

Studying Neutrophil Function in vitro: Cell Models and Environmental Factors

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Abstract: Neutrophils are the most abundant immune cell type in the blood and constitute the first line of defense against invading pathogens. Despite their important role in many diseases, they are challenging to study due to their short life span and the inability to cryopreserve or expand them in vitro. Thus, research into neutrophils has to rely on cells freshly isolated from peripheral blood of human donors, introducing donor-dependent variation in the experimental data. To counteract these problems, researchers tried to develop adequate cell models, such as cell lines. For those functional studies that cannot rely on cell models, a standardization of protocols regarding neutrophil purification and culturing could be a solution. In this review, we provide an overview of the most commonly used models for neutrophil function (HL-60, PLB-985, NB4, Kasumi-1 and induced pluripotent stem cells). In addition, we describe the effects of glucose concentration, pH, oxygen tension and temperature on neutrophil function.

Keywords: neutrophils, HL-60, PLB-985, NB4, Kasumi-1, induced pluripotent stem cells

Introduction

Neutrophils belong to the group of granulocytes, which are members of the innate immune system and constitute the most abundant leukocyte subtype circulating in blood. Neutrophils develop in the bone marrow from granulocyte-monocyte progenitor cells (GMPs), the same cells that give rise to monocytes, dendritic cells and other granulocytes. GMPs originate from hematopoietic stem cells and are committed to the myeloid lineage.¹ The stepwise development of neutrophils from GMPs is called granulopoiesis (Figure 1).¹ Terminally differentiated neutrophils have lost their capacity to proliferate and can therefore not be expanded in culture.

Neutrophils owe their name to their balanced uptake of hematoxylin and eosin, which distinguishes them from other granulocytes: eosinophils and basophils.¹ Another distinctive feature of neutrophils is their segmented nucleus and a cytoplasm filled with granules. Based on the biosynthesis of the granules during the maturation of neutrophils in the bone marrow, the granules can be classified into the following three categories: azurophilic (primary) granules, specific (secondary) granules and gelatinase (tertiary) granules. Examples of azurophilic granule proteins are myeloperoxidase (MPO), neutrophil elastase (NE) and cathepsin G; examples of specific granule proteins are lactoferrin and CD66b; and examples of gelatinase granule proteins are matrix metalloproteinase 9 (MMP-9) and CD11b. In addition, neutrophils contain secretory vesicles, which are mainly filled with surface receptors, ready to be mobilized to the plasma membrane.²

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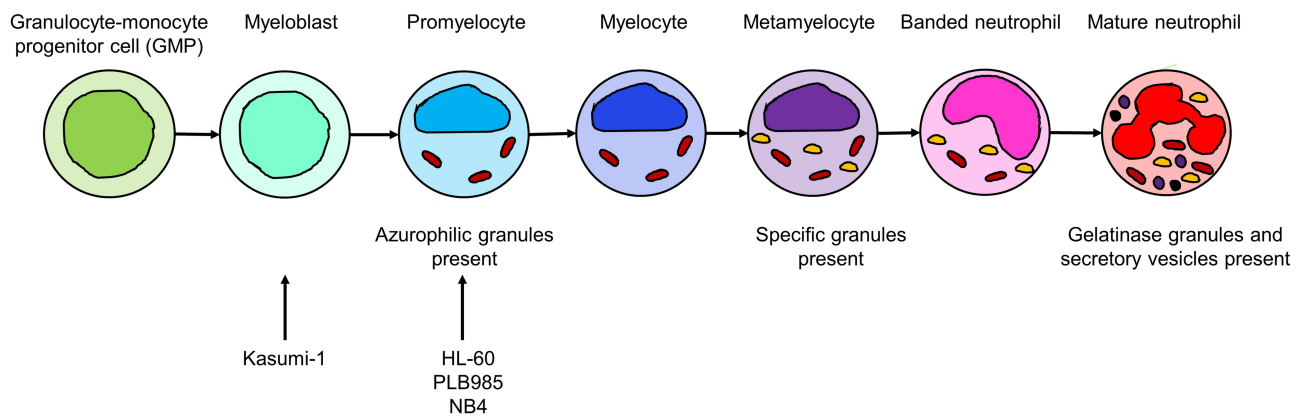


