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Neutrophils

Edited by Maitham Khajah



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Meet the editor



Dr. Maitham Khajah completed his BPharm degree from the Faculty of Pharmacy, Kuwait University, in 2003 and obtained his PhD degree in December 2009 from the University of Calgary, Canada (Gastrointestinal Science and Immunology). He was employed as an assistant professor in 2010 and was recently promoted to associate professor at Kuwait University, Faculty of Pharmacy, Department of Pharmacology and Therapeutics. He is currently the Chairman of the Department of Pharmacology and Therapeutics. His research interest is to study new molecular targets for the treatment of inflammatory bowel disease and breast cancer. He has supervised many students for the MSc Molecular Biology and Pharmaceutical Sciences Programs, College of Graduate Studies, Kuwait University. He joined Kuwait University in 2010, and received various grants such as PI and Co-I. He was awarded the Best Young Researcher award by Kuwait University, Research Sector, for the Year 2013–2014.

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Preface

Polymorphnuclear cells (or neutrophils) are the most abundant type of immune cells in the blood and play an integral role in innate and adaptive immunity towards various infectious and non-infectious triggers. These cells have the capability to perform various effector functions such as phagocytosis, degranulation, motility/chemotaxis, and the recently discovered function called neutrophil extracellular traps formation and subsequent microbial killing. *Neutrophils* provides recent evidence regarding the various properties of these immune cells in relation to various disease conditions aimed at future therapeutic targets.

Chapter 1 (Introductory Chapter: Background Summary Regarding Neutrophils) briefly discusses the basic effector functions of neutrophils, which provide the basis for the upcoming chapters in the book.

Chapter 2 (Cannabinoid Receptors as Regulators of Neutrophil Activity in Inflammatory Diseases) discusses how cannabinoids (binding through their receptors: CR-1 and -2) can modulate various activities of neutrophils, and act as a therapeutic target for various neutrophil-related inflammatory (e.g. arthritis, ischemic diseases, and colitis) and infectious (e.g. sepsis and mycobacterial infection) conditions.

Chapter 3 (Neutrophil Activation by Antibody Receptors) discusses the role of neutrophils in the adaptive immune response through their interactions with immunoglobulins (mainly IgG) secreted by B-cells. The main types of Fc γ Rs located on neutrophils and how particular Fc γ Rs can activate various signaling pathways to promote unique effector cell functions are also discussed in this chapter.

Chapter 4 (Remodeling of Phenotype CD16⁺CD11b⁺ Neutrophilic Granulocytes in Acute Viral and Acute Bacterial Infections) discusses the different expression patterns of the membrane receptors CD16 and CD11b in normal and pathological conditions such as in patients with acute viral and acute bacterial tonsillitis. The differential membranous expression profile of these receptors on the neutrophils may help in the early-stage diagnosis of these conditions.

Chapter 5 (Essence of Reducing Equivalent Transfer Powering Neutrophil Oxidative Microbicide Action and Chemiluminescence) discusses the physics of the NADPH oxidase system and the detection of the generated reactive oxygen species using various chemiluminogenic probes for circulating blood neutrophils, which can aid in *in vivo* state of host inflammatory activation.

We hope that the recent evidence described in this book provides a better understanding of the role of this important immune cell in various disease conditions and

forms the basis for future research activities aimed at providing better therapeutic approaches to treat various disease conditions.

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Introductory Chapter: Background Summary Regarding Neutrophils

Maitham Khajah

1. Neutrophils: important immune cells in health and diseases

Neutrophils are key players in the innate and the adaptive immunity and contribute to the pathogenesis of various infectious and noninfectious conditions. These cells have the capability of performing various effector functions and therefore are considered an important therapeutic target for many conditions. They are considered the fastest immune cells in our body and the first to arrive to the inflammatory site. This occurs in response to a wide variety of chemoattractive agents, such as CXC chemokines [keratinocyte-derived cytokine (KC), macrophage inflammatory protein-2 (MIP-2)], lipid mediators [leukotriene B₄ (LTB₄) and platelet-activating factor (PAF)], the complement split product (C5a), and the bacterial toxin formyl-met-leu-phe (fMLP)] [1]. Neutrophils use different intracellular signaling pathways in their migrative behavior which are dependent on the type of chemoattractant they encounter [1–3].

These immune cells also play an important role in the recognition of various pathogens through specific cell surface and cytoplasmic receptors including toll-like receptors (TLRs) and nucleotide oligomerization domains (NODs). In addition, they can also recognize opsonized particles through the complement receptors and mediate antibody-dependent cell cytotoxicity (ADCC) through their interaction with immunoglobulin receptors [4–6]. They can also mediate microbial killing through oxygen-dependent and oxygen-independent mechanisms. In response to various ligands, this results in a dramatic increase in oxygen consumption due to the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system leading to the generation of various reactive oxygen (e.g., superoxide anion radical) [7] and nitrogen (through nitric oxide synthase; NOS) intermediates [8]. This can not only aid in microbial killing but also contribute to the pathogenesis of various inflammatory and cancerous conditions (through the generation of peroxynitrite). The non-oxidative arm of neutrophil killing is mediated through the action of antimicrobial peptides and proteases present in various compartments of azurophilic (primary), specific (secondary), gelatinase, and secretory granules [9–13].


This book provides recent evidence regarding the role of cannabinoid receptors (CR-1 and CR-2) and different subtypes of the immunoglobulin receptor FcγRs in the pathogenesis, diagnosis, and treatment of various diseases of infectious and noninfectious origin. Furthermore, the differential expression pattern of CD16 + CD11b + receptors on the surface of neutrophils and their role in the diagnosis of acute viral and bacterial infections will also be highlighted. Finally, the utility of using different chemiluminogenic probes for the detection of NADPH activity for the circulating blood neutrophils and their role in determining the *in vivo* state of host inflammatory activation will be highlighted in this book.

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Cannabinoid Receptors as Regulators of Neutrophil Activity in Inflammatory Diseases

Mariana Conceição Souza and Elaine Cruz Rosas

Abstract

Cannabinoids are compounds present in *Cannabis sativa* (phytocannabinoids), endogenously produced (endocannabinoids) or synthesized, that bind to G protein-coupled receptors named cannabinoid receptors B1 and B2. They were first described as psychotropic compounds; however, cannabinoids are also potent immunoregulatory agents. Cannabinoids can modulate neutrophil activity in sterile and infectious inflammatory diseases. Concerning sterile inflammatory diseases as arthritis, ischemic diseases, and colitis, the use of CB2 agonist impairs the intracellular signaling pathways involved in the production of inflammatory mediators and expression of adhesion molecules. As a consequence, neutrophils did not release metalloproteinases either to adhere to endothelial cells, resulting in reduced tissue damage. A similar anti-inflammatory CB2 agonist mechanism of action in sepsis and mycobacterial infection models is observed. However, it is not clear if inflammation resolution promoted by cannabinoid treatment during infection is also related to microbial viability. Despite the growing literature showing the effects of cannabinoids on neutrophils, there are still some gaps that should be filled before proposing cannabinoid-based drugs to treat neutrophil-dependent diseases.

Keywords: cannabinoid agonist, inflammation, infection, endocannabinoids, phytocannabinoids, synthetic cannabinoids

1. Introduction

Neutrophils have been classically recognized as the most relevant cell during acute inflammatory responses and, more recently, in chronic inflammation [1]. On the one hand, neutrophils produce bactericidal molecules and coordinate the accumulation of pro-resolving cells. On the other hand, neutrophil over activation leads to tissue damage. In this context, several approaches have been proposed to regulate the accumulation and the activity of neutrophils in pathological conditions.

In parallel, the findings concerning the importance of the endocannabinoid system as the endogenous immunoregulatory mechanism raise questions on how it could be therapeutically used to treat inflammatory diseases. In this chapter, we discuss how the endocannabinoid system can be used to modulate the activity of neutrophils in sterile and infectious inflammatory diseases.

2. Cannabinoid system

Cannabis sativa (marijuana) is one of the oldest plants that produced psychoactive effects on humans. In addition, it has been used in medicine in controlling pain, convulsion, inflammation, and asthma [2, 3]. Cannabinoids are a group of lipophilic and pharmacologically active compounds present in *C. sativa*. The first component from cannabis identified was the tetrahydrocannabinol (Δ^9 -THC) and and, as other cannabinoids, binds to G protein-coupled receptors (GPCRs) named cannabinoid receptors [4, 5].

Cannabinoid receptor agonists are responsible for several biological effects, such as analgesic, antiemetic, antitumor, and anti-inflammatory [6–13], and are classified into three groups based on their origin: endogenous cannabinoids (endocannabinoids—**Figure 1a and b**), phytocannabinoids (**Figure 1c and d**), and synthetic cannabinoids (**Figure 1e and f**) [5].

Endocannabinoids (eCBs) are eicosanoids derived from polyunsaturated chain fatty acids, such as arachidonic acid, and comprise amides, esters, and ether [14]. Anandamide (AEA) was the first endocannabinoid described in the mammalian

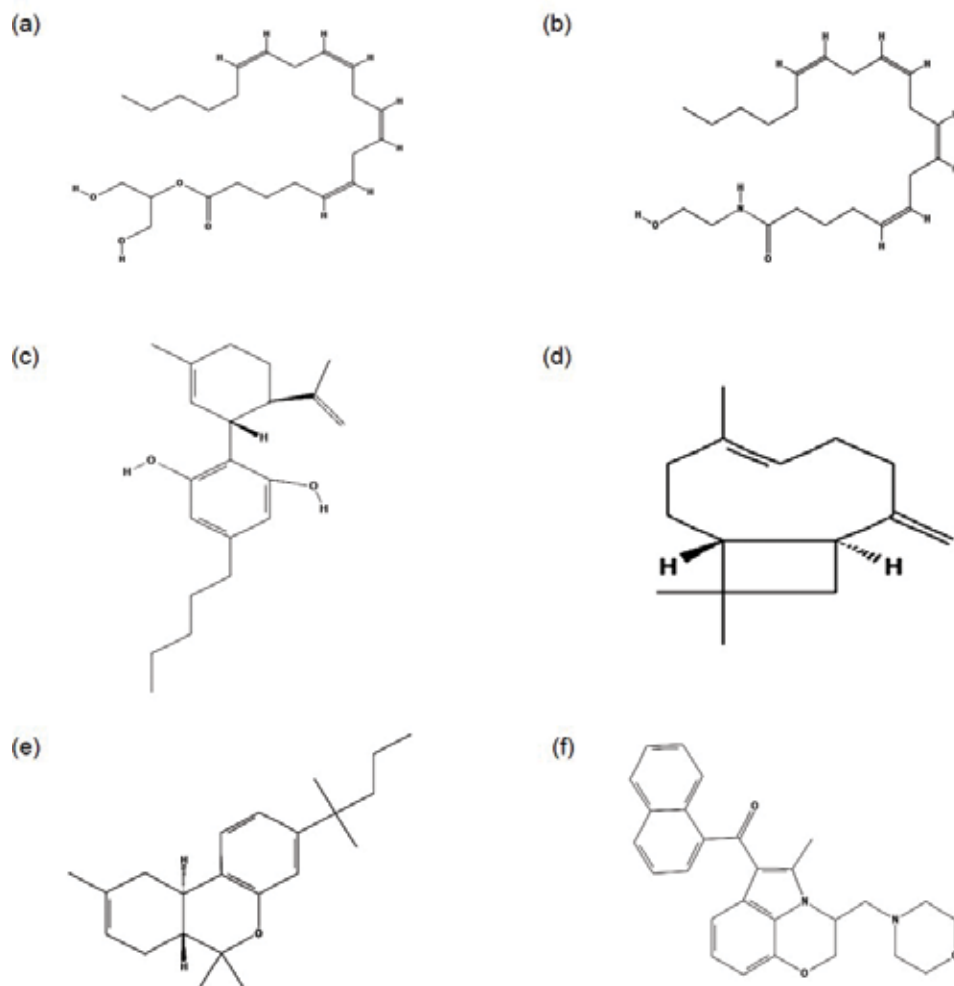


Figure 1. Chemical structure of some representatives of the cannabinoid agonists. (a) 2-AG, (b) AEA, (c) cannabidiol, (d) β -caryophyllene, (e) JWH-133, and (f) WIN 55212-2.

brain and other tissues [15], followed by 2-arachidonoylglycerol (2-AG) [16, 17]. The AEA and 2-AG represent the major substances of this class. The eCBs together with the cannabinoid receptors and the enzymes that regulate their biosynthesis and degradation constitute the “endocannabinoid system” [18]. Beyond the well-known psychotropic effects, the endocannabinoid system plays an essential immunomodulatory role by modulating the release of cytokines and on acute or chronic diseases through two main ways, neuro- and immunomodulation [19, 20].

The group of phytocannabinoids consists of active substances initially extracted from *Cannabis sativa*, whose pharmacological activity is associated with the terpene phenolic class. Phytocannabinoids are classified into two groups: psychoactive cannabinoids, such as Δ^9 -THC, and non-psychoactive such as cannabidiol and cannabitol [21, 22]. More recently, other molecules have been isolated from different plant species, which exert effects through cannabinoid receptors, such as the alkylamides derived from *Echinacea angustifolia* and *Otanthus maritimus* and sesquiterpene, as β -caryophyllene, found in some plant species from *Copaifera* genus [23–27].

The characterization of the chemical structure of Δ^9 -THC and endocannabinoids allowed the development of synthetic cannabinoids. From THC, it was possible to synthesize several compounds that have similar chemical structures with different levels of affinity for cannabinoid receptors [5, 28]. Synthetic cannabinoids have been used as a pharmacological tool for *in vivo* or *in vitro* studies to explore the therapeutic potential of the cannabinoid system. However, it has already been described that metabolites from dipyron and paracetamol exert its analgesic effects by inhibiting endocannabinoid biosynthesis and binding of cannabinoid receptors, respectively [29].

The cannabinoid receptors CB1 and CB2 are the main receptors of the cannabinoid system. Both belong to the family of GPCRs, specifically inhibitory G protein (Gi) [30]. The binding of agonists to cannabinoid receptors inhibits adenylate cyclase (AC) and modulates activation of different members of the MAPK family, including ERK1/ERK2, p38, and JNK1/2 (**Figure 2**) [30–37]. By inhibiting AC, the reduction of the second messenger cyclic adenosine monophosphate (cAMP) leads to the opening of rectifying potassium channels. CB1 also mediates the inhibition of N-type and P/Q-type calcium currents [22, 38]. Besides CB1 and CB2, the existence of a third cannabinoid receptor (CB3) has been suggested [39], and there are two orphan G protein-coupled receptors (GPCRs) which overlap with CB1 and CB2, named GPR18 and GPR55 [40]. In addition to activation via GPCRs, cannabinoids can perform their actions by activating PPARs, including PPAR γ [41–43].

CB1 is expressed in the central nervous system, especially by neurons [44] and modulates physiological processes, such as motor behavior, learning, memory and cognition, and pain perception [45]. This receptor is associated with the psychotropic effects of cannabinoid agonists, such as THC [46, 47].

CB2 is the peripheral receptor for cannabinoid agonists. It is mainly expressed in immune tissues such as the spleen and thymus as well as in blood cell subpopulations such as CD4 and CD8 lymphocytes, neutrophils, monocytes, natural killer (NK) cells, and B lymphocytes [47]. Nevertheless, CB2 is also found at low levels in neuronal and nonneuronal cells of the brain, but it does not produce psychoactive effects [47–49].

The CB2 expression intensity in immune cells depends on cell populations and activation state [50, 51]. Macrophages from different tissues increase CB2 expression after stimulation with interferon (IFN)- γ , which suggests that macrophages activated during an inflammatory process are more sensitive to the action of cannabinoid agonists than those in the resting state [52]. The first evidence that cannabinoids might modulate cytokine production was in the mid-1980s when murine

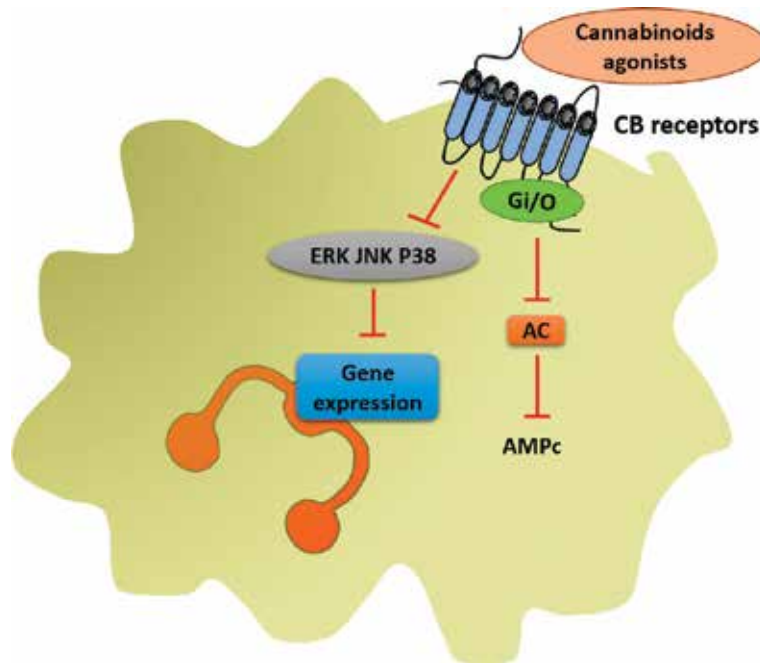


Figure 2.

Neutrophil activated expresses cannabinoid receptors (CB receptors). The cannabinoid receptors CB₁ and CB₂ belong to the family of GPCRs, specifically inhibitory G protein (Gi/o). The binding of agonists to cannabinoid receptors inhibits the activation of different members of the MAPK family (ERK_{1/2}, p38, and JNK_{1/2}), gene expression, as well as reduces cyclic adenosine monophosphate (cAMP) levels by inhibiting adenylate cyclase (AC) activity.

cells treated with agonist cannabinoid reduced the levels of type I interferons (IFN- α and IFN- β) after stimulation with LPS or polyinosinic-polycytidylic acid (polyI:C). Many subsequent studies have shown that cannabinoids inhibited the production of cytokines in innate and adaptive immune responses, both in animal models and in human cell cultures (to review [53]). In such a way, CB₂ has become an important target, especially in inflammatory conditions. The CB₂ receptor modulates immune cell functions, both *in vitro* and in animal models of inflammatory diseases. In this context, some studies have reported that mice lacking the CB₂ receptor have an exacerbated inflammatory phenotype (to review [19]). Besides, CB₂ agonists have an inhibitory effect on leukocyte migration and in the production of pro-inflammatory mediators *in vivo* and *in vitro*, showing a high anti-inflammatory potential [54]. Due to the lack of psychotropic effects, CB₂ agonists are considered a promising therapeutic strategy for the treatment of chronic inflammatory diseases. Preclinical studies showed the action of CB₂ agonists on different experimental models of inflammation, such as colitis, arthritis, cerebral ischemia, and sepsis [10, 53, 55–59], and in these studies, they showed that the action of CB₂ agonists modulated the neutrophil activity.

3. Neutrophils, cannabinoid system, inflammation, and infection

3.1 Neutrophils and cannabinoid system

Neutrophils play a crucial role in inflammatory processes, which are present in the pathology of different diseases. The neutrophil recruitment to the inflammatory site is an essential stage in the inflammatory responses; these cells are released from

the bone marrow to the periphery immediately after the first signal of inflammation. The mobilization of neutrophils from the bone marrow is conducted by the hematopoietic cytokine granulocyte colony-stimulating factor (G-CSF), which mobilizes neutrophils indirectly by shifting the balance between ligands to CXCR4 (CXCL12) and CXCR2 (CXCL1 and CXCL2) [60]. Neutrophil-active chemoattractant, as chemokines CXCL1 and CXCL2, is produced and released within the bone marrow and in inflamed tissue. In this context, chemokines from inflamed foci might make their way to the bone marrow and modulate neutrophil egress. Thus, CXCL1 and CXCL2 can act locally by inducing neutrophil recruitment from blood to peripheral tissue and systemically by inducing neutrophil mobilization from the bone marrow to the bloodstream [61].

Once in the peripheral blood, neutrophils can be rapidly recruited into inflamed or infected tissues. A panel of diverse stimuli, especially pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), activates macrophages, mast cells, or stromal cells to produce and release pro-inflammatory mediators as interleukin (IL)-1 β , tumor necrosis factor (TNF), CXCL8, CXCL1, CXCL2, and lipid mediators, such as leukotriene B₄ (LTB₄) and endocannabinoids [61, 62]. In inflammatory loci, neutrophils find antigen, molecules, and/or immune complexes that trigger different actions, such as phagocytosis, production of reactive oxygen species (ROS), and release of enzyme-rich granules (as collagenases, gelatinases, neutrophil myeloperoxidase (MPO), and elastase [63]). Neutrophils enter the tissue through surface molecules which interact with vascular endothelial cells. This process is accompanied by a regulated rearrangement of the cytoskeleton of neutrophils that lead to actin polymerization [64], which is mainly governed by members of the Rho family GTPases including RhoA, Rac1, and Cdc42 [65].

Concerning cannabinoid receptor expression, it was observed that neutrophil from healthy donors expresses low levels of CB2 [66]. In addition, it was shown that the CB2 receptor plays a crucial role in neutrophil differentiation, and it has been implicated in the development of leukemia [67, 68].

The role of the cannabinoid system in neutrophil migration is controversial. The activation of CB2 receptors by 2-AG did not induce polarization and migration of human blood neutrophils [69]. However, pretreatment of neutrophils with 2-AG inhibited the fMLP- and CXCL8-induced migration without affecting the polarization of the cells [69]. In contrast, McHugh and coworkers showed that 2-AG does not inhibit the migration of human neutrophils toward fMLP and does not show chemotactic effects by itself [70]. Furthermore, Balenga and coworkers showed that 2-AG induces migration of neutrophils toward inflammatory sites, through cross talk with activated GPR55 [62].

Despite the uncertainty regarding the involvement of CB2 agonist with the neutrophil migration and action, some studies show that there is a relationship between neutrophils and cannabinoid system in the pathogenesis of inflammatory or infection diseases (**Table 1**). In the next section, we will discuss the cannabinoid system and its action on the neutrophil participation in inflammatory and infection conditions [54, 62, 69–85].

