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Neutrophils, from cradle to grave and beyond

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Polymorphonuclear leukocytes, aka neutrophils, represent the predominant nucleated cell in human circulation, a status met by producing $\sim 10^{11}$ cells daily under basal conditions [reviewed in (1)], and serve as prominent effector cells in the innate arm of the immune system. As such, these amoeboid phagocytes constantly sample their environment for clues of microbial trespassers, to which they rapidly respond to track, ingest, kill and degrade potential pathogens (Figure). Until relatively recently, the longstanding view of neutrophils as quick but single-minded agents of microbial death defined the full extent of their cellular biology. However, ongoing work by investigators in the field reveals that neutrophils possess a much broader and more diverse repertoire of functional attributes and interact synergistically with soluble factors and other cells to integrate into the overarching network of the immune system (2, 3). Contemporary immunologists now recognize that neutrophils are transcriptionally active and produce many cytokines (4, 5), condition macrophages for long term immune responses (6), contribute actively to the resolution of inflammation (7), and *may* even have a memory, like other innate immune cells (8). With newly found roles for neutrophils in such a wide range of biological settings comes the challenge of unraveling the underlying mechanisms.

With this perspective in mind, this volume of *Immunological Reviews* provides you, the reader, with thoughtful reflections on selected aspects of the complex and many-splendored biology of neutrophils (Figure). By design, the overall scope of the volume is not exhaustive and several important properties of neutrophils were omitted from the list selected for inclusion. For example the phagocyte NADPH oxidase and the myeloperoxidase-dependent antimicrobial system, both required for optimal neutrophil-mediated host defense, are discussed within reviews of more specialized topics rather than in a chapter dedicated to them alone. My intention in selecting topics for this volume was to highlight the array of functional attributes that neutrophils exhibit in a variety of biological settings, with a focus on some behaviors that may surprise those unfamiliar with recent work in the field.

Just as the scope of topics included in this volume is broad, the contributing authors constitute a diverse group with respect to specific expertise, ranging from electrophysiology to clinical nephrology. Every author brings their unique perspective to construct the content of their review, applies their expertise to speculate when experimental data fall short of explaining observations and identifies the outstanding questions in their subject matter that

merit further exploration. Consequently, the reviews both inform and inspire and are arranged to summarize the life of a neutrophil, from cradle to grave and beyond.

The initial chapters review factors and systems that regulate granulopoiesis and support demands associated with the high daily turnover of neutrophils under basal conditions, as mentioned previously. Grounded in Borregaard's seminal work that links the timing of granule protein expression in neutrophil progenitors in the bone marrow with the delivery of granule proteins into different compartments, a process known as "targeting by timing" (9), Cowland and Borregaard provide an integrated view of granulopoiesis and granule content allocation as coupled events (10). From this fresh perspective, they present neutrophil-specific determination and committed granulopoiesis as two phases of granulocyte production in the bone marrow. They describe the transcriptional elements coordinating the process and highlight key proteins regulating cell cycle while introducing several concepts, including micro RNAs, the process of degranulation, and the notion of neutrophil subsets, that are discussed in greater detail in subsequent chapters.

Guo and colleagues in the Deng lab extend discussion of miRNAs to detail their contributions to neutrophil biology (11). After a brief overview of miRNAs, they focus specifically on miR-451, 4661, 142, and 223 in the context of neutrophil function and provide insights into the study of miRNAs in granulocyte differentiation, neutrophil recruitment, NLRP3 inflammasome activity, and other elements of the inflammatory response. In addition to reviewing the current state of knowledge regarding miRNAs and neutrophils, Guo et al. describe technical approaches to such work, including use of zebrafish, an experimental model that has already yielded novel insights into neutrophil biology (12).