Figure 1 Granulopoiesis. In the process of granulopoiesis, neutrophils develop stepwise from granulocyte-monocyte progenitor cells (GMPs). Each stage has a unique phenotype that is comprised of both morphologic features and cell surface markers. In the first step, the cell transitions into the myeloblast stage. The promyelocyte stage is characterized by the appearance of azurophilic granules. Specific granules start to develop at the myelocyte stage, and are fully present at the metamyelocyte stage; this is also the point at which the cell loses its proliferative capacity. After the metamyelocyte stage, the cell can be called a neutrophil; it acquires the distinctive banded nucleus and develops gelatinase granules and secretory vesicles.² The arrows indicate the stage of the cell lines that are discussed in this paper.

All of the granules are crucial for pathogen killing, as their contents can either be excreted into the extracellular space or fused with phagosomes, facilitating destruction of phagocytosed material inside the cell.² Azurophilic granules are mostly involved in phagosome formation; specific granules are involved in both phagosome formation and exocytosis; and gelatinase granules and secretory vesicles are mostly involved in exocytosis.³ The action of granules is supported by the production of reactive oxygen species (ROS), which can also damage pathogens both intracellularly and extracellularly. Another mechanism that neutrophils employ to fight infection, is the expulsion of neutrophil extracellular traps (NETs): strands of DNA mixed with histones and granule proteins, which can immobilize and kill pathogens. Finally, neutrophils can produce various cytokines, which attract other immune cells to the site of inflammation. Together, these mechanisms ensure efficient pathogen killing and host defense (Figure 2). For a more detailed overview of neutrophil function, the reader is referred to Ley et al.⁴

For a long time, neutrophils were considered to be a large pool of identical cells, destined to use their weapons on invading pathogens as fast as possible and quickly die afterwards. Now we know, however, that neutrophils are not identical but can be classified into different subsets based on parameters such as density, function and maturation stage.

Perhaps the clearest distinction can be made between immature, mature and activated neutrophils. Immature neutrophils mostly reside in the bone marrow but can be released into the blood stream under acute inflammatory

conditions following a process called emergency granulopoiesis.⁵ They can be distinguished from mature and aged neutrophils by the presence of a banded nucleus and the expression levels of CD16, CD62L and CD10 ($CD16^{low}/CD62L^{high}/CD10^{-}$).^{6,7} The exit of neutrophils from the bone marrow is regulated by the balance between CXCR2 and CXCR4 signaling. CXCR4 is the receptor for CXCL12, a chemokine highly expressed in the bone marrow. CXCR2 responds to multiple CXC chemokines, including CXCL8, the major neutrophil-attracting chemokine. As neutrophils mature in the bone marrow, they gradually lose surface expression of CXCR4 and upregulate CXCR2, allowing them to exit the bone marrow into the blood stream.⁸ Mature neutrophils constitute the majority of circulating neutrophils in the blood stream, and can be distinguished by a clearly segmented nucleus. They are $CD16^{high}/CD62L^{high}/CD10^{+}$.^{6,7} With time, mature neutrophils become aged, acquiring a hypersegmented nucleus and a $CD16^{high}/CD62L^{low}/CD10^{+}$ phenotype.^{6,7} Following a circadian rhythm, aged neutrophils upregulate CXCR4; this ensures that they can home back to the bone marrow, where they undergo apoptosis and are degraded by resident macrophages.^{8,9} Aged neutrophils can also be found in tissues, from where they can retreat back into the bloodstream in a process called reverse transendothelial migration.¹⁰

It has been suggested that neutrophils of different stages display different functional properties. For example, immature neutrophils show an improved intracellular killing of bacteria compared to mature or hypersegmented neutrophils, and aged neutrophils in mice have been

