Review

Neutrophils, from Marrow to Microbes

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Neutrophils are produced in the bone marrow from stem cells that proliferate and differentiate to mature neutrophils fully equipped with an armory of granules. These contain proteins that enable the neutrophil to deliver lethal hits against microorganisms, but also to cause great tissue damage. Neutrophils circulate in the blood as dormant cells. At sites of infection, endothelial cells capture bypassing neutrophils and guide them through the endothelial cell lining whereby the neutrophils are activated and tuned for the subsequent interaction with microbes. Once in tissues, neutrophils kill microorganisms by microbicidal agents liberated from granules or generated by metabolic activation. As a final act, neutrophils can extrude stands of DNA with bactericidal proteins attached that act as extracellular traps for microorganisms.

Introduction

Neutrophils are indispensable for defense against intruding microorganisms. They are generated in great number in the bone marrow and circulate in blood for a few hours. If microorganisms have successfully overcome the physical barriers provided by the skin and mucus membranes and gained access to tissues, signals generated by microbes and resident macrophages at sites of infection activate the local endothelial cells, which capture bypassing neutrophils and guide them across the endothelial cell lining. Products generated by live microorganisms and by their interaction with soluble proteins that recognize microorganisms guide neutrophils toward the microbes, which are taken up by phagocytosis and exposed to high concentrations of bactericidal substances in the phagocytic vacuole. Neutrophils in tissues produce agents that attract additional neutrophils and macrophages and T cells and regulate their activity. This review will focus on the mechanisms that control the generation of neutrophils and the formation of their distinct subsets of granules, the molecules that are involved in recruitment of neutrophils to sites of infection in the systemic circulation, the generation of bactericidal extracellular traps, and the resolution of inflammation.

Production of Neutrophils

Production of neutrophils is quantitatively the major activity of the bone marrow, where approximately two-thirds of the blood-cell-forming activity (hematopoiesis) is devoted to myelopoiesis, the term used for the collective production of monocytes and granulocytes. Hematopoietic stem cells localize to niches provided by osteoblasts and characterized by little blood flow and low oxygen tension, while the more mature and actively dividing stem cells reside closer to the abluminal side of the sinusoids, the special vascular structure of the bone marrow (Winkler et al., 2010). β_1-integrins such as $\alpha_4\beta_1$, $\alpha_6\beta_1$, and $\alpha_9\beta_1$ are expressed on hematopoietic stem cells and interact with osteoblasts in the stem cell niches and with the extracellular matrix (Qian et al., 2006, 2007; Schreiber et al., 2009; Grassinger et al., 2009). The chemokine receptor CXCR4 is essential for homing of stem cells and more mature neutrophils to the bone marrow (Lapidot and Kollet, 2002). CXCR4 binds CXCL12

(former stromal derived factor 1 [SDF1]), which is expressed by bone marrow stromal cells, including both osteoblasts and vascular endothelial cells in the bone marrow (Lapidot and Kollet, 2002).

The production of neutrophils is extensive in steady state with 1 to 2×10^{11} cells being generated per day in a normal adult human. G-CSF (granulocyte colony stimulating factor) is essential for tuning the production of neutrophils to meet the increased needs during infections, but G-CSF is not absolutely required for granulocytopoiesis because G-CSF null mice have approximately 25% residual granulocytopoiesis and generate fully mature neutrophils (Lieschke et al., 1994). The production of neutrophil is largely regulated by the rate of apoptosis of neutrophils in tissues. When macrophages and dendritic cells phagocytose apoptotic neutrophils in tissues, their production of interleukin 23 (IL-23) is reduced (Ley et al., 2006; Stark et al., 2005; von Vietinghoff and Ley, 2009). IL-23 stimulates production of interleukin 17A (IL-17A) by specialized T cells collectively called neutrophil regulatory T cells, which are largely localized in the mesenteric lymph nodes. These are largely gamma-delta T cells and NK-like T cells (Ley et al., 2006). IL-17A is an important stimulus for G-CSF production (Schwarzenberger et al., 2000). Hence, production of G-CSF is reduced when the number of neutrophils in tissues increases.

Release of Neutrophils from Bone Marrow

As mentioned, CXCR4 plays a crucial role for maintaining neutrophils in the bone marrow. Deletion of CXCR4 causes a shift in the pool of mature neutrophils from bone marrow to circulation without affecting the life-span of circulating neutrophils (Eash et al., 2009). Conversely, mutations of CXCR4 that result in increased signaling result in the clinical syndrome WHIM (warts, hypogammaglobulinemia, infections, myelokathexis) characterized by deficiency of neutrophils in circulation and by increased accumulation of mature neutrophils in the bone marrow (Hernandez et al., 2003). CXCR2 is another cytokine receptor expressed on myeloid cells. Its ligands, CXCL1 and CXCL2 (KC, and Groβ or MIP-2, respectively), are expressed by bone marrow endothelial cells (Eash et al., 2010). Deletion of CXCR2 also causes a myelokathexis phenotype with retention of mature neutrophils in the
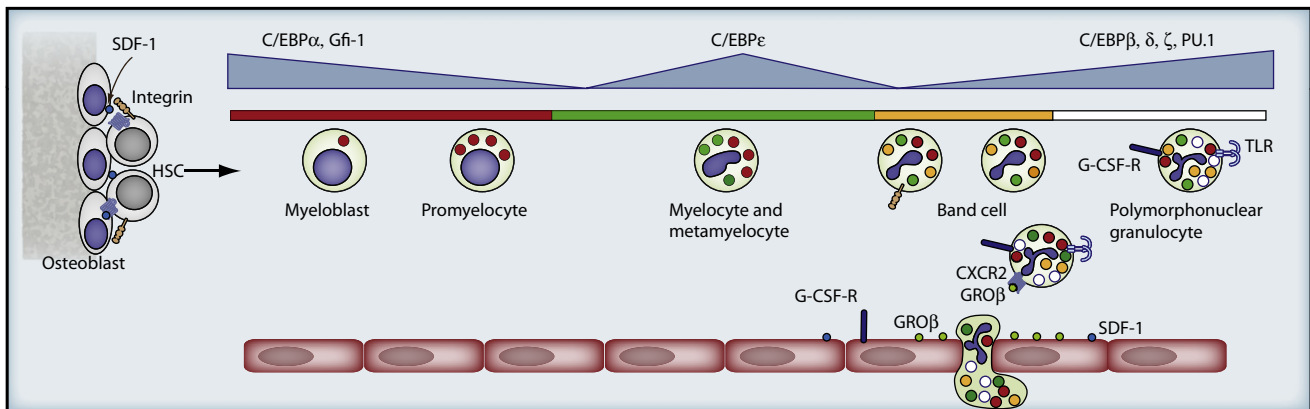
Figure 1. Granulocytopoiesis in the Bone Marrow
Stem cells localize to niches provided by osteoblasts and endothelial cells. The balance between PU.1 and C/EBPα and Gfi-1 expression determines the differentiation into the granulocytic or the monocytic pathways. The different subsets of granules (azurophil (red), specific granules (green), and gelatinase granules (yellow) and secretory vesicles (empty) are formed sequentially during maturation from promyelocytes, determined by the expression of transcription factors indicated on the top of the figure. Retention and release of cells is determined by the balance between CXCR4 (favoring retention) and CXCR2 (favoring release) and their ligands SDF-1, and KC and Groβ, respectively. G-CSF stimulates neutrophil release directly by effects on the neutrophil and indirectly by reducing the SDF-1 expression and enhancing the expression of Groβ on endothelial cells.