The dyssynchronous nature of granulopoiesis has been offered as an explanation, in part, for the perceived heterogeneity among neutrophils in circulation, earlier observed by Gallin (13, 14). However, the recent recognition that neutrophils can be prompted to be transcriptionally active and to alter expression of surface proteins prompts a more nuanced reflection on the question of neutrophil heterogeneity. During the past two decades since Cassatella's seminal observation that phagocytosing neutrophils produce and release IL-8 (15), investigators have increasingly recognized the transcriptional prowess of neutrophils in a wide range of biological settings, including production and release of cytokines and chemokines. In this way, neutrophils modulate the activities of neighboring cells and actively contribute to resolution of inflammation. Neutrophil transcriptional plasticity is manifested phenotypically as change in expression of surface molecules or activity. The presence of these changes in only a subset of neutrophils suggests that the circulating population is heterogeneous. Unfortunately, the pace at which apparent neutrophil heterogeneity has been recognized has outpaced the establishment of a consensus on criteria by which to define populations in circulation and in tissue. Furthermore, the durability of observed features of individual populations is not clear: are these *bona fide* stable cell subsets or the transient responses to trophic factors acting locally? Spazini *et al.* review this complex subject, highlighting ambiguities in nomenclature, functional attributes, and biological relevance (16). Their suggestion that categorization be based on specific master transcriptional factors

may prove a useful first step in clarifying the existence and importance of neutrophil subpopulations.

In response to danger, be it from infection or sterile inflammation, neutrophils exit the circulation, a process that necessitates traversing endothelium and, in some cases, epithelium. The initial barrier, endothelium, needs to be crossed in a manner that maintains vascular integrity. Muller details the series of reciprocal and finely coordinated interactions between neutrophils and endothelial cells in post-capillary venules that mediate the emigration from circulation (17). In a remarkably orchestrated crosstalk of signals and responses between neutrophils and endothelial cells, both participants modify their cellular architecture to permit egress of neutrophils across endothelium. Although much is known about transendothelial migration, Muller highlights several gaps in current understanding, particularly with respect to the lateral border recycling compartment, a critical endothelial element in transmigration.

Once in tissue, neutrophils migrate towards the source of inflammation in a process called “neutrophil swarming” (18) and recently appreciated because of the development and advances in two-photon vital microscopy. Kienle and Lämmerman clearly describe the sequential steps by which intricate biochemical crosstalk among the migrating neutrophils coordinates chemotaxis towards the noxious stimulus. Leukotriene B4 figures prominently in mediating the intercellular communication between neutrophils in the inflamed interstitium in a variety of physiologic and pathophysiologic settings. These events must be titrated precisely in order to favor host defense and avoid unchecked tissue damage.

During inflammation in mucous membrane-lined organ systems such as the airways or the gastrointestinal tract, neutrophils must cross the lining epithelium, a cellular barrier in addition to the endothelium. Brazil and Parkos (19) review the determinants for normal transepithelial migration and the mechanisms underlying their dysregulation. Understanding the molecular basis for derangements in transepithelial migration of neutrophils is prerequisite to designing novel therapies to abort and terminate unbridled inflammation in these organ systems. However, infiltrating neutrophils can promote reparation as well as damage of mucosal tissue, as discussed by Campbell and colleagues (20). When summarizing neutrophil behavior, we typically focus on the consequences of their consumption of oxygen, namely generation of superoxide anion, hydrogen peroxide and downstream products of the phagocyte NADPH oxidase. However, the decrease in tissue pO_2 mediated by the consumption of oxygen by transmigrating neutrophils creates a “physiologic hypoxia” locally, thereby stabilizing hypoxia-inducing factor and, consequently, inducing genes under its regulatory control. The subsequent metabolic events figure not only in the context of the inflammatory response but also in the composition of the tumor microenvironment

To serve effectively as responsive agents of host defense, neutrophils need to receive information from their environment and convert input signals into appropriate reactions. To that end, receptors expressed on the plasma membrane engage ligands that initiate biochemical pathways that drive the cellular state to promote or, in some cases, inhibit activation. Tyrosine kinases in the Src family and Syk tyrosine kinases mediate critical Fc

and adhesion receptor-dependent neutrophil functions (21). Futosi and Mocsai discuss the biochemical events driven by tyrosine kinases as well as the associated enzymes and adaptor proteins that promote receptor-dependent responses (22). Of course, these well-intended cellular responses must be regulated and inhibitor receptors contribute to that end. Benoit discusses the role of the inhibitory receptors that depend on the tyrosine-based inhibition motif for their action (23). These leukocyte immunoglobulin-like receptors and members of the sialic acid-binding immunoglobulin-type lectins multigene families rapidly respond in a negative fashion to temper neutrophil reaction to stimuli. Exploitation of the inhibitory receptors provides selected pathogens a novel avenue for subversion of neutrophil-mediated host defense.