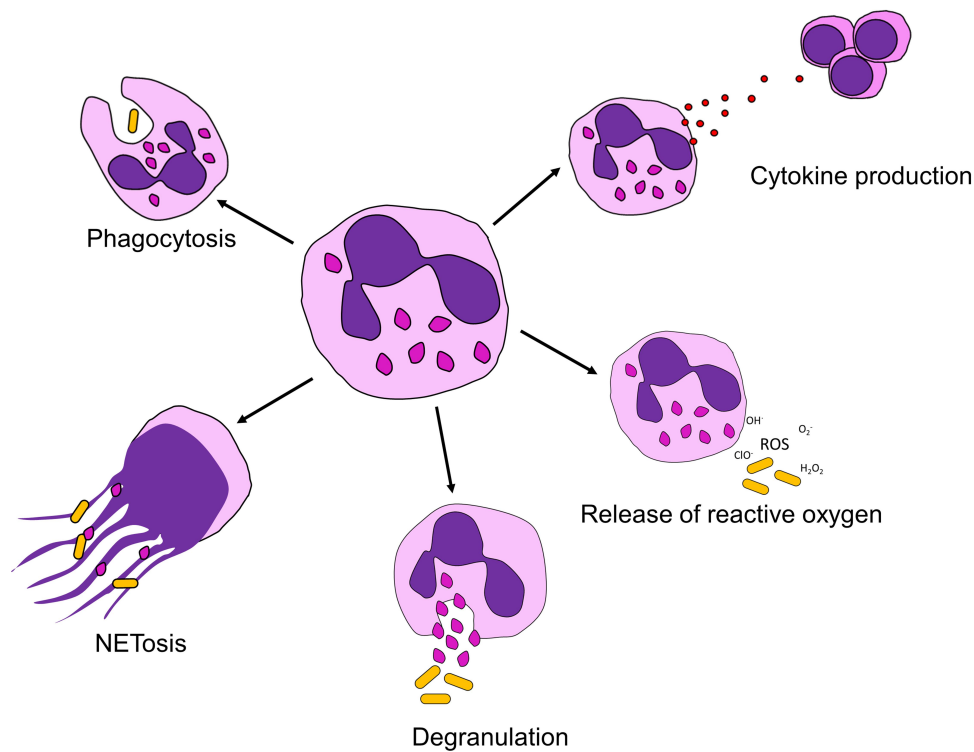


Figure 2 Neutrophil functions. To combat infection, neutrophils can execute various functions. Phagocytosis is the uptake of pathogens inside the cell. NETosis is a form of cell death in which neutrophils expulse their DNA together with histones and granular proteins (neutrophil extracellular traps). During degranulation, neutrophils release enzymes and antibacterial peptides which are normally stored inside granules. During the release of reactive oxygen species, neutrophils convert oxygen to highly reactive forms that are capable of damaging pathogens. Finally, neutrophils release cytokines, activating other immune cells and attracting them to the site of infection.

shown to be faster in reaching the tissues upon induction of acute inflammation.^{11,12} In addition, CD10⁻ neutrophils have been shown to promote T cell proliferation in vitro, whereas CD10⁺ neutrophils suppress it.¹³ Whether these differences in function have any clinical relevance remains to be discovered.

Another classification is based on the density of neutrophils. During density gradient-based neutrophil purification, whole blood is loaded onto a polysaccharide-containing solution with a density of 1.077 g/mL (eg, Ficoll). After centrifugation, granulocytes and erythrocytes can be found under the polysaccharide layer, whereas peripheral blood mononuclear cells (PBMC) form a band above it. Research has shown that a small percentage of neutrophils end up in the PBMC layer and are referred to as low-density neutrophils (LDNs), as opposed to the “conventional” normal-density neutrophils (NDNs).¹⁴ The percentage of LDNs in healthy individuals is very low, but in several diseases, including systemic lupus erythematosus (SLE) and rheumatoid arthritis, the proportion of LDNs is significantly increased.^{15,16} Various findings suggest that the LDNs can have different identities. LDNs can be either immature cells released from the bone marrow, or they can be mature cells

that have degranulated; in the latter case, the degranulation can have occurred in the blood stream, or it can have occurred in tissue, followed by reverse transmigration of the neutrophils into the bloodstream.¹⁴ Depending on the disease context, LDNs can have both pro-inflammatory and immunosuppressive functions, although more research is needed to understand what determines their functional profile.¹⁷

