP), a member of the inhibitor of apoptosis protein (IAP) family, can transform TNF- $\alpha$  induced apoptosis into necroptosis dependent on receptor-interacting protein kinase-1 (RIPK3) and mixed lineage kinase domain-like protein (MLKL).<sup>65</sup> The most upstream signaling activity required for induction of necroptosis by a TNF ligand family member is the protein kinase function of receptor-interacting protein kinase-1 (RIPK1).<sup>66,67</sup> The protein kinase RIPK3 acts downstream of RIPK1 in the necroptotic signaling pathway. RIPK3 mediates necroptosis by binding MLKL. Phospho-MLKL then causes cell lysis.<sup>68,69</sup> In apoptosis, caspases cleave substrate proteins orchestrate the cell-death process. In pyroptosis, other caspases cleave and hence activate gasdermin-D (GSDMD), thus causing death.<sup>70</sup> GSDMD, which also regulates necroptosis, was upregulated in the NET pathway in the corneas of mice with ocular graft-versus-host disease.<sup>71</sup> In both necroptosis and pyroptosis, the cell membrane ruptures, releasing cellular components that may trigger inflammation.<sup>70</sup> Mitochondria do not appear to be involved in TNF- $\alpha$ -mediated neutrophil apoptosis compared with Fas/FasL-mediated apoptosis, but there is a clear correlation between ROS production and TNF- $\alpha$ -induced apoptosis.<sup>61</sup>

There are few studies on the relationship between TNF- $\alpha$  and neutrophil polarization. TNF- $\alpha$  can upregulate the expression of CD11b/CD18 (Mac-1) on the surface of neutrophils, thereby mediating the chemotaxis and adhesion of neutrophils to inflammatory sites.<sup>72</sup> Similarly, the bacteria-derived chemoattractant fMLP also showed the chemotaxis and adhesion of neutrophils *in vitro*. In contrast, neutrophils induced by fMLP show a high degree of polarization, while TNF- $\alpha$ -induced neutrophils did not polarize. Further studies showed that the pro-adhesion effect of TNF- $\alpha$  was weakened and the polarization of neutrophils was enhanced after blocking p38 MAPK with SB203580 or SB202190. It can be concluded that TNF- $\alpha$  inhibits the polarization of neutrophils through p38 MAPK pathway.<sup>73</sup>

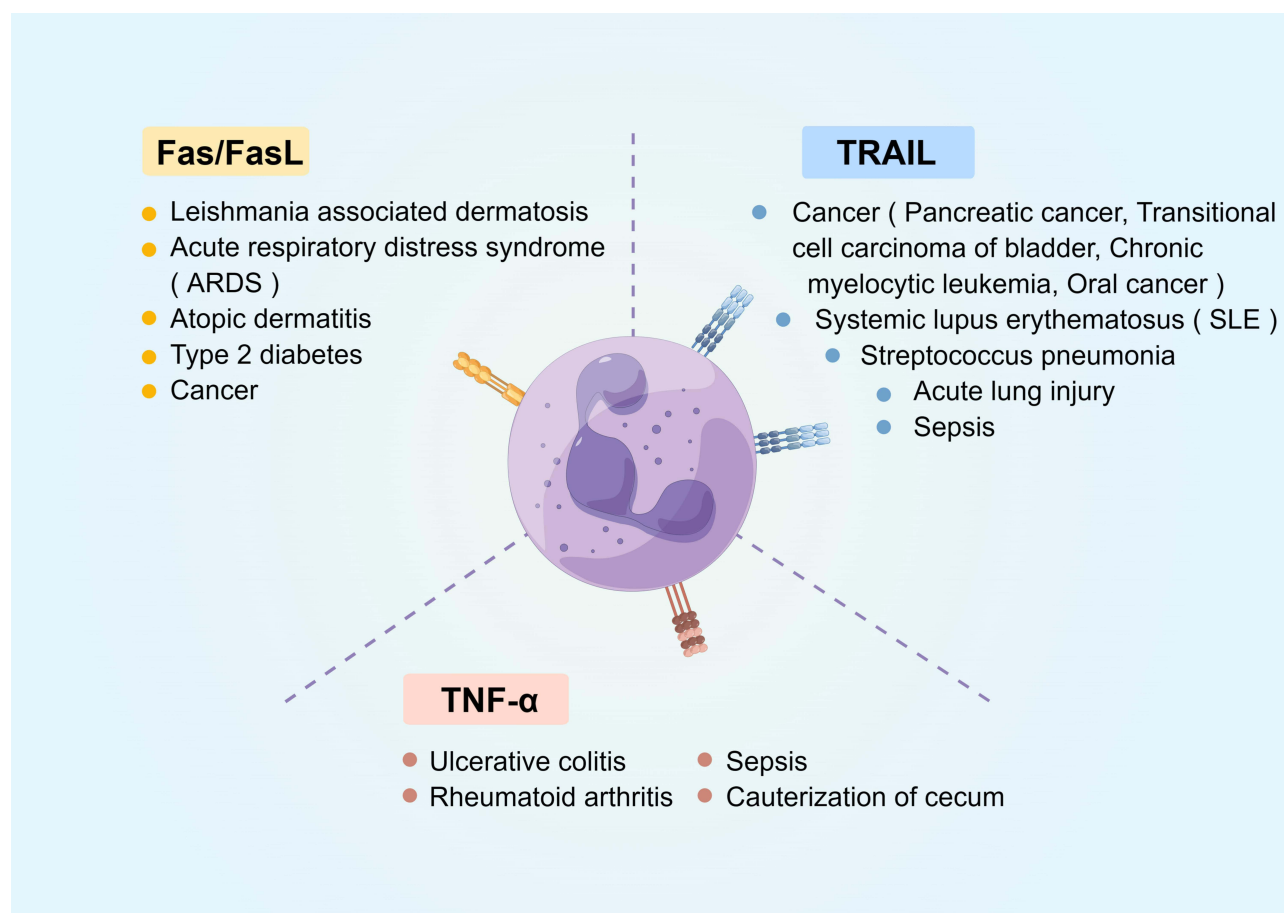
The regulation of TNF- $\alpha$  on neutrophils occurs in a variety of disease processes. In inflammatory diseases and injuries, such as cecal cauterization, the profibrotic ability of neutrophil NETs leads to postoperative adhesion. Preventive administration of TNF- $\alpha$  blocker etanercept can effectively reduce the release of neutrophil NETs after intestinal cauterization in mice and improve postoperative repair.<sup>74</sup> In ulcerative colitis, the release of NETs can be observed in neutrophils of patients with ulcerative colitis stimulated by TNF- $\alpha$ , and the same phenomenon can also be observed in neutrophils of healthy control group.<sup>75</sup> Notably, there seems to be a positive feedback relationship between TNF- $\alpha$  and the release of NETs. In the study of rheumatoid arthritis, the release of S100A11 was found to be dependent on NETosis, and the extracellular S100A11 also induced the release of TNF- $\alpha$  from neutrophils.<sup>76</sup> The increase of TNF- $\alpha$  secretion can be observed by stimulating the intestinal mucosal lamina propria mononuclear cells of patients with ulcerative colitis with NETs released by TNF- $\alpha$  stimulation.<sup>75</sup> In infectious diseases such as sepsis, TNF- $\alpha$  induces neutrophils to release exosomes by activating NF- $\kappa$ B pathway, and then targets SOCS-1 and SIRT1 through miR-30d-5p in exosomes to promote macrophage M1 polarization and pyroptosis, aggravating lung tissue injury<sup>77</sup> (Figure 3).