bone marrow, whereas double deletion of both CXCR2 and CXCR4 gives a phenotype similar to the CXCR4 deletion, i.e., inability to retain neutrophils in the bone marrow pool (Eash et al., 2010). Thus, CXCR4 is necessary for maintaining the neutrophil bone marrow pool ready for immediate release. Release may be effectuated by signals through CXCR2, G-CSFR or Toll-like receptors, all of which appear late during neutrophil maturation in the bone marrow (Theilgaard-Mönch et al., 2005a). Stimulation of these receptors does not cause additional neutrophil release from the bone marrow when CXCR4 is absent, demonstrating the fundamental role of CXCR4 signaling for homing of both immature and mature neutrophils in the bone marrow (Eash et al., 2009; Figure 1).

The expression of SDF-1 by bone marrow endothelial cells is downregulated by G-CSF, whereas G-CSF upregulates their expression of KC and Groβ, thus tilting the CCXR4- CXCR2 signaling in favor of CXCR2 and neutrophil release (Christopher et al., 2009; Eash et al., 2010) (Figure 1).

Terminal Granulocytopoiesis
Terminal granulocytopoiesis describes the production of mature granulocytes from committed progenitors. C/EBPα (CCAAT/ enhancer binding protein α) and PU.1 are both essential transcription factors necessary for terminal granulocytopoiesis. PU.1 is absolutely required for myeloid lineage commitment (Nerlov and Graf, 1998; Iwasaki et al., 2005). The subsequent decision between granulocyte and monocyte commitment is determined by the balance between C/EBPα and PU.1 (Reddy et al., 2002; Dahl et al., 2003; Laslo et al., 2006). High expression of PU.1 drives monocytic differentiation, whereas C/EBPα is absolutely required for granulocytopoiesis (Zhang et al., 1997; Radomska et al., 1998), but granulocytopoiesis can be rescued by C/EBPβ in C/EBPα-deficient myelopoiesis stimulated by IL-3 and GM-CSF (granulocyte-monocyte colony stimulating factor) (Hirai et al., 2006), and C/EBPβ is required for accelerated granulocytopoiesis in response to fungal infections (Hirai et al., 2006). MicroRNA 223 is an essential target for C/EBPα, which

displaces NFI-A from the miR223 promoter (Fazi et al., 2005), allowing for subsequent granulocytic differentiation, in part by downregulating the cell-cycle regulator E2F1 (Pulikkan et al., 2010), which is also inhibited directly by C/EBPα (D'Alo' et al., 2003). In accordance, miR-223-deficient mice have double production of neutrophils (Johnnidis et al., 2008). Recently, the NF-κB p50 subunit has been shown to activate C/EBPα expression, both alone and more so in complex with C/EBPα itself, resulting in enhanced G-CSF-induced granulocytopoiesis in vivo, thus illustrating that this factor that is critical for mediating inflammatory responses also regulates early granulocytopoiesis (Wang et al., 2009).

A transcription factor normally expressed during lymphopoiesis, Lef-1 (lymphoid enhancer-binding factor-1), has been suggested to regulate C/EBPα expression, and failure to express Lef1 has been proposed as a unifying mechanism for severe congenital neutropenia (Skokowa et al., 2006), caused by mutations in the neutrophil elastase gene (Dale et al., 2000) and the HAX1 gene (Klein et al., 2007). Although the mechanism for this is not entirely clear, studies of PFAAP5, a protein identified by 2-yeast hybrid screening with Lef-1 and elastase as baits, may provide some clues (Salipante et al., 2009).

The transcription factor Gfi-1 (growth factor independent-1) is necessary for neutrophil differentiation (Karsunky et al., 2002; Hock et al., 2003). Gfi-1 is upregulated during stem cell commitment to the granulocytic lineage (Velu et al., 2009). Upregulation of Gfi-1 represses genes encoding HoxA9, Meis1, and Pbx1, allowing for progression of differentiation (Horman et al., 2009), and restricts the proliferation of stem cells (Hock et al., 2004). Gfi-1 has additional effects on granulocytopoiesis by repressing expression of Egr2 (early growth response-2) (Laslo et al., 2006), a monocyte-promoting transcription factor, and Csf1 (Zarebski et al., 2008), the gene coding for the monocytopoietic cytokine CSF-1 (colony-stimulating factor 1). The effect of Gfi-1 is at least partially mediated by repression of miR21 and miR196b (Velu et al., 2009) which have promonocytopoietic and antigranulocytopoietic activities, respectively, in agreement with the effects

observed in Gfi-1-deficient mice (Karsunky et al., 2002; Hock et al., 2003). Mutations of Gfi-1 affecting its DNA binding site are responsible for some cases of severe congenital neutropenia (Person et al., 2003). The phenotype can be reproduced in mice. This has revealed that the N382S Gfi-1 mutation results in block of granulocytopoiesis because of overexpression of Csf-1. This block is lifted by removal of Csf-1 (Zarebski et al., 2008).