3.2 Neutrophils, cannabinoid system, and inflammation

The action of CB2 agonists on their receptors impairs the secretion of pro-inflammatory cytokines and chemokines and reduces the recruitment of neutrophils. As discussed previously, neutrophils play a significant role in inflammatory diseases, including acute, chronic, autoimmune, infectious, and noninfectious conditions. The relation between neutrophil and cannabinoid system in inflammatory disease was discussed in this section.

| Drugs | Mechanism of action on neutrophils | Reference |
|------------------------------------|---|----------------|
| Endocannabinoids | | |
| N-arachidonoyl-ethanolamine (AEA) | Decreases neutrophil migration in vitro | 70 |
| | Decreases neutrophil migration in vivo | 71 |
| 2-arachidonoylglycerol (2-AG) | Decreases neutrophil migration in vitro | 69 |
| | Increases neutrophil motility | 62, 72 |
| Phytocannabinoids | | |
| cannabidiol | Neutrophil deactivation in vitro | 70, 73, 74 |
| | Decreases neutrophil migration in vivo | 74, 75, 76, 77 |
| Delta 9-tetrahydrocannabinol (THC) | Decreases neutrophil migration in vivo | 77, 78, 79 |
| | Decreases neutrophil migration in vivo | 54, 80, 81, 82 |
| beta-caryophyllene | Decreases neutrophil migration in vivo | 83 |
| | Decreases neutrophil migration in vitro | 83 |
| Synthetic cannabinoid | | |
| HU-308 | Decreases neutrophil migration in vivo | 77 |
| JWH-133 | Neutrophil deactivation in vitro | 84 |
| WIN55212-2 | Decreases neutrophil migration in vivo and in vitro | 71 |

Table 1.
Cannabinoid agonists, which exert action on neutrophils.

Increased macro- and microscopic colon damage scores, a high number of macrophage and neutrophil and MPO activity, characterizes experimental colitis. The activation of CB receptors by their ligands produces a protective effect in experimental colitis by decreasing prostaglandin, ROS and nitric oxide production, and reduction of leukocyte accumulation as neutrophils, resulting in diminished of colon tissue inflammation. Besides, mice lacking functional CB receptors are less resistant to colon inflammation than wild-type animals [53, 86]. The synthetic non-psychotropic cannabinoids as JWH-133, cannabinoid from the plant as cannabigerol [87] and β -caryophyllene [54], and synthetic atypical cannabinoid O-1602 (non-CB1 and CB2 ligands) [88] have been able to inhibit neutrophil recruitment in colitis models. Thus, during inflammation, the CB2 receptor activation by endocannabinoids or synthetic cannabinoid provides a mechanism for the reestablishment of regular GI transit (to review [89]).

Neutrophils are also essential cells in the development of arthritis. Neutrophils are abundant in inflamed joints, and these cells are essential to the initiation and progression of rheumatoid arthritis (RA). Neutrophil effector mechanisms include the release of pro-inflammatory cytokines, reactive oxygen and nitrogen species

(ROS and RNS), and granules containing derivative enzymes, which can cause further damage to the tissue and amplify the inflammatory response [90]. In such way, it has already been described that in RA there is an increase of CB2 expression and elevated endocannabinoid levels, observed in the synovial tissue and fluid from a patient with this disease [91]. Synovial fibroblasts and macrophages are mainly responsible for endocannabinoid production. These cells also are essential in the production of chemokines (CXCR1 or 2 ligands, such as CXCL8), the C5a fragment of the complement system, and LTB₄ which are responsible for neutrophil mobilization to the synovial cavity [61]. In this context, the activation of CB2 receptors inhibits the production of pro-inflammatory mediators, like chemokines, which reduces the leukocyte migration, like neutrophils, to the synovial cavity and metalloproteinase release [92]. Moreover, the activation of CB2 receptors inhibits IL-1 β -induced activation of extracellular signal-regulated kinases 1 and 2 and p38 mitogen-activated protein kinase in RA fibroblast [92].

The role of cannabinoid in neutrophils was also studied in ischemic models. Endocannabinoids act via the CB2 receptor in the modulation of the inflammatory response and myocardial remodeling after infarction. CB2 receptor plays an essential role in the formation of infarction border zone, collagen deposition, and organization of stable scar during remodeling. Duerr and coworkers [93] showed increased numbers of neutrophils in the heart ischemic area of CB2 receptor-deficient (Cnr2 $-/-$) mice when compared with healthy mice. These results suggest that CB2 receptor modulates neutrophil migration to inflammatory infarction site [93]. In accordance, activation of the cannabinoid CB2 receptor by JWH-133 protects against atherosclerotic plaque formation and may also decrease neutrophil MMP-9 release, which reduces the vulnerability of ischemic stroke plaque in arteries. Together, the studies suggest that CB2 agonists represent a promising anti-atherosclerotic treatment [84].

Even though CB1 is the most prominent receptor in the CNS, CB2 modulates neuroinflammation. CB2 activation by JWH-133 reduced the number of neutrophils in the ischemic brain. Furthermore, CB2 activation *in vitro* inhibits adherence of neutrophils to brain endothelial cells. JWH-133 also interfered with the migration of neutrophils induced by the endogenous chemokine CXCL2 through activation of the MAP kinase p38. This effect on neutrophils is probably responsible for the neuroprotection mediated by JWH-133 [56].

3.3 Neutrophils, cannabinoid system, and infection

The relation between neutrophil and infection is well established [94]. On one hand, the exacerbation of neutrophil activation could lead to tissue damage. On the other hand, neutrophils control microorganism growth. In this context, it is essential to study the effect of cannabinoids on neutrophils during infections and evaluate if and how the cannabinoid system modulates neutrophil activity.

The interest in the relationship among cannabinoids and infections exists since the 1960s when studies regarding “hippie subculture” observed the increase of sexually transmitted infections by marijuana consumers [95]. By this time, the studies focused on consumer behavior and how it could increase susceptibility to infections but did not evaluate the effect of cannabinoids on host response to infections [96]. Nowadays, it is known that endocannabinoid system regulates and is regulated by host microbiota, a balance that protects the host from the infection-triggered inflammatory response [29]. However, the increase of studies showing the immunoregulatory role of the endocannabinoid system raises questions about if cannabinoids could modulate microbial viability and/or neutrophil response during infections.

Studies concerning the modulation of neutrophil activity by cannabinoids during infections are mostly addressed to experimental bacterial infection as sepsis model. Neutrophil activity during experimental sepsis is well characterized in studies *in vivo* (to review [97–99]). By using LPS sepsis model, Smith and coworkers showed that treatment with cannabinoid receptor agonists reduced the migration of neutrophils in the peritoneal cavity by inhibiting neutrophil chemoattractant production [59]. The authors conclude that CB2 was responsible for impairing neutrophil migration. In accordance, studies performed in CB2-deficient mice submitted to sepsis induced by cecal ligation and puncture showed increased production of neutrophil chemotactic chemokines and increased numbers of neutrophil in the bone marrow and lung tissue. Interestingly, despite the increase in neutrophil numbers, neutrophils from CB2-deficient mice were not able to activate the MAPK pathway neither control bacterial load [57, 100]. Furthermore, in mycobacteria model of infection, it was observed that CB2 agonism impairs neutrophil adhesion to endothelial cells probably by inhibiting actin polymerization [83].

There are few and controverting data concerning CB1 effects on neutrophils during infection. Leite-Avalca and coworkers showed that CB1 antagonist given to mice submitted to CLP increased the survival rate but not change neutrophil accumulation in the peritoneal cavity [101]. Nevertheless, Kianian and coworkers showed that antagonism of CB1 reduced the adhesion of leukocytes to intestinal submucosal venules [102]. It is noteworthy that in the sepsis model, the activation of CB1 increases the systemic arterial pressure and the flow and decreases the arterial oxygenation; however, it decreases the inflammatory cytokine production [103, 104]. In such a way, an indirect effect of CB1 on leukocyte behavior during sepsis cannot be ruled out.

The results regarding the immunomodulatory effects of cannabinoids in infection are in accordance with the host-directed therapy approach that aims to activate protective responses against microbes in addition to antimicrobial therapy [105]. However, it should be mentioned that antibiotics perturb gut microflora, which could result in the endocannabinoid system unbalance and, thus, in neuropsychiatric disorders [29]. Indeed, further studies are necessary to propose to activate the endocannabinoid system, especially the CB2 pathway, during infections.

4. Conclusion

An increasing amount of data concerning the immunoregulatory role of cannabinoids, especially the CB2 agonists, has been raising the interest in developing new therapeutic strategies for inflammatory diseases. It is already known that the mechanism of action of well-established anti-inflammatory drugs, like paracetamol, depends on the activation of the endocannabinoid system [29]. However, despite all studies showing that cannabinoids can modulate neutrophil biology, there is a long way to go to achieve cannabinoid-based drugs to treat neutrophil-dependent diseases such as arthritis and infection-induced acute lung injury.

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Conflict of interest

The authors declare no conflicts of interest.

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Neutrophil Activation by Antibody Receptors

Carlos Rosales and Eileen Uribe-Querol

Abstract

Neutrophils, the most abundant leukocytes in blood, are relevant cells of both the innate and the adaptive immune system. Immunoglobulin (Ig) G antibody molecules are crucial activators of neutrophils. IgGs identify many types of pathogens via their two Fab portions and are in turn detected through their Fc portion by specific Fc γ receptors (Fc γ Rs) on the membrane of neutrophils. Thus, antibodies bring the specificity of the adaptive immune response to the potent antimicrobial and inflammatory functions of neutrophils. Two types of Fc γ Rs with several polymorphic variants exist on the human neutrophil. These receptors are considered to be redundant in inducing cell responses. Yet, new evidence presented in recent years on how the particular IgG subclass and the glycosylation pattern of the antibody modulate the IgG–Fc γ R interaction has suggested that a particular effector function may in fact be activated in response to a specific type of Fc γ R. In this chapter, we describe the main types of Fc γ Rs on neutrophils and our current view on how particular Fc γ Rs activate various signaling pathways to promote unique effector cell functions, including phagocytosis, activation of integrins, nuclear factor activation, and formation of neutrophil extracellular traps (NETs).

Keywords: neutrophil, phagocytosis, degranulation, NETs, antibody, Fc receptors, integrins, NF- κ B

1. Introduction

Neutrophils are the most abundant cell type in human blood. They are produced in the bone marrow and then released into the circulation. At sites of infection or inflammation, neutrophils migrate to tissues, where they complete their functions. Finally, neutrophils die by apoptosis and are eliminated by macrophages. Neutrophils are an essential part of the innate immune system [1], with significant antimicrobial functions, including phagocytosis, degranulation, and the formation of neutrophil extracellular traps (NETs). These antimicrobial functions were believed to be the only goal of neutrophils. However, it has recently become clear that neutrophils display many functional responses that go beyond the simple killing of microorganisms. Neutrophils produce cytokines [2] and other inflammatory factors [3] that regulate the whole immune system [4, 5]. Consequently, neutrophils are also key effector cells of the adaptive immune system.

Immunoglobulin (Ig) G antibody molecules are an essential part of the adaptive immune system. IgGs recognize antigens via their two Fab portions and are in turn linked through their Fc portion to specific Fc γ receptors (Fc γ Rs) on the membrane of leukocytes [6, 7]. In this way, antibodies function as a bridge between the specific adaptive immune response and the potent innate immune functions of leukocytes. In the human neutrophil, two types of Fc γ R exist. Thus, antibodies are important activators of neutrophils. The Fc γ receptors on the neutrophil are considered to be redundant in inducing cell responses [8, 9]. However, recent findings on how a particular IgG subclass and the glycosylation pattern of the antibody regulate the IgG–Fc γ R interaction suggest that a particular effector function may in fact be activated in response to a specific type of Fc γ R. It is the purpose of this chapter to describe the Fc γ Rs on human neutrophils and present our current view of how particular Fc γ Rs activate various signaling pathways to promote unique effector cell functions.

2. Neutrophils

Neutrophils are the most abundant leukocytes in blood and because they are the first cells to appear at sites of inflammation and infection; they are regarded as the first line of defense of the innate immune system [10]. Neutrophils can rapidly move from the blood into affected sites through a process known as the leukocyte adhesion cascade. Once in the tissues, they perform important antimicrobial functions, including phagocytosis, degranulation, and formation of neutrophil extracellular traps (NETs) [11, 12].

2.1 Leukocyte adhesion cascade

Neutrophils leave the blood circulation at sites of infection or inflammation by binding to the endothelial cells and then transmigrating into the tissues [13]. This process known, as the leukocyte adhesion cascade (**Figure 1**), begins with the activation of endothelial cells at the affected site. Activated endothelial cells upregulate the expression of adhesion receptors such as E- and P-selectins. Neutrophils bind to these selectins via glycoprotein ligands on their membrane. As a consequence, neutrophils can then roll on endothelial cells. Next, neutrophils get activated by chemokines, which induce a high affinity state on integrins, another group of adhesion receptors. Binding of integrins with their corresponding ligands, such as intercellular adhesion molecule-1 (ICAM-1) and ICAM-2 on endothelial cells, results in slower neutrophil rolling and then firm adhesion that makes neutrophils stop. Finally, neutrophils transmigrate the endothelium into the tissues. Engagement of endothelial-cell adhesion molecules seems to provoke the opening of endothelial-cell contacts by redistributing junctional molecules in a way that promotes transmigration of neutrophils. Molecules that do not help neutrophil migration, such as vascular endothelial cadherin (VE-cadherin), are moved away from junctional regions. Other endothelial junctional molecules for which neutrophils express ligands concentrate on the endothelial cell luminal surface creating an adhesive environment for the neutrophil. Platelet/endothelial-cell adhesion molecule 1 (PECAM1) and CD99 support homophilic interactions between endothelial cells and neutrophils. While, junctional adhesion molecule (JAM)-1 and JAM-2 on the endothelial cell bind to the β 1 integrin VLA4, and the β 2 integrins LFA-1 and Mac-1 on the neutrophil, respectively. The endothelial cell-selective adhesion molecule (ESAM) is also involved in transmigration by binding to an unknown

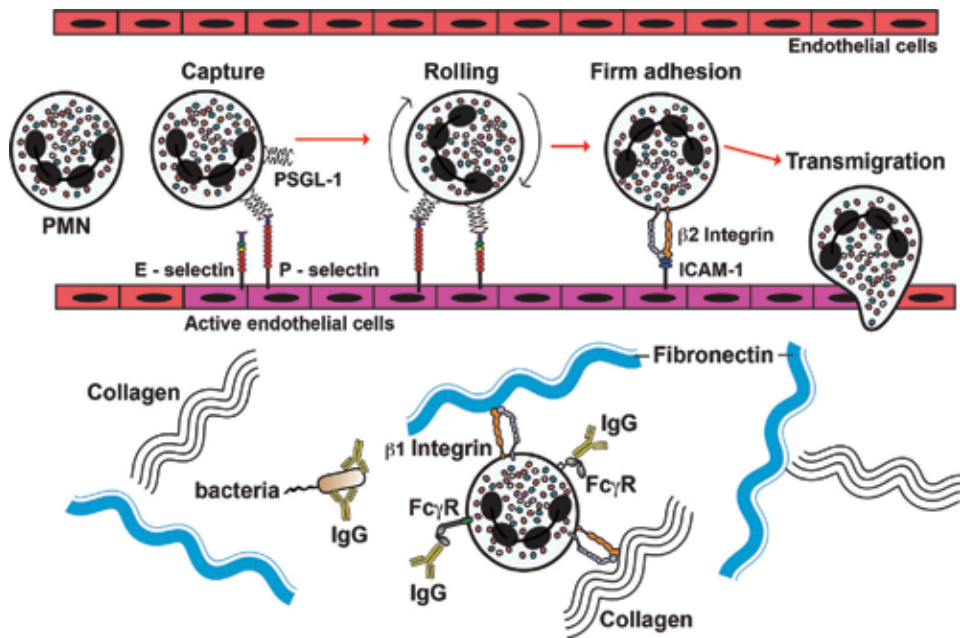


Figure 1. *Leukocyte adhesion cascade. Neutrophils, also known as polymorphonuclear (PMN) cells, move to sites of inflammation via a leukocyte adhesion cascade that includes activation of endothelial cells with upregulation of E- and P-selectins. Neutrophils bind to these selectins via glycoprotein ligands, such as P-selectin glycoprotein ligand-1 (PSGL-1), and begin rolling on endothelial cells. Next, neutrophils get stimulated by chemokines and activate their $\beta 2$ integrins, which bind to their corresponding ligands, such as intercellular adhesion molecule-1 (ICAM-1). Integrin binding induces firm adhesion and transmigration of neutrophils into tissues. Once in tissues, neutrophils move following chemoattractant gradients to reach affected sites using now adhesion of $\beta 1$ integrins to proteins of the extracellular matrix, such as collagen and fibronectin. Antibodies (IgG) bind to microorganisms (bacteria) and are in turn recognized by Fc γ receptors (Fc γ R) on the membrane of neutrophils.*

ligand on neutrophils [12, 14]. Once neutrophils move into tissues, they follow chemoattractant gradients to reach affected sites using now adhesion of $\beta 1$ integrins to proteins of the extracellular matrix, such as collagen and fibronectin [15] (Figure 1). Important chemoattractants for neutrophils are activated complement components, such as the anaphylatoxin C5a, bacterial components, such as formyl-methionyl-leucyl-phenylalanine (fMLF) and cytokines, such as interleukin (IL) 8.

2.2 Antimicrobial mechanisms of neutrophils

Neutrophils recruited from the circulation into infected tissues can eliminate microorganisms by phagocytosis, by releasing antimicrobial substances or by forming NETs [11, 12] (Figure 2).

2.2.1 Phagocytosis

Phagocytosis is the process by which particles larger than 5 μm get internalized by the cell into a vacuole called the phagosome. Neutrophils recognize pathogens directly through pattern-recognition receptors (PAMPs), or indirectly through opsonin receptors. Opsonins are host proteins, such as antibody molecules or complement components, that bind to microorganisms and facilitate their detection and destruction by leukocytes [16, 17]. After internalization, the nascent phagosome matures by fusing with lysosomes [18]. During maturation, antimicrobial

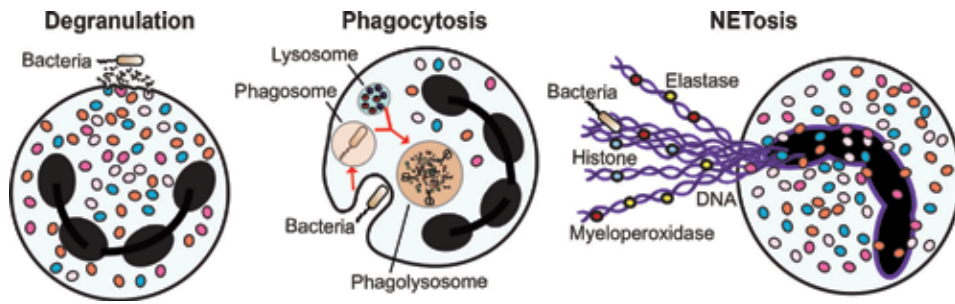


Figure 2. Antimicrobial mechanisms of neutrophils. Neutrophils can destroy microbial pathogens, such as bacteria by (a) degranulation, (b) phagocytosis, and (c) NETosis. During degranulation, antimicrobial proteins are released outside the neutrophil. In phagocytosis, the pathogen is ingested in a vacuole named phagosome, which then fuses to lysosomes and becomes a phagolysosome, where the pathogen is destroyed. During NETosis, DNA fibers decorated with histones and granular proteins, such as elastase and myeloperoxidase are released in structures known as neutrophil extracellular traps (NETs).

molecules are delivered into the phagosomal lumen, and the vesicle is transformed into a phagolysosome [19]. In the phagolysosome, reactive oxygen species (ROS) are produced by the NADPH oxidase on the phagosomal membrane, and the pH inside drops to 4.5–5. Also, hydrogen peroxide (H_2O_2) is converted to hypochlorous acid (HOCl) in a reaction catalyzed by myeloperoxidase (MPO) [20]. Together, these actions form a toxic environment for the microorganism.

2.2.2 Degranulation

During neutrophil formation in the bone marrow, immature neutrophils synthesize proteins that are sorted into different granules [10]. Granules are classified into three different types based on their content. Azurophilic granules contain mainly myeloperoxidase, elastase, and cathepsin G. Specific granules contain mainly collagenase, lactoferrin, and lysozyme. Gelatinase granules contain mainly gelatinase, lysozyme, and cytochrome b558 [21]. Neutrophils also form secretory vesicles at the last step of their differentiation. These secretory vesicles contain several important receptors on their membrane, including complement receptors (CR1), Fc receptors (CD16), lipopolysaccharide (LPS) receptors (CD-14), and fMLF receptors. Granule heterogeneity is due to the controlled expression of the granule protein genes [22]. Mature neutrophils are released into the circulation and when they reach sites of infection, neutrophils can degranulate in order to deliver their antimicrobial proteins. Secretory vesicles present the greatest predisposition for extracellular release, followed by gelatinase granules, specific granules, and azurophil granules [23]. The hierarchical mobilization of neutrophil granules and secretory vesicles depend on intracellular Ca^{2+} -level [24].

2.2.3 Neutrophil extracellular traps (NETs)

When neutrophils cannot ingest large microorganisms, they can display another antimicrobial strategy [25]. Neutrophils can release long chromatin fibers that are decorated with proteins from their granules. These fibers can trap microorganisms, and therefore, they have been called neutrophil extracellular traps (NETs) [26]. The process of NETs formation is called NETosis [27]. NETosis has been described as a special form of programmed cell death. The complete mechanisms of NETs formation are still unknown; it seems that NETosis requires NADPH oxidase activation, reactive oxygen species (ROS) production, myeloperoxidase (MPO), and neutrophil elastase (NE) release [28, 29] (Figure 2).

3. Fc γ receptors

Antibodies produced by the adaptive immune response are mainly of the IgG class. These antibodies present higher affinity and greater specificity for their particular antigen. Thus, IgG antibodies are key for controlling infections from all types of pathogens, including viruses, bacteria, fungi, and protozoa [30]. However, IgG molecules do not directly damage the microorganisms they recognize. It is in fact, the cells of the innate immune system, which are responsible for the anti-microbial functions of these antibodies. Although, some antibodies can activate complement, which is then deposited on microorganisms to promote phagocytosis via complement receptors [17, 31], or to induce bacterial lysis via the formation of the membrane attack complex [32], most IgG antibodies bind to specific receptors on the membrane of leukocytes [7, 8]. These receptors recognize the fragment crystallizable (Fc) portion of IgG molecules and are therefore known as Fc γ receptors (Fc γ R). Cross-linking of Fc γ R on the surface of cells activates several antimicrobial functions [6].

3.1 Human Fc γ receptors

Human Fc γ receptors comprise a family of glycoproteins expressed on the membrane of immune cells [7, 8]. These receptors can bind to the various IgG subclasses with different affinities [7], and induce different cellular responses [6]. Fc γ R can be classified as activating receptors (Fc γ RI/CD64, Fc γ RIIa/CD32a, Fc γ RIIIa/CD16a, and Fc γ RIIIb/CD16b), and one inhibitory receptor (Fc γ RIIb/CD32b) [7, 9, 33, 34] (**Figure 3**).

Fc γ RI is a high affinity receptor, having three Ig-like extracellular domains. It binds mainly monomeric IgG [9]. In contrast, Fc γ RII and Fc γ RIII are low-affinity receptors, having two Ig-like extracellular domains. They bind only multimeric immune complexes [9, 35]. Fc γ RI is associated with a dimer of the common Fc receptor γ chain, which contains an immunoreceptor tyrosine-based activation motif (ITAM) sequence (**Figure 3**). The ITAM sequence is important for receptor signaling [36].