Neutrophils exert the bulk of their antimicrobial action within the confines of the phagosome, a membrane-bound compartment created *de novo* as part of the engulfment process. Phagocytosis, the process by which neutrophils ingest targets, requires extensive cellular modification and membrane remodeling, and Levin *et al.* describe with impressive clarity the step-wise progression of intricate biochemical changes and structural remodeling that are required for successful entrapment of a target into the phagosome (24). Although much of current understanding of the molecular and cell biology of phagocytosis comes from Grinstein's elegant analysis of uptake by macrophages, the authors compare and contrast what is known about these events in neutrophils and in macrophages. Of note, the authors' analysis extends beyond those events essential to killing ingested prey and includes steps critical to degradation of microbial remnants, which would otherwise serve as a source of continued immune activation, and to resolution of the phagosome itself.

Confined within phagosomes, ingested microbes undergo attack by oxidants generated *in situ* by the NADPH oxidase and by granule proteins recruited by fusion of intracellular granules. Soluble factors present during inflammation, such as TNF α and GM-CSF, can promote more robust NADPH oxidase activity, a process referred to as "priming". El Benna and colleagues delineate the biochemical steps in priming of the activity of the NADPH oxidase, with particular attention focused on the phosphorylation of Ser³⁴⁵ in p47 $phox$, subsequent binding of proline isomerase Pin 1 and the resulting conformational change in p47 $phox$ that facilitate access of other functionally important targets to phosphorylation by protein kinase C (25). By virtue of this cascade of reactions, agents that would be present during inflammation can augment the output of oxidants that contribute to antimicrobial action.

Fundamentally, the NADPH oxidase operates as an electron transferase, shuttling electrons from cytosolic NADPH to molecular oxygen in the extracellular space or phagosome to generate superoxide anion (26). Given the electrogenic nature of the phagocyte oxidase, sustained activity requires a mechanism to compensate for the membrane depolarization that would otherwise occur and terminate oxidant generation. DeCoursey chronicles the torturous journey of discovery that culminated in identification of Hv1, the voltage-gated proton channel (27–29) as the predominant mediator of charge compensation for the electrogenic phagocyte NADPH oxidase (30). DeCoursey's recounting of the saga highlights many of the missteps, each of which was identified and corrected by rigorous experimentation by several laboratories. In the end, the explication of the integrated

functional relationships between Hv1 and the phagocyte oxidase resolved the question of how the oxidase sustains activity in the face of robust electron transfer across plasma or phagosomal membranes. As a corollary, the tale of investigation of charge compensation in neutrophils underscores the importance of diligently challenging published observations, even those featured in high impact journals, when independent investigations fails to confirm or demonstrates them to be incorrect. Although resistance to publishing negative or contradictory findings poses a significant barrier, the integrity of the scientific venture depends on rigorous examination and cross-examination of findings.

The ability of neutrophils to utilize the granule protein myeloperoxidase and hydrogen peroxide generated by the NADPH oxidase to produce the potent microbicide hypochlorous acid (HOCl) makes them ideally suited to serve in the first-line of defense against infection (31). Just as sustained oxidase activity requires specialized biochemical adaptations, production of HOCl necessitates a continuous supply of chloride ion, as the amount incidentally internalized from the extracellular space during phagocytosis would be rapidly consumed (32). Wang reviews the mechanisms by which chloride redistributes from cytosol, where the concentration is ~ 80 mM, into the phagosomes via chloride transporters, including cystic fibrosis transmembrane conductance regulator (CFTR), the protein dysfunctional in cystic fibrosis (33). Unstimulated neutrophils express CFTR in the membrane of secretory vesicles and not in the plasma membrane (34). Hence, concomitant with phagocytosis, fusion of secretory vesicles with the nascent phagosome delivers CFTR to support the chloride transport necessary to sustain production of HOCl (35).

Myeloperoxidase represents only one of the many granule proteins that contribute to neutrophil action. Prominent among proteins in neutrophil granules are the four neutrophil serine proteases, namely human neutrophil elastase (HNE), PR3, cathepsin G, and NSP4, each of which contributes to antimicrobial action and to tissue degradation. Kettritz's review focuses on the biology of the three better known neutrophil serine proteases (*i.e.* HNE, PR3, and cathepsin G) and their roles in neutrophil biology and in human disease (36). PR3 merits particular recognition, as it serves as an autoantigen in systemic necrotizing vasculitis. Kettritz explores the complex genetic and epigenetic regulation of PR3 expression and links to the necrotizing vasculitis that occurs in small vessels.

