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Review

The Balancing Act of Neutrophils

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Neutrophils are endowed with a plethora of toxic molecules that are mobilized in immune responses. These cells evolved to fight infections, but when deployed at the wrong time and in the wrong place, they cause damage to the host. Here, we review the generalities of these cells as well as the difficulties encountered when trying to unravel them mechanistically. We then focus on how neutrophils develop and their function in infection. We center our attention on human neutrophils and what we learn from clinical immunodeficiencies. Finally, we use autoimmune disease to illustrate the harmful potential of dysregulated neutrophil responses.

Introduction

Neutrophils are the most abundant white blood cells, and deficiencies in these cells, inherited or acquired, often result in severe infections (Klein, 2011). Curiously, in spite of their obvious relevance in immunity, in comparison to other immune cells, we know relatively little about how they function. Neutrophils are easy to recognize because of their uniquely lobulated nucleus, which has earned these cells the alternative name of polymorphonuclear cells (PMNs), and we use these two names indistinctively. They contain different types of granules packed with molecules that allow them to fulfil their antimicrobial function. Neutrophils develop in the bone marrow and emerge as terminally differentiated cells in circulation, where they live a short life (whether it is hours or days is currently debated) (Pillay et al., 2010; Tak et al., 2013), unless called into action at an inflammatory site (Nathan, 2006; Amulic et al., 2012).

Neutrophils are recruited from the circulation to an infection site in response to the call of microbial molecules and cytokines produced by tissue-resident cells, like interleukin-1β (IL-1β), IL-6, and tumor necrosis factor α (TNF-α), or chemokines, like IL-8. Neutrophils are the first cells to arrive at an inflammatory site, and they do that in massive numbers. T cells and other immune cells also recruit neutrophils during more chronic inflammation, for example by secreting IL-17. Regardless of the cue, neutrophils in circulation first recognize signals in the endothelium close to an inflammatory site and, after rolling on the endothelium, extravasate into the tissue in a process that has been well described and reviewed (Borregaard, 2010; Kolaczkowska and Kubes, 2013). Once entered into tissues, neutrophils are fully equipped to fight infections and to interact with other cells of the immune system.

When a neutrophil meets a microbe, it can respond through various mechanisms, and here we will concentrate on degranulation, phagocytosis, or the generation of neutrophil extracellular traps (NETs). Microbes might also trigger other mechanisms, like autophagy, apoptosis, or pyroptosis, which we will not review here because of space limitations. Degranulation is an exocytosis process, whereby neutrophil granules fuse with the cytoplasmic membrane, releasing an arsenal of enzymes, antimicrobial peptides, and other molecules into the surrounding tissue. These include proteases that degrade virulence factors and toxins, lysozyme that degrades the bacterial cell wall, and antimicrobials like bactericidal/permeability-increasing protein (BPI), cathelicidins, and defensins that can kill bacteria directly, at least at high concentrations and in buffer solutions in vitro. Once released, these molecules have powerful antimicrobial capacities, but they also harm the host tissue by collateral damage. During phagocytosis, microbes or other particles are recognized by pattern recognition receptors (PRRs) or, even more efficiently, by antibody or complement receptors if the particles are opsonised. When recognized, particles are first engulfed in a phagosome, which later fuses with granules to make a phagolysosome. In this process, the NADPH oxidase is assembled to convert oxygen into oxidizing molecules like superoxide, hydrogen peroxide, and halic acids, collectively called reactive oxygen species (ROS). The combination of granule contents and ROS leads to an efficient killing of microbes, and it is likely that these processes act in concert and potentiate each other. Besides phagocytosis and degranulation, neutrophils stimulated by microbes or by specific antibodies can also undergo an unusual form of cell death where chromatin gets processed, studded with antimicrobial proteins, and released in the form of NETs. Indeed, the extrusion of chromatin likely occurs through different mechanisms, including the fascinating nuclear exclusion recently described (Yipp et al., 2012). NETs expose a concentrated form of antimicrobials, including histones, which can trap and kill microbes as well as activate other immune cells. Neutrophils might be triggered to respond differently to distinct microbes, and the relevance of these antimicrobial processes in specific diseases is not entirely clear.

The occurrence of life-threatening infections in neutropenic patients illustrates the importance of neutrophils in antimicrobial defense. In addition, several rare immune deficiencies have been described as affecting particular antimicrobial functions of neutrophils (Bouma et al., 2010). Patients with such deficiencies often suffer from infections caused by opportunistic pathogens that rarely cause severe infections in healthy individuals. These "experiments of nature" show that neutrophils are crucial cells in host defense against microbes. The specific phenotypes of these patients can help us understand neutrophil function.

Traditionally, based on their impressive antimicrobial capacity in vitro and the susceptibility of patients with few or defective neutrophils, we think of PMNs primarily as microbe hunters. This view might well be correct, but in the last decade neutrophils

are emerging, not surprisingly, as instructors of other immune cells like dendritic cells (DCs), macrophages, natural killer (NK) cells, B cells, and T cells. These advances were recently reviewed (Mócsai, 2013), and we will only touch upon them in the context of neutrophils as the culprit of jumpstarting autoimmunity after infections. Due to these diverse functions, on top of the collateral damage occurring during their antimicrobial action, neutrophils are now implicated in many diseases, including cancer, metabolic diseases, and circulatory disturbances.

In this review, we will examine the mechanism of neutrophil development, the role of neutrophils in infections (with a focus on immunodeficiency), and how neutrophil activation can also be detrimental to the host, as demonstrated by their involvement in the development of autoimmunity. However, before proceeding, a few cautionary words are necessary. Neutrophils are short lived, do not divide, and cannot be genetically modified. There are few neutrophil cell lines, and mice are an imperfect model organism since they do not fully mimic human neutrophils in function, morphology, or physiology. In vivo, murine neutrophils can be depleted with antibodies, but this depletion is transient, as low neutrophil numbers trigger the production of new PMNs. On the positive side, neutrophils are abundant in human blood, and it is relatively easy to obtain populations allowing short-term experiments. The limitations in interpreting results with human neutrophils are that, since the experiments can be performed only in vitro, the cells rest on artificial substrates and media. Thus, it might be important to consider these caveats while reading this review.