Fc γ RIIIa contains its own ITAM within its cytoplasmic tail. In contrast, the inhibitory receptor Fc γ RIIb contains an immunoreceptor tyrosine-based inhibition

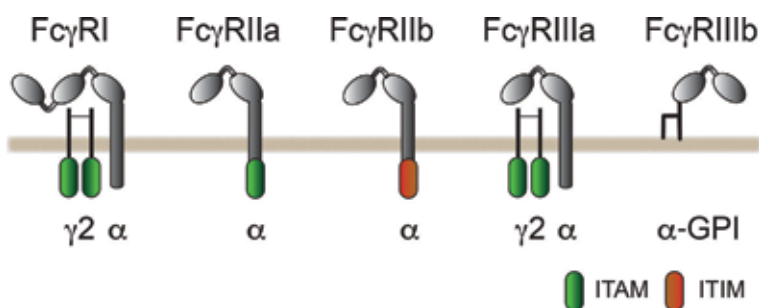


Figure 3. Human Fc γ receptors. Schematic illustration of human receptors for IgG. Fc γ receptors are shown relative to the cell membrane (brown line). The IgG-binding chain (α) is expressed together with their respective γ 2 signaling subunits. Fc γ RI is a high affinity receptor, having three Ig-like extracellular domains. Fc γ RII and Fc γ RIII are low-affinity receptors, having two Ig-like extracellular domains. Fc γ RIIIb is expressed exclusively on neutrophils, and it is a glycosylphosphatidylinositol (GPI)-linked receptor missing a cytoplasmic tail. ITAM, immunoreceptor tyrosine-based activation motif with consensus sequence YxxI/Lx₍₆₋₁₂₎YxxI/L [36] (green oval); ITIM, immunoreceptor tyrosine-based inhibition motif with the consensus sequence I/V/L/SxYxxL/V [39] (red oval).

motif (ITIM) within its cytoplasmic tail (**Figure 3**). The Fc γ RIIb negatively regulates various cell functions including antibody production by the B cell [37], proliferation, degranulation, and phagocytosis in other leukocytes when it is cross-linked with activating Fc γ Rs [38, 39]. Most leukocytes express both activating and inhibitory Fc γ Rs, hence simultaneous cross-linking establishes a threshold for cell activation [40] that maintains a balanced immune response [41, 42].

Fc γ RIII has two isoforms: Fc γ RIIIa is a receptor with a transmembrane domain and a cytoplasmic tail, associated with an ITAM-containing homodimer of Fc receptor γ chains (**Figure 3**). It is expressed mainly on macrophages, natural killer (NK) cells, and dendritic cells [7, 8]. In contrast, Fc γ RIIIb is expressed exclusively on neutrophils and it is a glycosylphosphatidylinositol (GPI)-linked receptor missing a cytoplasmic tail. Also, no other subunits are known to associate with it (**Figure 3**). It is important to mention that human Fc γ RIIIa and Fc γ RIIIb are exclusive receptors that are not found in other species [33, 43].

4. IgG binding to Fc γ receptors

As mentioned before, there is one high-affinity Fc γ receptor, Fc γ RI (CD64), and two groups of low-affinity Fc γ receptors, Fc γ RII and Fc γ RIII (**Figure 3**). This causes that a single IgG molecule cannot bind to most Fc γ receptors. However, when IgG molecules form antigen-antibody (immune) complexes, they can have many low affinity interactions with Fc γ receptors. Thus, only immune complexes are able to induce the cross-linking of Fc γ R required for the activation of various antibody-mediated cell functions. It is clear then that depending on the nature of the immune complex, the interaction with various Fc γ R will change. Several factors have been identified as having an important influence on the affinity of antibody molecules for particular Fc γ Rs. These factors include the type of IgG subclass [7, 44], the IgG glycosylation pattern [45, 46], and receptor polymorphisms.

4.1 The type of IgG subclass

There are four subclasses of IgG (IgG1, IgG2a, IgG2b, and IgG3 in mice; and IgG1, IgG2, IgG3, and IgG4 in humans) [47]. This leads to the formation of different types of immune complexes. Several *in vivo* studies have indeed suggested that different IgG subclasses can activate particular cell responses. For example, in mice, IgG2b was better than IgG1 at eliminating B cell [48] and T cell lymphomas [49]. Also, antierythrocyte antibodies of IgG2a and IgG2b subclasses were better than antibodies of IgG1 and IgG3 subclasses in mediating phagocytosis of opsonized erythrocytes [50]. In humans, it was shown that most Fc γ Rs bind primarily IgG1 and IgG3 over the other subclasses of IgG [6, 7]. Together, these reports confirm that different IgG subclasses mediate different cellular responses *in vivo*, and suggest that different cellular activities result from cross-linking different Fc γ Rs. However, the mechanism used to generate this IgG-Fc γ R selectivity is not completely understood. Accordingly, a great interest exists for determining which type of IgG binds to which Fc γ R and what particular receptor is involved in mediating a certain cellular function.

Obviously, this selectivity depends mainly on the affinities of different IgG subclasses to particular Fc γ receptors. For this reason, detailed studies to measure the affinities of IgG subclasses to the various Fc γ receptors have been conducted both for mice Fc γ Rs [51] and for all human Fc γ Rs [35]. Through these studies, it was found that IgG1 and IgG3 bind to all Fc γ R. IgG2 binds mainly to Fc γ RIIa (H₁₃₁ isoform),

and Fc γ RIIIa (V₁₅₈ isoform), but not to Fc γ RIIIb [35]. IgG4 binds to many Fc γ Rs [35]. Thus, it is clear that different IgG subclasses engage different Fc γ receptors depending on the relative affinity of these receptors for a particular IgG class [33].

4.2 The IgG glycosylation pattern

All IgG molecules are glycoproteins with an N-glycosylated carbohydrate side chain that is important for antibody function [52]. Deletion of this carbohydrate (sugar) side chain results in poor binding to Fc γ Rs [53]. The N-glycans are heterogeneous in their sugar composition and are attached to asparagine 297 (Asp²⁹⁷) in the Fc portion of the IgG [54]. The carbohydrate side chain may contain sugar residues such as galactose, fucose, and sialic acid in straight or branching patterns [46], and the differences in the glycosylation pattern seem to regulate IgG activity [55].

Many IgG antibodies present a fucose residue linked to an N-acetylglucosamine residue [56]. When this residue is removed, IgG molecules present an increased affinity to the Fc γ RIIIa [57], and also an increase in antibody-dependent cell cytotoxicity (ADCC) activity against various tumor cells [51, 57, 58]. Based on these findings, recombinant IgG antibodies with low fucose levels have been produced in order to increase their ADCC activity. Several of these antibodies are now in clinical trials to test their therapeutic potential [59].

Many IgG antibodies also present a carbohydrate side chain that terminates with sialic acid residues [60]. Contrary to antibodies without fucose, terminal sialic acid usually correlates with low affinity for Fc γ Rs and also with lower ADCC activity [61, 62]. Interestingly, these sialic acid-rich antibodies seem to preferentially bind other receptors different from Fc γ Rs. The receptor dendritic cell specific ICAM-3 grabbing nonintegrin (DC-SIGN) was identified as a receptor for sialic acid-rich IgG [63]. Therefore, terminal sialic acid can modify IgG activity by promoting less binding to Fc γ Rs and more binding to other receptors [45].

4.3 Polymorphisms of receptors

Another factor influencing the affinity of antibody molecules is the existence of several polymorphisms for the unique Fc γ RIIIa and Fc γ RIIIb present on human neutrophils [64]. There are two isoforms for Fc γ RIIIa with different amino acids at position 131. These are identified as low-responder (H₁₃₁) and high-responder (R₁₃₁) [65]. Similarly, for Fc γ RIIIb two isoforms exist differing at four positions, NA1 (R36 N65 D82 V106) and NA2 (S36 S65 N82 I106) [66], and with different glycosylation patterns [67]. In addition, another Fc γ RIIIb isoform named SH is generated by a point mutation (A78D) in the NA2 allele [68]. These multiple Fc γ R isoforms display diverse binding affinity for different IgG classes [35], creating variable cell responses to different antibodies.

5. Fc γ receptor signaling

The human neutrophil expresses two unique activating Fc receptors: Fc γ RIIIa and Fc γ RIIIb. Fc γ RIIIa is a receptor containing ITAM sequences [36, 69], and it signals similarly to other typical immunoreceptors, such as the antigen receptor of T lymphocytes (TCR) and the antigen receptor of B lymphocytes (BCR) [70]. The initial signaling steps for all immunoreceptors are alike and involve first cross-linking of the receptors on the membrane of the cell, followed by the activation of Src family tyrosine kinases (**Figure 4**). These kinases lead to activation of spleen

tyrosine kinase (Syk), which in turn phosphorylates tyrosines within the ITAM sequence. Phosphorylated ITAM then becomes a binding site for Syk. After binding to the receptor, Syk phosphorylates multiple substrates leading to different cell responses [6, 31, 71] (**Figure 4**). Syk can phosphorylate and activate phospholipase C γ (PLC γ), which in turn generates diacylglycerol (DAG) and inositol triphosphate (IP $_3$). DAG also activates protein kinase C (PKC), an important serine/threonine kinase that can lead to the activation of MAP kinases extracellular signal-regulated kinase (ERK) and p38 (**Figure 4**). IP $_3$ induces release of intracellular calcium from the endoplasmic reticulum. Calcium regulates several proteins such as calmodulin and calcineurin. Syk can also induce activation of phosphatidylinositol-3 kinase (PI3K), which produces phosphatidylinositol 3,4,5-trisphosphate (PIP $_3$). This phospholipid is relevant to the activation of small GTPases, such as Rho and Rac, which are involved in cytoskeleton remodeling for phagocytosis. Rac also leads to activation of the MAPK/ERK kinase (MEK)—ERK pathway, and to activation of c-Jun N-terminal kinases (JNK). These kinases are important for activation of nuclear factors, such as Elk-1, AP-1, and nuclear factor of activated T cells (NFAT) (**Figure 4**). These nuclear factors induce the expression of cytokines important for inflammation and immune regulation, such as IL-2, IL-6, IL-8, IL-10, tumor necrosis factor α (TNF- α), and IFN- γ [72–74] (**Figure 4**).

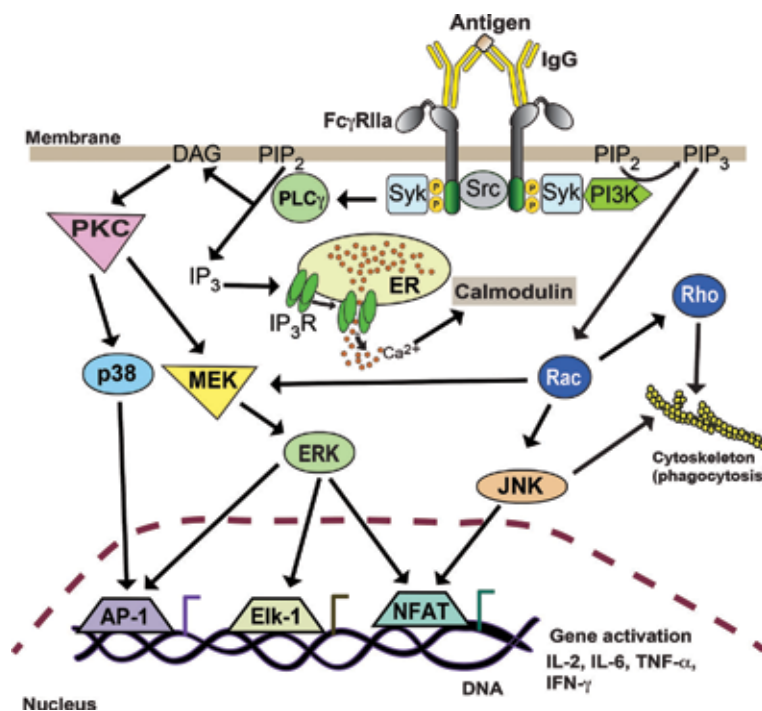


Figure 4.

Signaling transduction pathway of the neutrophil Fc γ RIIIa. Engagement of activating Fc γ RIIIa by IgG-antigen immune complexes induces receptor cross-linking and phosphorylation of tyrosine residues in the immunoreceptor tyrosine-based activation motif domains (green oval) by Src family kinases. Phosphorylated tyrosines then become docking sites for Syk, which in turn phosphorylates multiple substrates leading to different signaling pathways that ultimately activate various cell responses. See text for details. P represents a phosphate group; Syk, spleen tyrosine kinase; PI3K, phosphatidylinositol-3 kinase; PIP $_2$, phosphatidylinositol 4,5-bisphosphate; PIP $_3$, phosphatidylinositol 3,4,5-trisphosphate; JNK, c-Jun N-terminal kinase; NFAT, nuclear factor of activated T cells; PLC γ , phospholipase C γ ; DAG, diacylglycerol; IP $_3$, inositol triphosphate; ER, endoplasmic reticulum; PKC, protein kinase C; MEK, MAPK/ERK kinase; ERK, extracellular signal-regulated kinase; p38, p38 MAP kinase; AP-1, activator protein 1; Elk-1, Ets LiKe gene1 (ETS) transcription factor; IL-2, interleukin-2; IL-6, interleukin-6; TNF- α , tumor necrosis factor α ; and IFN- γ , interferon- γ .

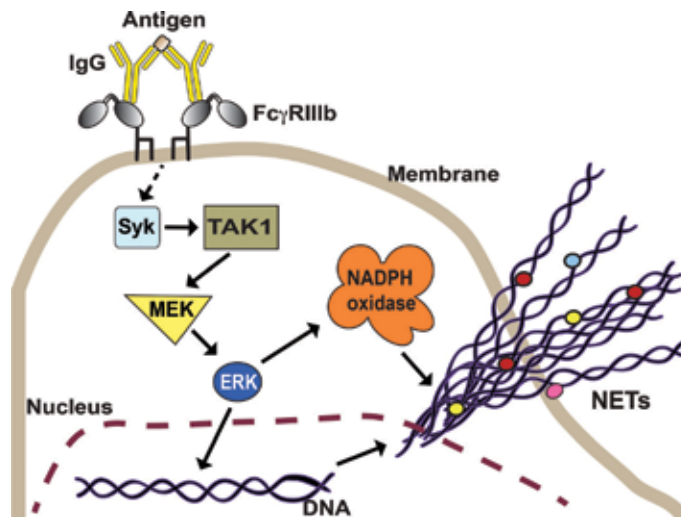


Figure 5. Signaling transduction pathway of the neutrophil Fc γ RIIIb. Cross-linking of the human Fc γ RIIIb by IgG-antigen immune complexes induces activation of spleen tyrosine kinase (Syk) by a mechanism not yet described. Syk then activates transforming growth factor- β -activated kinase 1 (TAK1). TAK1 is in turn required for activation of ERK kinase (MEK) and extracellular signal regulated kinase (ERK). Activated ERK signals to the nucleus and contributes to activation of NADPH oxidase, which together lead to formation of neutrophil extracellular traps (NETs).

In contrast, the human Fc γ RIIIb is a GPI-linked receptor that lacks an intracellular portion. Thus, it is not clear how it can connect to intracellular signaling molecules. However, there is no doubt that Fc γ RIIIb is an activating receptor inducing several neutrophil responses such as increase in calcium concentration [75], activation of the respiratory burst [76], activation of integrins [77], and induction of NETosis [78, 79]. Despite, the initial signaling mechanism for Fc γ RIIIb remains unknown, the signaling pathway for this receptor engages Syk and then transforming growth factor- β -activated kinase 1 (TAK1), as well as the MEK/ERK cascade [80] (Figure 5). One possibility to connect Fc γ RIIIb with Syk is that the receptor could link with signaling molecules such as Src family tyrosine kinases on the plane of the cell membrane. Because GPI-linked proteins, like the Fc γ RIIIb, concentrate in lipid rafts on the cell membrane together with Src kinases [81, 82], we can imagine that after cross-linking Fc γ RIIIb, it associates somehow with these kinases and activates Syk. A possible connection is the binding of the receptor, within the lipid rafts, to a putative ITAM-containing molecule [83]. Many steps are still unknown and future research will help in completely elucidate this signaling pathway.

6. Each Fc γ R leads to unique cellular responses

The signaling pathways activated by immune complexes binding to Fc γ receptors stimulate different neutrophil responses including phagocytosis, respiratory burst, cytokine and chemokine production, and antibody-dependent cellular cytotoxicity (ADCC) [7, 8, 33]. However, our understanding of what particular function is activated in a cell responding to an individual type of Fc γ R is still very limited. This lack of knowledge is due, in part, to the fact that each cell expresses several types of Fc γ R and all receptors can bind to more than one type of IgG. Thus, it is not clear whether each receptor leads to a particular response or the average signaling from various receptors activates a predetermined cell response. Traditionally, it

has been thought that each cell is set to activate a particular cell function after Fc γ R cross-linking. More recently, however, another interpretation has been considered: each Fc γ R activates a particular signaling pathway leading to a unique cell response. In the traditional view, each cell is already programmed to perform a particular cell function after Fc γ R cross-linking, independently of the receptor used. This idea is not really supported by experimental evidence. As indicated above, different IgG subclasses bind particular Fc γ receptors with different affinity, leading to unique cell functions *in vivo* [42]. In the most recent view, each Fc γ R activates a distinctive signaling pathway leading to an individual cell function. This view is supported by recent reports, where individual Fc γ R on human neutrophils initiate particular cell responses [77, 78, 84–86].

The idea that particular Fc γ receptors could activate unique cell functions was initially published more than 20 years ago. It was found that the neutrophil Fc γ RIIIb induced actin polymerization in a Ca²⁺-dependent manner, while Fc γ RIIa did not [87]. This initial report was not followed by similar reports and the idea of one receptor one response was forgotten. However, with time, other reports have provided new evidence that supports this idea. Some years later, it was reported that Fc γ RIIa, but not Fc γ RIIIb caused shedding of L-selectin expression [88] (**Figure 6**). Consequently, it was proposed that binding of antibodies to Fc γ RIIIb could induce a proadhesive phenotype of neutrophils [88]. More recently, new evidence supporting this idea was found. When each receptor was selectively activated with specific monoclonal antibodies, Fc γ RIIIb but not Fc γ RIIa, was able to activate β 1 integrins [77] (**Figure 7**). This activation resulted from an increase in binding affinity to fibronectin [77]. Thus, after neutrophils leave the circulation, engagement of Fc γ RIIIb could lead to activation β 1 integrins, allowing the cells to adhere to extracellular matrix proteins and migrate into tissues [89] (**Figure 1**). In contrast, for antibody-mediated phagocytosis [17], Fc γ RIIa was the main Fc γ receptor mediating this response, while Fc γ RIIIb contribution to phagocytosis was minimal [86]. Therefore, at least in human neutrophils, each Fc γ receptor initiates particular cell functions. Fc γ RIIa induces phagocytosis (**Figure 6**), while Fc γ RIIIb promotes an adhesive phenotype via activation of β 1 integrins (**Figure 7**).

In addition, it was also reported that Fc γ RIIIb signals to the neutrophil nucleus more efficiently than Fc γ RIIa. Fc γ RIIIb, but not Fc γ RIIa, induced a large increase

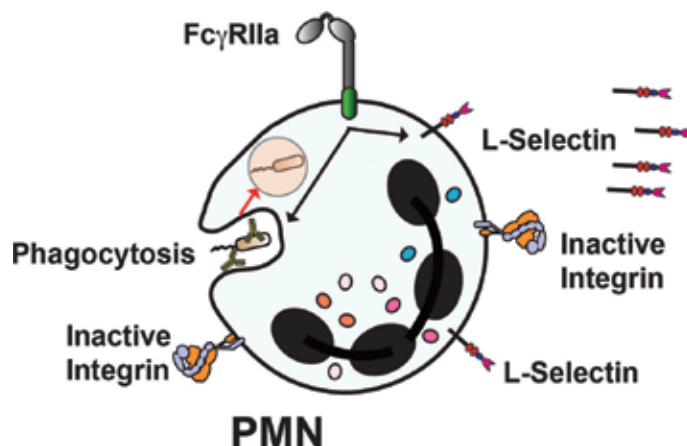


Figure 6. Neutrophil functions activated by Fc γ RIIa. In human neutrophils, Fc γ RIIa signaling induces L-selectin shedding from the cell membrane, and also activates efficient phagocytosis. The oval represents IgG-opsonized bacteria.

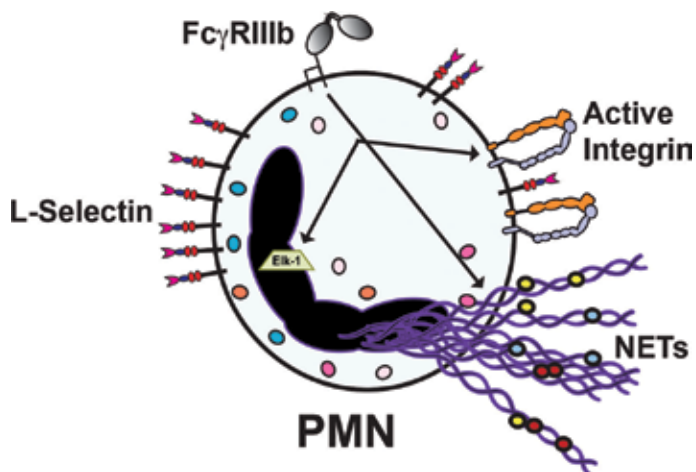


Figure 7. Neutrophil functions activated by Fc γ RIIIb. Cross-linking of the human Fc γ RIIIb stimulates activation of β 1 integrins promoting in this way a proadhesive neutrophil phenotype. Fc γ RIIIb also induces activation of the nuclear factor Elk-1 and formation of neutrophil extracellular traps (NETs).

in phosphorylated ERK in the nucleus, and also efficient phosphorylation of the nuclear factor Elk-1 [84] (**Figure 7**). Interestingly, Fc γ RIIa also induced phosphorylation of ERK in the cytosol [84, 90], but this active ERK seems to function mainly in enhancing phagocytosis and not in nuclear signaling [91] (**Figure 4**).

A recently discovered antimicrobial function of neutrophils is the formation of neutrophil extracellular traps (NETs) [92, 93]. NETs are induced by several pathogens, including virus, bacteria, fungi, and parasites [94]. Also, pro-inflammatory stimuli such as IL-8, TNF- α , and phorbol-12-myristate-13-acetate (PMA) are efficient inducers of NETs [95]. Because, antigen-antibody complexes are also capable of inducing NET formation [96]; it was clear that Fc γ R were involved in NET formation. Recently, it was found that Fc γ RIIIb, but not Fc γ RIIa, is the receptor responsible for NET formation [78–80] (**Figure 8**).