In mice, tumor-associated neutrophils have been shown to polarize towards an N1 or N2 phenotype.¹⁸ N1 neutrophils are pro-inflammatory and highly cytotoxic, producing vast amounts of ROS and activating the adaptive immune system. In contrast, N2 neutrophils are immunosuppressive and promote tumor growth and metastasis by producing angiogenic factors and enzymes that degrade extracellular matrix. Tumors can actively recruit neutrophils and transform them to the N2 phenotype through the production of transforming growth factor β (TGF- β). Blocking TGF- β or administering interferon β (IFN- β) promotes neutrophil polarization towards N1.¹⁸

In addition to pro-tumoral N2 neutrophils, tumor growth can also be promoted by myeloid-derived suppressor cells (MDSCs).¹⁹ MDSCs are generally categorized,

based upon expression of monocytic or granulocytic markers, into M-MDSCs and PMN-MDSCs, respectively, but are best defined by immune regulatory activity, eg, suppression of the proliferation and activation of cytotoxic T lymphocytes.^{20,21} PMN-MDSCs are typically immature and phenotypically very similar to other neutrophils. Several MDSC-specific markers have been described, including lectin-type oxidized LDL receptor-1 (LOX-1) and secreted protein acidic and rich in cysteine (SPARC).^{22,23} MDSCs develop in the bone marrow, but can be actively recruited by solid tumors, where they are activated and directed against T lymphocytes.²¹ Various ongoing clinical trials employ inhibition of MDSC pro-tumorigenic activity to treat cancer.¹⁹ Apart from cancer, MDSCs are involved in a range of other diseases, including infectious diseases and autoimmunity.^{24–26}

Other neutrophil subsets have been proposed, including CD177⁺ (approximately half of peripheral blood neutrophils) and OLFM4⁺ (approximately a quarter of peripheral blood neutrophils) cells; however, more research is needed to determine their functional properties.¹⁷

While neutrophils play an important role in many diseases, including infection, autoimmunity and cancer, they pose a challenge for those trying to study them.^{27–29} The average half-life of a neutrophil in the blood stream comprises only 6–8 h, and after purification of neutrophils from the blood the cells quickly go to apoptosis.^{30,31} Neutrophils do not proliferate, making it impossible to expand them *in vitro*; and cryopreservation of these cells has so far not been proven successful.¹ Thus, research into neutrophils has to rely on cells freshly isolated from peripheral blood of human donors. This introduces donor-dependent variation in the experimental data. To counteract these problems, researchers tried to develop adequate neutrophil models, such as cell lines. For those functional studies that cannot rely on cell models, a standardization of protocols regarding neutrophil purification and culturing could be a solution. In this review, we provide an overview of the most commonly used models for neutrophil research. In addition, we describe the effects of glucose concentration, pH, oxygen tension and temperature on neutrophil function.

The HL-60 Cell Line as a Model for Human Neutrophils

The most commonly used cell line in neutrophil research is the HL-60 cell line, derived from a female Caucasian patient with acute promyelocytic leukemia.^{32,33} HL-60 is

a suspension cell line which does not require growth supplements and proliferates relatively fast (doubling time 20–45 hours). A big advantage of HL-60 cells is that they are permissive to a range of genetic editing techniques, including lentiviral transduction, lipofectamine transfection, electroporation and nucleofection.^{34–38} Importantly, HL-60 cells can be differentiated into neutrophil-like cells by treating them with all-trans retinoic acid (ATRA), polar-planar compounds (eg, dimethyl sulfoxide [DMSO] and dimethylformamide [DMF]), actinomycin D or dibutyryl cyclic AMP (dbcAMP).^{39–43} Other compounds (eg, vitamin D) may lead to differentiation towards a more monocytic phenotype.⁴⁴

Differentiation of HL-60 Cells into Granulocytes

The uncontrolled growth and lack of differentiation in HL-60 cells is largely dependent on the c-Myc gene.⁴⁵ c-Myc is a transcription factor that controls cell differentiation, proliferation and apoptosis. While expression of c-Myc is high in all proliferating cells, it is rapidly downregulated upon terminal differentiation; not surprisingly, therefore, dysregulated c-Myc has been found in many types of cancer.⁴⁶ In HL-60 cells, there is a more than ten-fold genomic amplification of c-Myc, which can be present both inside the chromosomes and in extrachromosomal structures.^{45,47,48} Interestingly, the extra copies have been shown to be more stable in later passages of HL-60 cells, correlating with a reduction in doubling time.⁴⁸ A small percentage of the cell population differentiates spontaneously into granulocyte-like cells; these spontaneous granulocytes have been shown to have less c-Myc DNA than their undifferentiated sister cells.⁴⁹ It is likely, however, that c-Myc is not the only player in the cancerous phenotype of HL-60 cells, as inhibition of c-Myc alone reduces proliferation and survival but does not necessarily result in granulocytic differentiation.^{50,51}