## Neutrophil and FasL

FasL appears on the membrane of activated T cells (CTL, NK), and the binding of FasL to Fas can induce apoptosis. FasL is available in membrane-bound (mFasL) and soluble (sFasL) forms. The extracellular region of mFasL can be detached from the cell surface to form sFasL under the action of metalloproteinases, while neutrophils constitutively express FasL.<sup>78,79</sup> In the past, it has been believed that FasL is only involved in the process of neutrophil apoptosis, but recent studies have shown that FasL is also involved in triggering neutrophil-mediated immune responses<sup>80</sup> (Figure 3).

FasL/Fas is involved in the aggregation of neutrophils to inflammatory sites in tumor grafts.<sup>81,82</sup> Subsequently, the gradient chemotactic model of sFasL on neutrophils further confirmed that sFasL has the chemotactic effect of neutrophils.<sup>83</sup> Notably, neutrophil infiltration was also observed in L5178Y-R lymphoma cells transfected with mFasL by intraperitoneal injection into DBA/2 mice, the chemotactic effects were still present in Fas knockout mice. It is speculated that the chemotactic effect of mFasL on neutrophils may be induced by some proinflammatory agents.<sup>84</sup> Peter J Dupont et al failed to detect the ROS upregulation of FasL-stimulated neutrophils.<sup>85</sup> On the contrary, FasL has also been reported to exert its pro-inflammatory effects via neutrophil recruitment but not activation.<sup>85</sup> Chen et al observed activation of mitogen-activated protein kinase in FasL-induced neutrophils.<sup>86</sup> At the same time, mRNA levels of NF- $\kappa$ B, caspase-1, IL-1  $\beta$  in neutrophils were increased in patients with type 2 diabetes induced by sFasL.<sup>87</sup>





**Figure 3** TNF- $\alpha$ , FasL and TRAIL are involved in inflammatory diseases by regulating neutrophils (By Figdraw). TNF- $\alpha$  is involved in the development of ulcerative colitis, sepsis, rheumatoid arthritis and postoperative repair of cecal cauterization by regulating neutrophil function. TRAIL is involved in the development of cancer (pancreatic cancer, bladder transitional cell carcinoma, chronic myeloid leukemia, ovarian cancer), systemic lupus erythematosus, acute lung injury, sepsis, streptococcal pneumonia by regulating neutrophil function. FasL is involved in the development of leishmaniasis-associated skin diseases, acute respiratory distress syndrome, type II diabetes, cancer, and atopic dermatitis by regulating neutrophil function.

FasL/Fas plays an important role in both spontaneous apoptosis of neutrophils and clearance of neutrophils during inflammation.<sup>87</sup> The pro-apoptotic effect of FasL/Fas has been proved to be caspase-dependent. However, recent experiments have shown that inhibition of caspase does not block FasL/Fas mediated apoptosis in mouse neutrophils, but leads to RIPK3-dependent necroptosis, which is not observed in human neutrophils.<sup>88</sup> In addition, it has been proved that mitochondria participate in the response of neutrophils to Fas apoptosis signal. In mice with knockout of Bcl-2 family member A1, neutrophils showed higher survival rate after FasL stimulation than wild-type mice,<sup>89</sup> while knockout of BID delayed FasL-mediated neutrophils apoptosis.<sup>88</sup>

In view of the multifaceted regulation of FasL/Fas on the physiological and pathological activities of neutrophils, it is closely related to a variety of inflammatory diseases, infectious diseases and even tumors. In tumors, FasL no longer plays a role in regulating the function of neutrophils, but regulates the expression of cell cycle checkpoint proteins by expressing on the surface of neutrophils and binding to Fas receptors expressed by tumor cells, leading to early cell cycle arrest, thereby achieving anticancer effects. In vitro, Fas antagonist can restore the growth activity of A549 cells in the co-culture system. Similarly, when Fas was knocked out in A549 cells, the anticancer effect of neutrophils was weakened. Therefore, FasL/Fas may be one of the anti-cancer mechanisms of neutrophils, and this anticancer effect is independent of pro-apoptotic effect of FasL/Fas.<sup>90</sup>

In addition, the expression of sFasL has been detected in the serum of patients with many inflammatory diseases, such as systemic lupus erythematosus, rheumatoid arthritis, Sjogren's syndrome, myocarditis, alcoholic liver disease,<sup>91</sup> and type 2 diabetes.<sup>87</sup> Increased levels of soluble FasL (sFasL) have been determined in various immunological and non-

immunological diseases, it has been suggested that sFasL might serve as a prognostic or diagnostic marker. Circulating sFasL levels predict the severity and outcome of burn injury,<sup>92–97</sup> tuberculosis, sepsis, lung cancer, spontaneous intracerebral hemorrhage, esophageal carcinomas, autoimmune thyroid disease. Serum levels of sFasL as a marker of thyroid dysfunction in children with autoimmune thyroid disease.<sup>97</sup> FasL/Fas has also been confirmed to play a role in a variety of diseases. For example, in atopic dermatitis, the dermal thickening and neutrophil infiltration observed in Fas or FasL knockout mice were more obvious than those in wild-type mice using (OVA) sensitized model mice. Therefore, FasL/Fas may alleviate the symptoms of atopic dermatitis by promoting neutrophil apoptosis.<sup>98</sup> In type 2 diabetes, FasL/Fas can enhance the inflammatory response of neutrophils without increasing apoptosis, thereby exacerbating disease damage.<sup>87</sup> Similarly, FasL/Fas is also responsible for lung tissue injury in acute respiratory distress syndrome.<sup>99</sup> In ulcerative dermatosis caused by *Leishmania* protozoa, FasL plays a role in chemotactic neutrophils to the site of infection. When FasL was neutralized, the number of neutrophils in the infected site decreased significantly and the local inflammatory response was alleviated.<sup>100</sup>