Development of Neutrophils

The Development of Granulocyte Precursors

Neutrophils that leave the bone marrow and enter the bloodstream are terminally differentiated cells with a short lifespan. To maintain a stable number of neutrophils in circulation, they are produced at the staggering rate of 1×10^{11}–2×10^{11} per day in humans. The development of granulocyte precursors as well as the terminal differentiation of neutrophils are complex processes that are controlled by transcriptional regulation, growth factors, cytokines, microRNAs, and other regulatory systems. Understanding hematopoietic development necessitates the use of mice; in this section, we deviate from a clinical perspective and report mainly the work of many colleagues in experimental animals.

The initial precursors of neutrophils are hematopoietic stem cells (HSCs), which reside in low numbers in bone marrow niches (Figure 1). These slowly dividing cells are capable of self-renewal and are maintained in these niches by interacting with stromal cells such as osteoblasts (Orkin and Zon, 2008). In the first step of their development, HSCs lose their self-renewing potential and give rise to multipotent precursors (MPPs) that can develop into all blood cell lineages. Several models have been proposed about the lineage choice of MPPs. It is currently assumed that MPPs develop into either lymphomyeloid or erythromyeloid progenitors (Görgens et al., 2013a). Interestingly, neutrophils arise from the lymphomyeloid progenitors, whereas the other granulocyte subtypes, eosinophils and basophils, are generated from the erythromyeloid lineage (Görgens et al., 2013b). The decision of MPPs to undergo differentiation into either the lymphomyeloid or the erythromyeloid direction depends largely on the balance between the transcription factors GATA-1 and PU.1. These factors antagonize each other, and high levels of PU.1 are crucial to generate the lymphomyeloid lineage (Arinobu et al., 2007; McKercher et al., 1996).

Lymphomyeloid precursors can give rise to granulocyte/monocyte precursors (GMPs), a decision tightly controlled by the transcription factor family of CCAAT enhancer-binding proteins (C/EBP). C/EBP-α is absolutely required for neutrophil development, regulating expression of proteins necessary for neutrophil differentiation such as granulocyte colony-stimulating factor receptor (G-CSFR) (Radomska et al., 1998). Indeed, G-CSF is the most important cytokine during neutrophil differentiation, although a minor amount of functional neutrophils can still be generated in the absence of this growth factor (Lieschke et al., 1994; Zhang et al., 1997).

Neutrophil Terminal Differentiation

Neutrophil precursors first develop into a myeloblast, a relatively small (10 μm) cell that does not express granule proteins. Myeloblasts give rise to promyelocytes, which further differentiate into myelocytes, metamyelocytes, band cells (which are generally considered immature immune cells), and finally segmented neutrophils, which are then able to leave the bone marrow and enter the bloodstream (Bainton et al., 1971) (Figure 1). After the promyelocyte stage, the differentiating neutrophils exit the cell cycle. Granules are formed continuously during the differentiation process from the promyelocyte stage onward. Primary (or azurophilic) granules are made in promyelocytes, secondary (or specific) granules in myelocytes, tertiary (or gelatinase) granules in band cells, and secretory vesicles in segmented neutrophils (Borregaard, 2010). Proteins do not carry a specific signal sequence targeting them as granule cargo. Therefore, the content of a granule most likely reflects the gene expression pattern of the differentiation stage during which the granule was formed. This unspecific loading also suggests that the classification into distinct granule subtypes may not be absolute and there might be granules containing proteins of different subtypes.

Similarly to the development of granulocyte precursors, terminal differentiation of neutrophils is regulated by a balance between different transcription factors. C/EBP-α, as well as Gfi-1 or Lef-1, is abundant in myeloblasts, but its expression decreases as differentiation progresses (Bjerregaard et al., 2003). In contrast, C/EBP-ε peaks during the myelocyte/metamyelocyte stages, and the expression of C/EBP-β, C/EBP-γ, and C/EBP-δ continuously increases during neutrophil maturation (Bjerregaard et al., 2003). Interestingly, as described below, infections or inflammation triggers granulopoiesis, which depends on transcription factors different from those depended upon in homeostatic conditions.

Although transcriptional regulation is essential for neutrophil terminal differentiation, other regulatory mechanisms also influence the process. For example, it has been shown that some neutrophil mRNAs are able to retain their introns by a poorly characterized mode of alternative splicing called intron retention (IR), a phenomenon that increases during differentiation (Wong et al., 2013). These retained introns might result in premature stop codons, and the mRNAs could therefore be targeted for degradation by the cell's nonsense-mediated mRNA decay machinery. Indeed, several gene products seem to be down-regulated through an IR-dependent mechanism during the