Together, all these reports strongly reinforce the modern view that each Fc γ R induces a particular signaling pathway that activates a single cellular function. Elucidating the conditions that engage a single type of Fc γ R to activate a particular cellular response would be very helpful in the future for controlling some of cellular

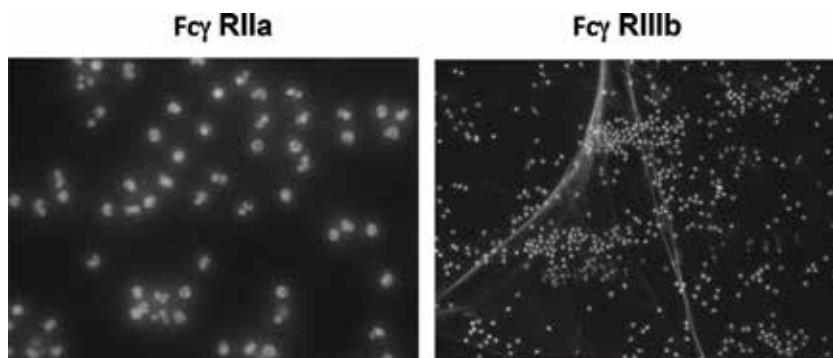


Figure 8. Neutrophil Fc γ RIIIb, but not Fc γ RIIa, induces neutrophil extracellular traps (NETs) formation. Human neutrophils were stimulated by cross-linking Fc γ RIIa with the specific monoclonal antibody IV3, or by cross-linking Fc γ RIIIb with the specific monoclonal antibody 3G8. After 4 hours, neutrophils were fixed and stained for DNA.

functions in clinical settings. For example, in intense infections, it may be important to activate phagocytosis. Because IgG2 binds better to Fc γ RIIa than to Fc γ RIIIb [33, 35], it is likely that IgG2 antibodies would activate phagocytosis by neutrophils much better than other IgG subclass antibodies. In consequence, promoting IgG2 antibodies against certain pathogens would result in better phagocytosis against them.

7. Conclusion

Fc γ receptors expressed in different immune cells are capable of activating different cellular responses important not only for controlling microbial infections but also for regulating immunity [71, 97]. Different subclasses of IgG antibodies bind the various Fc γ receptors with different affinities [33, 35] and can activate various cellular functions of great importance for host defense and for immune regulation. In the human neutrophil, it is clear that a specific Fc γ receptor activates particular cellular responses. Fc γ RIIa induces efficient phagocytosis [86], while Fc γ RIIIb signals to the nucleus for nuclear factor activation [84] and for NETs formation [78]. Therefore, in principle, a particular cell response could be induced or inhibited by engaging or blocking the corresponding Fc γ R. Information similar to the one described for neutrophil Fc γ receptors on other immune cells, such as monocytes or dendritic cells, is not available. Future research is needed in this area.

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Remodeling of Phenotype CD16⁺CD11b⁺ Neutrophilic Granulocytes in Acute Viral and Acute Bacterial Infections

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Abstract

Neutrophilic granulocytes (NGs) are very important cells of innate immunity that can very quickly realize antibacterial and antiviral defense. Until the present time, the phenomenon of different levels of presentations of membrane receptors CD16 and CD11b NG in normal and pathological conditions wasn't studied. We had studied the population of CD16⁺CD11b⁺NG in two groups of patients with acute viral and acute bacterial infections in the models of acute bacterial tonsillitis (ABT) and acute viral tonsillitis-EBV infection (AEBVI), having the same clinical symptoms in early stages of the disease. Comparative analysis of the redistribution of equipment intensity of CD16 and CD11b has detected three subpopulations of CD16⁺CD11b⁺NG population—CD16^{bright}CD11b^{bright}, CD16^{bright}CD11b^{dim}, and CD16^{dim}CD11b^{bright}—in normal and pathological conditions. It was found that subpopulation CD16^{bright}CD11b^{dim}NG dominates in healthy individuals; subpopulation CD16^{bright}CD11b^{bright}NG dominates in patients with acute viral infection; subpopulation CD16^{dim}CD11b^{bright}NG dominates in patients with acute bacterial infections. We had demonstrated that the study of CD16⁺CD11b⁺NG subpopulations allows in early stage of diseases to diagnose acute viral and acute bacterial infections. Our studies have demonstrated the positive effects of eukaryotic DNA sodium salt on the negatively altered phenotype subpopulation CD16⁺CD11b⁺NG, in particular, through the remodeling of the expression of CD11b on NG membrane.

Keywords: neutrophilic granulocytes, subset, phenotype, receptors, acute viral and bacterial infections, eukaryotic DNA sodium salt

1. Introduction

Neutrophil granulocytes (NGs) are the most mobile and numerous populations of innate immunity cell, which reacts lightly to any aggression, which also carries out powerful anti-infectious protection.

The surprising universality and multifunctionality of this cell, once again, underline the existence of heterogeneity within the NG population, that is,

the presence of subpopulations with different immunological roles. The use of monoclonal antibodies made it possible to confirm the existence of NG subpopulations using phenotypic characteristics. In 1998, the first nomenclature of human neutrophil antigens (HNAs) was created on the basis of membrane-expressed glycoprotein groups: HNA-1 (FcγRIIIb, CD16), HNA-2 (CD177), HNA-3 (CTL2), HNA-4 (CD11b/CD18, Mac-1, CR3), and HNA-5 (CD11a/CD18) [1]. The concept of heterogeneity of NG was discussed by scientists for more than 20 years and was confirmed with the accumulation of evidence on the presence of subsets of NG with various functions both in healthy subjects and in various diseases. Various methods have been used to detect the subpopulations of NG, such as cell maturity, functional activity, and localization, including receptors or markers of the cell surface.

2. Neutrophil granulocyte receptors

Cell populations and subpopulations of NG show a high degree of plasticity and functional heterogeneity depending on the characteristics of the course of physiological or pathological scenarios of the immune response, which, first of all, is due to potent receptor equipment. The membrane complex of NG expresses adhesion molecules, receptors for different ligands: cytokines, immunoglobulins, other cell membrane molecules, etc. NGs are capable to express MHC-1, selectins (CD62L), selectin receptors (CD162 (PSGL-1)), integrins (CD18 (β2-integrin), CD11a (LFA-1), CD11b (CR3), CD11c (CR4), CD11d), integrin receptors (ICAM receptors for β2-integrins - ICAM-1 (CD50), ICAM-3 (CD54)). NG expresses receptors for chemoattractants (PFPR and FPLR for fMLP), receptors for chemokines (CXCR1, CXCR2, CCR1), FcR receptors (CD16 (FcγRIII), CD32 (FcγRII), CD64 (FcγRI), CD89 (FcαRI), FcεR), receptors for complement components (CR1 (CD35), CR3 (CD11b), CR4 (CD11c), C5aR, C3aR, C5L2), receptor for LPS and endotoxins (CD14), cell adhesion receptor (CD15). NG receptors are involved in binding bacteria, in angiogenesis and apoptosis (CD17), in cell proliferation and differentiation (CD24), in PAMP recognition (TLR 1, 2, 4-10; NOD - receptors). In addition, on NG membrane there is a costimulatory receptor for B- lymphocytes (CD28), apoptosis activation/induction receptor (CD95), IL-2 receptor (CD25), which is NG activation marker; there are also molecules that determine the ability of NG to be APC (CD40, CD80, CD86, MHC II). NGs have multiple receptors for cytokines (IL-8, TNFα, IL-1, IL-2, IL-15, IL-17, IFNα, IFNγ, G-CSF, GM-CSF, etc.), hormones, neuropeptides, histamine, and kinases. The recently revealed expression of TCR-like (TCRL, TCRαβ) receptors on NG membrane, present throughout the life of a person and decreasing in old age, opens up new, previously unknown immune mechanisms for the functioning of NG [2] (**Figure 1**).

NGs are equipped with receptors that recognize endogenous molecules of “danger” alarms or danger-associated molecular patterns (DAMPs)—extracellular ATP, fragments of the extracellular matrix, heat shock proteins, nucleic acids (DNA and RNA fragments of its own cells), nuclear protein HMGB-1, and others—through which activation of the cell takes place and its inclusion in the inflammation reaction [3]. It has been established that the initiation of apoptosis of NG in clinically healthy individuals is under the influence of TNFα, sTRAIL, and IL-4 ligand [4]. Recently, new ways of activating the NG signal via ITAM/Syk-CARD9 have been described in the interaction of β-glycans with dectin-1, which triggers the synthesis of the cytokine IL-23 inducing the formation of Th17 cells [5]. NG receptor pool is located on intracellular membrane of secretory vesicles, gelatinase and specific granules, these receptors are translocated to surface membrane of NG only under the action of inducing stimuli [6]. Thus, the membrane expression of NG not only reflects the

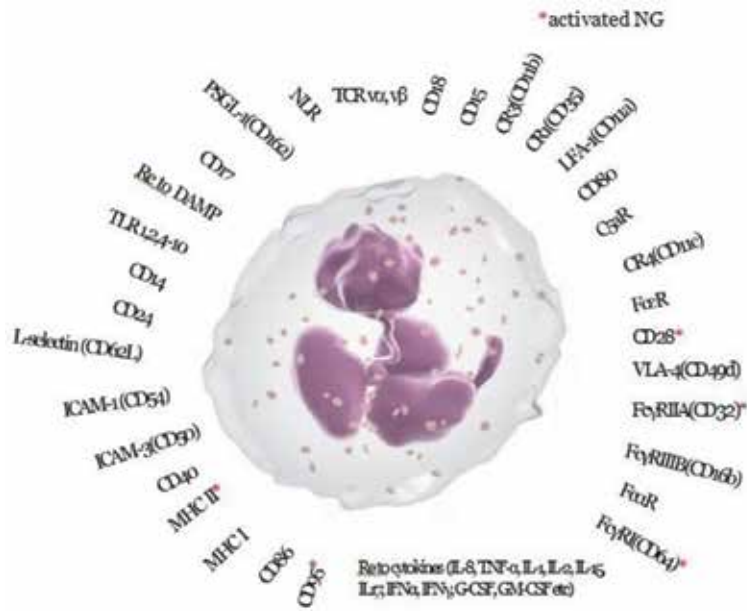


Figure 1.
 Surface membrane receptors of neutrophilic granulocytes.

processes occurring during the life cycle of the cell but also allows us to evaluate the functional priming by reorganizing the surface cytoplasmic membrane of NG.

3. Phenotypic profile and functional features of neutrophil granulocytes

The study of the subpopulations of NG presents a new approach to the determination of functional activity of NG, allowing to assess the adequacy of the inclusion of NG in the implementation of the immune response, as well as to diagnose and predict the outcome of the disease. It is known that various phenotypic profiles and the level of equipment with surface receptors are associated with morphological features and determine the functional potential of NG-cytokine production, transendothelial migration, intracellular and extracellular killing, and formation of NET [7–9]. The existence of a sufficiently large number of NG subpopulations with different possibilities is demonstrated. NGs that receive complex cytokine influences not only acquire new features but also undergo different stages of activation and differentiation while expressing MHCII antigens, CD80, CD86, ICAM-1, and LFA-1 [7, 10, 11]. It has been shown that inducing cytokine stimuli differentiates NG in a unique hybrid subpopulation with dual phenotypic and functional properties characteristic of both NG and dendritic cells (DC) involved in innate and adaptive immune responses [12]. We have identified in our earlier works the following subpopulations of NG: regulatory; suppressor; pro-inflammatory, initiating inflammatory reaction; inflammatory with a positive microbicidal potential (antibacterial, antiviral, antifungal); inflammatory with negative cytotoxic potential, “aggressive”; anti-inflammatory, regulating inflammation regression; antineoplastic, TAN1; and pro-tumor, TAN2 and hybrid [13]. Phagocytic and microbicidal function and virucidal activity of NG are directly dependent on phenotypic features: the number and density of such expressed receptors as CD11b/CD18, CD10, CD15, CD16, CD32, CD64, CD35, etc. [6]. Expression on NG membrane of CD32 and CD16 is important

in the realization of phagocytic function and antibody-dependent cellular cytotoxicity (ADCC), which is associated with CD11b-/CD18-dependent enhancement of adhesion, degranulation, and killing [14]. CD64, CD32, and CD16 are triggering molecules that induce immune phagocytosis and killing processes [15].

The variants of remodeling of NG phenotype simultaneously expressing functionally significant receptors CD64, CD32, CD11b, and CD16 in patients with infectious and inflammatory diseases, including newborns of different gestational ages [15], patients with neoplastic processes [10, 16], women of reproductive age with genital and extragenital infectious-inflammatory diseases [17] have great diagnostic and prognostic significance. When we study the variability of the simultaneous presentation of NG receptors CD64, CD32, CD16, and CD11b on the membrane, it was established that in healthy adults and children of different ages in the peripheral blood, there is one major subpopulation of CD64⁻CD32⁺CD16⁺CD11b⁺ and five minor subpopulations of NG, CD64⁻CD32⁺CD16⁺CD11b⁻, CD64⁻CD32⁻CD16⁺CD11b⁺, CD64⁺CD32⁺CD16⁺CD11b⁺, CD64⁺CD32⁺CD16⁺CD11b⁻, and CD64⁺CD32⁻CD16⁺CD11b⁺, with different equipment and density of studied receptors. We detected a significant increase in NG subpopulation with CD64⁺CD32⁺CD16⁺CD11b⁺ phenotype with a high expression density of CD11b and CD16 in newborns with infectious and inflammatory diseases of bacterial etiology (congenital pneumonia, neonatal sepsis) (**Figure 2**).

The observed increase in this subpopulation of NG in the peripheral blood is directly related to the severity of the infectious-inflammatory process: the more clinically severe the disease, the greater the number of NG with this phenotype CD64⁺CD32⁺CD16⁺CD11b⁺ is in circulation [18]. In fertile age women with genital and extragenital infectious and inflammatory diseases planning pregnancy, the phenotypic variability of NG—the appearance of a subpopulation of CD16⁺CD32⁺CD11b⁻—was also revealed, which indicates a persistent violation of their receptor function and the need for its adequate correction consisting in restoring the phenotypic composition of NG. Thus, the provision of pre-gravity training with the inclusion of immunotherapy has a positive clinical and immunological effect, which consists in normalizing the receptor function of NG, which

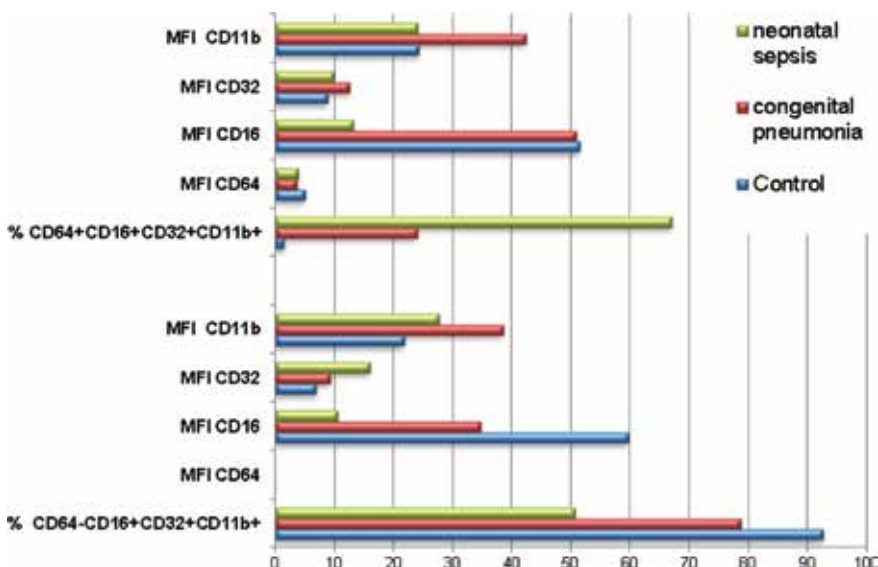


Figure 2. Phenotypic profiles of CD64⁺CD32⁺CD16⁺CD11b⁺ NG in children with congenital pneumonia and neonatal sepsis.

correlates with an increase in the percentage of women who become pregnant [17]. The obtained data allow us to develop criteria for monitoring the course of associated viral infections and bacterial pro-inflammatory diseases, to diagnose and/or predict the aggravation of their severity, and to optimize immunotherapy methods aimed at correcting NG dysfunction. Multiple increases in the subpopulation of CD64⁻CD32⁻CD16⁺CD11b⁺ NG have been shown in children with repeated acute respiratory viral infections associated with herpesviral mono- or mixed infection. There was a significant replicative activity of herpesviruses such as HSV I/HSV II, CMV, EBV, and HHV VI [18, 19]. In addition, authors of this article put together all information into the table for the period from 2010 to 2016, about NG subpopulation phenotype according to their studies (Table 1).

Specific NG subpopulation composition and also adequate level of corresponding surface membrane marker expression density is important for the proper NG function. Thus, Pillay et al. found several subpopulations of NG with different phenotypes, which differed in the number and density of equipment with receptors: mature NG with the phenotype CD16^{high}CD62L^{high}, immature NG with the phenotype CD16^{low}CD62L^{high}, suppressive NG with the phenotype CD16^{high}CD62L^{low}, and NG precursors with the phenotype CD16^{low}CD62L^{low} [11]. Circulating NG subpopulation with the phenotype CD16^{low}CD62L^{high} was observed in children with respiratory syncytial viral infection, as well as in viral and bacterial coinfection

| Group | Subpopulation | Functions/diagnostic significance |
|---|--|--|
| Healthy adults and children | CD64 ⁻ CD32 ⁺ CD16 ⁺ CD11b ⁺ | -Anti-inflammatory and antitumor effect |
| | CD16 ^{bright} CD11b ^{dim} CD62L ^{bright} CD63 ^{dim} CD62L ^{dim} CD63 ^{dim} (1:1) | -Major subpopulations in healthy individuals -Full implementation of ADCC, microbicidal activity |
| Purulent-septic diseases in children and adults | CD64 ⁺ CD32 ⁺ CD16 ⁺ CD11b ⁺ | -Marker of severity of bacterial infection process |
| | CD62L ^{dim} CD63 ^{mid} CD62L ^{dim} CD63 ^{dim} | - Minor subpopulation in healthy individuals - Activated NG in vivo by bacterial antigens (significant increase in circulation) |
| Acute bacterial infection in adults | CD16 ^{dim} CD11b ^{bright} | -Marker of acute process of the bacterial infection - Major subpopulation (significant increase in circulation) |
| Respiratory and herpes infections in children | CD64 ⁻ CD16 ⁺ CD32 ⁻ CD11b ⁺ | -Prognostic sign of adverse course of viral infection |
| | | - Minor subpopulation in healthy individuals -Significant increase at viral respiratory and herpetic infection - Depression of NG phagocytic and microbicidal activity |
| Acute EBV infection in adults | CD16 ^{bright} CD11b ^{bright} | - Marker of severity of viral infection process - Prognostic sign of concomitant bacterial infection - Major subpopulation (significant increase in circulation) - High level of ADCC reaction and ROS-dependent inhibition of T-cell proliferation |

Table 1. Neutrophilic granulocyte subpopulation phenotypes and their function and diagnostic significance (Nesterova I.V. et al., 2010–2016).

[20, 21]. It is shown that the subpopulation of immature NG did not possess the ability to protect against microorganisms. Activated mature NG with immunosuppressive properties was found in patients with HIV infection [22]. Suppressive NG can cause paralysis of the immune system, as a result of which anti-infective protection is disrupted, which facilitates the occurrence of bacterial complications and the emergence of viral and bacterial coinfection [20, 23]. The appearance of CD16^{high}CD62L^{low} NG significantly increases with bacterial infection or viral and bacterial coinfection, and at the same time, in the lower respiratory tract, in lungs this subpopulation is practically not detected in patients with viral infection [11, 20]. Neutrophilic subpopulation characterized by the phenotype CD16^{low}CD62L^{low} was observed in children with severe viral respiratory infection without bacterial coinfection and in patients with bacterial sepsis [20, 21]. Using flow cytometry in combination with a visual evaluation of cells, it was shown that a large number of myelocytes and metamyelocytes are included in this subpopulation, so NG with the phenotype CD16^{low}CD62L^{low} was called a subpopulation of NG precursors. A sequential increase in the number of NG precursors was statistically significant ($p < 0.001$) and did not depend on the presence of bacterial coinfection [20]. It was suggested that the NG precursors originate from a heterogeneous family of granulocyte myeloid-derived suppressor cells (G-MDSCs), which include granulocyte cells with the property of immune inhibition [20, 24]. Significant differences in the number of markers of activation and degranulation of CD11b, CD54, CD63, and CD66b in the above four subpopulations (mature NG, immature NG, suppressor NG, and NG “precursors”) in viral infections and in bacterial coinfections in newborns with severe viral infection, practically, are not revealed. It was noted that the activation and degranulation of suppressor NG revealed a high level of expression of CD11b and CD63, whereas in NG “precursors,” the highest level of expression of CD63 and CD66b and a low level of expression of CD11b and CD54 were observed [20]. Interestingly that NG number in the bloodstream equipped with CD62L on the surface membrane is larger than CD62L NG obtained from bronchoalveolar lavage, which is presumably associated with the loss of this receptor during migration. Pillay et al. [11, 24] discovered the existence of a new subpopulation CD11c^{bright}CD62L^{dim}CD16^{bright}CD11b^{bright} NG—mature hypersegmented human NG with immunosuppressive activity. This subpopulation was able to suppress the proliferation of T cells through the release of active forms of the oxygenate and showed high expression of CD11b. Earlier studies by Woodfin A. and co-authors demonstrated that suppressive NGs—mature cells with hypersegmented nucleus, expressing high levels of ICAM-1—have the ability to reverse transendothelial migration (TEM) [25]. Later, Cortjens and his colleagues in 2017 [20] showed that in severe respiratory viral infection in infants, the expression of the activation marker CD11b was significantly increased in the suppressor subpopulation of NG. These suppressor subpopulations of NG, which appeared in viral and bacterial coinfections in newborns, also had the highest expression of CD63 molecules on surface membranes, which indicated active degranulation of NG.

4. Neutrophil granulocytes in infectious diseases

Defective functioning NGs (deficiency of NG amount; violation of phagocytic function; deficiency of myeloperoxidase, defensins, lactoferrin, glucose-6-phosphate dehydrogenase, NADPH oxidase, etc.; defects in the formation of NET) do not provide adequate antimicrobial protection, which leads to the development of atypically occurring infectious and inflammatory diseases, sepsis, acute haematogenous osteomyelitis, recurrent purulent infections, chronic bacterial infections,

etc. Adequate response of NG in contact with various aggressive pathogens (viruses, bacteria, fungi) can develop in different ways. At the same time, the lack of functional activity of NG is a risk factor for the development of many pathological conditions. Changes in both quantitative and functional characteristics of different subsets of NG are recorded in pathological conditions, that is, NG phenotype is transformed due to a multivariate change in the expression of various receptors. In this case, the defectiveness of microbicidal and regulatory functions of NG leads to a violation of antigen elimination and, as a result, to the aggravation of the course of acute or chronic bacterial, viral, and fungal infections [13]. At present, various dysfunctions of NG are described, which can proceed according to different scenarios in patients with infectious diseases with atypical current: (1) hypofunction and NG deficiency in recurrent and persistent-relapsing purulent processes and chronic infectious diseases, viral and bacterial etiology, not amenable to standard treatment; (2) blockade of functional activity of NG, manifested by the development of an inadequate response up to the state of non-response in chronic sluggish infectious and inflammatory processes with a protracted course of exacerbations in socially significant infections and sepsis; and (3) hyperfunctioning of NG (e.g., extracellular production of oxygen radicals in a high concentration), which can lead to suppression of the T cells and other members of the immune system and damage different organs and tissues in chronic immune-dependent diseases or septic shock [26–28]. Atypically occurring infectious and inflammatory diseases against the background of immune system disorders, and, in particular, against the background of NG dysfunction, lead to increased morbidity, partial and sometimes complete loss of ability to work, and high lethality in sepsis, both in adult subjects and in children especially in the neonatal period [29, 30].