## Neutrophil and TRAIL

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), also called Apo2 ligand (Apo2L) is one of several members of the TNF gene superfamily that induces apoptosis engagement of death receptors.<sup>101</sup> It is found when searching for TNF homologous molecules from expressed sequence tags (ESTs). Compared with TNF- $\alpha$  and FasL, there is few study on the regulation of TRAIL on neutrophils. In the absence of inflammation, TRAIL is involved in maintaining the stable circulation number of neutrophils. Stromal cell-derived factor 1 (SDF-1) interacts with CXCR4 expressed on senescent neutrophils, promotes senescent neutrophils to return to bone marrow and upregulates the expression of TRAIL receptors, thereby increasing the sensitivity of neutrophils to TRAIL, and then completes the clearance of senescent neutrophils under the action of paracrine or autocrine TRAIL.<sup>102</sup>

In inflammatory diseases, the accumulation of neutrophils often aggravates tissue damage, and TRAIL can effectively remove infiltrating neutrophils in inflammatory tissues by inducing neutrophil apoptosis, thereby controlling the development of the disease. In mouse acute lung injury models, TRAIL has been proved to induce neutrophil apoptosis without damaging epithelial cells, thus reducing the damage of acute inflammation to lung tissues.<sup>103</sup> Similarly, in sepsis characterized by excessive inflammatory response, TRAIL promotes neutrophil apoptosis in the later stage of disease development, thereby inhibiting inflammation and protecting organs from damage.<sup>104</sup> In systemic lupus erythematosus (SLE), the killing effect of TRAIL on neutrophils may be the culprit of the disease. Studies have shown that apoptosis of neutrophils may be one of the causes of SLE. The serum of young SLE patients was incubated with normal neutrophils, showing a higher neutrophils apoptosis rate, and the serum TRAIL level of patients was also significantly increased (Figure 3). These results suggest that TRAIL may be involved in the process of abnormal apoptosis of neutrophils in SLE patients.<sup>105</sup>

In infectious diseases and tumors, TRAIL, as a weapon of neutrophils, plays a role in resisting invasive pathogens and maintaining the normal function of the body. Neutrophils inhibit the replication of murine cytomegalovirus by expressing membrane-bound TRAIL to kill fibroblasts.<sup>106</sup> TRAIL is also a powerful tool for neutrophils in pancreatic cancer,<sup>107</sup> bladder cancer,<sup>108</sup> oral cancer,<sup>109</sup> and chronic myelogenous leukemia.<sup>110</sup> A new treatment that stimulates neutrophils to release TRAIL and achieve anti-tumor effects has been applied in the treatment of transitional bladder cancer. Experiments have proved that BCG injection can directly stimulate neutrophils to release TRAIL, which plays a role in the treatment of transitional bladder cancer.<sup>72</sup> In addition, neutrophils release NETs under the stimulation of tumor-derived IL-8 (Figure 3). And the TRAIL-modified NETs demonstrated a more powerful killing effect for tumors without affecting the sterilization effect.<sup>111</sup>

## Neutrophil and Others

PD-L1 is a ligand for programmed death protein 1 (PD-1), and its expression has been shown to be upregulated in neutrophils harvested from septic patients. PD-L1 is a suppressor molecule expressed on hematopoietic and nonhematopoietic cells that helps maintain T-cell homeostasis, but it is upregulated in tumor cells, which helps to immune escape.<sup>112</sup> PD-L1 activation inhibits neutrophil apoptosis, whereas PD-1 activation promotes apoptosis of T lymphocytes,

which leads to immunosuppression.<sup>113</sup> Increased PD-L1 expression on neutrophils has an important relationship with poor prognosis of patients with sepsis. PD-L1 overexpression on neutrophils delays cellular apoptosis through triggering PI3K-dependent AKT phosphorylation to drive lung injury and increase mortality during clinical and experimental sepsis.<sup>114</sup> Single-cell RNA sequencing revealed that multiple subclusters of neutrophils were differentiated after lipopolysaccharide (LPS) stimulation. Further observations showed that LPS mediates PD-L1 over expression through p38 $\alpha$ -MSK1/-MK2 pathway in neutrophils.<sup>115</sup> NETs were generated by neutrophils stimulated with phorbol 12-myristate 13-acetate (PMA) or LPS.<sup>116</sup> LPS markedly increased neutrophil infiltration in lungs and inflammatory cytokines in bronchoalveolar lavage fluid.<sup>117</sup>

Integrins are critical for neutrophil functions, especially for their recruitment to sites of inflammation or infections,<sup>118</sup> as their activation and related signaling pathways mediate neutrophil arrest, trans-endothelium migration, and in-tissue migration to the site of infection or inflammation.<sup>118</sup> Integrin activation is required for neutrophil functions such as crawling, and migration during vascular inflammation.<sup>119</sup> Impaired integrin activation on neutrophils is the hallmark of leukocyte adhesion deficiency syndrome in humans.<sup>120</sup>

## Conclusions

Apoptosis inducing ligand TNF- $\alpha$ , FasL and TRAIL can programmatically induce neutrophil apoptosis, regulate neutrophil recruitment / infiltration and exercise functions, and participate in the process of infectious or sterile diseases. Apoptosis-inducing ligands are the key targets of drug development. How to deal with the relationship between the ligands and neutrophils is very important for the treatment of different diseases. Although great breakthroughs have been made in the functional research of apoptosis-inducing ligands, the side effects in clinical application should not be ignored. The regulation of apoptosis-induced ligands on neutrophils should be further clarified to provide new ideas and therapeutic strategies for the treatment of inflammatory diseases.