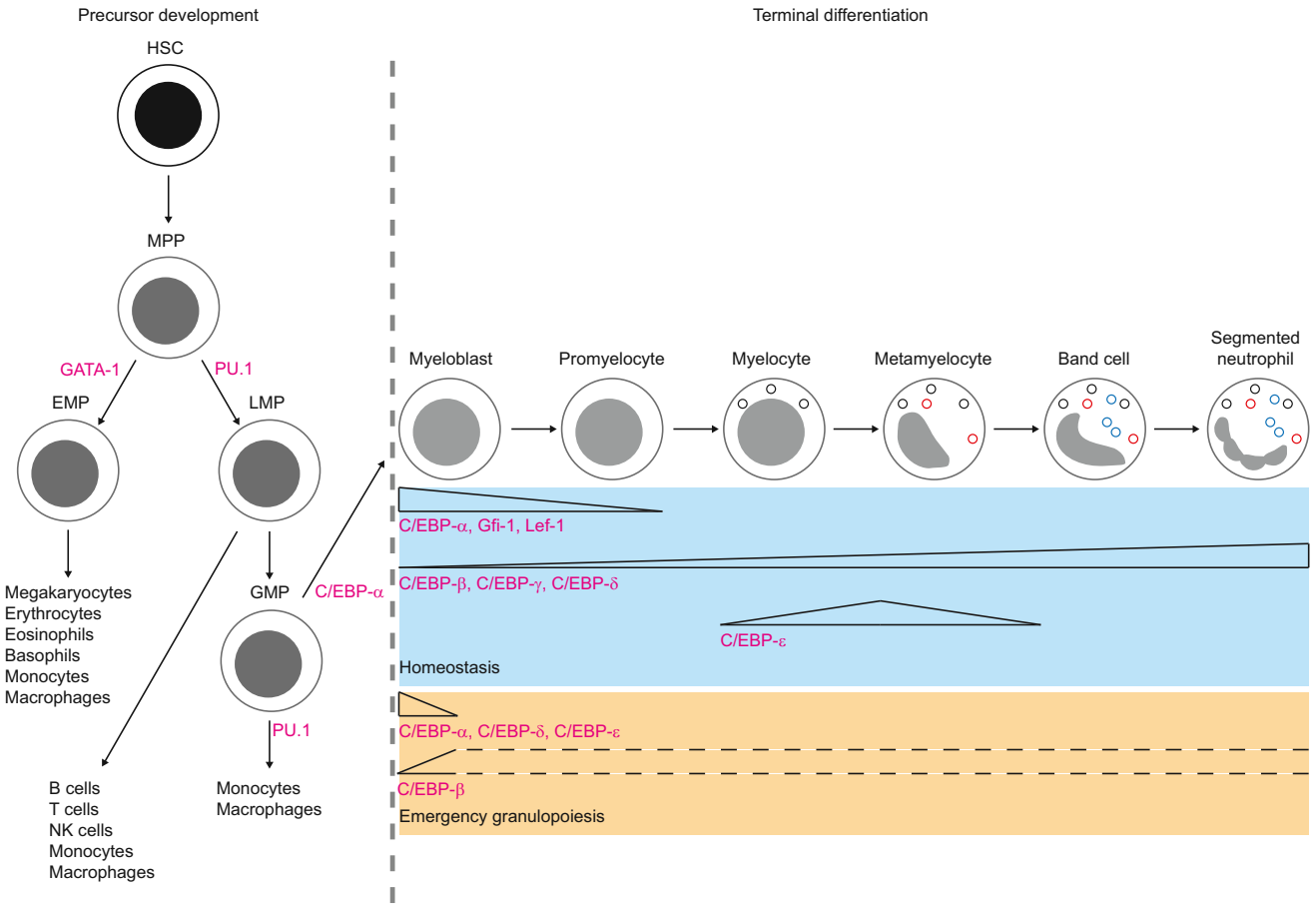

Figure 1. The Development of Neutrophils from Hematopoietic Stem Cells
Precursor development and terminal differentiation of neutrophils from hematopoietic stem cells. Essential transcription factors are highlighted in pink. HSC, hematopoietic stem cell; MPP, multipotent precursor; EMP, erythromyeloid precursor; LMP, lymphomyeloid precursor; GMP, granulocyte-macrophage precursor. Please refer to the Development of Neutrophils section for details. Adapted from Borregaard (2010).

terminal differentiation of murine neutrophils, including lamin B1 (Lmnb1), which regulates nuclear morphology. Interestingly, expression of a nondegradable mutant of Lmnb1, containing no introns, results in an aberrant differentiation and the alteration of the characteristic shape of the neutrophil nucleus (Wong et al., 2013).

Neutrophil Development during Homeostasis and Infections

Neutrophils are highly efficient when it comes to killing microorganisms. However, their modes of action are rather unspecific and capable of harming the host as well as the invading pathogen, a recurrent theme in this review. It therefore makes sense that both the production and release of neutrophils from the bone marrow are tightly regulated. Indeed, the amount of neutrophils in peripheral tissues influences the production rate of new precursors in the bone marrow through a negative feedback loop. Neutrophils of mice deficient for adhesion molecules are not able to pass the endothelium. The resulting low level of neutrophils in peripheral tissues triggers tissue-resident macrophages and DCs to produce IL-23. IL-23 induces T helper 17 (Th17) cells to make IL-17, leading to G-CSF expression, which enhances neutrophil differentiation (Stark et al., 2005). In wild-type animals, neutrophils are abundant in peripheral tissues, where they regularly undergo apoptosis. It has been suggested that the clearance of apoptotic neutrophils by macrophages and DCs blunts the production of IL-23 and is therefore one of several possible pathways by which to regulate granulopoiesis (Stark et al., 2005). However, mice deficient for T cells, B cells, and NK cells can still produce enhanced G-CSF and increase granulopoiesis in response to neutrophil depletion, indicating that neutrophil development is occurring in the absence of T cell-produced IL-17. Granulopoiesis is most likely regulated by various and possibly redundant mechanisms (Bugl et al., 2012, 2013). Furthermore, a recent study showed that "old" neutrophils in circulation return to the bone marrow, where they are phagocytosed by resident macrophages. In turn, macrophages stimulate the release of new progenitors to maintain the number of neutrophils in circulation. Interestingly, this cycle of neutrophil release and clearance oscillates in circadian rhythms, with aged neutrophils being cleared from the circulation at the end of the resting period of mice (Casanova-Acebes et al., 2013).

The production of neutrophils is even further enhanced during infections by a process called emergency granulopoiesis. When neutrophils combating pathogenic microorganisms die

at inflammatory sites, the ensuing neutropenia enhances granulopoiesis, thus satisfying the organism's cellular requirement to combat infections. Notably, and similarly to homeostasis, emergency granulopoiesis depends on C/EBP transcription factors; however, during emergency, C/EBP-β seems to be the driving force behind granulopoiesis, whereas C/EBP-α is dispensable (Hirai et al., 2006). However, C/EBP-β is required for terminal differentiation rather than for precursor development (Cain et al., 2011). Surprisingly, although G-CSF is a major regulator of homeostatic neutrophil development, it is not necessary for an emergency response to infection with the fungal pathogen *Candida albicans* (Basu et al., 2000). Conversely, other reports demonstrated that G-CSF is crucial for emergency granulopoiesis, for example during infections with the intracellular bacteria *Listeria monocytogenes*. The role of G-CSF in emergency granulopoiesis therefore seems to be context dependent, and there could be other cytokines regulating neutrophil development in the absence of G-CSF (Panopoulos and Watowich, 2008). G-CSF can be induced by an inflammatory milieu, which leads to mobilization of neutrophils from the bone marrow. The ensuing neutropenia in the bone marrow triggers the production of new precursors (Cain et al., 2011). This suggests that granulopoiesis is influenced not only by the previously mentioned abundance of neutrophils in peripheral tissues, but also by neutrophil density in the bone marrow.