Neonatal NG characterized qualitative and quantitative deficit compared to adult NG. Neonatal sepsis is a global problem because it has the most severe consequences and is characterized by high mortality. This occurs against the background of impairments in the functioning of the immune system and defective NG, which contributes to the rapid dissimulation of the infection and, as a result, to the death of the newborn [31]. Thus, three important violations of NG that contribute to the emergence of severe neonatal sepsis and septic shock are described: neutropenia, decreased plasticity, and delayed apoptosis [32]. In the case of sepsis or the syndrome of a systemic inflammatory reaction, a large number of immature forms of NG appear in circulation. NGs are characterized by a decrease in phagocytic functional activity, a decreased production of ROS, a defective expression level of CD14 receptors, and a violation of the migratory ability. Immature NGs are characterized by a high basal level of intracellular TNF- α /IL-10 ratio, which confirms their pro-inflammatory phenotype. They have a longer life cycle, are resistant to spontaneous apoptosis, and can mature *ex vivo* [33]. Patients with sepsis (a more severe inflammatory reaction) have a more pronounced decrease in some receptors, in particular TREM-1, which has a key role in amplifying the production of inflammatory cytokines than patients suffering from a noninfectious systemic inflammatory reaction syndrome [34].

NGs affect the adaptive immune response in viral infection [13, 22] through antigen presentation, translocation of pathogenic viruses to the lymph nodes, suppressor modulation of the T-cell response, and expression of Toll-like receptors recognizing the herpesvirus DNA (TLR-9) [35–37]. NGs are important elements of antiviral immunity, realizing their capabilities through the process of phagocytosis, the formation of active forms of oxygen (ROS), the formation of NET, and the ability to synthesize and secrete cytokines, defensins, and interferons [18, 38–40]. Recent studies have shown that on the one hand, NG can perform antiviral protection and on the other hand, many viruses, in particular herpesviruses, can

negatively affect the function of the NG, transform their phenotype, and influence the formation of populations/subpopulations with different functional properties [22]. Herpes viruses block NG antiviral activity, increase NG apoptosis, which leads to neutropenia. Damage to the NG by herpesviruses disrupts their functioning and leads in combination with other factors to disruption of adaptation reactions [13, 22, 36, 41, 42]. In recent years, it has been shown that in chronic herpesviral infection, there are numerous subpopulations of the NG, characterized by different phenotypes with different receptor equipment, possessing different functional properties: the ability to restructure chromatin, express cytokine genes and secrete cytokines, realize the activity of the granular apparatus, produce active oxygen species, and form NET and cytotoxicity.

4.1 CD16⁺CD11b⁺ phenotype of neutrophilic granulocytes in acute viral and acute bacterial infections

The first reports of the heterogeneity of CD16 expression (FcγIII) on NG membrane (induces oxidative burst and phagocytosis) appeared more than 25 years ago [25, 43, 44], but only recently the mechanisms and functional consequences of this heterogeneity have been studied. In particular we have identified different CD16⁺CD11b⁺ NG phenotypes with individual characteristics in patients with acute viral (acute viral Epstein-Barr (EBV) infection) and acute bacterial infectious-inflammatory diseases (acute bacterial tonsillitis) [45]. Summarizing the obtained data, it should be noted that in healthy subjects, CD16^{bright}CD11b^{dim}NG subpopulation was major. The NG of this subpopulation in trace amounts was detected with acute viral infection and was completely absent in acute bacterial infection. In acute viral infection, the number of NGs of a highly equipped subpopulation—CD16^{bright}CD11b^{bright}NG—significantly increased, whereas in acute bacterial infection, there was a significantly lower increase in the number of NGs of this subpopulation. At the same time, in healthy individuals the subpopulation of CD16^{bright}CD11b^{bright}NG was minor. In the case of acute bacterial infection, the CD16^{dim}CD11b^{bright} NG subpopulation, which was absent in conditionally healthy individuals, became dominant, and in the case of acute viral infection, it appeared in an insignificant amount. Subpopulation CD16^{dim}CD11b^{dim} was detected only in healthy individuals (Figure 3).

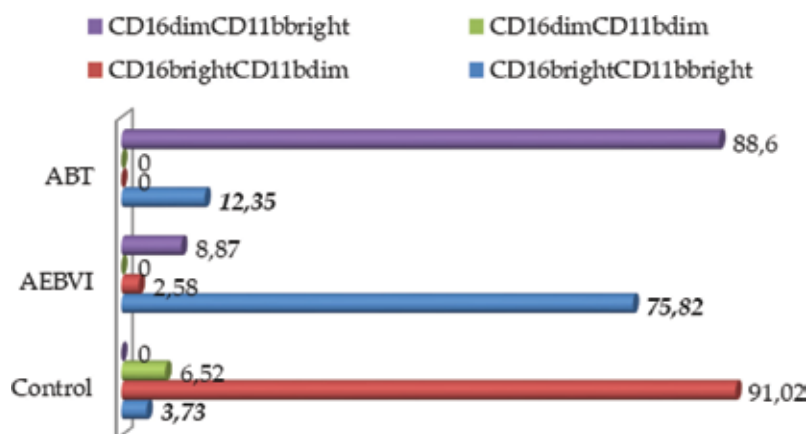


Figure 3. Phenotypic profiles of CD16⁺CD11b⁺NG in acute viral (AEBVI) and acute bacterial (ABT) infections.

Apparently, this is a reserve nonactivated pool of circulating NG, since it is known that in the resting state, the NG is insignificantly equipped with membranes CD16 and CD11b. It should be noted that there are certain difficulties and differences in interpreting the data concerning the reasons for the low equipment of the NG CD16 receptor. Thus, early works of Elghetany M. T. (2002) [6] states that, in inflammation, the expression level of CD16 decreases and explain this phenomenon by shading this receptor; then Elghetany and Lacombe note that surface antigen expression of granulocytes depends on age, sex, race, and the presence of stress [46]. Pillay et al. [24] noted the appearance of a “paradoxical” NG population of low-membrane CD16-CD16^{dim} NG in the experiment with the introduction of LPS in vivo. The authors linked this phenomenon to the release of immature forms of granulocytes in the blood, reinforcing this conclusion with morphological studies: CD16^{dim}NG demonstrates the morphology of “young” band nuclear NG [47]. Thus, it is possible that the appearance of the prevalent major population of CD16^{dim}CD11b^{bright} in the acute bacterial infection of the pharyngeal lymphoid ring in patients in a state of moderate severity or with a severe condition is associated with the release of immature forms of the NG into circulation, which is a stereotype response of the NG in severe bacterial infection. The predominant subpopulation of CD16^{bright}CD11b^{bright}NG in patients with acute viral infection has a high cytotoxic antiviral potential due to the high level of CD16 and CD11b expression. According to Kushner and Cheung [48], the detectable enhanced expression of CD16 on NG in a viral infection may be due to the greater functional significance of cytotoxic NGs expressing FcγRIII (CD16) for the implementation of ADCC associated with CD11b-dependent increase in adhesion and degranulation [14].

The clinical picture of many infectious diseases of viral or bacterial etiology in the early stages of the disease can proceed according to a similar scenario. In this case, when verifying the diagnosis of an acute infectious process of viral or bacterial etiology, there are often certain difficulties that prevent timely proper selection of etiotropic therapy. Conducting an express analysis that allows us to specify dominant NG subpopulation—CD16^{bright}CD11b^{bright} or CD16^{dim}CD11b^{bright} can contribute to the differential diagnosis of acute viral and acute bacterial processes of the lymphogenous ring, which will allow timely optimization of etiotropic therapy. On the other hand, it is possible that the evaluation of these subpopulations of NG can be used for early differential diagnosis of various acute viral and acute bacterial processes of other localizations; however, this requires further study.

Thus, CD16^{bright}CD11b^{dim} NG subpopulations prevail in healthy subjects from 80 and up to 99.9%. In acute viral infection of the lymphogenous ring—infectious mononucleosis associated with EBV—the predominance of the subpopulation CD16^{bright}CD11b^{bright}NG is detected in an amount of 40% or more. In acute bacterial infection of the lymphatic pharynx, a subpopulation CD16^{dim}CD11b^{bright} NG predominates—from 40% and higher. The observed phenomenon of different dynamics of presentation of CD16 and CD11b membrane receptors in the CD16⁺CD11b⁺ NG population in healthy individuals and acute inflammation in the region of the lymphopharynx ring reflects the differentiated response of NG to acute viral and acute bacterial infections.

4.2 Evaluation of the effects of the sodium salt of eukaryotic DNA and ODN2395 on CD16⁺CD11b⁺NG phenotype in patients with acute viral and bacterial infection

The use of drugs to improve the accuracy of exposure to target cells and selectively trigger the type of effector reaction is currently considered topical;

in particular, the use of exogenous oligomers of RNA and DNA, in the process of metabolism of which nucleotides and deoxynucleotides are formed, is promising [49]. Nucleic acid preparations of immunomodulating action of various natures are widely used in practical medicine. Pharmacopoeial preparations are known: sodium nucleate (RNA derived from yeast) [50], sodium deoxyribonucleate (sodium salt of native DNA isolated from sturgeon fish milt) [51], and placentex-integro (DNA from trout milt) [52]. Now, the team of authors [49] showed that the substance of sodium deoxyribonucleate mainly contains short and medium DNA chains ending in the CpG motif. Recognition of CpG motifs by the immune system occurs through their interaction with the Toll-like receptor 9 (TLR9), which acts on the cells as “alarm,” activating innate and acquired immunity and many times enhancing the body’s response even to low-immunogenic antigens [53] with effects of increased proliferation, maturation, and secretion of a number of biologically active molecules—cytokines, costimulatory molecules, molecules of the main histocompatibility complex, etc. [51, 54]. Inflammation is directly related to neutrophilic granulocytes (NGs), which express almost all known TLRs [55], which explains their crucial role in the regulation of phagocytic cells. It should be noted that activation of TLR-4 induces production of pro-inflammatory cytokines and chemokines (IL1 β , IL8, TNF α), TLR-2 activation induces production of chemokine MCP-1, and synchronous activation of TLR-4 and TLR-9 is accompanied by a pronounced respiratory burst a change in the expression of NG adhesion molecules [56, 57]. In the current literature, there are data on the cooperation of TLR9 with the functionally significant receptor of phagocytes—CD11b in the process of recognition of pathogens, even at a low level of exposure to such pathogens [58]. CD11b is also known to both positively and negatively regulate TLR9-mediated mechanisms: control TLR9-triggered NK cell cytotoxicity and macrophage inflammatory responses [59, 60]. On the other hand, it was found that bacterial DNA enhances expression of CD11b genes, while TLR9 expression in NG does not change under the influence of bacterial DNA [61].

Comparative evaluation of the effect of *in vitro* sodium deoxyribonucleate and the TLR9 agonist (ODN2395) on CD16⁺CD11b⁺ subpopulation composition of NG in both healthy individuals and infectious diseases was of interest [62].

In particular, it was shown that when the peripheral blood of conventionally healthy volunteers with sodium deoxyribonucleate is incubated *in vitro*, the density of surface-localized CD11b and CD16 receptors is increased, which is expressed by a significant increase in the content of CD16^{br}CD11b^{br}NG. The effect of the TLR9 agonist (ODN2395) on this subpopulation in patients with AEBVI allowed us to identify a tendency to decrease its relative content, the effects of the TLR9 agonist and sodium deoxyribonucleate did not affect the relative content of CD16^{br}CD11b^{br}NG in patients with ABT (**Figures 4–6**).

The assessment of the content of the CD16^{br}CD11b^{br}NG subpopulation in the incubation of the peripheral blood of patients with acute viral and acute bacterial processes made it possible to reveal a significant immunomodulating effect only in the experiment with sodium deoxyribonucleate. When the blood of patients with AEBVI was incubated, a significant decrease in the initially high relative content of CD16^{br}CD11b^{br}NG was found.

In acute bacterial infection (ABT), there was an increase in the percentage of CD16^{br}CD11b^{dim}NG and a decrease in the initially predominant subpopulation of CD16^{dim}CD11b^{dim}NG (as in incubation with sodium deoxyribonucleate and with the TLR9 agonist), whereas in acute EBV infection, an increase in CD16^{br}CD11b^{dim}NG was observed under the influence of sodium deoxyribonucleate *in vitro* (**Figure 4**).

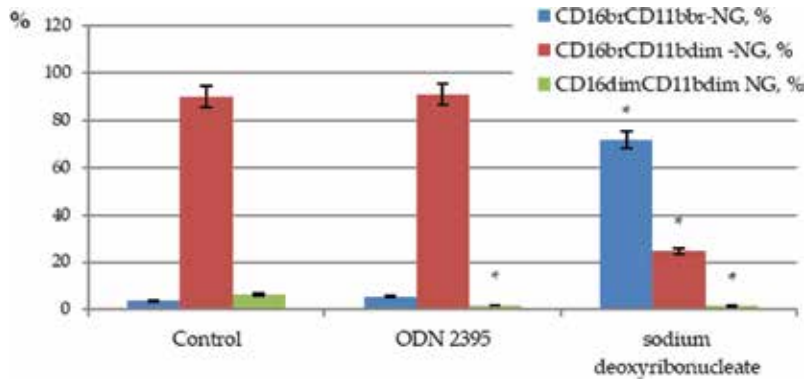


Figure 4. Comparative analysis of the effect of the TLR9 agonist and sodium deoxyribonucleate in vitro on the subpopulation of CD16⁺CD11b⁺NG of healthy volunteers.

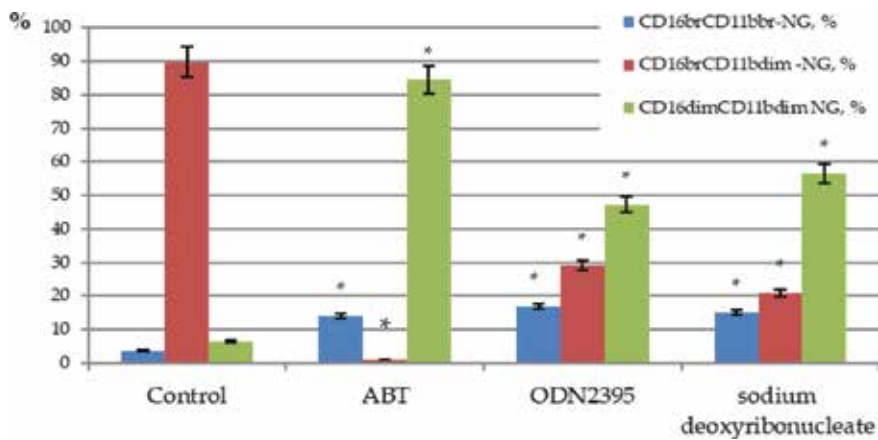


Figure 5. Comparative analysis of the effect of the TLR9 agonist and sodium deoxyribonucleate in vitro on the subpopulation composition of CD16⁺CD11b⁺NG in patients with ABT.

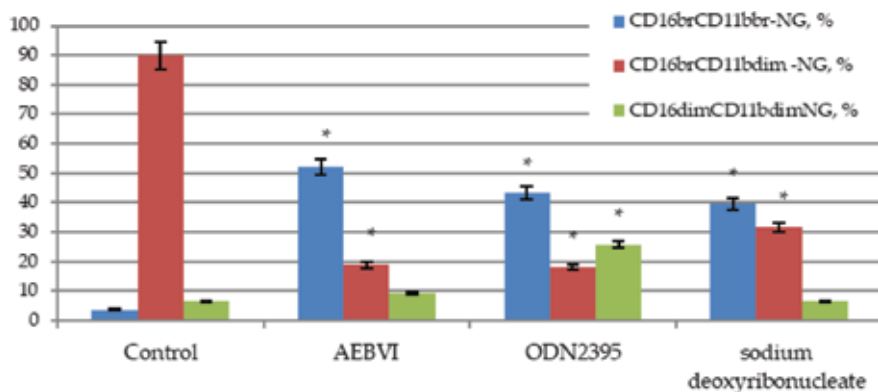


Figure 6. Comparative analysis of the effect of the TLR9 agonist and sodium deoxyribonucleate in vitro on the subpopulation of CD16⁺CD11b⁺NG in patients with acute EBV infection.

It is important to note that the redistribution of NG subpopulation composition occurring under the action of both agonist and, especially, sodium deoxyribonucleate has a modulating nature, which suggests the involvement of Toll-like type 9 receptors in the regulation of functional NG activity in infectious processes.

5. Conclusion

The classical view of the NG, as short-lived finally differentiated cells, which carry out only phagocytosis, killing, and elimination of extracellular pathogens, is convincingly refuted by numerous recent studies. New scientific facts obtained during the last 10–15 years have demonstrated that NGs possess certain regulatory influences of activating, modulating, and suppressive nature, practically on all cells, both innate and adaptive immunity. The development of new diagnostic technologies allowed us to expand and deepen our understanding of the role of the NG in immune homeostasis and to evaluate the dynamic interrelation of the functional potential of the cell with gene expression and phenotypic polarization of the NG in response to inducing signals of intra- and extracellular environment. It is important to note that, to date, not all NG subpopulations have been identified.

Today it is well known that the population of CD16⁺CD11b⁺NG plays an important role in the reactions of phagocytosis and ADCC in infectious processes of various natures. It is also known that CD11b and CD16 NG are the most important triggers inducing the cascade of activation and regulatory processes of the NG. The resting unactivated NGs express the low levels of CD11b and CD16 membrane molecules. After activation additional translocation of intracellular CD16 and CD11b molecules to the NG membrane takes place [6, 36, 63]. Our studies showed that the subpopulation CD16^{bright}CD11b^{dim}NG prevailed in healthy people, and the NG with the phenotype CD16^{bright}CD11b^{bright} was absent in healthy volunteers but appeared and dominated in patients with acute EBV infection. It has been established that CD16^{bright}CD11b^{dim}NG subpopulation predominates in healthy individuals, subpopulation CD16^{bright}CD11b^{bright}NG prevails in patients with acute viral infection, and CD16^{dim}CD11b^{bright}NG subpopulation dominates in patients with acute bacterial infection. Identified by us in acute bacterial infection (acute bacterial tonsillitis), emergence of the prevalent population of CD16^{dim}CD11b^{bright}NG indicated, in our opinion, the release into circulation of immature forms of NG in a bacterial attack. At the same time, CD16^{bright}CD11b^{bright}NG subpopulation predominated in patients with acute viral infection (acute EBV infection). We hypothesized that on the one hand, the appearance of CD16^{bright}CD11b^{bright}NG with high cytotoxicity (high levels of CD16 expression) and with a suppressive effect on T-cell proliferation (high levels of CD11b molecules) is necessary for the implementation of antiviral activity of the NG in their fight against EBV infection. CD16^{bright}CD11b^{bright}NG should have high antiviral activity. On the other hand, their suppressor properties (high levels of CD11b expression) may lead to various complications in the form of secondary bacterial infections. Thus, in severe acute EBV infection, we revealed the transformation of the NG phenotype and the appearance of a new subpopulation of CD16^{bright}CD11b^{bright}NG with high cytotoxicity and suppressive effects. Further studies are needed to determine the functional significance of the CD16^{bright}CD11b^{bright}NG subpopulation for both EBV infection and other herpesvirus infections. In addition, early diagnosis of the etiological factors that cause an acute infectious process of a viral or bacterial nature is extremely important for the appointment of early etiopathogenetic therapy. The results of the present study demonstrate that the determination of various subpopulations of the NG in the early stages of an acute infectious process can contribute to the early differentiation of an acute viral process in which the CD16^{bright}CD11b^{bright}NG subpopulation dominates and the acute bacterial process dominated by the CD16^{dim}CD11b^{bright}NG subpopulation. On the other hand, it is extremely important to search for new substances that have immunomodulatory effects on the “negatively transformed” phenotype of the NG with the possibility of positive remodeling, which can prevent the attachment of serious complications, both in viral and bacterial infections [63].

Our studies of the eukaryotic DNA sodium salt effect on the expression of functionally significant CD16 and CD11b NG receptors in healthy individuals and in infectious diseases of viral and bacterial etiology have demonstrated the potential for transformation of the negatively altered phenotype of the NG, in particular, by remodeling the expression of CD11b on the NG membrane [64]. The obtained data open certain prospects for the development of new therapeutic strategies that allow correcting the negatively transformed phenotype of various subpopulations of defective functioning NG in severe infectious and inflammatory processes, both viral and bacterial etiologies.

Conflict of interest


Authors declare that there is no conflict of interest.

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Essence of Reducing Equivalent Transfer Powering Neutrophil Oxidative Microbicidal Action and Chemiluminescence

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Abstract

Neutrophil leukocytes provide first-line phagocytic defense against infection. Phagocyte locomotion to the site of infection, identification, and phagocytosis of the infecting microbe results in metabolically driven O_2 -dependent combustive microbicidal action. NADPH oxidase activity controls this respiratory burst metabolism. Its flavoenzyme character allows semiquinone-mediated crossover from two reducing equivalents (2RE) to 1RE transfer, as is necessary for univalent reduction of O_2 to the acid hydroperoxyl radical (HO_2) and its conjugate base, superoxide anion (O_2^-). RE transfer dynamics is considered from the perspectives of quantum and particle physics, as well as frontier orbital interactions. Direct disproportionation of HO_2 - O_2^- yields electronically excited singlet molecular oxygen ($^1O_2^*$) and hydrogen peroxide (H_2O_2). Myeloperoxidase catalyzes H_2O_2 -dependent 2RE oxidation of chloride (Cl^-) to hypochlorite (OCl^-). Direct nonenzymatic reaction of OCl^- with an additional H_2O_2 yields Cl^- , H_2O , and $^1O_2^*$. Thus, for two 2RE metabolized through NADPH oxidase, a total of three $^1O_2^*$ are possible. H_2O_2 , OCl^- , and $^1O_2^*$ generated are all singlet multiplicity reactants and can participate in spin-allowed combustive oxygenations yielding light emission, that is, luminescence or chemiluminescence. The sensitivity of luminescence for measuring neutrophil redox activities is increased several orders of magnitude by introducing chemiluminogenic probes. Probes can be selected to differentiate oxidase from haloperoxidase activities.

Keywords: neutrophil, respiratory burst, reducing equivalent, combustion, frontier orbital, spin quantum number, NADPH oxidase, myeloperoxidase, Wigner spin conservation, Hund's maximum multiplicity rule, boson, fermion

1. Introduction

There is a complicated hypothesis, which usually entails an element of mystery and several unnecessary assumptions. This is opposed by a more simple explanation, which contains no unnecessary assumptions. The complicated one is always the popular one at first, but the simpler one, as a rule, eventually is found to be correct. This process

frequently requires 10–20 years. The reason for this long time lag was explained by Max Planck. He remarked that scientists never change their minds, but eventually they die.