## Abbreviations

BG,  $\beta$ -glucan; C5a, Complementary 5a; CaI, Carbonic anhydrase inhibitor; DAMPs, Damp-related molecular patterns; DISC, Death-inducing signal complex; GO, Glucose oxidase; GPCR, G-protein-coupled receptors; GSDMD, gasdermin-D; LPS, lipopolysaccharide; MLKL, mixed lineage kinase domain-like protein; MPO, Myeloperoxidase; NE, Neutrophil elastin; NETs, Neutrophil extracellular traps; PAMPs, Pathogen-related molecular patterns; PANs, Angiogenic neutrophils; PD-1, programmed death protein 1; PMA, Phorbol-12-myristate-13-acetate; PMNs, polymorphonuclear leukocytes; RIPK1/3, receptor-interacting protein kinase-1/3; ROS, Reactive oxygen species; SLE, Systemic lupus erythematosus; TRAIL, Tumor necrosis factor-related apoptosis-inducing ligand.

## Acknowledgment

The authors would like to express their gratitude to Figdraw ([www.figdraw.com/static/index.html](http://www.figdraw.com/static/index.html)) for the expert graphic services provided.

## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

## Funding

This work was supported by the Science and Technology Project of Henan Province (232102311055 to Dan Zhao and 252102310245 to Zhiming Song), the Key Scientific Research Projects of Medicine in Henan Province (SBGJ202402083 to Zhiming Song) and the Science and Technology Project of Kaifeng City (2207001 to Zhiming Song).

## Disclosure

The authors declare that there are no conflicts of interest in this work.

## References

- Klopf J, Brostjan C, Eilenberg W, Neumayer C. Neutrophil extracellular traps and their implications in cardiovascular and inflammatory disease. *Int J mol Sci*. 2021;22(2):559. doi:10.3390/ijms22020559
- Carlos S-R, Braster Q, Ortega-Gomez A, Soehnlein O. Neutrophils as regulators of cardiovascular inflammation. *Nat Rev Cardiol*. 2020;17(6):327–340. doi:10.1038/s41569-019-0326-7
- Zhang G. Tumor necrosis factor family ligand-receptor binding. *Curr Opin Struct Biol*. 2004;14(2):154–160. doi:10.1016/j.sbi.2004.03.003
- Hirota Y, Hirohata T, Fukuda K. Association of alcohol intake, cigarette smoking, and occupational status with the risk of idiopathic osteonecrosis of the femoral head. *Am J Epidemiol*. 1993;137(5):530–538. doi:10.1093/oxfordjournals.aje.a116706
- Castanheira F, Kubes P. Neutrophils and NETs in modulating acute and chronic inflammation. *Blood*. 2019;133(20):2178–2185. doi:10.1182/blood-2018-11-844530
- Peveri P, Walz A, Dewald B, Baggiolini M. A novel neutrophil-activating factor produced by human mononuclear phagocytes. *J Exp Med*. 1988;167(5):1547–1559. doi:10.1084/jem.167.5.1547
- Yoshimura T, Matsushima K, Tanaka S, et al. Purification of a human monocyte-derived neutrophil chemotactic factor that has peptide sequence similarity to other host defense cytokines. *Proc Natl Acad Sci USA*. 1987;84(24):9233–9237. doi:10.1073/pnas.84.24.9233
- Oliveira C, Navarro-Xavier R, Anjos-Vallota E, et al. Effect of plant neutrophil elastase inhibitor on leucocyte migration, adhesion and cytokine release in inflammatory conditions. *Br J Pharmacol*. 2010;161(4):899–910. doi:10.1111/j.1476-5381.2010.00924.x
- Bae G, Lee H, Jung Y, et al. Identification of novel peptides that stimulate human neutrophils. *Exp Mol Med*. 2012;44(2):130–137. doi:10.3858/emmm.2012.44.2.008
- Hazeldine J, Hampson P, Opoku F, Foster M, Lord J. N-Formyl peptides drive mitochondrial damage associated molecular pattern induced neutrophil activation through ERK1/2 and P38 MAP kinase signalling pathways. *Injury*. 2015;46(6):975–984. doi:10.1016/j.injury.2015.03.028
- Russo R, Garcia C, Teixeira M, Amaral F. The CXCL8/IL-8 chemokine family and its receptors in inflammatory diseases. *Expert Rev Clinical Immunol*. 2014;10(5):593–619. doi:10.1586/1744666x.2014.894886