Surprisingly, germ-free mice are severely neutropenic, to a higher extent than G-CSF- or G-CSFR-deficient animals, suggesting that microflora also regulate granulopoiesis (Bugl et al., 2013). Neutrophil depletion leads to an increased production of G-CSF and subsequent neutrophil development. However, this response is abolished in animals deficient for the PRR Toll-like receptor 4 (TLR4) or its adaptor TRIF, indicating that TLR4 ligands provided by the microflora induce granulopoiesis (Bugl et al., 2013). These ligands could also be provided by invading pathogens, and therefore the TLR4-TRIF axis might be a common regulator of homeostatic and emergency granulopoiesis.

The observation that hematopoietic stem and progenitor cells (HSPCs) are able to migrate from the bone marrow to skin wounds, where they differentiate into mature neutrophils and exert their defense functions, added further complexity to the regulation of granulopoiesis (Granick et al., 2013). The number of HSPCs migrating to the site of injury was markedly enhanced in the context of *Staphylococcus aureus*-infected skin wounds and depended on the expression of TLR2 and its adaptor MyD88 in HSPCs. The induction of TLR2/MyD88 signaling resulted in production of prostaglandin E_2 in HSPCs, thereby supporting their survival and proliferation (Granick et al., 2013). Differentiation of functional neutrophils can therefore occur at sites of infection, possibly helping establish localized responses against pathogens.

It is clear that neutrophil development is dynamic and regulated by a variety of factors, such as genetic background, cytokine milieu, or the amount of neutrophils present in peripheral blood or tissues, that influence each other. Furthermore, inflammation or infections are able to trigger enhanced granulopoiesis in order to fulfil the organism's need for an increase in the number of immune cells. The mechanisms of homeostatic and emergency granulopoiesis have been extensively studied, but there are still open questions, especially regarding how the two processes are linked and where they differ from each other.

Neutrophils in Infection

As outlined above, neutrophils are the first line of host defense against pathogens, as they are recruited in great numbers to the site of infection. Therefore, the absence of neutrophils or impairment of neutrophil activation can lead to severe infections. The most common pathogen in patients with neutropenia or specific neutrophil function disorders is *S. aureus* (Winkelstein et al., 2000; Picard et al., 2010). Fungi like *Candida* and *Aspergillus* species also cause frequent problems when one or all of the neutrophil antimicrobial defense systems are impaired (Smeekens et al., 2013). Microbes evolved virulence factors to avoid or target the neutrophil, as illustrated by the extensive arsenal that *S. aureus* acquired against this host immune cell (Spaan et al., 2013). *S. aureus* blocks neutrophil activation, PRR recognition, and produces a golden pigment with antioxidant properties to protect itself against ROS damage within the phagosome (Liu et al., 2005). Although *S. aureus* neutralizes almost all weapons of neutrophils, healthy individuals can deal with this bacterium quite efficiently. In this section, we will focus on immunodeficiencies that affect the microbial recognition and antimicrobial functions of neutrophils.

Microbial Recognition

TLRs recognize a variety of highly conserved microbial ligands that are usually essential for microbial fitness. Human neutrophils express almost all TLRs, except TLR3 and TLR7 (Janke et al., 2009). They also recognize microbial ligands via C-type lectins and sense intracellular microbial danger signals via the inflammasome. TLRs and C-type lectins trigger neutrophil migration, phagocytosis, programmed cell death, ROS generation, and cytokine production. Upon TLR activation, the intracellular receptor domain recruits adaptor proteins like MyD88. MyD88 recruits interleukin-1 receptor-associated kinase (IRAK) complex, including the two kinases IRAK1 and IRAK4. Activated IRAK1 initiates further downstream signaling, leading to phosphorylation of the inhibitor of κB (IκB) by the IκB kinase complex (IKK) and release of NF-κB. The IKK complex consists of three subunits: IKK-α, IKK-β, and NF-κB essential modulator (NEMO). NF-κB induces expression of different inflammatory genes that trigger further neutrophil activation and secretion of proinflammatory cytokines. In contrast to other cells, neutrophils signal only via the MyD88 pathway and not via TRIF (Tamassia et al., 2007). TLR3 and TLR4 activate this MyD88-independent pathway in other cells; however, TRIF signaling is not induced upon TLR4 ligation in neutrophils.

A deficiency in IRAK4 was found in patients with recurrent pyogenic infections (Picard et al., 2010). These individuals were mostly suffering from *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, and *S. aureus* infections, whereas they had normal resistance against many other bacteria and fungi. Neutrophils isolated from these patients fail to produce an oxidative burst in response to different TLR ligands. Another study, which included a single IRAK4-deficient patient, showed that these neutrophils also fail to respond to TLR ligands, but killing of bacteria and fungi was unaffected (van Bruggen et al., 2010). This suggests that recognition of microbial ligands is redundant in neutrophils or that some bacteria developed strategies to