Compounds most commonly used to differentiate HL-60 cells into granulocytes are ATRA and DMSO.^{39,40} ATRA is a derivative of vitamin A, which binds preferentially to the retinoic acid receptor A (RAR α).⁵² Of note, vitamin A can be metabolized into both ATRA and 9-cis-retinoic acid, both of which have different physiological properties. However, older studies describing HL-60 differentiation often make no distinction between the two, using the more generic term retinoic acid (RA). The effects of ATRA on normal adult hematopoiesis seem to

differ depending on the context; thus, it can both induce and inhibit proliferation and differentiation of neutrophil precursors.⁵³ ATRA-induced differentiation mechanisms in HL-60 cells can be divided into receptor-dependent and receptor-independent effects. Receptor-dependent effects are mediated by RAR α . RAR α is a nuclear receptor, which initiates gene transcription upon ligand binding.⁵² Activation of RAR α by ATRA initiates multiple signaling pathways, among which the MAPK and the PI3K/Akt pathways.^{54,55} This ultimately results in growth arrest and cytoskeletal reorganizations needed for differentiation.⁵⁶ In addition to these receptor-mediated effects, ATRA has been shown to block the transcription of c-Myc in a receptor-independent fashion.⁵⁷

The mechanisms by which DMSO induces HL-60 differentiation are much less clear. It is known that DMSO can induce a transcription elongation block of the c-Myc gene and inhibit its splicing upon prolonged exposure.⁵⁸ In addition, DMSO has the capacity to eliminate extrachromosomal structures containing amplified c-Myc gene.⁵⁹ Finally, DMSO has been shown to upregulate several kinases involved in intracellular signaling pathways, including Ras (involved in the MAPK pathway) and protein kinase C (PKC).^{60,61}

Further in this paper, we will refer to undifferentiated HL-60 cells as uHL-60, and to differentiated HL-60 as dHL-60, either or not preceded by the specific

differentiation agent (eg, DMSO-dHL-60). The complete overview of functions executed by dHL-60 cells is summarized in Table 1.

HL-60 Cells to Study Chemotaxis Chemotaxis in Neutrophils – An Overview

Chemotaxis is the directional movement of a cell along a chemical gradient. In order to migrate, the cell first needs to sense the presence of an inflammatory environment. While travelling through the blood vessels, neutrophils constantly attach and detach from the endothelial cell wall, a process called rolling. Rolling is mediated by three types of selectins: L-selectin (CD62L, present on leukocytes), E-selectin (present on endothelial cells) and P-selectin (present on endothelial cells and activated platelets). Selectins can bind to various ligands, the most studied of which is P-selectin glycoprotein ligand-1 (PSGL-1).⁶² Selectin-mediated interaction between neutrophils and endothelial cells is relatively weak, but during inflammation, release of various mediators (eg, tumor necrosis factor α [TNF- α], interleukin-1 β [IL-1 β]) upregulates the expression of E-selectin, resulting in stronger binding.⁶² The process of rolling ensures that neutrophils can come in contact with chemokines (chemotactic cytokines), which are captured on glycosaminoglycans (GAGs) on the surface of endothelial cells.⁶³ The chemokines then bind to their receptors on the

Table 1 Functions Which Differentiated HL-60 Cells are Capable of Executing

Function	Stimulus	Differentiation Agent	References
Cell polarization	fMLF CXCL8	DMSO, DMF ATRA, DMSO	[80,86] [86]
Chemotaxis	fMLF CXCL8 LTB4 C5a	DMSO, dbcAMP ATRA, DMSO RA dbcAMP	[81,86,87] [86] [87] [88]
ROS production	PMA fMLF Opsonized zymosan	DMSO, ATRA DMSO DMSO	[126,127] [126] [127]
NETosis	Cls PMA	DMSO, ATRA, DMF ATRA, DMF	[134,141–143] [142,143]
Phagocytosis	Saccharomyces Cerevisiae Latex beads Antibody-coated bacteria Complement-coated bacteria	RA DMSO, RA DMF DMF	[146] [87] [114,115,147] [148]