- Neel N, Barzik M, Raman D, et al. VASP is a CXCR2-interacting protein that regulates CXCR2-mediated polarization and chemotaxis. *J Cell Sci*. 2009;122(11):1882–1894. doi:10.1242/jcs.039057
- Richard CC, Kim ND, Sadik CD. Lipid-cytokine-chemokine cascade drives neutrophil recruitment in a murine model of inflammatory arthritis. *Immunity*. 2010;33(2):266–278. doi:10.1016/j.immuni.2010.07.018
- Oliver S, Lindbom L. Phagocyte partnership during the onset and resolution of inflammation. *Nat Rev Immunol*. 2010;10(6):427–439. doi:10.1038/nri2779
- Christopher DB, Ross EA, McGettrick HM. Identification of a phenotypically and functionally distinct population of long-lived neutrophils in a model of reverse endothelial migration. *J Leukocyte Biol*. 2006;79(2):303–311. doi:10.1189/jlb.0905496
- Gabrilovich DI. Myeloid-Derived Suppressor Cells. *Cancer Immunol Res*. 2017;5(1):3–8. doi:10.1158/2326-6066.CIR-16-0297
- Shi X, Pang S, Zhou J, Yan G, Sun J, Tan W. Feedback loop between fatty acid transport protein 2 and receptor interacting protein 3 pathways promotes polymorphonuclear neutrophil myeloid-derived suppressor cells-potentiated suppressive immunity in bladder cancer. *Mol Biol Rep*. 2022;49(12):11643–11652. doi:10.1007/s11033-022-07924-x
- Zhou J, Jiang S, Wang W, Liu R. Research progress of tumor-associated neutrophils and lung cancer. *Zhongguo Fei Ai Za Zhi*. 2019;22(11):727–731. doi:10.3779/j.issn.1009-3419.2019.11.07
- Greifenberg V, Ribechini E, Rossner S, Lutz MB. Myeloid-derived suppressor cell activation by combined LPS and IFN-treatment impairs DC development. *Eur J Immunol*. 2009;39(10):2865–2876. doi:10.1002/eji.200939486
- Buckley CD, Ross EA, McGettrick HM, et al. Identification of a phenotypically and functionally distinct population of long-lived neutrophils in a model of reverse endothelial migration. *J Leukoc Biol*. 2006;79(2):303–311. doi:10.1189/jlb.0905496
- Herro R, Grimes HL. The diverse roles of neutrophils from protection to pathogenesis. *Nat Immunol*. 2024;25(12):2209–2219. doi:10.1038/s41590-024-02006-5
- Wang C, Zheng X, Zhang J, et al. CD300ld on neutrophils is required for tumour-driven immune suppression. *Nature*. 2023;621(7980):830–839. doi:10.1038/s41586-023-06511-9
- Zilionis R, Engblom C, Pfirschke C, et al. Single-cell transcriptomics of human and mouse lung cancers reveals conserved myeloid populations across individuals and species. *Immunity*. 2019;50(5):1317–1334e10. doi:10.1016/j.immuni.2019.03.009
- Chiang CC, Cheng WJ, Korinek M, Lin CY, Hwang TL. Neutrophils in Psoriasis. *Front Immunol*. 2019;10:2376. doi:10.3389/fimmu.2019.02376
- Aitken EH, Alemu A, Rogerson SJ. Neutrophils and Malaria. *Front Immunol*. 2018;9:3005. doi:10.3389/fimmu.2018.03005
- Rui C. The critical role of cell metabolism for essential neutrophil functions. *Cell Physiol Biochem*. 2020;54(4):629–647. doi:10.33594/000000245
- Kobayashi S, DeLeo F. Role of neutrophils in innate immunity: a systems biology-level approach. *Wiley Interdiscip Rev Syst Biol Med*. 2009;1(3):309–333. doi:10.1002/wsbm.32
- Shephard R, Shek P. Autoimmune disorders, physical activity, and training, with particular reference to rheumatoid arthritis. *Exercise Immunol Rev*. 1997;3:53–67.
- van Rees D, Szilagyi K, Kuijpers T, Matlung H, van den Berg T. Immunoreceptors on neutrophils. *Semin Immunopathol*. 2016;28(2):94–108. doi:10.1016/j.smim.2016.02.004
- P J, den Braber I, Vrsek N. In vivo labeling with  $^2\text{H}_2\text{O}$  reveals a human neutrophil lifespan of 5.4 days. *Blood J Am Soc Hematol*. 2010;116(4):625–627. doi:10.1182/blood-2010-01-259028
- Nguyen GT, Green ER, Mecsas J. Neutrophils to the ROScues: mechanisms of NADPH Oxidase Activation and Bacterial Resistance. *Front Cell Infect Microbiol*. 2017;7:373. doi:10.3389/fcimb.2017.00373