The further differentiation beyond promyelocytes is regulated by several transcription factors. C/EBPε becomes expressed at the myelocyte stage (Morosetti et al., 1997; Yamanaka et al., 1997a, 1997b; Bjerregaard et al., 2003). C/EBPε is required for transcription of granule proteins at the myelocyte stage and beyond, as evidenced by C/EBPε gene-targeted mice (Yamanaka et al., 1997a; Lekstrom-Himes and Xanthopoulos, 1999). Mutations of C/EBPε leading to nonexpression of the protein account for some cases of the rare condition termed specific granule deficiency (Gombart et al., 2001). Interaction with Gfi-1 may be essential for full activity of C/EBPε (Khanna-Gupta et al., 2007), although Gfi-1 expression is at a nadir when C/EBPε expression is at its peak (Bjerregaard et al., 2003). The transition from promyelocyte into myelocyte is associated with exit from cell cycle (Klausen et al., 2004). This is in part regulated by C/EBPε, which binds Rb protein and E2F1 (Gery et al., 2004) and represses their transactivating effect on genes that drive the cell cycle. This effect, combined with the aforementioned effect of miR223 on E2F, may explain the exit from cell cycle during this stage of myelopoiesis. Expression of PU.1, essential for the initial commitment to myelopoiesis, increases during maturation from the promyelocyte stage, and its coactivator c-jun and its dimerization partner c-fos both increase in myelocytes and metamyelocytes (Bjerregaard et al., 2003). Accordingly, PU.1-deficient mice demonstrate defects in terminal granulocytic development in addition to the deficiency of committed stem cells. Whereas C/EBPα expression gradually diminishes from the myeloblast stage and up, and C/EBPε peaks at the myelocyte-metamyelocyte stage, C/EBPβ, C/EBPγ, C/EBPδ, and C/EBPζ all increase from the metamyelocyte stage and on during maturation (Bjerregaard et al., 2003). As the C/EBPs form both homodimers and heterodimers and their activity is further regulated by phosphorylation (Chumakov et al., 2007), they provide basis for a highly individualized expression of granule proteins during terminal granulocytic differentiation.

Formation of Granules and of Granule Proteins
Granules, the hallmark of granulocytes (eosinophils, basophils, and neutrophils) are stores of proteins that can kill microbes and digest tissues. Appearance of granules marks the transition from myeloblast to promyelocyte and formation of granules continues until the segmented stage of maturation is reached. Granules may be filled passively by bulk flow of proteins coming from the more proximal Golgi compartments. In agreement with this, a variety of proteins that are constitutively secreted by the liver, but also produced by neutrophil precursors, have been identified as genuine granule proteins of the human neutrophil such as haptoglobin (Theilgaard-Mönch et al., 2006a), α1-acid glycoprotein (Theilgaard-Mönch et al., 2005b), and α1-antitrypsin (Johansson et al., 2001). However, not all granule proteins are stored with equal efficiency. Lysozyme, an antibacterial protein, is made by neutrophil precursors from the early promye-

locyte and up to band cells (Lollike et al., 1995), and most newly formed lysozyme is spilled out of the cells, while α-defensins are efficiently withheld when formed in promyelocytes, but largely spilled out when formed in the more mature myelocytes, indicating that some selectivity does exist in the routing between constitutive secretion and diversion to storage in granules (Arnljots et al., 1998). Considering the size of the myeloid compartment in the bone marrow, this is potentially a major organ for secretion of proteins that are not efficiently targeted to granules such as lysozyme and the cathelicidin antimicrobial protein hCAP-18 (whose C-terminal part is known as LL-37), both of which are found in high amounts in plasma (Lollike et al., 1995; Sørensen et al., 1999).

Neutrophil granules are classified into three distinct subsets based on the presence of characteristic granule proteins: primary (azurophil) granules (myeloperoxidase [MPO]), secondary (specific) granules (lactoferrin), and tertiary (gelatinase granules) (gelatinase). These are formed sequentially during granulocytic differentiation in the bone marrow i.e., at the promyelocyte stage, the myelocyte-metamyelocyte stage, and the band cell stage (Borregaard et al., 1995; Borregaard and Cowland, 1997). This distinction is operative because it not only reflects differences in content of granules (Lominadze et al., 2005), but also differences in their mobilization with the granules formed latest being the ones to be released earliest (Borregaard and Cowland, 1997).The distinction is, however, artificial because other marker proteins identify subsets of granules that are formed at somewhat different stages of differentiation than the classical three granule subtypes (Faurschou et al., 2002; Rørvig et al., 2009; Udby et al., 2002). The heterogeneity of neutrophil granules is best comprehended by assuming that granules are formed constitutively from the early promyelocyte and up until the segmented stage in the bone marrow and filled with proteins, synthesized when the granules are formed. Because the windows of expression of the individual granule proteins are highly divergent, granules formed at different stages during maturation carry a different cargo of matrix and membrane proteins. We have named this mechanism for explaining granule heterogeneity "targeting by timing of biosynthesis" (Le Cabec et al., 1996). Proteins synthesized in neutrophils after exit from the bone marrow do not seem to be packed in granules (Theilgaard-Mönch et al., 2004; Lapinet et al., 2000), but the mechanisms for initiation and cessation of granule formation are not known.

Profiling the gene expression during myelopoiesis in relation to stage of maturity of the cells has consequently allowed us to confirm the relationship between gene expression of known granule proteins and their granule localization (Theilgaard-Mönch et al., 2005a). It has further permitted us to predict the localization of proteins not previously known to be granule proteins of neutrophils and confirm their localization, e.g., haptoglobin (Theilgaard-Mönch et al., 2006a), pentraxin 3 (Jaillon et al., 2007), arginase 1 (Jacobsen et al., 2007), and ficolin 1 (Rørvig et al., 2009).

Secretory Vesicles
The view of granules as storage organelles for proteins to be liberated into phagosomes or outside the cell changed by the discovery that the membrane components of the NADPH oxidase, now known as the $p22^{phox}$-$gp91^{phox}$ complex, is
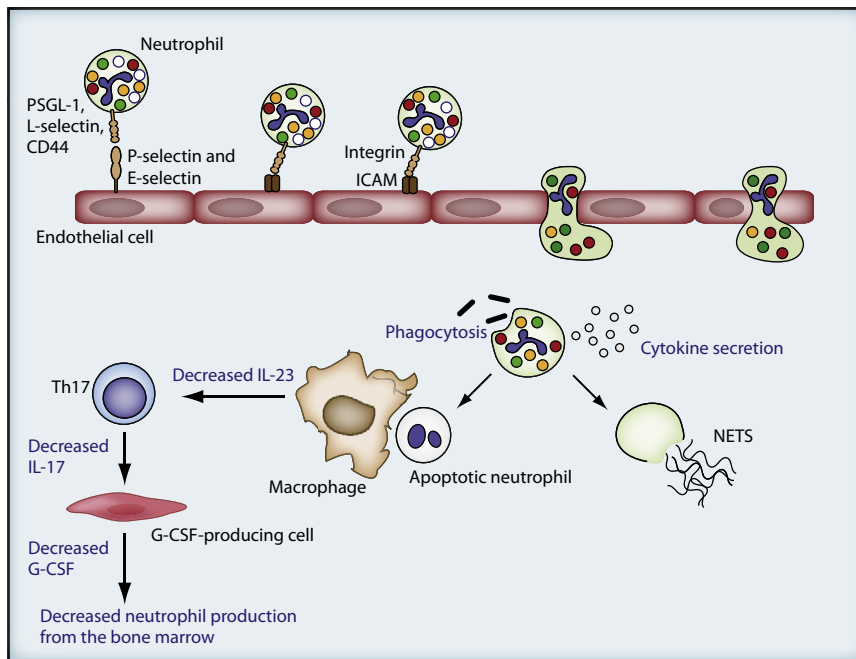
Figure 2. Neutrophils in Tissues