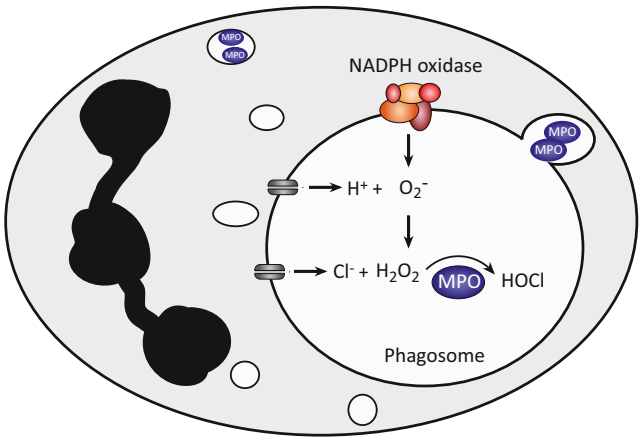

Figure 2. Schematic Overview of ROS Production in the Phagosome
MPO is stored in azurophilic granules within the neutrophil. After phagocytosis, the granules fuse and release MPO into the phagosome. The NADPH oxidase complex generates superoxide that is converted into hydrogen peroxide. MPO uses hydrogen peroxide to catalyze the production of potent antimicrobial molecules, such as hypochlorous acid.

circumvent TLR recognition. Patients with a deficiency in MyD88 suffer from bacterial infections similar to those of IRAK4-deficient individuals (von Bernuth et al., 2008). Invasive infections in IRAK4- and MyD88-deficient individuals have a 30% mortality rate in infants. The susceptibility to infections decreases with age, suggesting that other parts of the immune system take over later in life.

Analogous effects were observed in neutrophils from patients with a mutation in NEMO (Singh et al., 2009). However, NEMO-deficient patients suffer from infections with a broader range of microbes, as seen in MyD88 and IRAK4 deficiencies. This probably reflects the involvement of NEMO in regulation of other pathways that activate NF-κB, such as the T cell receptor and TNF receptor family (Picard et al., 2011).

A recent study reported a link between caspase recruitment domain-containing protein 9 (CARD9) deficiency and *Candida* infections (Drewniak et al., 2013). CARD9 deficiency was identified previously in a family with a history of *Candida* infections. Dectin-1 recognizes various beta-glucans from plants and fungi, including *Candida* lectins, and activates downstream signaling via a CARD9-containing complex to NF-κB. Neutrophils isolated from a CARD9-deficient patient suffering from chronic invasive *Candida* infection of the brain showed an impaired killing of non-opsonized *Candida*. Nonopsonized *Candida* is especially relevant in brain infections, since complement proteins are absent in this part of the body. In addition, CARD9-deficient neutrophils stimulated with *C. albicans* form abnormal phagolysosomes. Neutrophils from patients with deficiencies in microbial recognition pathways clearly show impaired detection and killing of microbes. Nevertheless, these deficiencies affect a wide variety of cells that use these signaling pathways, and infections observed in these patients are not necessarily caused by defects in the antimicrobial response of neutrophils.

Reactive Oxygen Species

Neutrophils phagocytose microbes and subsequently kill them by generating ROS within the hostile environment of the phagolysosome. The NADPH oxidase complex initiates the oxidative burst by converting oxygen into superoxide, which dismutates, with the help of superoxide dismutases, to hydrogen peroxide (Figure 2). Hydrogen peroxide, in turn, is used by myeloperoxidase (MPO) to form more toxic ROS. Chronic granulomatous disease (CGD) patients have a strongly reduced NADPH oxidase activity due to a genetic defect in one of the components that forms the NADPH complex (Kuhns et al., 2010). They suffer from life-threatening infections and need chronic antibiotic or antifungal therapy to fight microbes. Other characteristics of CGD patients are the formation of granulomas and development of a variety of inflammatory diseases, such as inflammatory bowel disease.

S. aureus often causes diseases in CGD patients (around 30% of all infections), followed by *Aspergillus*, *Salmonella*, *Candida*, and *Serratia* species (Winkelstein et al., 2000; van den Berg et al., 2009). The localization and outcome of the infections differ depending on the infecting microbe: *Aspergillus* species mostly cause infections in the lungs and brain with a high mortality rate. The disease severity depends on the residual activity of the NADPH complex, which in CGD patients can be reduced to 0.1% of its activity in healthy individuals (Kuhns et al., 2010). Neutrophils of CGD patients show normal antimicrobial activity when hydrogen peroxide is introduced exogenously, indicating that CGD neutrophils function normally, except for superoxide production.

In contrast to *S. aureus*, infections with the fungus *Aspergiullus nidulans* are less common in neutropenic patients (Henriet et al., 2011). Interestingly, CGD patients suffer from severe infections with this fungus. An obvious explanation would be that a defect in the NADPH oxidase-mediated oxidative burst protects *A. nidulans* from neutrophil-mediated killing. However, neutrophils maintain their antifungal activity to *A. nidulans* upon chemical inhibition of the oxidative burst (Henriet et al., 2012). Neutrophils from CGD patients showed a delayed recruitment to the site of infection, suggesting an intact antimicrobial function of the neutrophil itself. However, the oxidative burst also triggers the formation of NETs, and indeed neutrophils isolated from CGD patients fail to expel their DNA and to reduce *A. nidulans* growth. Repairing the NADPH oxidase function by gene therapy restored NET formation and the ability to prevent *A. nidulans* growth and allowed the patient to clear the infection (Bianchi et al., 2009). Monocytes and macrophages, like neutrophils, phagocytose and degrade bacteria in their phagolysosome. A functional NADPH oxidase also contributes to bacterial killing in these cells. Indeed, monocytes and macrophages isolated from CGD patients also have an impaired NADPH oxidase activity, and these cells should also be considered when studying infections in CGD patients. In summary, it is likely that the NADPH oxidase regulates several pathways, including the production of cytokines, and these diverse functions contribute to CGD.

Subsequent to phagocytosis, azurophilic granules fuse with and release MPO into the phagosome, which generates hypochlorous acid out of hydrogen peroxide and chloride (Figure 2). Hypochlorous acid has powerful antimicrobial activity in vitro, suggesting that MPO plays an important role in the antimicrobial defense of neutrophils (Klebanoff et al., 2013). Screening of neutrophils for MPO activity revealed that a functional deficiency in MPO activity occurs in 1 out of 3,000 individuals (Parry et al., 1981; Kutter, 1998). Several mutations in the gene encoding for

MPO have been described that affect MPO production, activity, or trafficking, leading to variations in residual MPO activity among these patients, which may influence the risk for severe infection. Some people with a MPO deficiency suffer from *Candida* infections; however, most of them are apparently healthy.