32. Pham C. Neutrophil serine proteases fine-tune the inflammatory response. *Int J Biochem Cell Biol.* 2008;40(6–7):1317–1333. doi:10.1016/j.biocel.2007.11.008
33. Winterbourn C, Kettle A, Hampton M. Reactive oxygen species and neutrophil function. *Annu. Rev. Biochem.* 2016;85(1):765–792. doi:10.1146/annurev-biochem-060815-014442
34. Samuni A, Black CD, Krishna CM, Malech HL, Bernstein EF, Russo A. Hydroxyl radical production by stimulated neutrophils reappraised. *J Biol Chem.* 1988;263(27):13797–13801. doi:10.1016/S0021-9258(18)68313-9
35. Zorov DB, Juhaszova M, Sollott SJ. Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. *Physiol Rev.* 2014;94(3):909–950. doi:10.1152/physrev.00026.2013
36. Fossati G, Moulding DA, Spiller DG, Moots RJ, White MR, Edwards SW. The mitochondrial network of human neutrophils: role in chemotaxis, phagocytosis, respiratory burst activation, and commitment to apoptosis. *J Immunol.* 2003;170(4):1964–1972. doi:10.4049/jimmunol.170.4.1964
37. Shekhova E. Mitochondrial reactive oxygen species as major effectors of antimicrobial immunity. *PLoS Pathog.* 2020;16(5):e1008470. doi:10.1371/journal.ppat.1008470
38. Herring SE, Mao S, Bhalla M, Tchalla EYI, Kramer JM, Bou Ghanem EN. Mitochondrial ROS production by neutrophils is required for host antimicrobial function against *Streptococcus pneumoniae* and is controlled by A2B adenosine receptor signaling. *PLoS Pathog.* 2022;18(11):e1010700. doi:10.1371/journal.ppat.1010700
39. Deniset JF, Surewaard BG, Lee WY, Kubes P. Splenic Ly6G(high) mature and Ly6G(int) immature neutrophils contribute to eradication of *S. pneumoniae*. *J Exp Med.* 2017;214(5):1333–1350. doi:10.1084/jem.20161621
40. Standish AJ, Weiser JN. Human neutrophils kill *Streptococcus pneumoniae* via serine proteases. *J Immunol.* 2009;183(4):2602–2609. doi:10.4049/jimmunol.0900688
41. Volker B, Reichard U, Goosmann C. Neutrophil extracellular traps kill bacteria. *Science.* 2004;303(5663):1532–1535. doi:10.1126/science.1092385
42. Thiam H, Wong S, Wagner D, Waterman C. Cellular mechanisms of NETosis. *Annu Rev Cell Dev Biol.* 2020;36(1):191–218. doi:10.1146/annurev-cellbio-020520-111016
43. Sung PS, Peng YC, Chiu SP, Chiu CH, Hsieh SL. CLEC5A is critical in *Pseudomonas aeruginosa*-induced NET formation and acute lung injury. *JCI Insight.* 2022;7(18):156613. doi:10.1172/jci.insight.156613
44. Fuchs T, Abed U, Goosmann C, et al. Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol.* 2007;176(2):231–241. doi:10.1083/jcb.200606027
45. Dömer D, Walther T, Möller S, Behnen M, Laskay T. Neutrophil extracellular traps activate proinflammatory functions of human neutrophils. *Front Immunol.* 2021;12:636954. doi:10.3389/fimmu.2021.636954
46. Dąbrowska D, Jabłońska E, Iwaniuk A, Garley M. Many ways-one destination: different types of neutrophils death. *Int Rev Immunol.* 2019;38(1):18–32. doi:10.1080/08830185.2018.1540616
47. Greenlee-Wacker MC. Clearance of apoptotic neutrophils and resolution of inflammation. *Immunol Rev.* 2016;273(1):357–370. doi:10.1111/imr.12453
48. Noseykina E, Schepetkin I, Atochin D. Molecular mechanisms for regulation of neutrophil apoptosis under normal and pathological conditions. *J Evolutionary Biochem Physiol.* 2021;57(3):429–450. doi:10.1134/s0022093021030017
49. María Laura G, Trevani AS, Sabatté J, Geffner J. Mechanisms regulating neutrophil survival and cell death. *Semin Immunopathol.* 2013;35(4):423–437. doi:10.1007/s00281-013-0364-x
50. Vanden Berghe T, Vanlangenakker N, Parthoens E, et al. Necroptosis, necrosis and secondary necrosis converge on similar cellular disintegration features. *Cell Death Differ.* 2010;17(6):922–930. doi:10.1038/cdd.2009.184
51. Krysko D, Vanden Berghe T, Parthoens E, D’Herde K, Vandenabeele P. Methods for distinguishing apoptotic from necrotic cells and measuring their clearance. *Methods Enzymol.* 2008;442:307–341. doi:10.1016/s0076-6879(08)01416-x
52. Krysko D, Vanden Berghe T, D’Herde K, Vandenabeele P. Apoptosis and necrosis: detection, discrimination and phagocytosis. *Methods.* 2008;44(3):205–221. doi:10.1016/j.ymeth.2007.12.001
53. Golstein P, Kroemer G. Cell death by necrosis: towards a molecular definition. *Trends Biochem Sci.* 2007;32(1):37–43. doi:10.1016/j.tibs.2006.11.001
54. Li M, Carpio D, Zheng Y, et al. An essential role of the NF- $\kappa$ B/Toll-like receptor pathway in induction of inflammatory and tissue-repair gene expression by necrotic cells. *J Immunol.* 2001;166(12):7128–7135. doi:10.4049/jimmunol.166.12.7128
55. Savill J, Henson P, Haslett C. Phagocytosis of aged human neutrophils by macrophages is mediated by a novel “charge-sensitive” recognition mechanism. *J Clin Invest.* 1989;84(5):1518–1527. doi:10.1172/jci114328
56. Rydell-Törmänen K, Uller L, Erjefält J. Direct evidence of secondary necrosis of neutrophils during intense lung inflammation. *Europ Resp J.* 2006;28(2):268–274. doi:10.1183/09031936.06.00126905
57. Lily C. The roles of neutrophils in cytokine storms. *Viruses.* 2021;13(11):2318. doi:10.3390/v13112318
58. de Boer O, Li X, Teeling P, et al. Neutrophils, neutrophil extracellular traps and interleukin-17 associate with the organisation of thrombi in acute myocardial infarction. *Thrombosis Haemostasis.* 2013;109(2):290–297. doi:10.1160/th12-06-0425
59. Futosi K, Fodor S, Mocsa A. Neutrophil cell surface receptors and their intracellular signal transduction pathways. *Int Immunopharmacol.* 2013;17(3):638–650. doi:10.1016/j.intimp.2013.06.034
60. van den Berg JM, Weyer S, Weening JJ, et al. Divergent effects of tumor necrosis factor on a poptosis of human neutrophils.pdf>. *J Leukocyte Biol.* 2001;69:1.
61. Geering B, Simon HU. Peculiarities of cell death mechanisms in neutrophils. *Cell Death Differ.* 2011;18(9):1457–1469. doi:10.1038/cdd.2011.75
62. Brandt E, Petersen F, Flad H. Recombinant tumor necrosis factor- $\alpha$  potentiates neutrophil degranulation in response to host defense cytokines neutrophil-activating peptide 2 and IL-8 by modulating intracellular cyclic AMP levels. *J Immunol.* 1992;149(4):1356–1364. doi:10.4049/jimmunol.149.4.1356
63. Lu Y, Huang C, Huang Y, et al. Tumor necrosis factor  $\alpha$ -dependent neutrophil priming prevents intestinal ischemia/reperfusion-induced bacterial translocation. *Dig Dis Sci.* 2017;62(6):1498–1510. doi:10.1007/s10620-017-4468-3