As with oxidase assembly and activation, intracellular events tightly regulate granule fusion with target membranes, both plasma and phagosomal. Understanding the mechanisms underpinning the process of degranulation poses special challenges, given both the rapidity of the response and the presence of multiple, distinctly different intracellular granules. Ramadass and Catz present evidence linking regulated trafficking of intracellular vesicles to the activity of small GTPases and their associated proteins (37). Identification of the molecular principles governing regulated secretion and vesicle fusion with phagosomes will provide the rational basis for therapeutic interventions to temper inflammatory processes.

Killing and degradation of organisms trapped within phagosomes reflect irreversible biochemical changes in vulnerable structures or systems that are essential for the integrity of specific microbes. Differential susceptibility of substrates to modification by toxic agents produced by neutrophils creates a hierarchy in the ease with which targets react. For

example, thiol-containing targets such as methionine and cystine are more than 10^7 -fold more susceptible to oxidation by HOCl than by H_2O_2 [reviewed in (38)]. The chemical composition and structural organization of organisms that cause infection include a remarkable spectrum, including the surfaces of Gram negative vs Gram positive bacteria, the waxy coating of mycobacteria, glycocalyxes of yeasts, and the membranes of protozoa, to name only a few. Adding to the number and complexity of potential substrates for attack in phagosomes are soluble proteins incidentally ingested during phagocytosis as well as any products secreted by ingested microbes. Of course, reactive agents attack potential targets equally, whether of microbial or host origin, thereby making granule proteins and lipids released into the phagosome subject to modification. In fact, the predominance of chlorinated proteins within phagosomes are of host origin (39). Given the multitude of potential substrates, the wide range of critical targets within individual types of microbes, and variability in soluble elements released into the phagosome during degranulation, neutrophil-mediated killing should not be viewed as a monolithic process, as it is often portrayed in textbooks and reviews. The fact that patients with chronic granulomatous disease, who lack an active phagocyte NADPH oxidase, are especially susceptible to infection with a relatively small number of pathogens [*i.e. S. aureus, Burkholderia, Serratia, Nocardia, and Aspergillus* (40)], underscores the variability of interactions between microbes and neutrophils. Three chapters present variations on the theme of host-microbe interactions and discuss critical issues related to the engagement and fate of neutrophils and their targets.

Kincaid and Allen review studies from the Allen lab that have identified several maneuvers which *Francisella* employs to thwart neutrophil-mediated killing and disturb neutrophil cell death pathways (41). *Francisella* not only survives within neutrophils but also globally interferes with oxidase activation (42). Furthermore, neutrophils fed *Francisella* fail to undergo the accelerated apoptosis that typically accompanies phagocytosis (43) and exhibit prolonged survival, thereby allowing intraphagosomal organisms to replicate and escape into the cytosol (44,45).

The broad spectrum of outcomes from neutrophil-microbe interactions reflects the diversity of both microorganism and sites of infection in the host. The oral cavity, particularly the periodontal space, houses a complex microbiome especially adapted to the low redox environment and regulated in large part by neutrophils (46,47). Uriarte draws attention to the central role of neutrophils in maintenance of periodontal health and to the consequences of failed neutrophil surveillance in the oral cavity (48). Whereas interactions between neutrophils and normal oral commensals maintain homeostasis and healthy tissues, colonization by pathogens such as *Porphyromonas gingivalis*, which resists neutrophil-mediated oxidant killing, disrupts the balance (49). The resultant dysbiotic state results in exuberant neutrophil activity and extensive collateral damage to periodontal tissues, often with loss of teeth. The high prevalence of periodontitis and the epidemiologic links between the associated persistent inflammation and cardiovascular disease (50,51) provide strong incentive to elucidate the underlying pathogenic mechanisms.

As mentioned earlier, *Aspergillus* represents a very common cause of infection in patients with chronic granulomatous disease (40) and thus illustrates the important role of

neutrophils in defense against fungi. Gazendern *et al.* utilize neutrophils from patients with defined immunodeficiencies as probes to decipher how normal neutrophils respond to *Aspergillus* and to *Candida*, both common human pathogens (52). This experimental approach has already yielded unanticipated insights into normal phagocyte biology and promises to be a rich source of new information going forward.