Neutrophils are captured by endothelial cell P-selectins. Bonds between P-Selectin and E-selectin on endothelial cells and PSGL-1, L-selectin, ESL-1, and CD44 on neutrophils mediate rolling and activation of the neutrophil integrins, which interact with ICAMs expressed on endothelial cells. The trans-endothelial migration is either paracellular or transcellular. The tight junctions are loosened by neutrophils that present alternative binding partners PECAM-1, JAM-A, LFA-1, and Mac-1, thus unzipping the tight junctions and zipping themselves between the endothelial cells to mediate paracellular migration. In transcellular migration, transmigratory cups high in ICAM-1 and VCAM-1 capture crawling neutrophils that then make their way through the cells. Once out in tissues, a burst of transcriptional activity in part mediated by local G-CSF production initiates generation of cytokines by neutrophils. The neutrophils phagocytose microorganisms and degranulate and may then either undergo apoptosis or netosis. Uptake of apoptotic neutrophils by macrophages downmodulates the production of IL-23. Thereby, less IL-17 is generated from T cells. Hence, less G-CSF is produced by fibroblast and endothelial cells to stimulate the neutrophil production in the bone marrow. Lipid mediators, lipoxins, resolvins, and protectins are generated through sequential steps involving neutrophils, endothelial cells, and macrophages. They downmodulate neutrophil activation and endothelial cell activation while enhancing uptake of apoptotic cells by macrophages.

located in the membrane of specific granules and becomes incorporated into the surface membrane during activation of neutrophils (Borregaard et al., 1983; Borregaard and Tauber, 1984). This was further expanded when secretory vesicles were discovered as storage organelles of membrane proteins wrapped around a matrix of plasma proteins (Borregaard et al., 1992). They are created by endocytosis during the late maturation of neutrophils in the bone marrow since they contain membrane proteins whose genes are transcribed at the band and segmented stage (Borregaard et al., 2007; Theilgaard-Mönch et al., 2006b), including the marker protein of secretory vesicles, CD35 (Sengeløv et al., 1994). Secretory vesicles incorporate their membrane into the surface membrane of neutrophils in response to chemotactic peptides (Borregaard et al., 1987) and are fully integrated into the surface during transendothelial migration of neutrophils (Sengeløv et al., 1995). A complete characterization of the protein content of secretory vesicles has not yet been performed.

Control of Granule Exocytosis

Although the structural heterogeneity of neutrophils granules can be seen and understood as a consequence of differences in expression of granule proteins during maturation of myeloid cells in the bone marrow, it is less apparent how the hierarchy for mobilization of neutrophils granules is established. Secretory vesicles are mobilized completely by several stimuli that are relevant in the interaction of neutrophils with activated endothelium, such as signaling by selectins and their cognate proteins, and by chemokines, such as IL-8 and fMLP. In this way, the secretory vesicles can furnish the surface membrane with receptors and other functional proteins, such as integrins, for use in

transmigration without liberating potential harmful granule proteins (Borregaard et al., 1992, 1993, 1994; Sengeløv et al., 1993). Gelatinase granules have a somewhat higher threshold for exocytosis than secretory vesicles (20% mobilized by fMLP (Kjeldsen et al., 1992, 1993), specific granules again have a higher threshold than gelatinase granules and azurophil granules can only be mobilized partially (Sengeløv et al., 1993; Faurschou et al., 2002).

Neutrophil-Endothelial Cell Interactions

The neutrophils need to cross the vascular wall to arrive at the site of entry of microorganisms (Figure 2). The crossing takes place largely at postcapillary venules. Here, the vessel wall is rather thin, and the diameter of the vessel is sufficiently small that the neutrophils can make contact with the vessel wall, but sufficiently large not to be occluded by neutrophils when they arrest and make firm contact with the endothelium. Leukocyte recruitment into many systemic organs is unique, and it is not possible to review this herein. The issue is the subject of several excellent reviews (Vestweber, 2007; Zarbock and Ley, 2009a, 2009b; Alcaide et al., 2009; Woodfin et al., 2010; Fernandez-Borja et al., 2010; Petri et al., 2008; Engelhardt, 2008). The mechanism for diapedesis of neutrophils described in the following pertains to neutrophil transendothelial migration in response to inflammation in the systemic vascular bed.

Rolling

Initial attachment of neutrophils to endothelial cells is determined by the endothelial cells, which react to stimuli such as TNFα, IL-1β, and IL-17 that are generated during infection or inflammation. Such stimulation results in expression of P-selectin (also

expressed by activated platelets), E-selectin, as well as members of the integrin superfamily (the ICAMs and VCAMs, detailed later) on their luminal surface. These selectins bind PSGL-1 (*P*-selectin *l*igand *1*) and L-selectin, which are expressed constitutively on the tips of neutrophil microvilli, a location organized by the ERM proteins ezrin, radixin, and moesin that connect to the actin cytoskeleton (Bruehl et al., 1997; Steegmaier et al., 1997; Buscher et al., 2010). E-selectin also binds ESL-1 (*E*-selectin *l*igand 1) present on the side of microvilli and CD44 present on the cell body (Buscher et al., 2010). Binding of PSGL-1 to P-selectin and E-selectin establishes the initial contact between neutrophils and activated endothelial cells. E-selectin and ESL-1 mediate the slower rolling, while E-selectin binding to CD44 mediates a redistribution of PSGL-1 and L-selectin to form clusters (Hidalgo et al., 2007) concomitant with further reduction in the speed of rolling. The selectin mediated bonds form and detach sequentially, but sufficient strength to mediate attachment only develops during the shear stress that is created by the laminar flow of blood in vessels (Ley et al., 2007; Pospieszalska and Ley, 2009).