64. Ferrante A. Tumor necrosis factor alpha potentiates neutrophil antimicrobial activity: increased fungicidal activity against *torulopsis glabrata* and *candida albicans* and associated increases in oxygen radical production and lysosomal enzyme release. *Infect Immun.* 1989;57(7):2115–2122. doi:10.1128/iai.57.7.2115-2122.1989
65. Wicki S, Gurzeler U, Wei-Lynn Wong W, Jost P, Bachmann D, Kaufmann T. Loss of XIAP facilitates switch to TNF $\alpha$ -induced necroptosis in mouse neutrophils. *Cell Death Dis.* 2016;7(10):e2422. doi:10.1038/cddis.2016.311
66. Holler N, Zaru R, Micheau O, et al. Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. *Nat Immunol.* 2000;1(6):489–495. doi:10.1038/82732
67. Degterev A, Hitomi J, Gemscheid M, et al. Identification of RIP1 kinase as a specific cellular target of necrostatins. *Nat Chem Biol.* 2008;4(5):313–321. doi:10.1038/nchembio.83
68. Cho YS, Challa S, Moquin D, et al. Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. *Cell.* 2009;137(6):1112–1123. doi:10.1016/j.cell.2009.05.037
69. He S, Wang L, Miao L, et al. Receptor interacting protein kinase-3 determines cellular necrotic response to TNF- $\alpha$ . *Cell.* 2009;137(6):1100–1111. doi:10.1016/j.cell.2009.05.021
70. Wallach D, Kang TB, Dillon CP, Green DR. Programmed necrosis in inflammation: toward identification of the effector molecules. *Science.* 2016;352(6281):aaf2154. doi:10.1126/science.aaf2154
71. Asai K, Lee HK, Sato S, et al. The necroptosis pathway is upregulated in the cornea in mice with ocular graft-versus-host disease. *Invest Ophthalmol Vis Sci.* 2024;65(10):38. doi:10.1167/iovs.65.10.38
72. Simons MP, O'Donnell MA, Griffith TS. Role of neutrophils in BCG immunotherapy for bladder cancer. *Urol Oncol Available in PMC.* 2009;26:341–345.
73. Lokuta MA, Huttenlocher A. TNF- $\alpha$  promotes a stop signal that inhibits neutrophil polarization and migration via a p38 MAPK pathway. *J Leukoc Biol.* 2005;78(1):210–219. doi:10.1189/jlb.0205067
74. Sudo M, Iida K, Tsutsui H, et al. Blockade of tumor necrosis factor by etanercept prevents postoperative adhesion formation in mice. *Cellular Physiol Biochem.* 2020;54(5):1041–1053. doi:10.33594/0000000286
75. Navrátilová A, Bečvář V, Baloun J, et al. S100A11 (calgizzarin) is released via NETosis in rheumatoid arthritis (RA) and stimulates IL-6 and TNF secretion by neutrophils. *Sci Rep.* 2021;11(1):6063. doi:10.1038/s41598-021-85561-3
76. Dinallo V, Marafini I, Di Fusco D, et al. Neutrophil extracellular traps sustain inflammatory signals in ulcerative colitis. *J Crohn's Colitis.* 2019;13(6):772–784. doi:10.1093/ecco-jcc/jjy215
77. Jiao Y, Zhang T, Zhang C, et al. Exosomal miR-30d-5p of neutrophils induces M1 macrophage polarization and primes macrophage pyroptosis in sepsis-related acute lung injury. *Crit Care.* 2021;25(1):356. doi:10.1186/s13054-021-03775-3
78. Gregory-Ksander M, Marshak-Rothstein A. The FasLane to ocular pathology-metalloproteinase cleavage of membrane-bound FasL determines FasL function. *J Leukoc Biol.* 2021;110(5):965–977. doi:10.1002/JLB.3RI1220-834R
79. Liles WC, Kiener PA, Ledbetter JA, Aruffo A, Klebanoff SJ. Differential expression of Fas (CD95) and Fas ligand on normal human phagocytes: implications for the regulation of apoptosis in neutrophils. *J Exp Med.* 1996;184(2):429–440. doi:10.1084/jem.184.2.429
80. Jaber BL, Perianayagam MC, Balakrishnan VS, King AJ, Pereira BJ. Mechanisms of neutrophil apoptosis in uremia and relevance of the Fas (APO-1, CD95)/Fas ligand system. *J Leukoc Biol.* 2001;69(6):1006–1012. doi:10.1189/jlb.69.6.1006
81. Arai H, Gordon D, Nabel E, Nabel G. Gene transfer of Fas ligand induces tumor regression in vivo. *Proc Natl Acad Sci USA.* 1997;94(25):13862–13867. doi:10.1073/pnas.94.25.13862
82. Seino K, Kayagaki N, Okumura K, Yagita H. Antitumor effect of locally produced CD95 ligand. *Nature Med.* 1997;3(2):165–170. doi:10.1038/nm0297-165
83. Ottonello L, Tortolina G, Amelotti M, Dallegri F. Soluble Fas ligand is chemotactic for human neutrophilic polymorphonuclear leukocytes. *J Immunol.* 1999;162(6):3601–3606. doi:10.4049/jimmunol.162.6.3601
84. Hohlbaum A, Moe S, Marshak-Rothstein A. Opposing effects of transmembrane and soluble Fas ligand expression on inflammation and tumor cell survival. *J Exp Med.* 2000;191(7):1209–1220. doi:10.1084/jem.191.7.1209
85. Dupont PJ, Warren AN. Fas ligand exerts its pro-inflammatory effects via neutrophil recruitment but not activation. *Immunology.* 2007;120(1):133–139. doi:10.1111/j.1365-2567.2006.02504.x
86. Chen -J-J, Sun Y, Nabel GJ. Regulation of the proinflammatory effects of Fas ligand (CD95L). *Science.* 1998;282(5394):1714–1717. doi:10.1126/science.282.5394.1714
87. Margaryan S, Witkowitz A, Arakelyan A, Partyka A, Karabon L, Manukyan G. sFasL-mediated induction of neutrophil activation in patients with type 2 diabetes mellitus. *PLoS One.* 2018;13(7):e0201087. doi:10.1371/journal.pone.0201087
88. Wicki S, Gurzeler U, Corazza N, Genitsch V, Wong W, Kaufmann T. Loss of Bid delays FASL-induced cell death of mouse neutrophils and aggravates dss-induced weight loss. *Int J mol Sci.* 2018;19(3):684. doi:10.3390/ijms19030684
89. Schenk R, Gangoda L, Lawlor K, O'Reilly L, Strasser A, Herold M. The pro-survival Bcl-2 family member A1 delays spontaneous and FAS ligand-induced apoptosis of activated neutrophils. *Cell Death Dis.* 2020;11(6):474. doi:10.1038/s41419-020-2676-9
90. Sun B, Qin W, Song M, et al. Neutrophil suppresses tumor cell proliferation via fas /fas ligand pathway mediated cell cycle arrested. *Int J Biol Sci.* 2018;14(14):2103–2113. doi:10.7150/ijbs.29297
91. Seino K, Iwabuchi K, Kayagaki N, et al. Chemotactic activity of soluble Fas ligand against phagocytes. *J Immunol.* 1998;161(9):4484–4488. doi:10.4049/jimmunol.161.9.4484
92. Kozłowski M, Kowalczyk O, Sulewska A, et al. Serum soluble Fas ligand (sFasL) in patients with primary squamous cell carcinoma of the esophagus. *Folia Histochem Cytobiol.* 2007;45(3):199–204.
93. Lin JC, Chen ZH, Chen XD, Wang SB. Circulating sFasL levels predict the severity and outcome of burn injury: a prospective observational study. *J Surg Res.* 2021;265:1–10. doi:10.1016/j.jss.2021.01.012
94. Lorente L, Martin MM, Ortiz-Lopez R, et al. Association between serum sFasL concentrations and sepsis mortality. *Infect Dis.* 2021;53(1):38–43. doi:10.1080/23744235.2020.1819560
95. Lorente L, Martin MM, Ortiz-Lopez R, et al. Serum sFasL concentrations and mortality prediction in patients with sepsis. *Infect Dis.* 2021;53(8):643–646. doi:10.1080/23744235.2021.1901982