Abbreviations: ATRA, all-trans retinoic acid; C5a, complement component 5a; Cls, calcium ionophores; CXCL8, CXC motif chemokine ligand 8; dbcAMP, dibutyryl cyclic AMP; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; fMLF, N-formyl-methionine-leucyl-phenylalanine; LTB4, leukotriene B4; PMA, phorbol 12-myristate 13-acetate; RA, retinoic acid; ROS, reactive oxygen species.

surface of the neutrophils, initiating arrest, firm adhesion and transmigration through the endothelial layer.⁶⁴

Apart from chemokines, three other groups of neutrophil chemoattractants have been described: lipid chemoattractants (eg, Leukotriene B4 [LTB4], which binds to Leukotriene B4 receptor 1 and 2 [BLT1 and BLT2, respectively]); peptides (eg, N-formyl-Met-Leu-Phe [fMLF], which binds to formyl peptide receptors 1 and 2 [FPR1 and FPR2, respectively]); and complement anaphylatoxins (eg, C5a, which binds to C5a receptors 1 and 2 [C5aR1 and C5aR2, respectively]).⁶⁵ All chemoattractant receptors are G-protein coupled receptors (GPCRs) and converge into the same signaling pathways leading to migration.

The GPCR is coupled to a trimeric G-protein bound to GDP. Upon ligand binding, the G-protein is activated, exchanging the GDP for GTP. This leads to dissociation of the G-protein into a G α and a G $\beta\gamma$ subunit. Subsequently, the G $\beta\gamma$ subunit initiates two independent intracellular signaling pathways, each of which contributes to neutrophil chemotaxis.⁶⁶ First, the PLC/DAG pathway leads to activation of Rap1 and consequent mobilization of integrins, which ensure firm adhesion of the neutrophil to the endothelial wall.⁶⁷ Second, the PI3K pathway leads, via the generation of PIP₃, to the activation of Rac and Cdc42, which promote the accumulation of polymerized actin (filamentous actin [F-actin]) at the leading edge of the cell. The polymerized actin accumulates at the front of the cell (leading edge) and pushes the membrane forward.⁶⁷ Meanwhile, at the rear end of the cell (uropod), the Rho-activated protein ROCK activates myosin II, which binds to the actin filaments and ensures retraction of the uropod.⁶⁸ To cross the endothelial layer (diapedesis), the neutrophil needs to either crawl between the cells (paracellular route) or go directly through them (transcellular route). In both cases, the neutrophils activate ICAM-

1 molecules on the surface of the endothelial cells, which either induces the formation of intracellular channels (employed in the transcellular route) or, together with the endothelial molecule VCAM-1, destabilizes VE-cadherin, a major component of adherens junctions between the endothelial cells.⁶⁹

Chemoattractant Receptors on dHL-60 Cells

Upon granulocytic differentiation, HL-60 cells have been shown to upregulate receptors for fMLF, LTB4, C5a, C3a, platelet-activating factor (PAF) and CXC chemokines (Table 2).⁷⁰⁻⁷⁷ The levels of CXCR1 and CXCR2 have been reported to be relatively low compared to wild-type neutrophils.⁷⁷

Interestingly, a study by Jacobs et al showed that the G-protein/receptor complexes for fMLF, LTB4 and C5a on DMSO-dHL-60 have a different affinity for GTP binding, resulting in different potency of the chemoattractant. The authors demonstrate that DMSO-dHL-60 cells preferentially respond to fMLF, less readily to C5a and least readily to LTB4.⁷⁸