The toxic action of neutrophil-generated HOCl extends beyond invading microbes and host proteins to tumor cells as well, as noted earlier by Clark (53,54). More recently, the identification of additional properties of neutrophils in the tumor microenvironment has extended understanding of their behavior in malignancy. Two chapters within this volume explore neutrophil phenotype and function in tumors. Analogous to the situation for neutrophil-microbe interactions, heterogeneity among tumors with respect to cell type, tissue environment, and stage of disease makes general principles often difficult to identify. As reviewed by Treffers *et al.*, neutrophils can promote tumor progression and spread or, in other situations, participate in host defense against malignancy (55). Neutrophils enlist many of the same enzyme systems prominent in antimicrobial action to attack nascent tumors. Furthermore, neutrophils figure prominently in therapeutic antibody use, acting as potent effectors of tumor cytotoxicity.

From a perspective slightly different from that of Treffers *et al.*, Singel and Segal discuss the complexities of neutrophil-driven platelet activation and thrombosis, angiogenesis, tissue remodeling, and modulation of cell-mediated immunity in anti-tumor responses (56). In addition, they incorporate into their thinking the consequence of iatrogenic factors, such as chemotherapy and changes in the gastrointestinal microbiome resulting from empiric use of broad-spectrum antibiotics in febrile cancer patients.

As mentioned earlier, neutrophils survive a relatively short time, with a half-life in circulation of less than twenty four hours (57–59). In the absence of stimulation, neutrophils typically undergo apoptosis, a process accelerated by phagocytosis (60). However, circumstances can modify the usual apoptotic pathways and extend neutrophil survival, as mentioned earlier in the discussion of neutrophils that have ingested *Francisella* or *Neisseria gonorrhoeae* (61). Study of the key determinants and regulatory signals in the cell death pathways of neutrophils represents a rapidly developing field, and Witko-Sarsat reviews the cellular mechanisms that govern neutrophil fate, with particular emphasis on proliferating cell nuclear antigen (PCNA) (62). PCNA, a protein recognized to reside [WITKO-SARSAT], exists exclusively in the cytoplasm of neutrophils, where it appears to modulate neutrophil survival (63). Rather than existing as part of the cell cycle-dependent nuclear machinery, cytosolic PCNA exists in a multi-component protein complex that redirects neutrophils from programmed cell death to prolonged survival. How PCNA and other cytosolic elements direct neutrophils to distinct cell fates remains to be determined.

Exhausted by aging or spent after executing attacks on targets, the effete neutrophil needs to die by apoptosis and be cleared away in an immunologically silent fashion, thereby avoiding prolonged inflammation and restoring homeostasis. To that end, macrophages ingest apoptotic neutrophils by a process known as efferocytosis, meaning “to carry to the grave” (64,65). Greenlee-Wacker provides an overview that focuses on clearance of *human*

neutrophils by *human* macrophages and dendritic cells (66). Many distinct cell surface receptors and membrane-bound ligands and bridging molecules form the phagocyte synapse between apoptotic neutrophils and macrophages that drives efferocytosis. The multiple and redundant determinants of efferocytosis underscore its importance for resolution of inflammation and restoration of homeostasis. When overwhelmed or dysregulated, failed efferocytosis allows apoptotic cells to undergo secondary necrosis, thereby releasing intracellular contents that acts as danger-associated molecular patterns, which in turn fuel more inflammation (67). Elucidation of the molecular regulation of normal efferocytosis may provide a blueprint for the design of therapeutics to intervene when exuberant inflammation creates clinical disease.

In summary, the remarkable plasticity of neutrophils serves as a dominant *leitmotif* throughout the chapters in this volume, demonstrating time and again that context matters. Phenotypic features contribute to the observed heterogeneity among neutrophils in circulation, within swarms, and in specialized microenvironments, such as in tumors. Trophic factors within distinct tissues and other biological settings dictate both the functional attributes and the fate of neutrophils. Whether the array of plasma membrane proteins and functional properties of neutrophils under diverse settings are durable enough to represent evidence for the existence of *bona fide* subsets appears to be unresolved. Nonetheless, contributions to this volume indicate directions for exciting work to come. I am confident that readers will be rewarded, as I was in reviewing each contribution, with an experience that is informative, lucidly presented, and thought-provocative, and will agree that the contents of the volume both inform and inspire.

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Figure.
Interaction of human neutrophils with (A) *Staphylococcus aureus* (red) or (B) *Escherichia coli* (green). Images provided courtesy of Frank DeLeo (NIAID).