John H. Northrop, 1961 [1].

Appreciating why combustion is not spontaneous, how electrons are transferred biologically, and the unusual nature of oxygen reactivity were difficult for me as a student. So, in addition to biochemical studies, my mentor Richard Steele suggested I study the writings of Herzberg and others. Although challenging, such exposure shook open the door to other perspectives. Fundamental quantum and particle physics considerations were entertained with regard to oxygen and biologic electron transfer. My epiphany was in recognizing that the polymorphonuclear neutrophil, a leukocyte familiar to me from clinical laboratory experience, might realize the electronegative potential of oxygen for combustive microbicidal action by changing the spin multiplicity of oxygen. The following, taken from a symposium abstract presented in 1972, succinctly describes that position [2]. “Recently, a chemiluminescence (CL) has been observed when human polymorphonuclear leukocytes (PMN) phagocytize bacteria or particulate matter. The CL response correlates well with the stimulation of the hexose monophosphate shunt, which results in the generation of NADPH. The PMN possesses both CN^- -insensitive NADH and NADPH oxidases. Flavoproteins oxidases of this type are capable of univalent reduction of O_2 . The reduced oxygen ($\cdot\text{O}_2^-$, $\cdot\text{O}_2\text{H}$) may then disproportionate yielding HOOH and singlet molecular oxygen $^1\text{O}_2$. The PMN also possesses a CN^- -sensitive peroxidase, myeloperoxidase, which has microbicidal activity in the presence of HOOH and halide. In this reaction, the HOOH is reduced to OH^- with the oxidation of the halide to a reactive halogonium species. In cases where the halogonium formed is Cl^+ or Br^+ , there is potential for further reaction with HOOH resulting in the generation of a haloperoxy anion. This unstable species can disintegrate to yield the original halide and $^1\text{O}_2$. $^1\text{O}_2$ has been demonstrated to be a potent microbicidal agent. Therefore, the biochemical generation of $^1\text{O}_2$ by the PMN might be closely associated with microbicidal activity. The CL response may be the result of the relaxation of excited carbonyl groups generated via $^1\text{O}_2$ -mediated oxidations.”

Neutrophil leukocytes and monocytes play an essential role in innate phagocytic defense against infection. Immune surveillance mechanisms detect the presence of potentially pathologic microbes and generate the chemical signals that mobilize circulating neutrophils and prime the expression of receptors necessary for neutrophil navigation and phagocytosis. Contact of a primed neutrophil with activated endothelium is followed by neutrophil diapedesis into the tissue interstitial space, and locomotion to the site of infection guided by concentration gradients of complement anaphylatoxin, microbial products, cytokines, and lipid factors. Once an immunologically primed neutrophil contacts an opsonin-labeled pathogen, phagocytosis occurs. Phagocytosis is associated with a constellation of metabolic changes classically referred to as the “respiratory burst” [3]. This presentation focuses on the neutrophil redox mechanisms necessary for microbicidal action, especially the roles of NADPH oxidase and myeloperoxidase (MPO) in lethal microbicidal oxygenations. The Merriam-Webster dictionary defines combustion as a chemical reaction that occurs when oxygen combines with other substances to produce heat and usually light. By changing the spin multiplicity of oxygen from triplet to doublet, and then to singlet, neutrophils remove the spin barrier to direct oxygenation, enabling direct oxygen combustive microbicidal action with associated light emission, that is, chemiluminescence or luminescence [4].

2. Respiratory burst

The neutrophil “respiratory burst” describes the large increases in glucose consumption via the hexose monophosphate shunt (aka, pentose pathway) [5, 6], and in nonmitochondrial molecular oxygen (O_2) consumption [7] associated with phagocytosis, and required for microbicidal action. Appreciating the underlying necessity for such metabolic changes provides perception into oxygen chemistry and biochemistry, radical reactivity and combustion in general. The character of electron transfer mediated by the dehydrogenases of the hexose monophosphate (HMP) shunt is common to cytoplasmic redox reactions. Such oxidation-reduction transfers typically involve movement of two reducing equivalents (2RE), that is, 2 electrons (e^-) and 2 protons (H^+), from an organic substrate catalyzed by a dehydrogenase. In turn, the dehydrogenase mobilizes the 2RE by transfer to nicotinamide adenine dinucleotide (phosphate) $NAD(P)^+$ generating its reduced form $NAD(P)H$. The cofactors NADPH and NADH serve as the cytoplasmic redox carriers for 2RE transfers between dehydrogenases and oxidases, and are common to various pathways of cytoplasmic metabolism. Consumption of 2RE carried by NADPH returns it to $NADP^+$. Availability of $NADP^+$ is rate limiting for HMP shunt dehydrogenase activity. Dehydrogenation is a type of oxidation that does not require or directly involve O_2 . Glucose-6-phosphate (G-6-P) dehydrogenase, the initiator enzyme of the HMP shunt removes a total of 2RE and transfers the 2RE to $NADP^+$ producing NADPH. The point for emphasis is that 2RE are transferred, not one. Such 2RE transfer, sometimes referred to as hydride ion (H^-) transfer, is the rule for cytoplasmic redox reactions [8].

Respiratory burst metabolism results from the activation of NADPH oxidase. Like many oxidases, NADPH oxidase is a flavoenzyme. Flavoenzymes are mechanistically unique in that 2RE reduction, by cofactors such as $NAD(P)H$, is followed by a series of 1RE oxidations. In its 1RE form, the riboflavin prosthetic group of flavin adenine dinucleotide (FAD) is in the semiquinone state [9, 10]. This semiquinone capability, usually in combination with a cytochrome component, allows the oxidase to transition from 2RE transfer to 1RE transfers. As such, flavoenzymes are the junction enzymes where 2RE transfer proceeds as 1RE cytochrome transfers, for example, the mitochondrial electron transport system or the microsomal cytochrome-P450 mixed-function oxidase system [10, 11]. Flavoprotein oxidases are also capable of catalyzing the 1RE reduction of O_2 [12, 13]. As such, phagocytosis-associated activation of NADPH oxidase opens the possibility for univalent, that is, 1RE, reduction of O_2 .

The molecular oxygen we breathe has unique physical-chemical characteristics. In its ground, that is, lowest energy state, oxygen is a diradical, paramagnetic molecule with triplet spin multiplicity [3O_2 ; the preceding superscripted (3) indicates multiplicity]. These spin characteristics guarantee a tendency for 3O_2 to participate in 1RE reduction yielding the doublet multiplicity hydroperoxyl radical (2HO_2) and its conjugate base, the superoxide anion radical ($^2O_2^-$) [2, 4, 14, 15]. Such reduction does not produce radical character; it decreases such character.

2.1 Bosonic character of coupled fermionic electron transfer

Movement of 2RE is the transfer of an electron couple, that is, an orbital pair of electrons. Such 2RE transfers are the rule in cytoplasmic redox reactions. Considered from the perspective of particle physics, movement of a single electron (1RE) is quite different from paired electron (2RE) movement. Transfer of 1RE is a fermionic transfer. An electron is a fermion, and fermions have wave functions that are antisymmetric to exchange of particles; that is, $\Psi(a, b) = -\Psi(b, a)$. Fermions anti-commute; that is, $a \times b \neq b \times a$. Rotating a fermion through 360° ,

$\Psi \rightarrow 360^\circ \rightarrow -\Psi$, changes the phase, but does not return the fermion to its original state. An additional 360° rotation, $-\Psi \rightarrow 360^\circ \rightarrow \Psi$, is required to return the anti-symmetric particle to its original state [16]. Fermions obey Fermi-Dirac statistics.

A fermionic electron is defined by its five quantum numbers: n , l , m_l , s , and m_s [17]. The spin number, s , describes the intrinsic angular momentum of the electron independent of orbital motion, and has a value of $1/2\hbar$ (abbreviated to $1/2$). This quality has no analogy in classical physics. The total spin angular momentum, S , of an atom or molecule is expressed by the equation $S = \sqrt{s(s+1)}\hbar$. s gives rise to the quantum number m_s , and only two values are allowed. When $m_s = 1/2$, the e^- is described as spin up (\uparrow); when $m_s = -1/2$, the e^- is described as spin down (\downarrow). The Pauli exclusion principle states that the total wave function for a system must be antisymmetric to the exchange of any pair of electrons. Differently stated, no two electrons of a given atom or molecule can have identical quantum numbers, and for two electrons to occupy an orbital, each electron must have opposite spins, that is, one orbital e^- must have an $m_s = 1/2$ (\uparrow), the other orbital e^- must have an $m_s = -1/2$ (\downarrow). Consequently, the total spin quantum number, S , for a filled orbital electron-couple is $1/2 + (-1/2) = 0$ ($\uparrow\downarrow$).

Bosons obey Bose-Einstein statistics, and have wave functions that are symmetric to exchange of a pair of particles; that is, $\Psi(a, b) = \Psi(b, a)$. They obey ordinary commutation, that is, $a \times b = b \times a$. Rotating a boson through 360° , $\Psi \rightarrow 360^\circ \rightarrow \Psi$, returns it to its original state. Bosons, for example, photons are symmetric particles with integral spin. Likewise, a spin-balanced composite of fermionic particles, for example, an alpha particle with an S of 0, is bosonic. With regard to biochemical redox reactions, the coupling of antisymmetric fermions, for example, the coupled electrons of an orbital pair, result in a $S = 0$ state with bosonic symmetry. The transfer of 2RE describes the movement of a coupled electron pair with an $S = 0$ and is in essence a bosonic transfer.

2.2 Bosonic versus fermionic frontier orbital interactions

Chemistry is about the frontier orbital interactions of atoms and molecules [18]. The focus of frontier orbital theory is on the initial orbital conditions of the reactants and on reactive transition to product(s) with emphasis on the highest occupied atomic or molecular orbital (HO(A)MO) and the lowest unoccupied atomic or molecular (LU(A)MO) orbital. The frontier orbital of a radical reactant is neither empty nor completely filled, and as such, is described as a singly occupied atomic or molecular orbital (SO(A)MO or SOMO). Atomic and molecular orbitals, including frontier orbitals, can have bosonic or fermionic character [19, 20]. A HO(A)MO has an $S = 0$. Such an atom or molecule has singlet spin multiplicity with nonradical, diamagnetic character. A radical SO(A)MO has an $S = 1/2$ or $-1/2$, and has doublet spin multiplicity with radical, paramagnetic character.

The bosonic character of the HOMO of a nonradical reactant differs fundamentally from the fermionic character of the SOMO of a radical reactant. The fermionic nature of a SOMO limits overlap possibilities with bosonic HOMO. If such reaction occurs, the fermionic character must be preserved in the product. The electronegative Fenton radical (^2OH) can extract 1RE from the HOMO of a singlet multiplicity nonradical substrate ($^1\text{substrate}$) yielding singlet multiplicity $^1\text{H}_2\text{O}$, but in the process the HOMO of the substrate is converted to a SOMO, that is, the substrate becomes a doublet multiplicity radical ($^2\text{substrate}$). The symmetry of the reactants is preserved in the products. If a fermionic (doublet)-bosonic (singlet) reaction occurs, symmetry will be retained in the bosonic (singlet)-fermionic (doublet) products. Consistent with the Wigner-Witmer rules described in **Table 1**, spin symmetry is conserved [19–22].

The fermionic character of two radical reactants is eliminated in reactive bonding yielding a bosonic product. As described in **Table 1**, fermionic radical-radical, SOMO-SOMO reaction yields bosonic nonradical product. Simply stated, radicals

tend to react with radicals, and such doublet-doublet annihilations yield nonradical, that is, bosonic, product. Such reaction is responsible for terminating radical chain propagation reactions.

Molecular oxygen in its ground state has unique triplet spin multiplicity [23]. Its two degenerate, that is, equal energy, frontier orbitals are each populated by a single electron. These two SOMO electrons obey Hund's maximum multiplicity rule, that is, the electron in each degenerate SOMO will have the same spin [24]. As illustrated in **Figure 1**, the *S* value for molecular oxygen is $\frac{1}{2} + \frac{1}{2}$ or $-\frac{1}{2} + -\frac{1}{2}$, and thus, the multiplicity is triplet, that is, $2|1 \text{ or } -1| + 1 = 3$. This bi-radical, bi-fermionic character is responsible for the paramagnetic character of $^3\text{O}_2$. The high electronegativity of $^3\text{O}_2$ predicts potential for highly exergonic reactions with nonradical, singlet multiplicity organic molecules, but thermodynamic potential does not guarantee reactivity, and combustion is not spontaneous. Taking a different perspective, it is

| Reactants | Products |
|--|-------------------------|
| Singlet + Singlet bosonic + bosonic | Singlet bosonic |
| Singlet + Doublet bosonic + fermionic | Doublet fermionic |
| Singlet + Triplet bosonic + bi-fermionic | Triplet bi-fermionic |
| Doublet + Doublet fermionic + fermionic | Singlet bosonic |
| Doublet + Triplet fermionic + bi-fermionic | Doublet fermionic |
| Triplet + Triplet bi-fermionic + bi-fermionic | Singlet bosonic |

Spin multiplicity states with regard to the bosonic-fermionic character of reactants and products.

Table 1.
Spin conservation rules.

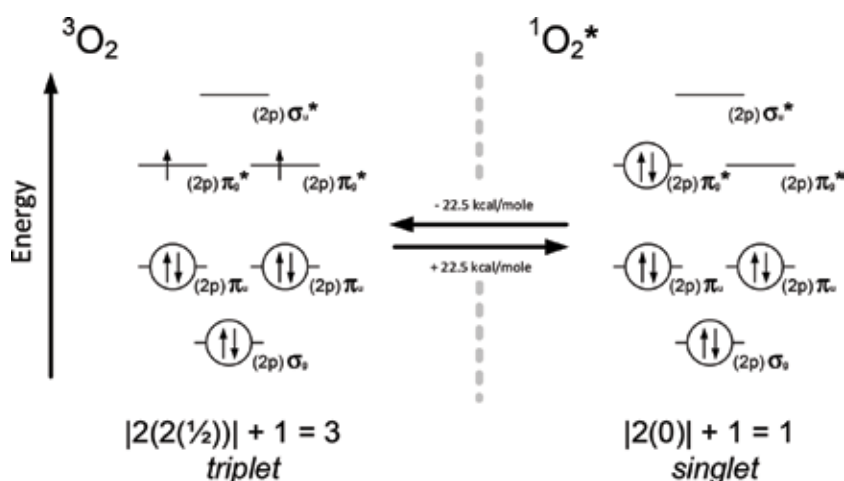


Figure 1.
Triplet and electronically excited singlet molecular oxygen with emphasis on the π^* (pi antibonding) frontier orbitals. The two π^* are degenerate (same energy level). Hund's maximum multiplicity rule predicts lowest energy is achieved when each SOMO electron has the same spin, that is, the triplet state ($^3\text{O}_2$). The electronic energy of $^1\text{O}_2^*$ is 22.5 kcal/mol (94.2 kJ/mol) above $^3\text{O}_2$.

the bi-fermionic, bi-radical nature of $^3\text{O}_2$ that restricts its reactive potential. As per **Table 1**, the reaction of $^3\text{O}_2$ with a bosonic 1 substrate molecules is spin symmetry restricted, and could only result in the improbable generation of a bi-fermionic, triplet multiplicity product(s). However, the reaction of bi-fermionic $^3\text{O}_2$ with a fermionic (doublet multiplicity) radical can proceed via SOMO-SOMO overlap. As per **Table 1**, such a doublet-triplet reaction will generate a fermionic (doublet multiplicity) radical product. Thus, $^3\text{O}_2$ can participate in and be a necessary reactant in radical propagation reactions.

3. NADPH oxidase

NADPH oxidase controls “respiratory burst” metabolism, microbicidal action, and chemiluminescence [15, 25]. The oxidase (Nox2) is a complex flavoenzyme, and a member of the Nox family of enzymes involved in various biochemical activities [26–29]. More specifically, NADPH oxidase is a flavocytochrome enzyme composed of a large membrane-bound glycoprotein (gp91^{phox}) subunit associated with a smaller protein (p22^{phox}). The C-terminal portion of gp91^{phox} subunit contains the NADPH and flavin adenine dinucleotide (FAD) binding sites and an N-terminal portion that binds two heme groups. The activation of the oxidase is complex and involves other components. Association with the p67^{phox} component is essential for full activity. The present treatment will focus on the central role of the semiquinone state of the riboflavin component of FAD and heme involvement in splitting the 2RE from $^1\text{NADPH}$ and facilitating 1RE reduction of $^3\text{O}_2$.

As illustrated in **Figure 2**, the product of 1RE reduction of $^3\text{O}_2$ is the acid hydroperoxyl radical ($^2\text{HO}_2$) with an acid dissociation constant $\text{p}K_a$ of 4.8 [30]. For comparison, the $\text{p}K_a$ of $^1\text{H}_2\text{O}_2$ is 11.7. As the pH of the phagolysosomal space approaches the $\text{p}K_a$, the ratio of $^2\text{HO}_2$ to its conjugate base, the superoxide anion ($^2\text{O}_2^-$) approaches unity, and acid disproportionation, that is, reaction of $^2\text{HO}_2$ with $^2\text{O}_2^-$, approaches maximum reaction rate. At unity, anionic repulsion is no longer a problem. The rate constant for the reaction is $4.5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ at pH 7.0 and reaches a maximum of $8.5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ at pH 4.8 [30, 31]. From the frontier orbital perspective, this is a SOMO-SOMO reaction that yields the nonradical (singlet multiplicity) products $^1\text{H}_2\text{O}_2$ and $^1\text{O}_2^*$. As per **Table 1**, doublet-doublet annihilation yields single products [15, 32]. The reaction is sufficiently exergonic to yield $^1\text{O}_2^*$ with an energy of 22.5 kcal/mol (94.1 kJ/mol) above ground state $^3\text{O}_2$.

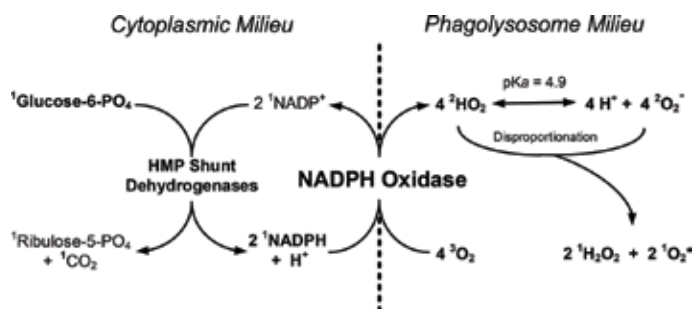


Figure 2. Schema illustrating the central role of membrane-associated NADPH oxidase in respiratory burst metabolism. In the activated state, the Michaelis constant (K_M) of the oxidase for NADPH is decreased. NADP⁺ availability controls the activities of glucose-6- PO_4 dehydrogenase and 6-phosphogluconate dehydrogenase of the HMP shunt. Each pass of the cycle generates two NADPH, that is, two 2RE. In the schema, the spin multiplicities of each molecule are indicated by the superscripted number preceding the molecular description, that is, 1 , 2 , and 3 for singlet, doublet, and triplet multiplicity, respectively.

In **Figure 2**, note that all reactions in the cytoplasmic milieu are singlet multiplicity nonradical reactions and that radical production is confined to the phagolysosomal milieu. The 2RE nature of cytoplasmic redox transfer provides a bosonic barrier to reaction with bi-fermionic $^3\text{O}_2$. Transfer of an orbital electron couple is nonradical, bosonic, and singlet multiplicity. In an atmosphere that is 20.9% $^3\text{O}_2$, the presence of a doublet multiplicity molecule is an opportunity for SOMO-SOMO overlap. The 2RE transfer from the HOMO of a reductant to the LUMO of an oxidant maintains the bosonic $S = 0$ condition.

The $S = 0$ condition is described by Dirac's statement that "If a state has zero total angular momentum, the dynamical system is equally likely to have any orientation, and hence spherical symmetry occurs" [33]. In addition to providing protection from the reactive consequences of fermionic 1RE transfer in an atmosphere high in $^3\text{O}_2$, 2RE transfer of a bosonic orbital electron couple may have additional advantage. Heisenberg's uncertainty principle states that the uncertainty of momentum (Δp) multiplied by the uncertainty of position (Δx) is always equal to or greater than $\frac{1}{2}\hbar$, that is, $\Delta p \Delta x \geq \frac{1}{2}\hbar$ [17]. With regard to 2RE transfer, the bosonic orbital electron couple has $S = 0$. Consequently, the positional uncertainty of the electron-couple must be proportionally large. The $S = 0$ nature of HOMO-LUMO redox transfer involving a 2RE orbital electron-couple opens the possibility that such transfer is facilitated by quantum tunneling. The nature of such transfer would be analogous to the emission of a bosonic $S = 0$ alpha particle from an atomic nucleus in alpha radiation decay [19, 20].

4. Myeloperoxidase

Myeloperoxidase (MPO) is a unique green cationic homo-dimeric glycosylated heme-a protein that is highly expressed in neutrophil leukocytes, making up about 5% of its dry mass [34, 35]. It is also synthesized to a lesser degree in monocytes and serves as a cellular marker for both neutrophils and monocytes. MPO synthesis occurs only during the promyelocyte phase of neutrophil development [36]. During the promyelocyte phase, MPO and other cationic lysosomal proteins are synthesized and stored in the azurophilic (aka primary) granules. Each mitotic division during the following myelocyte phase of development dilutes the azurophilic granule content per neutrophil by a half. Under normal conditions of hematopoietic production, these myelocytic phase mitoses are the rule, but under condition of neutrophil inflammatory consumption or G-CSF-stimulated marrow production, the promyelocyte pool is expanded, and there are fewer mitoses in the myelocyte phase of development. Neutrophils released into the circulation following a few days of myelopoietic stimulation show the effect of decreased myelocyte mitoses. These neutrophils are significantly increased in size due to greater azurophilic granule retention, and the MPO activity per neutrophil is severalfold higher than normal [37].

4.1 Electrochemistry of halide oxidation-reduction

MPO, like eosinophil peroxidase, lactoperoxidase and thyroperoxidase, is a haloperoxidase (XPO). However, MPO is unique in its ability to catalyze the pH-dependent oxidation of chloride [38–40]. Based on the Allen scale, fluorine (F) is the most electronegative element with a value of 4.19, followed by oxygen with a value of 3.61, then chlorine with a value of 2.87, bromine with a value of 2.69, and iodine with a value of 2.36 [41].

With regard to chloride oxidation, the Nernstian electrochemical possibilities and limitations are as follows [11, 42].

$$E = E_o - (RT/nF) \ln [\text{reduced}]/[\text{oxidized}] \quad (1)$$

where E is observed potential (in volts), E_o is the standard potential (in volts), R is the gas constant, T is the absolute temperature, F is a faraday (23 kcal/absolute volt equivalent), and n is the number of electrons/gram equivalent transferred.