Selectins bind sialyl Lewis X carbohydrate structures expressed on their ligands (Somers et al., 2000). Inherited defects in intracellular fucose transport disabling the construction of Lewis X result in *l*eukocyte *a*dhesion *d*eficiency type *II* (LAD II) (Lühn et al., 2001; Phillips et al., 1995). The signaling induced by binding of ligands to PSGL-1 and CD44 involves activation of Src family kinases Hck, Fgr, and Lyn (Yago et al., 2010), which phosphorylate and activate ITAMs (*i*mmunoreceptor *t*yrosine-*b*ased *a*ctivation *m*otifs) on two adaptor proteins, DAP12 (NDAX activation protein of 12 kDa) and FcRγ (γ chain of immunoglobulin *Fc r*eceptors) (Zarbock et al., 2008). These recruit Syk (*s*pleen *t*yrosine *k*inase) that becomes activated by phosphorylation. Activated Syk in turn activates Bruton tyrosine kinase (Yago et al., 2010; Mueller et al., 2010), which mediates the further activation of PLC-γ (phospholipase C γ), PI3K (Phosphoinositide 3-kinase), and p38 mitogen-activated protein kinase, resulting in integrin activation and cytoskeletal rearrangements in the neutrophil (Ley et al., 2007; Woodfin et al., 2010; Barreiro and Sánchez-Madrid, 2009; Scheiermann et al., 2010).

Firm Adhesion
The key molecules mediating firm adhesion of neutrophils to the activated endothelium are the β_2 integrins LAF-1 ($\alpha_L\beta_2$) and Mac-1 ($\alpha_M\beta_2$), present on neutrophils, and their ligands, members of the immunoglobulin superfamily, ICAM-1 (*i*ntercellular *a*dhesion *m*olecule-*1*) and ICAM-2, present on the endothelial cells. Deficiency of CD18 the β_2-integrin chain causes Leukocyt Adhesion Deficiency type I, characterized by inability to establish firm adhesion and diapedesis of neutrophils (Bunting et al., 2002).

Integrins can adopt three states of activation: a bent form incapable of ligand binding, an extended, but not fully open, form with intermediate ligand binding capacity, and an extended and open form with full avidity for ligand binding. The molecular mechanism of integrin activation has been delineated in great detail and recently reviewed (Evans et al., 2009; Abram and Lowell, 2009b). Activated phospholipase Cγ causes production of diacyl glycerol, which activates protein kinase C and inositol-3-phosphate that induces a rise in intracellular Ca^{2+}. These activate the guanine exchange factor CalDAG-GEF1, which turns the

small GTPase, Rap-1, into its active GTP associated form. Activated Rap-1 binds *R*ap-1-med*i*ated *a*daptor *m*olecule (RIAM), which recruits talins to break salt bridges between the cytoplasmic tails of the integrin β and α chains by binding to a transmembrane-near phosphotyrosine domain, NPxY, in the β chains, thus twisting the two integrin chains apart. This results in extension of the extracellular domains and opening for ligand binding (Anthis et al., 2009). At the same time, talins tether the integrins to the actin cytoskeleton. Kindlins, like talins, contain domains for binding to NPxY sites. Kindlin3, which is expressed exclusively in cells of hematopoietic origin, is recruited to the integrin β chains, but at more distal NxxY sites. Kindlin 3 gene-targeted mice display defective integrin activation (Moser et al., 2008, 2009a, 2009b) Mutations of kindlin 3 were recently discovered as cause of congenital leukocyte adhesion deficiency LAD III. In contrast to LAD I, LAD III also displays hemostatic defects because of dysfunctional platelets as result of defective activation of all β integrins (McDowall et al., 2003, 2010; Kuijpers et al., 2009; Svensson et al., 2009).

LFA-1 is the principal adhesion molecule on neutrophils that mediates transition from rolling to adhesion on the endothelial cell surface (Phillipson et al., 2006). Rolling on E- or P-selectin induces the extended, but not high affinity form of LFA-1 (Kuwano et al., 2010), enabling the LFA-1 to mediate rolling, but not firm adhesion, to ICAM-1. This activation of LFA-1 is induced by signals from PSGL-1 and CD44 (Zarbock et al., 2008; Yago et al., 2010). Full LFA-1 activation is mediated by activation of the chemokine receptors on neutrophils (Zarbock et al., 2007), which pick up ligands secreted by endothelial cells or presented by the endothelial cell glycosaminoglycans or by the Duffy antigen-receptor for chemokines as discussed in Middleton et al. (2002) and Zarbock and Ley (2009a). Fully activated LFA-1 mediates stop of rolling and firm adhesion together with Mac-1 (Woodfin et al., 2010).

The activated (by tumor-necrosis factor α [TNFα], Interleukin-1 [IL-1], and IL-17) endothelial cells produce IL-8 and MIP-2 that activate neutrophils. Macrophage migration inhibitory factor generation by endothelial cells has been shown to enhance the production of IL-8, P-Selectin, and ICAM-1 by cultured TNFα stimulated HUVECS (Cheng et al., 2010).

Concomitant with cessation of rolling and induction of firm adhesion, the neutrophils polarize into a leading edge lamellipodium, where receptors for chemokines and for phagocytosis are concentrated. This is organized by F-actin and controlled by G_i type G protein-coupled receptors via signals from phosphoinositol 3 phosphate (PIP3) and the Rho GTPase, Rac. The tail end of polarized neutrophils contains a contractile actomyosin system controlled by Gα12 and Gα13 type G protein coupled receptors, CDC42, Rho A, Rho-dependent kinase, ROCK, and myosin II (Xu et al., 2003; Van Keymeulen et al., 2006). This polarization is regulated by Ly49Q, an ITIM (*i*mmunoreceptor *t*yrosine-based *i*nhibitory *m*otif)-containing receptor, which, when located in the surface membrane, recruits the phosphotyrosine phosphatase SHP1 and maintains the neutrophil round and suppresses activation but, when active, recruits SHP2 and reorganizes lipid rafts and their associated signaling molecules to the perinuclear region and induces the polarized shape via the signaling molecules mentioned above (Sasawatari et al., 2010).