Surprisingly, despite the lack of infections in most patients, MPO-deficient neutrophils have a clearly reduced antimicrobial activity. Neutrophils isolated from MPO-deficient patients show delayed and reduced killing of *S. aureus* and *C. albicans*. Patients with MPO deficiencies have a very mild phenotype compared to CGD patients. This is striking, since MPO is the most abundant protein produced by neutrophils; a significant portion of the cell's protein content consists of MPO, and the enzyme directly acts downstream of NADPH oxidase (Winterbourn et al., 2006). A difference between MPO deficiency and CGD patients is that neutrophils and monocytes are the main producers of MPO, whereas NADPH oxidase plays an important role in other phagocytes. In addition, hydrogen peroxide serves different purposes aside from the generation of hypocholorous acid by MPO.

Antimicrobial Proteins

Neutrophils utilize antimicrobial molecules such as MPO, neutrophil elastase (NE), cathepsin G, and defensins to combat infection. However, although most of these proteins have clear antimicrobial effects in vitro, their biological relevance in controlling infections is less clear. A mutation in the transcription factor C/EBP-ε, essential for neutrophil differentiation, causes the rare neutrophil-specific granule deficiency (SGD) (Shiohara et al., 2004). Neutrophils from SGD patients have morphological abnormalities and functional defects. They lack primary, secondary, and tertiary granule proteins, including defensins and BPI, but are sufficient in MPO and NADPH oxidase activity. These patients suffer from infections with *S. aureus*, *P. aeruginosa*, and *Klebsiella pneumonia*. However, the mutation also affects monocytes and macrophages, and therefore infections in these patients cannot exclusively be linked to the absence of neutrophil granule proteins.

The azurophilic granule protein NE cleaves a wide variety of proteins including bacterial virulence factors (Weinrauch et al., 2002). Activation of the serine proteases NE and cathepsin G requires cleavage of the N-terminal dipeptide by dipeptidyl peptidase 1 (DPPI) (Korkmaz et al., 2010). Loss-of-function mutations in the gene encoding DPPI have been strongly associated with the Papillon-Lefèvre Syndrome (PLS), in which patients suffer from skin problems and severe periodontitis. Neutrophils isolated from PLS patients lack NE and cathepsin G activity and display a reduction in phagocytic and chemotactic capacity; still, DPPI might have other undiscovered substrates beyond NE and cathepsin G that are important for the phenotype of PLS patients. The absence of activity of another protease in PLS neutrophils, proteinase-3 (PR3), results in a decreased level of the antimicrobial peptide LL-37 (de Haar et al., 2006). PR3 normally processes this protein into its active form. Neutrophils from PLS patients have a reduced killing activity against the periodontic pathogen *Actinobacillus actinomycetemcomitans*, which causes periodontitis and loss of teeth in PLS patients, and the lack of active neutrophil serine proteases may contribute to the inability of neutrophils to clear the infection. Another study showed that neutrophils isolated from PLS patients kill *E. coli* and *S. aureus* as effectively as control neutrophils (Pham et al., 2004). This suggests that serine proteases don't play a crucial role in the neutrophil defense mechanisms against these microbes.

Antimicrobial effects of NE are mainly based on in vitro data and infection models in mice showing that NE and cathepsin G protect against Gram-positive, Gram-negative, and fungal infections. Humans with NE mutations suffer from severe infections; however, this is not directly linked to NE activity. Mutations in NE cause half of the cases of severe congenital neutropenia, a disease characterized by a low number of circulating neutrophils (Bouma et al., 2010). There is no link between a particular mutation in the gene encoding NE and the clinical outcome, except for one mutation that is in proximity of the NE active site and likely alters the biological activity of the enzyme. Individuals with this mutation have a severe form of the disease, with neutrophil counts close to zero. The mechanism by which defects in NE cause a decrease in the neutrophil number is still under investigation. A role for the unfolded protein response, which is activated by misfolded proteins in the endoplasmic reticulum (ER), has been proposed, leading to apoptosis (Grenda et al., 2007). Several mutations in NE lead to improper trafficking of the protein, which may cause the accumulation of the misfolded protein in the ER.

Genetic diseases that impair neutrophil function have a low prevalence of less than 1 in 200,000 individuals, underscoring the relevance of neutrophils in defense. An exception is MPO deficiency, with a higher prevalence and relatively minor defects in antimicrobial defense. It is important to note that the type of infections that patients with neutrophil-affecting immunodeficiency are susceptible to might be biased by the low incidence of these inherited diseases and by the fact that many of them live in areas with high hygienic standards. In addition, genetic defects associated with neutrophil antimicrobial function sometimes also affect other cell types of the immune system. Nevertheless, it is clear that individuals with neutrophil functional defects or neutropenia are more prone to infections. Microbes causing such infections may benefit from inactivation of specific neutrophil functions. However, infections with certain microbes may also be a result of exposure. For instance, many people carry *S. aureus* in the nose, whereas the chance of exposure to other microbes is much lower. Interestingly, viral infections in patients with impaired neutrophil function are not often reported. Although studies indicate that neutrophils participate in clearing viral infections (Gabriel et al., 2013), this may be because neutrophils play a minor role in the defense against viral infections or because patients with neutrophil immunodeficiencies are rarely exposed to viruses. Collectively, the valuable data supplied by immunodeficiency in patients, mice, and in vitro studies have revealed several potential additional antimicrobial pathways and molecules of neutrophils.