Also, appreciate that hydrogen ion concentration, $[H^+]$, has an effect on redox chemistry.

$$E = (RT/F) \ln [H^+]/[P_{H_2}]^{1/2} \quad (2)$$

P_{H_2} is the partial pressure of H_2 gas

$$E = (2.3RT/F) \log [H^+] = 0.06 \log [H^+] = -0.06 \text{ pH} \quad (3)$$

For the reaction, $A_{\text{red}} + B_{\text{ox}} \leftrightarrow B_{\text{red}} + D_{\text{ox}}$, the half reaction equations become:

$$E = E_o^A - (RT/nF) \ln [A_{\text{red}}]/[A_{\text{ox}}] \quad (4)$$

$$E = E_o^B - (RT/nF) \ln [B_{\text{red}}]/[B_{\text{ox}}] \quad (5)$$

$$E_o^B - E_o^A = (RT/nF) [\ln [B_{\text{red}}]/[B_{\text{ox}}] - \ln [A_{\text{red}}]/[A_{\text{ox}}]] \quad (6)$$

$$\Delta E_o = (RT/nF) [\ln [A_{\text{ox}}][B_{\text{red}}]/\ln [A_{\text{red}}][B_{\text{ox}}]] \quad (7)$$

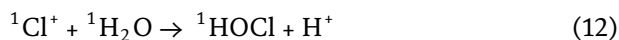
$$\Delta E_o = (RT/nF) \ln K_{\text{eq}} \quad (8)$$

K_{eq} is the equilibrium constant. The change in potential (ΔE) can be expressed in terms of Gibbs free energy (ΔG).

$$\Delta G^0 = -RT \ln K_{\text{eq}} \quad (9)$$

$$\Delta G^0 = -nF \Delta E_o \quad (10)$$

The schema of **Figure 3** depicts the MPO-catalyzed H_2O_2 oxidation of Cl^- to HOCl. Chloride serves as the reductant and undergoes a 2RE oxidization yielding a chloronium intermediate (Cl^+) that reacts with 1H_2O to generate hypochlorous acid with a pKa of 7.5.



Note that 1H_2O_2 is the oxidant for the MPO-catalyzed reaction undergoing 2RE reduction yielding two waters. One 1H_2O is consumed in the reaction described by Eq. (12).



The reactants and products of this MPO-catalyzed redox reaction are exclusively singlet multiplicity, that is, nonradical [2, 15].

As depicted in **Figure 4**, increasing acidity, that is, lowering pH, increases the ΔE (i.e., $E_{\text{H}_2\text{O}_2} - E_{\text{X}^-}$) and the Gibbs free energy for all halides. The exergonicity of MPO-catalyzed 2RE dehydrogenation of Cl^- increases with increasing acidity. The required potentials for the various halides are consistent with their electronegativities. Dehydrogenation of Cl^- is more difficult than Br^- , but dehydrogenation of I^- is relatively easy. Whereas MPO is capable of dehydrogenating Cl^- , Br^- , and I^- , eosinophil peroxidase (EPO), lactoperoxidase, and thyroperoxidase are only capable of dehydrogenating Br^- and I^- .

The plots of **Figure 4** illustrate that increasing acidity increases the exergonicity of MPO-catalyzed ${}^1\text{H}_2\text{O}_2$ -dependent oxidation of halides. This is especially important for MPO-catalyzed oxidation of chloride. Conversely, increasing alkalinity increases the exergonicity of the nonenzymatic ${}^1\text{OCl}^-$ - ${}^1\text{H}_2\text{O}_2$ reaction yielding ${}^1\text{O}_2^*$ as depicted in **Figure 5**. The combined ${}^1\text{H}_2\text{O}_2$ -driven haloperoxidase plus ${}^1\text{H}_2\text{O}_2$ -driven ${}^1\text{OCl}^-$ generation of ${}^1\text{O}_2^*$ can be considered as a net disproportionation reaction, as depicted in **Figure 6**. ${}^1\text{H}_2\text{O}_2$ is the reactant common to both MPO-catalyzed reaction of **Figure 4** and the chemical reaction of **Figure 5**. The Gibbs free energies shown in

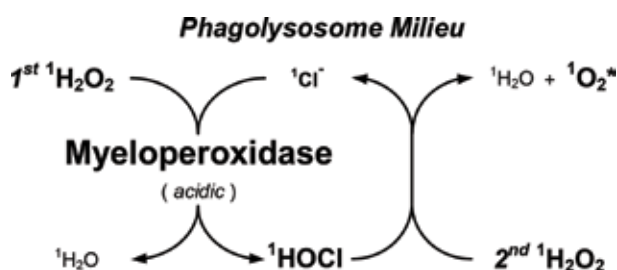


Figure 3. Schema depicting myeloperoxidase-catalyzed H_2O_2 -dependent oxidation of chloride to hypochlorite, and its reaction with a second H_2O_2 to generate ${}^1\text{O}_2^*$. The spin multiplicity of each molecule is indicated by the superscripted number preceding the molecular description.

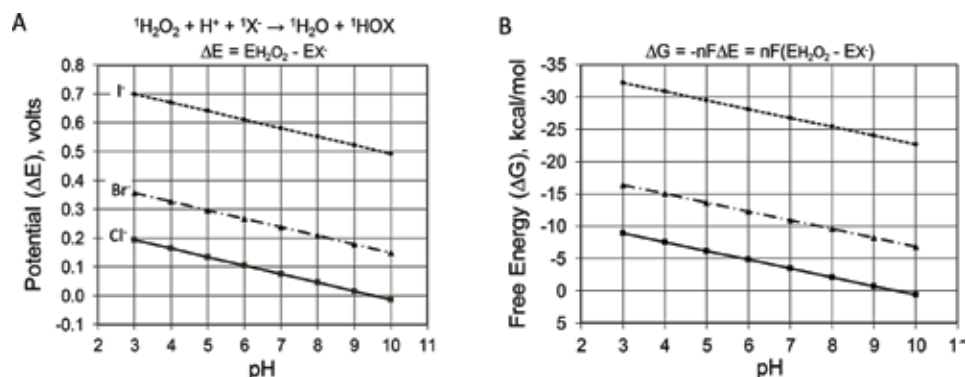


Figure 4. Graph **A** plots changes in potential (ΔE) and graph **B** plots change in Gibbs free energy against pH for various halides. From bottom to top, the plotted lines represent chloride (lowest), bromide (middle) and iodide (highest).

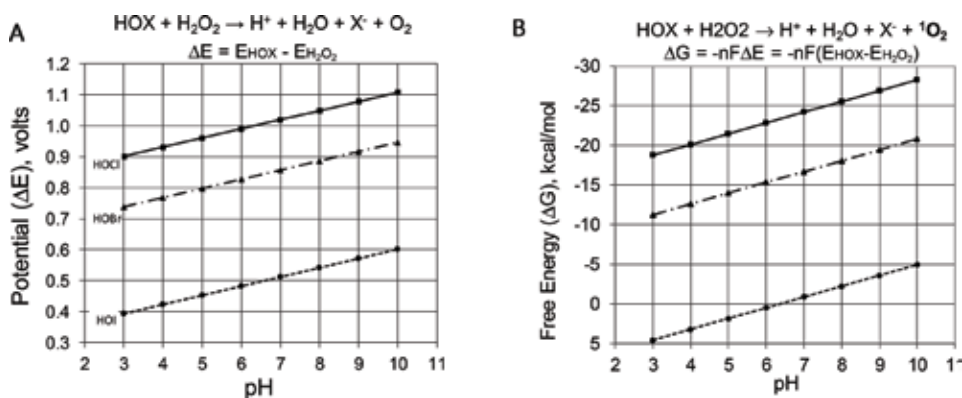


Figure 5.

Graph A plots changes in potential (ΔE) and graph B plots change in Gibbs free energy with respect to pH for various halides for the reaction of ${}^1\text{H}_2\text{O}_2$ with ${}^1\text{OCl}^-$. In graph B the Gibbs free energies are adjusted for the $22.5 \text{ kcal mol}^{-1}$ retained as the electronic energy of ${}^1\text{O}_2^*$, that is, the difference separating ${}^3\text{O}_2$ from ${}^1\text{O}_2^*$.

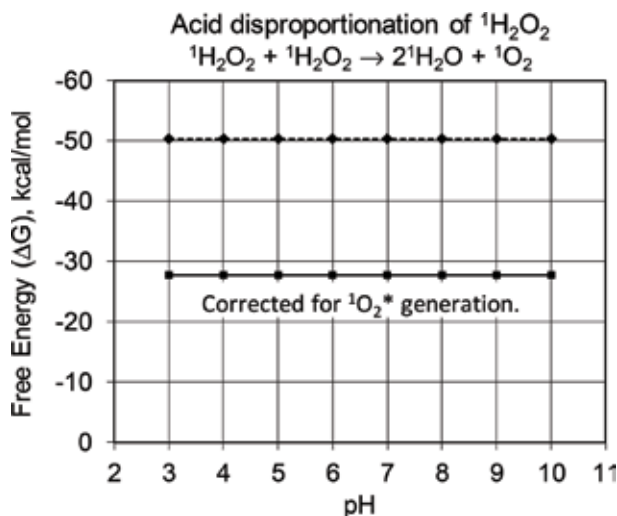


Figure 6.

Plot of free energy against pH for the net ${}^1\text{H}_2\text{O}_2$ disproportionation reaction as described in Figure 2. The free energy results are expressed with ($\Delta G = -27.8 \text{ kcal mol}^{-1}$) and without ($\Delta G = -50.3 \text{ kcal mol}^{-1}$) adjustment for the energy electronically conserved in oxygen excitation ($\Delta G = -22.5 \text{ kcal mol}^{-1}$).

Figure 6 have been adjusted to reflect the energy conserved in electronically excited ${}^1\text{O}_2^*$. The overall net free energy is independent of the halide employed and independent of pH.

Since the reactants involved are all singlet multiplicity, the products of reaction, that is, ${}^1\text{H}_2\text{O}$, ${}^1\text{Cl}^-$, and ${}^1\text{O}_2^*$, are all singlet multiplicity. This provides a spin symmetry explanation as to why pouring bleach (${}^1\text{OCl}^-$) into ${}^1\text{H}_2\text{O}_2$ causes rapid reactive release of ${}^1\text{O}_2^*$ gas and a red chemiluminescence [23]. Caution, rapid release of gas is potentially explosive. When the concentration of ${}^1\text{O}_2^*$ is sufficiently high, ${}^1\text{O}_2^* - {}^1\text{O}_2^*$ collision with simultaneous relaxation yields red chemiluminescence. The relaxation of one ${}^1\text{O}_2^*$ emits a 1270 nm photon; simultaneous relaxation of two ${}^1\text{O}_2^*$ emits a 635 nm photon. As such, this red emission is second order with respect to ${}^1\text{O}_2^*$, that is, $dh\nu_{635\text{nm}}/dt = k[{}^1\text{O}_2^*]^2$, and is relatively short-lived.

The double dehydrogenation of $^1\text{glucose-6-PO}_4$ produces $^1\text{ribulose-5-PO}_4$ plus $^1\text{CO}_2$ plus two 2RE, that is, two bosonic electron couples carried as 2NADPH. As illustrated in **Figure 2**, NADPH oxidase reduces four $^3\text{O}_2$ in four one 1RE reduction steps, ultimately yielding two $^1\text{O}_2^*$ and two $^1\text{H}_2\text{O}_2$. As illustrated in **Figure 3**, MPO uses one $^1\text{H}_2\text{O}_2$ for oxidation of Cl^- to OCL^- , and this OCL^- reacts with the other $^1\text{H}_2\text{O}_2$ to generate an additional $^1\text{O}_2^*$. Thus, two NADPH have the potential to drive the generation of three $^1\text{O}_2^*$. Steinbeck et al. have reported experiments using glass beads coated with 9,10-diphenylanthracene, a $^1\text{O}_2^*$ -specific trap, for measurements of neutrophil $^1\text{O}_2^*$ production [43]. Neutrophils were allowed to phagocytose the beads for an hour. The endoperoxide trapped indicated that at least $11.3 \pm 4.9 \text{ nmol } ^1\text{O}_2^*/1.25 \times 10^6$ neutrophils were produced. When the neutrophils were chemically activated with phorbol-12-myristate-13-acetate (PMA), at least $14.1 \pm 4.1 \text{ nmol } ^1\text{O}_2^*/1.25 \times 10^6$ neutrophils were produced. Based on their trapping results, $^1\text{O}_2^*$ production accounted for at least $19 \pm 5\%$ of the total oxygen consumed. Although the quantities of $^1\text{O}_2^*$ measured using this difficult trapping approach are lower than expected; this study provides direct empirical evidence of significant neutrophil $^1\text{O}_2^*$ production.

Quantifying cellular production of $^1\text{O}_2^*$ by measuring the 1270 nm near-infrared photon emitted on $^1\text{O}_2^*$ relaxation to $^3\text{O}_2$ is also problematic. Although highly specific for $^1\text{O}_2^*$, this infrared proton emission approach is highly insensitive in biological system measurements. The fact that a 1270 nm photon is measured is proof that $^1\text{O}_2^*$ did not participate in chemical reaction. Considering the variety of reactive substrates available in biological milieu, electrophilic reaction is favored over relaxation.

4.2 Myeloperoxidase-binding specificity focuses combustive activity

$^1\text{O}_2^*$ is a potent electrophilic reactant with a high probability for participation in spin-allowed reaction with electron-dense biological substrates. The lifetime of metastable electronically excited $^1\text{O}_2^*$ restricts its reactive possibilities [44]. In biological milieu, $^1\text{O}_2^*$ has a reactive lifetime of about 4–6 microseconds [45, 46]. This lifetime restricts reactivity to within a radius of about 0.2–0.3 μm (microns) from its point of generation. In the case of MPO generation of $^1\text{O}_2^*$, these temporal and spatial restrictions can be advantageous.

MPO selectively binds to all gram-negative bacteria and most gram-positive bacteria tested, but MPO binding is weak for gram-positive lactic acid bacteria (LAB) [44, 47]. LAB are common members of the normal flora of the mouth, vagina, and colon, and include streptococci, lactobacilli, and bifidobacteria. These LAB cannot synthesize cytochromes and produce lactic acid as a metabolic end product. They are typically microaerophilic, and often produce $^1\text{H}_2\text{O}_2$ as a metabolic product. The green hemolysis associated with colonies of viridans streptococci on blood agar plates results from the production of $^1\text{H}_2\text{O}_2$ by the streptococci. When a pathogen, such as *Staphylococcus aureus* or *Escherichia coli*, is contacted with a nonpathogen LAB, such as *Streptococcus viridans*, the pathogen overwhelmingly inhibits the LAB, but when a small quantity of MPO is added to a mixture, the pathogen is inhibited allowing LAB dominance. This phenomenon repeats even when erythrocytes are added to the mix at a ratio of 10 erythrocytes per bacteria. MPO selectively binds to the *S. aureus* and *E. coli* with essentially no binding to $^1\text{H}_2\text{O}_2$ -producing *Strep. viridans*. Thus, LAB-produced $^1\text{H}_2\text{O}_2$ drives MPO microbicidal action that is restricted to the surface of the MPO-bound pathogen. MPO combustive microbicidal action is focused on the pathogen with minimum damage to the $^1\text{H}_2\text{O}_2$ -producing LAB, and without hemolytic damage to the added erythrocytes, that is, no bystander injury.

Specificity of MPO binding results in specificity of microbicidal action. Binding specificity allows synergistic MPO-LAB interaction and suppression of pathogens. It also suggests a role for MPO in the selection and maintenance of LAB in the normal flora [48]. Healthy human adults release about a hundred billion MPO-rich neutrophils into the circulating blood each day. The circulating lifetime of the neutrophil is reportedly less than a day. The neutrophils then leave the blood and enter a tissue and body cavity phase lasting a few days [36]. Migration of MPO-rich neutrophils into the mouth and vagina is well-known [49, 50]. When quantified, the neutrophil count of the mouth is proportional to the blood neutrophil count. These spaces typically provide an acidic milieu. Neutrophil disintegration with MPO release may provide LAB with a selective advantage in such body spaces.

5. Microbicidal combustion and chemiluminescence

Reactions of $^1\text{O}_2^*$ with singlet multiplicity substrates (^1Sub) are spin-allowed and highly exergonic. The exergonicities of most biochemical reactions are sufficient for rotational and vibrational excitation, but not electronic excitation. Dioxygenation reactions are sufficiently exergonic for electronic excitation. Oxygenations producing singlet multiplicity endoperoxide and dioxetane intermediates are excellent candidates for luminescence [51]. The disintegrations of such intermediates generate $n\pi^*$ electronically excited products, that is, an electron from the nonbonding (n) orbital of oxygen populates the pi antibonding (π^*) orbital of the carbonyl. Singlet multiplicity $n\pi^*$ excited molecules have short lifetimes. Electronic transition from the π^* of the carbonyl to the n of oxygen with photon emission is spin-allowed.

In addition to direct reaction of $^1\text{O}_2^*$ with ^1Sub , other singlet multiplicity reactants such as $^1\text{OCl}^-$ can react with ^1Sub to yield chloramine products ($^1\text{Sub-Cl}$) or dehydrogenated products ($^1\text{Sub}_{-2\text{RE}}$). Such products can in turn react with $^1\text{H}_2\text{O}_2$ yielding endoperoxide or dioxetane intermediates with subsequent disintegration to $n\pi^*$ -excited carbonyl products relaxing by photon emission [52, 53]. The fundamental principle is that all reactants and products are singlet multiplicity nonradicals.

Dioxygenations yielding intermediate endoperoxide and dioxetanes disintegrate yielding an $n\pi^*$ electronically excited carbonyl. **Figure 7** illustrates the energy and orbital differences that characterize the carbonyl states. Physical generation of a $n\pi^*$ electronically excited carbonyl occurs when a fluorescent compound in its ground state absorbs a photon of appropriate energy. Because the ground state of the carbonyl is singlet, an electronically excited singlet multiplicity carbonyl undergoes rapid spin-allowed relaxation to ground state with a lifetime of less than 10^{-8} second [51]. Fluorescence describes photon-generated excitation followed by photon emission. Chemiluminescence or luminescence describes chemically generated electronic excitation followed by photon emission.

The metabolic changes of the respiratory burst describe the movement of RE required to change the spin multiplicity of $^3\text{O}_2$ from triplet to doublet ($^2\text{HO}_2$), and ultimately to singlet, that is, $^1\text{H}_2\text{O}_2$ and $^1\text{O}_2^*$. MPO catalyzes the 2RE oxidation of $^1\text{Cl}^-$ to $^1\text{HOCl}$ followed by chemical reaction with a second $^1\text{H}_2\text{O}_2$ to generate $^1\text{O}_2^*$. Changing the bi-fermionic $^3\text{O}_2$ to bosonic $^1\text{O}_2^*$ eliminates the spin barrier to direct dioxygenation of bosonic singlet multiplicity biological molecules. If intermediate endoperoxides and dioxetanes are generated, their disintegration yields electronically excited $n\pi^*$ carbonyl functions that relax by photon emission. By changing the spin multiplicity of oxygen, neutrophil leukocytes realize its electronegative potential for combustive microbicidal action. Such combustion generates electronically excited products emitting light in the visible range of the spectrum.

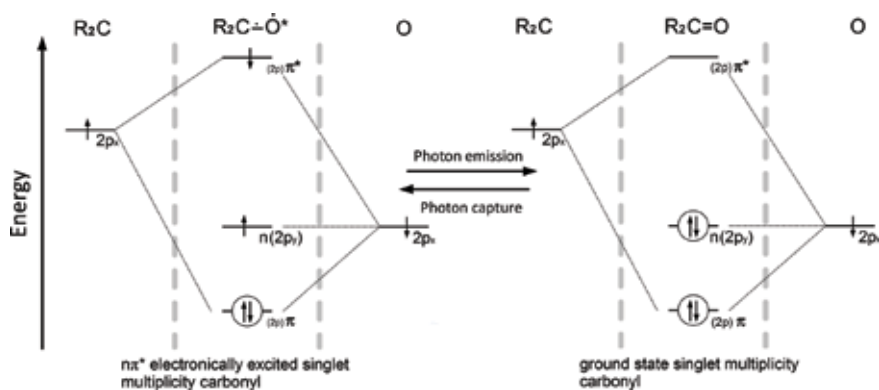


Figure 7. Orbital diagram plot depicting the $n\pi^*$ electronically excited singlet state and the singlet ground state of a carbonyl. The gray dashed brackets indicate the carbonyl with the participating carbon and oxygen atoms shown on to the left and right, respectively. In the carbonyl diagram on the left, the $n\pi^*$ notation indicates that an electron of the nonbonding (n) orbital of the carbonyl oxygen atom has been excited to the π antibonding (π^*) orbital of the carbonyl. Although excited, the electrons remained paired and the excited state is singlet multiplicity. Electron relaxation from π^* -to- n yields photon emission.

6. Chemiluminogenic probes

The native chemiluminescence of neutrophils is proportional to respiratory burst activity [4, 54]. Since the luminescence resulting from microbicidal combustion is proportional to dioxygenations, especially those yielding endoperoxide and dioxetane intermediates, it follows that native neutrophil luminescence is influenced by the molecular composition of the microbe combusted. Native luminescence from phagocytosing neutrophils can be detected using less than a million neutrophils. For perspective, a milliliter of normal human blood contains about 4 million neutrophils. The native luminescence product of neutrophil combustive action is of low intensity. However, electronic excitation and the resultant luminescence is unambiguous evidence of neutrophil combustive dioxygenation action. Native luminescence has been usefully applied to measurement of neutrophil metabolic defects, e.g., chronic granulomatous disease [54, 55], and neutrophil responsiveness to humoral immune factors, such as complement and immunoglobulins [56].

Inclusion of high quantum yield chemiluminogenic substrates as probes (CLP) of neutrophil dioxygenation activities greatly increases the sensitivity and, to some degree, the specificity for detecting such activities [52, 57, 58]. With regard to increasing sensitivity, a CLP must be susceptible to neutrophil dioxygenation activities. This is achieved when endoperoxide or dioxetane intermediate are produced. The breakdown of such intermediates yields electronically excited $n\pi^*$ carbonyl functions that relax by light emission. Use of a CLP typically increases the sensitivity for detecting dioxygenation activity by several orders of magnitude. Selecting a CLP with reactive specificity also provides information with regard to the nature of neutrophil activity measured.

6.1 Probing reductive oxygenation activity with lucigenin

Phagocytic or chemical activation of neutrophil respiratory burst metabolism can be tested using the dye nitro-blue tetrazolium (NBT) [59]. The NBT reaction measures neutrophil reduction activity, not neutrophil oxidation activity. A positive NBT result requires neutrophil respiratory burst activity resulting in reduction of

the tetrazolium ring of the dye to a dark blue water-insoluble formazan precipitate. NBT is a large complex nitrogen heterocyclic compound with abundant resonance and electron delocalization possibilities. That NBT reduction might be linked to neutrophil univalent reduction of molecular oxygen was considered, and we observed that adding a small grain of potassium superoxide (KO_2) to a solution of NBT resulted in immediate reduction of the dye to a dark blue formazan precipitate [15]. Normal neutrophils reduce NBT upon activation of NADPH oxidase. The neutrophils of chronic granulomatous disease patients have defective NADPH oxidase, and as such, are incapable of NBT reduction [60].