Transendothelial Migration

Once firm adhesion is established, two roads can be taken for transendothelial migration: the transcellular road, whereby neutrophils penetrate the individual endothelial cell, or the paracellular road, whereby neutrophils squeeze between endothelial cells. The key players involved in guidance toward paracellular or transcellular migration are again the major neutrophil β_2 integrins LFA-1 and Mac-1 and their ligands ICAM-1 and ICAM-2. ICAM-1 is concentrated to the recently described tetraspanin-enriched microdomains to form so-called endothelial adhesive platforms (Barreiro et al., 2008). This is independent on the cytoskeleton and independent on activation of ICAM-1. Binding to LFA-1 on neutrophils generates signals from the cytoplasmic tail of ICAM-1 (Fernandez-Borja et al., 2010; van Buul et al., 2010a). These induce activation of the GTPases RhoG and RhoA and induce actin polymerization (van Buul et al., 2010a), leading to formation of small membrane protrusions called apical cups or docking structures where ICAM-1, and VCAM-1 (a ligand for β_1 integrins) are concentrated (van Buul et al., 2007; Barreiro et al., 2002), most likely via ezrin, radixin, and moesin that, when activated, tether ICAM-1 and V-CAM-1 to the actin cytoskeleton (Sato et al., 1992; Tsukita and Yonemura, 1997, 1999; van Buul et al., 2007; Oh et al., 2007). Accumulation of ICAM-1 may by itself recruit VCAM-1 to the clusters (van Buul et al., 2010b). Tyrosine phosphorylation of cortactin by Src further enhances the ICAM-1 F-actin association and helps transforming adhesion platforms to adhesion cups (Yang et al., 2006a, 2006b). α-actinins recruited to ICAM-1 cytoplasmic tail domains further strengthen the ICAM-1 F-actin interaction and stabilize the latter (Yang et al., 2006a; Celli et al., 2006). These cups are necessary for efficient transcellular migration both via the transcellular route and via the paracellular route (Carman and Springer, 2004). ICAM-2 and PECAM-1 are concentrated at endothelial cell junctions (Woodfin et al., 2009). $\alpha_9\beta_1$ is the major β_1 integrin upregulated on (human) neutrophils by recruitment from intracellular stores not yet identified (Mambole et al., 2010). This allows adhesion to VCAM-1 (Taooka et al., 1999; Mambole et al., 2010) but plays a minor role in neutrophil transendothelial migration compared to LFA-1 and Mac-1 (Woodfin et al., 2010).

Mac-1, the other major β_2 integrin on neutrophils, mediates crawling of neutrophils along endothelial cells to points of paracellular migration (Phillipson et al., 2006; Woodfin et al., 2010). Mac-1 interacts to a large extent with the same ligands on endothelial cells as LFA-1, i.e., ICAM-1 and ICAM-2. Vav1, a guanine exchange factor and activator for the Rho family GTPases, Rac and Cdc42, which are central for downstream signaling from LFA-1, is essential for this active migration of neutrophils toward endothelial cell-cell borders mediated by Mac-1 as observed in vivo during flow (mouse cremaster) (Phillipson et al., 2009).

It was recently suggested that stimulated neutrophils release a truncated form of the cytosolic protein annexin A1, lacking the N-terminal 26 amino acids as result of proteolysis by calpain. This 33 kDa peptide activates endothelial cells via extracellular signal regulated kinase (ERK1 and 2) to support transendothelial cell migration as demonstrated in vitro in a trans-well system using confluent HUVECs (*h*uman *u*mbilical *ve*ne *e*ndothelial *c*ells) (Williams et al., 2010).

The molecular mechanisms mediating the paracellular migration of neutrophils have been worked out in great detail. The main players on endothelial cells are ICAM-1; ICAM-2; PECAM-1 (*p*latelet *e*ndothelial *c*ell *a*dhesion *m*olecule 1); JAM (*j*unctional *a*dhesion *m*olecule)-A, -B, and -C; ESAM (*e*ndothelial cell-*s*elec-tive *a*dhesion *m*olecule); PVR (*p*oliovirus *r*eceptor—all belonging to the immunoglobulin superfamily—CD99; CD99L2; and VE-Cadherin. PECAM-1; JAM A, -B, and -C; ESAM; CD99; and VE-cadherins form homotypic contacts to stabilize the endothelial cell junctions, but with the exception of VE-cadherin and ESAM, these adhesion molecules are also expressed on the neutrophil (PECAM-1, JAM A, CD99) and are capable of binding to proteins expressed on the neutrophil surface and, thus, assist the neutrophils in passage between the endothelial cells.

The following sequence has been demonstrated: ICAM-1 and ICAM-2 accumulate at the cell-cell junctions mediating neutrophil contact via their β_2 integrin partners (LFA-1 and Mac-1). ICAM-1 at the endothelial cell junctions guides neutrophils to these structures (Alcaide et al., 2009). Signals from ICAM-1 activate Src and Pyk-2 tyrosine kinases, which phosphorylate VE-Cadherin and destabilize the VE-Cadherin bonds, probably by preventing VE-Cadherin from associating with β-catenin that mediates binding to the actin cytoskeleton via α-catenin (van Buul et al., 2005, 2010a; van Buul and Hordijk, 2008; Allingham et al., 2007), thus loosening the endothelial cell-cell junctions. ESAM may also play a role in loosening of the endothelial cell junctions. Lack of ESAM, as demonstrated in the $Esam^{-/-}$ mouse, results in decreased activity of the Rho signaling pathway in endothelial cells (Wegmann et al., 2006), which is known to destabilize endothelial cell junctions (Stamatovic et al., 2003). Lack of ESAM results in decreased neutrophil trans-endothelial cell migration as observed in both the mouse cremaster model (Wegmann et al., 2006) and in an ischemia reperfusion mouse liver model (Khandoga et al., 2009).

ICAM-2 concentrated at the endothelial cell junctions further guides neutrophils to enter the endothelial cell junctions. Neutrophils accumulate at the entrance between endothelial cells if ICAM-2 is blocked or absent and successful transmigration is inhibited correspondingly (Woodfin et al., 2009).

Further penetration between the cells is mediated by JAM-A as neutrophils accumulate deeper down between the endothelial in $Jama^{-/-}$ mice than in $Icam2^{-/-}$ mice as observed in vivo in IL-1β stimulated mouse cremaster muscle endothelial cells. Finally, neutrophils are observed to accumulate between the endothelial cells and the basement membrane in $Pecam1^{-/-}$ mice, indicating that PECAM-1 is essential for the neutrophils to slide through this compartment (Woodfin et al., 2009). Upregulation of the laminin receptor $\alpha_6\beta_1$ induced by PECAM-1 signaling assists the further migration through the perivascular basement membrane (Dangerfield et al., 2002). Endothelial cell PECAM-1 may also interact with the GPI-linked CD177 (NB1-antigen), which is upregulated on neutrophil surfaces from specific granules to facilitate transmigration (Sachs et al., 2007). The CD177-PECAM-1 interaction facilitates neutrophil transmigration with an efficacy that depends on a dimorphism of the PECAM-1 antigen (Bayat et al., 2010).

CD99 and the distantly related CD99L2 are both small, approx. 100 amino acid long, but highly *O*-glycosylated proteins. They are both expressed at endothelial cell-cell junctions and on leukocytes including neutrophils (Bixel et al., 2010). Both induce cell-cell aggregation (Dufour et al., 2008; Bixel et al., 2007, 2010),

and transcellular migration of neutrophils is inhibited by blocking antibodies in in vivo studies (Bixel et al., 2007; Dufour et al., 2008). Blocking antibodies against CD99 and CD99L2 lead to neutrophil accumulation between the endothelial cells and the basement membrane, similar to the stop in diapedesis observed in the absence of PECAM-1 (Bixel et al., 2010).