Neutrophils in Autoimmunity

Autoimmunity is characterized as a failure to distinguish self molecules from nonself molecules. The immune response initiated by this self-recognition results in major organ and tissue damage (Németh and Mócsai, 2012). A breakdown in the mechanism that ensures tolerance of cells to self molecules is responsible for the initiation of autoimmunity. Tolerance is the process by which

immune cells are instructed to not react to the presence of host molecules, thus ensuring that the immune system is not activated. A break in tolerance, which is often linked to infection (as reviewed by Bach, 2005), leads to the appearance of autoreactive T cells that are responsible for the first wave or "immunization phase" of self-recognition. Upon activation of the autoreactive T cells, a secondary immune response is initiated involving many other immune cells, such as B cells, NK cells, and neutrophils. This secondary response or "effector phase" can be instigated by the autoreactive T cells in several ways, including cytokine production or direct cell-cell contact.

A signature of autoimmunity is the generation of autoantibodies that can be directed against nuclear material such as dsDNA, ribonucleoproteins, and histones. A question of great interest in the field of autoimmunity is the source of these self molecules that activate autoreactive cells and lead to an adverse immune response. Until recently, the debate centered on whether apoptotic or necrotic cell debris were the source of this self material that activated the immune system. However, in the last decade a new potential source of self molecules has come to light with the discovery of NETs. Indeed, molecules released during NETosis are found as autoantigens in many autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus, and vasculitis.

Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a prototypical autoimmune disease that affects between 0.5% and 1% of the population in the developed world. Both genetic and environmental factors influence the onset of RA, which is characterized by the chronic swelling and destruction of distal synovial and larger joints. Arthritic joints exhibit increased synovial fluid volume, high concentrations of proinflammatory cytokines, and large cellular infiltrates that include DCs, neutrophils, and macrophages (Németh and Mócsai, 2012). RA is routinely diagnosed based on the presence of rheumatoid factor, an autoantibody generated against the Fc portion of immunoglobulins, and anti-citrullinated protein antibodies (ACPAs) (Bugatti et al., 2007; De Rycke et al., 2004).

Neutrophils are the most abundant cell type found in the synovial fluid. They also accumulate in the arthritic joints, where most of the tissue damage occurs (Mohr et al., 1981). Animal models of autoantibody-induced arthritis showed that neutrophils migrate to affected areas early in disease progression (Nandakumar et al., 2003; Wipke and Allen, 2001), where they produce enhanced oxidative responses to several stimuli (Dularay et al., 1988) and are, at least partially, responsible for the progression and severity of the disease. Several studies looking at cell recruitment to arthritic/inflamed joints in mice have revealed a signaling cascade initiated by the complement C5a receptor and Fcγ receptors, resulting in the release of the inflammatory mediator leukotriene B_4 (LTB_4) and IL-1β into the joint and subsequent neutrophil recruitment (Kim et al., 2006; Chou et al., 2010; Sadik et al., 2012).

Recently, it was proposed that neutrophils undergoing NETosis may also be the source of self antigens, such as ACPAs, that give rise to autoantibodies in RA (Khandpur et al., 2013). Consistent with this hypothesis, five known proteins released by NETs are considered autoantigens in RA (Darrah and Andrade, 2012), and neutrophils isolated from RA patients have been shown to exhibit enhanced NETosis (Khandpur et al., 2013). Analysis of NET components identified citrullinated vimentin, an important RA autoantigen that decorated NETs (Khandpur et al., 2013). Independently, Pratesi et al. (2013) demonstrated that RA patients generated antibodies against histone H4 and that the source of this citrullinated protein was NETs (Pratesi et al., 2013).

Peptidylarginine deiminase 4 (PAD4) citrullinates proteins during RA progression. PAD4, which is expressed by neutrophils, catalyzes the posttranslational conversion of peptidylarginine to citrulline on histones by deimination. This process is crucial for the generation of NETs (Wang et al., 2009). However, there is also a detrimental side to PAD4 activation, as RA patients have an abundance of ACPAs in their serum contributing to disease. Genetic studies show that RA patients with alleles linked to increased RA susceptibility have more stable PAD4 mRNA and as such may enhance PAD4 expression (Suzuki et al., 2003).

Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a prototypical autoimmune disease that affects around 0.1% of the world's population. Development of SLE results in multiorgan damage defined by a relapsing and remitting progression. Both genetic and environmental factors contribute to SLE development, which is characterized by the dysregulated activation of T and B lymphocytes and the production of autoantibodies directed against dsDNA, histones, and nucleosomes. In addition, higher levels of antineutrophil cytoplasmic antibodies (ANCA) directed against MPO, NE, and cathepsin G have been found in the serum of SLE patients compared with healthy control serum (Nässberger et al., 1990; Zhao et al., 1998). These antigens and their autoantibodies form immune complexes that are deposited in tissues, including the kidneys, skin, and joints. These immune complexes are also highly inflammatory and induce the production of IFN-α by plasmacytoid dendritic cells (Kaplan, 2011).

Neutrophils isolated from SLE patients display abnormalities, including increased aggregation, impaired phagocytosis, and an increased propensity to undergo NETosis. There is also a marked reduction in NET clearance, and as such, NETs may provide another mechanism by which DC activation occurs. Peripheral blood mononuclear cells (PBMCs) isolated from adult and pediatric SLE patients contain a subset of neutrophils that migrate at a lower density, which are appropriately called low-density granulocytes (LDGs) (Hacbarth and Kajdacsy-Balla, 1986). LDGs produce levels of proinflammatory cytokines higher than those of healthy donor controls or normal SLE neutrophils (Denny et al., 2010). These cells also form NETs more readily than healthy donors or even SLE neutrophils and thus release more neutrophil proteins and enzymes (Villanueva et al., 2011). Thus, molecules released from the NETs of SLE neutrophils or LDGs may be the source of autoantigens in SLE. Indeed, to date, at least eight neutrophil-derived molecules that are released upon NETosis, such as DNA, LL-37, or ribonucleoproteins, have been linked to SLE development (Darrah and Andrade, 2012).