Lucigenin (aka, bis-*N*-methylacridinium nitrate, or dimethyl biacridinium nitrate ($^1\text{DBA}^{+2}$)) is a heterocyclic organic compound known to generate chemiluminescence as a product of base-catalyzed peroxidation [61]. If sufficiently alkaline, singlet multiplicity $^1\text{lucigenin}$ reacts with the conjugate base of peroxide ($^1\text{HO}_2^-$) producing a dioxetane ($^1\text{lucigenin-dioxetane}$) intermediate that disintegrates to a $n\pi^*$ -excited carbonyl function that relaxes to ground state by π^* -to- n transition with photon emission. The $\text{p}K_a$ of $^1\text{H}_2\text{O}_2$ is 11.7. As previously considered, $^1\text{H}_2\text{O}_2$ is the sum product of two RE reductions of $^3\text{O}_2$. Consequently, lucigenin chemiluminescence is the product of reductive dioxygenation. Both lucigenin and peroxide are singlet multiplicity reactants. Spin restriction is not a problem. Alkalinity favors the formation of $^1\text{HO}_2^-$ and dioxygenation yielding a dioxetane.

Lucigenin is a heterocyclic compound with resonance and electron delocalization possibilities, and can undergo one RE reduction yielding a doublet multiplicity product ($^2\text{lucigenin}_{+RE}$). Such reduction may involve $^2\text{O}_2^-$ or some other 1RE reductant. The product radical, $^2\text{lucigenin}_{+RE}$, can now react with $^2\text{O}_2^-$ by SOMO-SOMO overlap, that is, a doublet-doublet annihilation, producing a singlet multiplicity product, the $^1\text{lucigenin-dioxetane}$ intermediate. As depicted in **Figure 8**, the disintegration of this unstable dioxetane yields chemiluminescence [52, 58, 62, 63].

Reduction of lucigenin by 2RE, that is, by a bosonic orbital electron couple, maintains singlet multiplicity. Such a reduced $^1\text{lucigenin}_{+2RE}$ can react with $^1\text{O}_2^*$, but not $^3\text{O}_2$, to produce chemiluminescence [64]. As shown in **Figure 8**, the state of lucigenin reduction determines the deoxygenating agent required. All reactions shown satisfy the spin conservation rules.

The radical product of 1RE reduction of lucigenin, $^2\text{lucigenin}_{+RE}$, can react with the radical product of NADPH oxidase, $^2\text{O}_2^-$, resulting in intermediate dioxetane formation with breakdown to a $n\pi^*$ electronically excited carbonyl with relaxation by light emission, and as such, lucigenin can be applied as a chemiluminogenic probe for measurement of NADPH oxidase activity [52, 58, 63]. MPO haloperoxidase activity does not yield lucigenin-luminescence.

Chicken blood phagocytes, that is, heterophil leukocytes, have oxidase activity, but are deficient in haloperoxidase. Chemical or phagocytic stimulation of these heterophil leukocytes results in lucigenin-dependent luminescence responses comparable to those observed from human neutrophils under similar test conditions and using similar stimuli [58, 65]. However, the luminol-dependent luminescence responses of MPO-deficient chicken heterophils are a hundredfold lower than those observed from MPO-rich human neutrophils. In addition, azide (N_3^-), a known inhibitor of MPO, inhibits the luminol-dependent luminescence responses of MPO-rich human neutrophil. Azide shows no inhibitory action against the luminol or the lucigenin luminescence responses of MPO-deficient chicken heterophils [66]. These chicken heterophil results plus the previously described macrophage results [57] experimentally support the position that luminol provides a very sensitive measure of MPO activity. However, the weaker luminol-luminescence measured is evidence for haloperoxidase-independent oxidase activity.

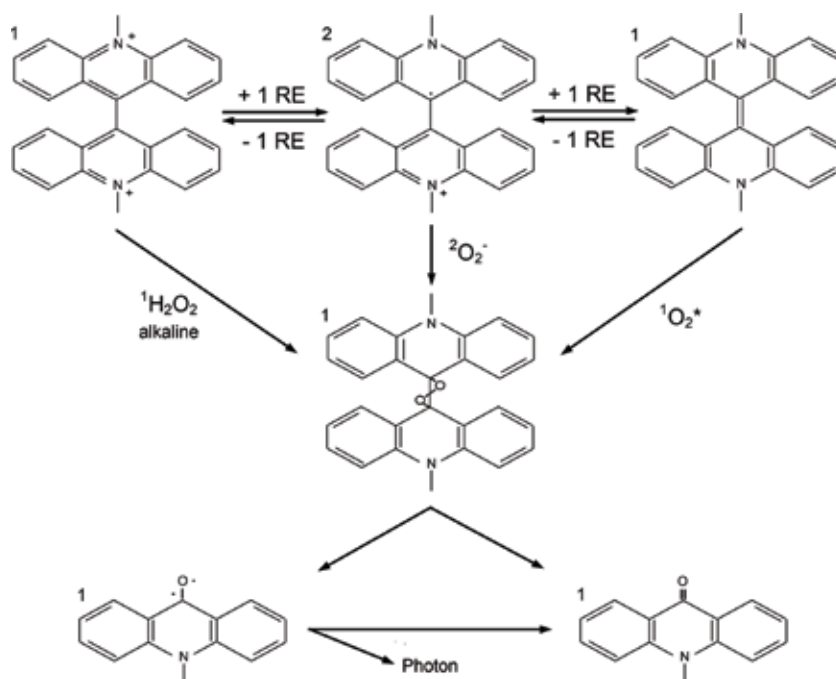


Figure 8. Oxygenating reactions yielding lucigenin chemiluminescence. Spin multiplicity is shown by the superscript value that precedes the reactant, and 1RE indicates one reducing equivalent.

6.2 Probing oxygenation activities with cyclic hydrazides

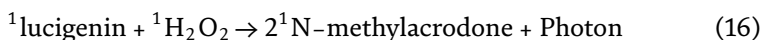
Luminol chemiluminescence is a well-established phenomenon, but the mechanisms responsible for luminol-luminescence are diverse [67]. Luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) is a nonradical, cyclic hydrazide [68]. Luminol dioxygenation is thought to involve an intermediate endoperoxide with disintegration yielding the $n\pi^*$ electronically excited aminophthalate that relaxes by photon emission. Albrecht first described the blood-catalyzed luminol-luminescence [69]. Like lucigenin, alkalinity and $^1\text{H}_2\text{O}_2$ are required, but luminol-luminescence has an additional requirement for a catalyst, for example, blood or peroxidase. To appreciate how these CLS differ, compare, and contrast the net reactions responsible for luminol-luminescence and lucigenin-luminescence. Luminol-luminescence is a dioxygenation:



The reaction of $^1\text{luminol}$ with $^3\text{O}_2$ (Eq. (14)) is not spin allowed, but reaction with $^1\text{O}_2^*$ (Eq. (15)) is spin allowed producing $n\pi^*$ electronically excited $^1\text{aminophthalate}^*$ plus $^1\text{N}_2$, and ultimately, ground state $^1\text{aminophthalate}$ plus a photon.



Lucigenin-luminescence is a reductive dioxygenation.

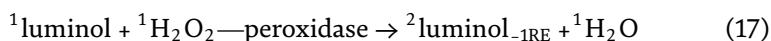


As per Eq. (16), lucigenin-luminescence requires the spin-allowed reactive addition of molecular oxygen plus 2RE, that is, $^1\text{H}_2\text{O}_2$. The product of this reductive

dioxygenation is a dioxetane intermediate that breaks down to one ground state ^1N -methylacridone and one $n\pi^*$ electronically excited ^1N -methylacridone*. Relaxation of the ^1N -methylacridone* yields a photon.

Luminol dioxygenation is not reductive. The net dioxygenation incorporates molecular oxygen to produce an endoperoxide intermediate with the breakdown release of $^1\text{N}_2$ and formation of a $n\pi^*$ electronically excited aminophthalate. As indicated by Eq. (14), $^1\text{luminol}$ does not react with ground state oxygen. Spin conservation and frontier orbital overlap problems restrict such direct reaction. As illustrated in **Figure 1**, the frontier orbitals of $^3\text{O}_2$ are its two degenerate π^* SOMOs. Hund's maximum multiplicity rule is satisfied when the electrons of each SOMO have the same spin. Each of the two π^* orbitals of $^3\text{O}_2$ have fermionic character that restricts overlap with the bosonic frontier orbitals of luminol. By contrast, the frontier π^* orbitals of $^1\text{O}_2^*$ are bosonic and include one LUMO π^* orbital and one HOMO π^* orbital. Overlap of the LUMO of $^1\text{O}_2^*$ with the HOMO of $^1\text{luminol}$ satisfies the symmetry requirements for reaction.

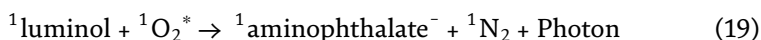
There are three mechanistic possibilities for $^1\text{luminol}$ reactions yielding luminescence. The fermionic (doublet multiplicity/radical) pathway requires two steps as illustrated by Eqs. (17) and (18).



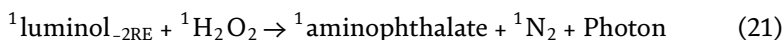
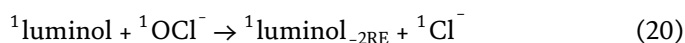
The radical $^2\text{luminol}_{-1\text{RE}}$ can participate in SOMO-SOMO reaction with superoxide ($^2\text{O}_2^-$) yielding singlet multiplicity electronically excited aminophthalate ($^1\text{aminophthalate}^*$) that relaxes with photon emission.



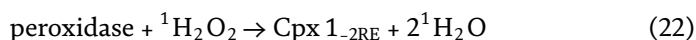
The bosonic (singlet multiplicity/nonradical) pathway can occur by a single reaction as illustrated by Eq. (19),



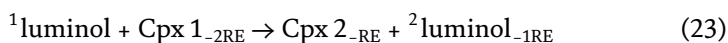
The bosonic (singlet multiplicity/nonradical) pathway can also occur by a two-step reaction as illustrated by Eqs. (20) and (21).



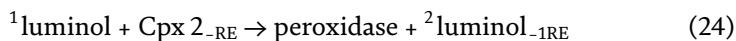
Although luminol is versatile with regard to reactive mechanism, dioxygenation is ultimately required for chemiluminescence. In an alkaline milieu, classical peroxidase or hemoglobin can catalyze $^1\text{H}_2\text{O}_2$ -dependent luminol-luminescence. The peroxidase-catalyzed mechanism of luminol-luminescence described by Dure and Cormier illustrates the kinetics of the fermionic pathway [70]. For such reaction, a classical peroxidase is first oxidized by $^1\text{H}_2\text{O}_2$, that is, 2RE are transferred to $^1\text{H}_2\text{O}_2$ producing two $^1\text{H}_2\text{O}$ as described in Eq. (22).



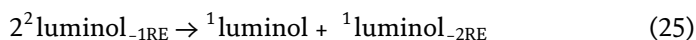
This 2RE oxidized peroxidase, referred to as complex 1 (Cpx 1), can now readily oxidize $^1\text{luminol}$ by removing 1RE producing $^2\text{luminol}_{-1\text{RE}}$, as per Eq. (23).



The reaction of complex 2 (Cpx 2_{-2RE}) with another ¹luminol is slow and rate limiting with regard to luminescence, but this reaction is necessary for regeneration of the starting peroxidase, as per Eq. (24).



Disproportionation of the two radical ²luminol_{-1RE} can proceed as a spin allowed SOMO-SOMO reaction, that is, a doublet-doublet annihilation, yielding the nonradical ¹luminol (starting reactant) and nonradical 2RE-oxidized luminol (¹luminol_{-2RE}).



As per Eq. (21), the spin-allowed reaction of ¹luminol_{-2RE} with ¹H₂O₂ yields electronically excited aminophthalate (¹aminophthalate*) that relaxes by photon emission.

Metalloenzymes and cytochromes are suited to 1RE transfers and under proper reaction conditions can catalyze the 1RE oxidation of a ¹substrate producing ²substrate_{-1RE}. The ¹H₂O₂-dependent oxidation of peroxidase to Cpx 1_{-2RE} allows it to catalyze the initial fermionic 1RE oxidation of luminol in an alkaline milieu. Hemoglobin has peroxidase activity under alkaline conditions, thus explaining the sensitivity of luminol-luminescence for detecting the presence of blood erythrocytes by alkaline peroxide methods. Luminol-luminescence by the classical plant peroxidase-catalyzed reactions of Eqs. (22)–(25) is sensitive to pH, decreasing with increasing acidity. Acidification of the reaction milieu to a pH of about 5 ± 1 effectively eliminates classical peroxidase-catalyzed luminol luminescence. This is quantitatively demonstrated in the Michaelis-Menten enzyme kinetic analyses of luminol-luminescence for myeloperoxidase and horse radish peroxidase presented in **Table 2** [71].

Alkaline pH favors the fermionic luminol-luminescence reactions catalyzed by plant peroxidase, hemoglobin, and heavy metals. The pKa of ¹H₂O₂ is 11.75. The ferri-cyanide-catalyzed luminol luminescence reaction is most efficient in the pH range from 10.4 to 10.8 [72]. In **Table 2**, note that no significant luminescence is observed from HRP-catalyzed luminol reaction at pH 4.9. The maximum luminescence velocity (*V*_{max}) values are low and standard errors (SE) are high. However, a relatively weak but significant luminescence is observed at pH 7.0, that is, Michaelis-Menten analysis of the HRP luminescence shows a low *V*_{max}, but an acceptable SE.

Of special note, Michaelis-Menten kinetic analysis indicates that the HRP-catalyzed luminol-luminescence velocity is first order with respect to H₂O₂ concentration, but second order with respect to luminol concentration, that is, the luminescence velocity is directly proportional to the square of the luminol concentration. These results are consistent with those reported by Dure and Cormier [70], and with the fermionic radical reactive pathway described in Eqs. (22)–(25) and Eq. (21).

Although luminol solubility becomes a problem at low pH, acidity favors the bosonic haloperoxidase luminol-luminescence catalyzed by MPO. Note that bosonic, haloperoxidase-catalyzed luminol luminescence is first order with respect to luminol, chloride, or bromide, but second order with respect to H₂O₂, that is, luminescence activity is proportional to the square of the H₂O₂ concentration.

The MPO-catalyzed luminol-luminescence kinetic finding is the opposite of those observed for HRP-catalyzed luminol-luminescence, and are consistent with the bosonic reactive pathway for luminol-luminescence via ¹O₂* reaction described

| | Substrate [S], variable (conc. range) | pH | Substrates, constant | | | Michaelis-Menten kinetics | | | |
|---|---|-----|------------------------------------|-------------------------|----------------------------|---------------------------|----------------------------------|--------------|------------|
| | | | H ₂ O ₂ , mM | Cl ⁻ , mEq/L | Br ⁻ , mEq/L | Luminol, μM | M-M equation | Km ± SE | Vmax ± SE |
| Haloperoxidase: | H ₂ O ₂ (0.01–1.4 mM) | 5.0 | variable | 90 | 0 | 77 | $v = V_{max}[S]^2/(K_m + [S])^2$ | 2.82 ± 0.05 | 3900 ± 1 |
| | Myeloperoxidase | 5.0 | variable | 0 | 4.5 | 77 | $v = V_{max}[S]^2/(K_m + [S])^2$ | 0.58 ± 0.03 | 2932 ± 2 |
| | Cl ⁻ (0.2–7.7 mEq/L) | 5.0 | 2.27 | variable | 0 | 45 | $v = V_{max}[S]/K_m + [S]$ | 7.60 ± 2.60 | 1105 ± 253 |
| | Br ⁻ (14–882 μEq/L) | 5.0 | 2.27 | 0 | variable | 45 | $v = V_{max}[S]/K_m + [S]$ | 0.68 ± 0.05 | 2280 ± 96 |
| | Luminol (0.0018–15 μM) | 4.9 | 2.27 | 90 | 0 | variable | $v = V_{max}[S]/K_m + [S]$ | 8.80 ± 0.77 | 1490 ± 70 |
| | Luminol (0.0018–0.47 μM) | 7.0 | 2.27 | 90 | 0 | variable | $v = V_{max}[S]/K_m + [S]$ | 0.10 ± 0.02 | 3252 ± 219 |
| Classical Peroxidase: Horse Radish Peroxidase | H ₂ O ₂ (0.01–1.4 mM) | 5.0 | variable | 90 | 0 | 77 | $v = V_{max}[S]/K_m + [S]$ | 31.02 ± 0.05 | 280 ± 55 |
| | H ₂ O ₂ (0.01–1.4 mM) | 5.0 | variable | 0 | 4.5 | 77 | $v = V_{max}[S]/K_m + [S]$ | 17.49 ± 3.84 | 156 ± 34 |
| | Cl ⁻ (0.2–900 mEq/L) | 5.0 | 2.27 | variable | 0 | 45 | $v = V_{max}[S]/K_m + [S]$ | 0.0 ± 0.7 | 0 ± 0 |
| | Br ⁻ (14–882 μEq/L) | 5.0 | 2.27 | 0 | variable | 45 | $v = V_{max}[S]/K_m + [S]$ | 6.23 ± 2.68 | 3 ± 0 |
| | Luminol (0.0147–30 μM) | 4.9 | 2.27 | 0 | 0 | variable | $v = V_{max}[S]^2/(K_m + [S])^2$ | 0.0 ± 0.0 | 0 ± 0 |
| | Luminol (0.0018–7.5 μM) | 7.0 | 2.27 | 0 | 0 | variable | $v = V_{max}[S]^2/(K_m + [S])^2$ | 0.90 ± 0.17 | 189 ± 2 |

Reaction milieu was 50 mM acetate buffer (pH 5.0, 4.9) or phosphate buffer (pH 7.0) in a 0.3 mL volume. The indicated conc. of Cl⁻ or Br⁻ was added in a 0.3 mL volume. The enzymes, 78 pmol MPO and 10 pmol HRP as indicated, were added in a 0.1 mL volume. The final concentration was 78 nM for MPO and 10 nM for HRP.

The luminescence reaction was initiated by injecting the indicated concentration of H₂O₂ in a 0.3 mL volume. The final volume was 1.0 mL.

Chemiluminescence velocity (v) and Vmax are expressed as peak kilocounts of relative light units (RLU × 10⁻³) per sec measured during the initial 20 sec post H₂O₂ injection.

Table 2. Michaelis-Menten enzyme kinetic analyses of classical peroxidase (horse radish peroxidase) and haloperoxidase (myeloperoxidase) activities with regard to H₂O₂, halide (Cl⁻ or Br⁻), luminol, and pH.

in Eq. (19) or the sequential bosonic pathway described in Eqs. (20), (21). By either pathway, and consistent with the second order findings, two $^1\text{H}_2\text{O}_2$ are required for luminol dioxygenation.

Under alkaline conditions, luminol-luminescence provides high sensitivity for detection of classical peroxidase catalysts or $^1\text{H}_2\text{O}_2$, but relatively low specificity. Under acid conditions, the luminol-luminescence provides a method for specific quantification of haloperoxidase-dependent dioxygenation activity. In **Table 2**, note that Cl^- or Br^- is required for MPO-catalyzed luminol-luminescence, that the requirement is first order with respect to halide, and that the Michaelis constant (K_M) for the more electronegative Cl^- is expectedly greater than that for Br^- . Haloperoxidase activity is exclusively bosonic. Reactants are all singlet multiplicity, involving HOMO-LUMO frontier orbital interaction.

Luminol was the first, and remains the most common, chemiluminogenic probe used for measurement of phagocyte oxygenation activities. Its original application was an attempt to amplify the relatively weak native luminescence signal from stimulated macrophages. Comparing the luminol-luminescence responses of neutrophils with those of macrophages illustrates that the MPO-rich neutrophils responses are several magnitudes greater than the luminol-luminescence responses from MPO-deficient macrophage [57].

Comparing MPO-rich human neutrophils with the MPO-deficient heterophile leukocytes of chickens further illustrates how chemiluminogenic probing can be used as a sensitive method for quantifying and differentiating the oxygenating activities of phagocytes [58, 65]. The luminol-dependent activities of MPO-positive human neutrophil leukocytes are a hundredfold higher than those of MPO-negative chicken heterophil leukocytes. Despite the diminution in luminol-luminescence, dioxygenation activity is still quantifiable from MPO-negative phagocytes. Such activity is not inhibited by the MPO inhibitor azide (N_3^-) [66]. In the absence of haloperoxidase, luminol-luminescence most probably reflects the type of fermionic oxidase-dependent reactions described in reactions Eqs. (17)–(18).

7. Circulating neutrophils reflect the state of inflammation

Under normal conditions, large numbers of neutrophils are produced by the hematopoietic marrow and released into the circulating blood each day, highlighting the importance of neutrophils for innate host protection against infection. To accomplish its microbicidal role, neutrophils undergo specific degranulation and mobilization of appropriate membrane receptors in response to a constellation of microbial peptides, complement activation products, cytokines, interleukins, and lipid activators. Such activities prepare neutrophils for phagocytosis, but do not directly trigger respiratory burst activity [73]. Priming actuates neutrophil locomotion and increases neutrophil recognition of and phagocytic response to opsonin-labeled microbes [56, 74, 75].

Activation of systemic inflammation in response to infection directly affects circulating blood neutrophils. The chemical signals of inflammation alter the state of neutrophil alert. As such, the state of neutrophil priming reflects the state of host immune activation [76]. Selective *in vitro* measurement of unprimed and maximally-primed circulating blood neutrophil activities by sensitive chemiluminogenic probing allows rapid multi-metric analysis using less than a half drop of anticoagulated whole blood. Analysis of such blood neutrophil luminescence metrics using classification statistical approaches, especially discriminant function analysis, allows assessment of the *in vivo* state of immune activation. The state of neutrophil priming gauges the state of host systemic inflammation [77, 78].

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Conflict of interest


I am the inventor of pending and issued patents related to diagnostic applications of chemiluminescence for quantifying neutrophil function and for gauging systemic immune activation, and patents related to therapeutic applications of haloperoxidases.

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This book highlights the numerous important properties of neutrophils and their role in various diseases, and as a possible therapeutic target as well. The first chapter briefly discusses the main effector neutrophil functions, which is followed by two chapters discussing the importance of different neutrophil receptors (cannabinoid and $Fc\gamma R$) and their role in various disease conditions. The fourth chapter discusses the differential expression profile of $CD16^+CD11b^+$ on the surface of neutrophils as a tool for the diagnosis of acute infections. The last chapter discusses the physics of the NADPH oxidase system and the use of different chemiluminogenic probes for the detection of various reactive oxygen intermediates of the circulating neutrophils.

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