As mentioned before, the neutrophils may exploit these molecules, which form part of the endothelial cell-cell junction by providing alternative homotypic binding because these molecules are also expressed on the activated neutrophil. This mechanism is demonstrated by the deficient transendothelial migration and accumulation of JAM-A-deficient neutrophils between endothelial cells that express JAM-A (Corada et al., 2005). In addition, LFA-1 can bind to JAM-A at a site distant from the site for homotypic interaction and, by doing so, loosen the homotypic binding (Wojcikiewicz et al., 2009). JAM-A may also be shed from the activated endothelial cells by ADAM17, and soluble JAM-A bound to LFA-1 on the neutrophils can inhibit transcellular migration in such negative feedback (Koenen et al., 2009).

The transcellular route is believed to be taken by some 20% of neutrophils but may vary greatly among different tissues and depend on the stimulation of the endothelial cells in in vitro set-ups (Woodfin et al., 2010). It seems that the lateral migration of neutrophils mediated by Mac-1 favors paracellular migration, and trancellular migration increases from 20% to 80% in the absence of Mac-1 as determined in a mouse cremaster muscle preparation (Phillipson et al., 2008). In that study, the endothelial cell docking structures were demonstrated to progress to domes that finally swept around the neutrophil in a process similar to phagocytosis, thus indicating a very active role of the endothelial cell in transcellular migration. An active role for the transmigrating cell has also been proposed. Podosome-like protrusions were described on lymphocytes that serve as probes for seeking out areas on the endothelial cell where transcellular migration would be favorable (Carman, 2009; Carman et al., 2007). Because ICAM-2, PECAM-1, and JAM-A, which are important for paracellular migration, are localized to the endothelial cell junctions, other proteins must be involved in guiding the cell across the endothelial cell body. It has been suggested that SNARE proteins essential in trafficking and fusion of intracellular organelles and exocytosis of granules and vesicles are essential in this process (Carman et al., 2007).

After crossing the endothelial lining, neutrophils must make their way through the basal membrane. The neutrophil is highly equipped with proteases capable of breaking down the basal membrane collagens and laminins such as elastase (azurophil granules), MMP8 (specific granules), MMP9, and the membrane attached matrix metalloproteinase MT6-MMP (gelatinase granules and secretory vesicles) (Kang et al., 2001). Evidence that these proteases are essential for neutrophil transendothelial migration is scarce, as only minimal effects are observed in gene-targeted mice and in studies using specific protease inhibitors (Kolaczkowska et al., 2009). It is possible that the same retraction of endothelial cells that opens the tight junctions subsequently allows passage through the basal membranes of a cell, capable of amoeboid migration, such as the neutrophil, without necessitating degradation of the matrix (Rowe and Weiss, 2008).

Tissue Neutrophils

Neutrophils that have migrated into tissues are more active as phagocytic cells than blood neutrophils (Sørensen et al., 2001) and activate a transcriptional program that results in generation of several chemokines, including IL-8 and Groα (Theilgaard-Mönch et al., 2004; Scapini et al., 2000, 2005), that recruit additional inflammatory cells. The initiating factor(s) responsible for this wave of transcriptional activity is not known, but local production of G-CSF may be important as indicated by its induction of synthesis and secretion of B-lymphocyte stimulator (BlyS) (Scapini et al., 2005, 2008) in neutrophil exudates. Also, engagement of the *triggering receptor expressed on myeloid cells* (TREM-1) (by ligands not yet identified; Ford and McVicar, 2009), which is upregulated (Bouchon et al., 2000, 2001) and signals through DAP12 (presented earlier) (Bouchon et al., 2000; Tessarz and Cerwenka, 2008), may lead to degranulation and transcriptional activity (Bouchon et al., 2000).

Activation of neutrophil respiratory burst and mechanisms of microbial killing have been reviewed recently (El-Benna et al., 2009; Robinson, 2008, 2009; Holland, 2010; Nauseef, 2007; Flannagan et al., 2009). It should be mentioned that a function was recently identified for the 3rd cytosolic component, p40^{phox}, which assembles with the other two cytosolic components, p47^{phox} and p67^{phox}, and the membrane bound p22^{phox}, gp91^{phox} complex to form the NADPH oxidase. P40^{phox} is necessary for assembly of the NADPH oxidase on intracellular membranes, including phagosomes into which the p22^{phox}, gp91^{phox} bearing granules fuse, and deficiency of p40^{phox} results in partial NADPH oxidase deficiency (Matute et al., 2009).

Whereas the presence of fully functional neutrophils in tissues is absolutely critical for our defense against microbial infections, as testified by the immunodeficiency associated with LAD (Abram and Lowell, 2009a; Abram and Lowell, 2009b; Bunting et al., 2002) and chronic granulomatous disease (Holland, 2010), it is equally important that the influx of neutrophils is controlled to prevent neutrophil-mediated tissue damage. This is controlled by negative feedback at several stages.

Intracellular negative feedback is provided by proteins carrying immunoreceptor tyrosine-based inhibitory motifs (ITIMs), which recruit phosphotyrosine phosphatases to deactivate receptors that are kept in signaling mode by phoshotyrosines (Daëron et al., 2008). Signal Inhibitory Receptor on Leukocytes-1 (Steevels et al., 2010), CEACAM1, and CD300a contain ITIMs of which the latter two are mobilized to the surface of neutrophils during activation (Ducker and Skubitz, 1992; Alvarez et al., 2008; Skubitz and Skubitz, 2008). Similarly, SOCS-3 (*suppressor of cytokine signaling 3*) downregulates G-CSF receptor signaling by blocking the phosphotyrosine on the activated receptor and preventing its interaction with STAT3 (*signal transducer and activator of transcription 3*), necessary for further signaling. The importance of this regulation is illustrated by the hyperactive neutrophils in SOCS3-deficient mice (Croker et al., 2004). MicroRNAs also regulate neutrophil activity. MiR-223 gene-targeted mice have hyperresponsive neutrophils that infiltrate organs and inflict tissue damage both spontaneously and after LPS stimulation (Johnnidis et al., 2008). MiR-9 is induced in LPS and TNF-α-stimulated neutrophils by NF-κB and exerts a feedback inhibition on NF-κB translation (Bazzoni et al., 2009).