A lack of NET degradation has also been linked to SLE. DNase I is a component of serum, and a Japanese study linked familial SLE progression to a heterozygous nonsense mutation in exon 2 of DNase I. This mutation led to lower DNase I enzymatic activity in the sera and B cells of these patients when compared with SLE patients without the mutation and healthy controls. The levels of

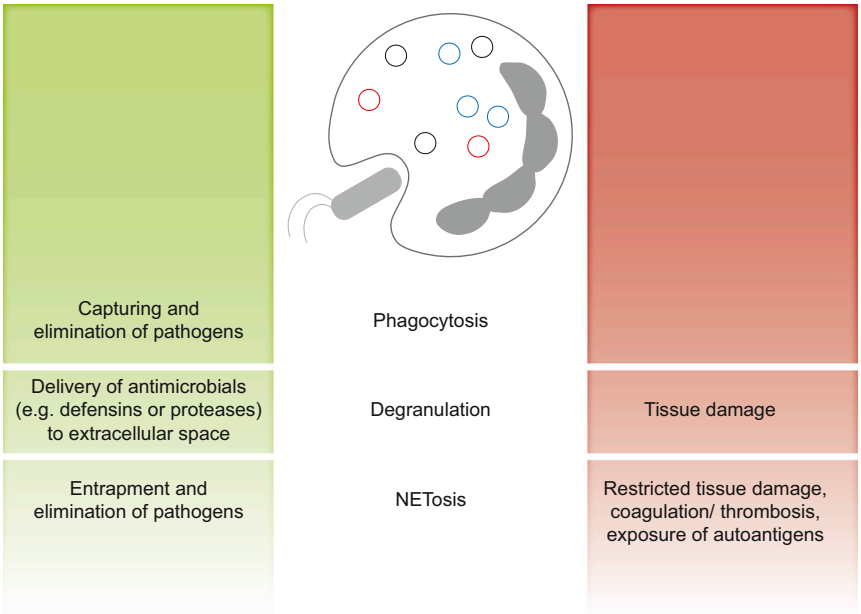

Figure 3. The Good and Bad Sides of Neutrophil Activation
The three main antimicrobial functions of neutrophils and their positive (green) and negative (red) aspects for the host.

immunoglobulin G (IgG) against nucleosomal antigens were also greatly increased in these DNase I mutant patients (Yasutomo et al., 2001). A subsequent investigation in pediatric SLE patients demonstrated that a mutation in the DNase I homolog DNase IL3 was also linked to SLE development (Al-Mayouf et al., 2011). These studies reveal that a lack of functioning DNase I is found in SLE patients. Alternatively, a decrease in DNase I activity in SLE patients can also occur due to the presence of antibodies against DNase I or NETs (Hakkim et al., 2010). The inability of DNase I to degrade NETs correlated with the development of severe glomerulonephritis.

Vasculitis

Vasculitis is the broad term used for a group of diseases that affect the blood vessels. It is characterized by necrotic inflammation of blood vessels, leading to thinning of the vessel walls and capillaries. The cause of onset of vasculitis is still somewhat unclear. However, it has been shown to occur as a result of infection, medication toxicity, cancer, or as a complication in RA and SLE patients.

Neutrophils play a role in the generation of the autoantibodies that result in the development of ANCA-associated systemic vasculitis. This subgroup of vasculitis is defined by the presence of autoantibodies generated against the neutrophil granule proteins PR3 and MPO (Niles et al., 1989). These autoantigens also induce NETosis in primed neutrophils and were found to be localized to DNA within the NET structures. Typical components of NETs such as DNA/histone complexes have been identified in the glomeruli and interstitium of kidney biopsies taken from vasculitis patients, revealing (like in SLE) that they may be involved in the development of glomerulonephritis. There is also a strong link to plasmacytoid dendritic cell activation by these autoantigen complexes and the release of IFN-α around the site of kidney damage (Kessenbrock et al., 2009).

Autoimmunity and Infection

While it is well documented that patients suffering from autoimmune diseases are more prone to infections that contribute to the morbidity and mortality of the diseases, the link between autoimmune onset and infection is less clear.

Investigations into infections and RA onset have not been fruitful to date. A study in Sweden revealed a high prevalence (45%) of prior infection in patients diagnosed with all forms of inflammatory arthritis. This screen examined many pathogens, including *Chlamydia trachomatis* and *pneumonia*, *Campylobacter jejuni*, *Borrelia burgdorferi*, parvovirus B19, and *Salmonella* Typhimurium. While *C. jejuni* was the most prevalent infection prior to onset, this was only clear in patients with reactive arthritis disease (Söderlin et al., 2003). As reviewed by Carty et al. (2003), while many infectious agents have been shown to be present prior to infection, there is no clear consensus that this is a causing factor of RA.

Pathogens utilize molecules similar to host molecules to evade the immune system, and as such in molecular mimicry, the immune response can eventually turn toward the self peptide as a result of cross-reactivity with subsequent activation of naive, autoreactive T cells. This can be responsible for initiation of autoimmune diseases such as SLE (Radic and Marion, 2013).

While neutrophils are central to host defense against pathogen attack, they are also clearly involved in the progression of autoimmunity. As many molecules found in neutrophils have been proven to be antigens in autoimmune diseases, methods to target these cells early in the onset of disease may provide a mechanism to prevent or dampen autoimmune disease progression.

Conclusion

Neutrophils are considered the front line of host defense against pathogen attack, as they are the first immune cells to arrive at the site of infection and are equipped with an arsenal of weapons to ensure successful clearance of pathogens. The importance of neutrophils in antimicrobial defense has been outlined here by the sheer numbers of new neutrophils generated on a daily basis to ensure the numbers required to clear infection are always available. This has been further confirmed by the fact that individuals with mutations in proteins important for neutrophil function or neutropenic patients succumb to certain infections more readily than healthy people. However, one must be aware that even though neutrophils aim to be helpful in the immune response, as they will do anything to ensure death of pathogens, this can also result in the release of potentially harmful self molecules that are known to be antigens in the progression of an autoimmune disease (Figure 3). Therefore, while it is clear that neutrophils are important in host defense, they must also be

thought of as potentially dangerous to the host and as a potential therapeutic target in autoimmunity.

AUTHOR CONTRIBUTIONS

All authors contributed equally to this work and are listed alphabetically in the author list.

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