96. Lorente L, Martin MM, Perez-Cejas A, et al. Mortality prediction of patients with spontaneous intracerebral hemorrhage by serum soluble Fas ligand concentrations. *Expert Rev Mol Diagn.* 2022;22(2):233–238. doi:10.1080/14737159.2022.2017775
97. Mikos H, Mikos M, Niedziela M. Diagnostic significance of serum concentrations of soluble Fas ligand (sFasL) in children with autoimmune thyroid disease. *Autoimmunity.* 2017;50(3):192–198. doi:10.1080/08916934.2017.1289180
98. Bieñ K, Żmigrodzka M, Orlowski P, et al. Involvement of Fas/FasL pathway in the murine model of atopic dermatitis. *Inflamm Res.* 2017;66(8):679–690. doi:10.1007/s00011-017-1049-z
99. Bruns B, Höhle T, Kellermann P, Ayala A, Perl M. Divergent effects of neutrophils on fas-induced pulmonary inflammation, apoptosis, and lung damage. *Shock.* 2017;47(2):225–235. doi:10.1097/shk.0000000000000685
100. Tasew G, Nylén S, Lieke T, et al. Systemic FasL and TRAIL neutralisation reduce leishmaniasis induced skin ulceration. *PLoS Negl Trop Dis.* 2010;4(10):e844. doi:10.1371/journal.pntd.0000844
101. Bucur O, Ray S, Bucur MC, Almasan A. APO2 ligand/tumor necrosis factor-related apoptosis-inducing ligand in prostate cancer therapy. *Front Biosci.* 2006;11(1):1549–1568. doi:10.2741/1903
102. Lum J, Bren G, McClure R, Badley A. Elimination of senescent neutrophils by TNF-related apoptosis-inducing [corrected] ligand. *J Immunol.* 2005;175(2):1232–1238. doi:10.4049/jimmunol.175.2.1232
103. L A, Marriott HM, Lawrie A. TNF-related apoptosis-inducing ligand (TRAIL) regulates inflammatory neutrophil apoptosis and enhances resolution of inflammation. *J Leukocyte Biol.* 2011;90(5):855–865. doi:10.1189/jlb.0211062
104. Beyer K, Poetschke C, Partecke L, et al. TRAIL induces neutrophil apoptosis and dampens sepsis-induced organ injury in murine colon ascendens stent peritonitis. *PLoS One.* 2014;9(6):e97451. doi:10.1371/journal.pone.0097451
105. Midgley A, McLaren Z, Moors R, Edwards S, Beresford M. The role of neutrophil apoptosis in juvenile-onset systemic lupus erythematosus. *Arthritis Rheum.* 2009;60(8):2390–2401. doi:10.1002/art.24634
106. Stacey M, Marsden M, Pham NT, et al. Neutrophils recruited by IL-22 in peripheral tissues function as TRAIL-dependent antiviral effectors against MCM. *Cell Host Microbe.* 2014;15(4):471–483. doi:10.1016/j.chom.2014.03.003
107. Hoshi H, Sawada T, Uchida M, et al. MUC5AC protects pancreatic cancer cells from TRAIL-induced death pathways. *Int J Oncol.* 2013;42(3):887–893. doi:10.3892/ijo.2013.1760
108. Brincks E, Risk M, Griffith T. PMN and anti-tumor immunity--the case of bladder cancer immunotherapy. *Semi Cancer Biol.* 2013;23(3):183–189. doi:10.1016/j.semcancer.2013.02.002
109. Jablonska E, Jablonski J, Marcinczyk M, Grabowska Z, Piotrowski L. The release of soluble forms of TRAIL and DR5 by neutrophils of oral cavity cancer patients. *Folia Histochemica Cytobiol.* 2008;46(2):177–183. doi:10.2478/v10042-008-0027-2
110. Tanaka H, Ito T, Kyo T, Kimura A. Treatment with IFN  $\alpha$  in vivo up-regulates serum-soluble TNF-related apoptosis inducing ligand (sTRAIL) levels and TRAIL mRNA expressions in neutrophils in chronic myelogenous leukemia patients. *Eur J Haematol.* 2007;78(5):389–398. doi:10.1111/j.1600-0609.2007.00834.x
111. Cao TM, King MR. Supercharged eGFP-TRAIL Decorated NETs to ensnare and kill disseminated tumor cells. *Cell Mol Bioeng.* 2020;13(4):359–367. doi:10.1007/s12195-020-00639-8
112. Gordon SR, Maute RL, Dulken BW, et al. PD-1 expression by tumour-associated macrophages inhibits phagocytosis and tumour immunity. *Nature.* 2017;545(7655):495–499. doi:10.1038/nature22396
113. Liu J, Song K, Lin B, et al. HMGB1 promotes neutrophil PD-L1 expression through TLR2 and mediates T cell apoptosis leading to immunosuppression in sepsis. *Int Immunopharmacol.* 2024;133:112130. doi:10.1016/j.intimp.2024.112130
114. Aarts CEM, Hiemstra IH, Tool ATJ, et al. Neutrophils as Suppressors of T Cell Proliferation: does Age Matter? *Front Immunol.* 2019;10:2144. doi:10.3389/fimmu.2019.02144
115. Qi X, Yu Y, Sun R, et al. Identification and characterization of neutrophil heterogeneity in sepsis. *Crit Care.* 2021;25(1):50. doi:10.1186/s13054-021-03481-0
116. Tamura K, Miyato H, Kanamaru R, et al. Neutrophil extracellular traps (NETs) reduce the diffusion of doxorubicin which may attenuate its ability to induce apoptosis of ovarian cancer cells. *Heliyon.* 2022;8(6):e09730. doi:10.1016/j.heliyon.2022.e09730
117. Xu M, Cao FL, Zhang YF, et al. Tanshinone IIA therapeutically reduces LPS-induced acute lung injury by inhibiting inflammation and apoptosis in mice. *Acta Pharmacol Sin.* 2015;36(2):179–187. doi:10.1038/aps.2014.112
118. Pulikott S, Hu L, Chen Y, Sun H, Fan Z. Integrin regulators in neutrophils. *Cells.* 2022;11(13):2025. doi:10.3390/cells11132025
119. Li Y, Xu X, Wang HJ, et al. Endoplasmic reticulum protein 72 regulates integrin mac-1 activity to influence neutrophil recruitment. *Arterioscler Thromb Vasc Biol.* 2024;44(3):e82–e98. doi:10.1161/ATVBAHA.123.319771
120. Boras M, Volmering S, Bokemeyer A, et al. Skap2 is required for beta(2) integrin-mediated neutrophil recruitment and functions. *J Exp Med.* 2017;214(3):851–874. doi:10.1084/jem.20160647