A complex and highly efficient mechanism to control and resolve inflammation operating at several stages has been identified after the deorphanization of the G protein-coupled receptor ChemR23 expressed on macrophages, dendritic cells, and recently also by activated endothelial cells (Kaur et al., 2010). Chem R23 was identified as receptor for chemerin, a 16 kD protein synthesized primarily by the liver. C-terminal peptides derived from chemerin are powerful agonists of ChemR23 and may be generated by proteolytic processing mediated by neutrophil proteases (Zabel et al., 2005, 2006). ChemR23 mediates a nonphlogistic activation of macrophages via PI3-K, Akt, and Syk signaling that greatly enhances the phagocytic capacity of macrophages, not the least for uptake of apoptotic neutrophils (Ohira et al., 2010; Cash et al., 2010). This reduces the risk of necrotic neutrophil death and also downregulates the production of G-CSF to limit neutrophil activation locally and release from the bone marrow as discussed previously.

A systemic analysis of lipid mediators generated in exudates identified two unique classes of anti-inflammatory lipid mediators, the resolvins and the protectins, both products generated from ω-3 fatty acids (Schwab et al., 2007; Serhan et al., 2008; Serhan, 2009). Resolvin E1 activates the ChemR23 and inhibits proinflammatory responses in macrophages (Ishida et al., 2010) and blocks the LTB_4 receptor BLT1, thus inhibiting LTB_4-mediated activation of neutrophils (Arita et al., 2007) and the neutrophil-induced increase in vascular permeability (Di Gennaro et al., 2009). Resolvin D1, which like resolvin E1 is also generated at the resolution phase of inflammation, activates the lipoxin A_4 receptor, ALX, known to mediate the anti-inflammatory actions of lipoxin A_4 (Maderna and Godson, 2009; Dufton et al., 2010; Maderna et al., 2010) and ChemR32. Like resolvin E1, resolvin D1 inhibits neutrophil activation (actin polymerization and $β_2$-integrin activation) (Serhan et al., 2008). Resolvin D2 has recently been shown to inhibit neutrophil activation and to augment the generation of nitric oxide by endothelial cells (Spite et al., 2009). In the same study, Resolvin D2 inhibited the adherence of neutrophils to activated endothelial cells in a mouse cremaster model. This was dependent of the induction of nitric oxide generation in accordance with the ability of nitric oxide to reduce neutrophil adherence to endothelial cells (Kubes et al., 1991). Recently, pentraxin 3, which is liberated from specific granules during transendothelial migration, has been shown to bind to P-selectin on endothelial cells and block its ability to capture neutrophils, thus serving as a narrow-looped negative feedback for neutrophil transmigration (Deban et al., 2010).

Neutrophil Extracellular Traps

During the last 6 years, it has been clear that neutrophils may extend their antimicrobial activity beyond life of the neutrophil. Formation of neutrophil extracellular traps (NETs) is an alternative to death by necrosis or apoptosis. During netosis, the nuclei swell and the chromatin is dissolved. Large strands of decondensed DNA are extruded from the cell, carrying along with them proteins from cytosol, from granules (which also disintegrated at the same time as the nuclei dissolve), and from chromatin (histones) (Brinkmann and Zychlinsky, 2007; Brinkmann et al., 2004; Fuchs et al., 2007). Mass spectrometry has identified 24 neutrophil proteins associated with NETs (Urban et al., 2009). NET proteins are primarily the cationic (thus, DNA-binding) bactericidal proteins: histones, defensins, elastase, proteinase 3, heparin binding protein, cathepsin G, lactoferrin, and myeloperoxidase (Urban et al., 2009), but also the pattern recognition molecule Pentraxin 3, is NET associated (Jaillon et al., 2007). Notably, the S100A8,9 complex known as calprotectin that constitutes 40% of the neutrophil's cytosolic protein (Murthy et al., 1993) is a prominent NET component and confers NETs with the ability to kill *Candida* species (Urban et al., 2009) and to impair growth of *Aspergillus fumigates* (McCormick et al., 2010).

The mechanism for formation of NETs is not yet completely known. Decondensation of chromatin, which is particularly dense in mature neutrophils, is associated with citrullination of Histone H3 by conversions of histone arginine to citrulline residues by *p*eptidyl*a*rginine *d*eiminase *4* (PAD4) (Wang et al., 2004), an enzyme which is particularly rich in mature neutrophils (Nakashima et al., 2002). This process seems central for NET formation because mice deficient in PAD4 fail to form NETs, and NET-dependent bacterial killing in vitro is correspondingly reduced. More important, the PAD4-deficient mice are more susceptible to necrotizing fasciitis induced by group A *Staphylococci* than wild-type mice (Li et al., 2010). Elastase released from azurophil granules degrades histones and synergize with myeloperoxidase to drive chromatin decondensation essential for NET formation (Papayannopoulos et al., 2010). NET formation is dependent on hydrogen peroxide generated by the NADPH oxidase and further metabolized by myeloperoxidase. CGD neutrophils and MPO-deficient neutrophils, therefore, do not form NETs (Bianchi et al., 2009; Metzler et al., 2010). Nitric oxide was recently demonstrated to induce NETs in a process that was dependent on myeloperoxidase, but the active species were not identified (Patel et al., 2010). Neutrophils from normal newborns are partly deficient in NET production, despite full respiratory burst activity, and demonstrate decreased extracellular bactericidal activity (Yost et al., 2009). The mechanism for this deficiency is unknown.

It was recently suggested that extracellular strands of DNA may also be generated from mitochondria from activated neutrophils in a process that was compatible with full neutrophil activity after NET formation, thus indicating an alternative to NET formation as a death mechanism of neutrophils (Yousefi et al., 2009).

NETs are cleared by DNases. *S*ystemic *l*upus *e*rythematosis (SLE) patients have autoantibodies against DNA-associated proteins such as the components of NETs. A subset of patients were recently identified with decreased activity of DNase-1, which is a NET-degrading protein found in plasma (Hakkim et al., 2010). These patients had enhanced risk of nephritis. Along the same lines, NETs were shown to induce thrombosis by stimulating platelets in vitro, and NET components were abundant in thrombi induced in a baboon model (Fuchs et al., 2010). NETs were recently found to damage activated human endothelial cells in culture (Gupta et al., 2010), all pointing to a potential proinflammatory role of NETs. The full contribution of NETs to overall antimicrobial activity versus the classical microbicidal activity of phagocytosing neutrophils in vivo has not yet been established. NETs may provide the biological basis for the old classification of pus into the viscous "pus bonum," i.e., the good pus (NETs), and the liquid "pus malle," i.e., the bad pus

(no NETs), known to be predictive of good and poor outcome of infections in the preantibiotic era.

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