Expression of Adhesion Molecules on HL-60 Cells

The leukocyte-specific CD62L is virtually absent on uHL-60 and only appears upon differentiation, although the expression levels do not reach those of peripheral blood neutrophils.⁸² By contrast, PSGL-1 is expressed on both dHL-60 and uHL-60 cells at similar levels.⁸³ The upregulation of CD62L upon differentiation is reflected in the rolling speed of the cells: dHL-60 cells rolled significantly faster than uHL-60 cells when placed in a flow chamber coated with P-selectin.⁸³ In addition, Sjögren et al reported that differentiation of HL-60 cells with DMSO induced the surface expression of CD11b, a component of the β_2 -integrin Mac-1 (CD11b/CD18).⁸² Since dHL-60 cells do

Table 2 Chemoattractant Receptors Expressed on the Surface of Differentiated HL-60 Cells

Receptor	Ligand	Differentiation Agent ^a	Reference
BLT	LTB4	DMSO, ATRA	[73,79]
C3aR	C3a	dbcAMP	[74]
C5aR	C5a	dbcAMP	[70,74]
CXCR1 (low)	CXCL6,8	ATRA, DMSO	[76]
CXCR2 (low)	CXCL1,2,3,5,6,7,8	DMSO	[77]
FPR	N-formyl peptides (eg fMLF)	dbcAMP, DMF, DMSO	[70,71,80,81]
PAF receptor	PAF	DMSO	[75]

Note: ^aCompounds that have been demonstrated to induce receptor expression on HL-60 cells.

Abbreviations: ATRA, all-trans retinoic acid; BLT, leukotriene B4 receptor; C3a, complement component 3a; C3aR, C3a receptor; C5a, complement component 5a; C5aR, C5a receptor; CXCL, CXC motif chemokine ligand; CXCR, CXC chemokine receptor; dbcAMP, dibutyryl cyclic AMP; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; fMLF, N-formyl-methionine-leucyl-phenylalanine; FPR, formyl peptide receptor; PAF, platelet-activating factor.

not have CD11b-containing specific granules, the authors suggest that the CD11b must be coming from a different intracellular source.⁸⁴ In addition, the same authors showed that while DMSO caused a strong upregulation of CD11b and CD62L, the effect of RA was much more limited, which was reflected in the reduced migratory capacity of RA-dHL-60.⁸² Finally, the expression of the β_2 -integrin CD11c/CD18 is slightly upregulated by differentiation with DMSO or RA, although the surface protein expression remains low.⁸⁵

Chemotaxis Function in dHL-60 Cells

It has been demonstrated that dHL-60 cells form actin filaments, visibly polarize and directionally migrate in response to fMLF, LTB₄, C5a and CXCL8, indicating that pathways downstream of the receptors are mostly intact.^{72,80,86–89} To confirm this, Hauert et al specifically verified that the PI3K and Rho pathways in dHL-60 cells are identical to those found in peripheral blood neutrophils.⁸⁹ Of note, a reduced response towards fMLF has been reported in ATRA-dHL-60.^{79,86,87,90}

Despite the chemotaxis pathways being fully functional in dHL-60 cells, most studies report that the average response is slightly lower than that of peripheral blood neutrophils, probably due to reduced expression levels of the receptors.^{77,80,89}

Many studies report the inability of undifferentiated HL-60 cells to migrate in response to chemotactic stimuli.^{80,82,89} While this is perfectly explainable by low expression of chemoattractant receptors and adhesion molecules (vide supra), another study has shown that HL-60 synthesize large amounts of actin upon differentiation.⁹¹ Perhaps the actin content in undifferentiated HL-60 cells is too low for efficient cell polarization and movement, contributing to their inability to transmigrate. This hypothesis is supported by the findings of Prossnitz et al, who transfected uHL-60 cells with FPR and observed increased but insufficient actin polymerization in response to fMLF stimulation. By contrast, other responses to fMLF, such as mobilization of intracellular calcium, were intact in this study.⁹²

Transgenic HL-60-Derived Sublines for Chemotaxis Studies

Certain limitations of dHL-60 cells (eg, low receptor expression or affinity) can be overcome by creating transgenic sublines. For example, Kikuchi-Ueda et al established an HL-60 cell line overexpressing CXCR1.⁷⁶ When

differentiated with ATRA, these cells showed a fourfold increase in chemotaxis towards CXCL8 as compared to conventional dHL-60 cells. Similarly, HL-60 cells overexpressing CXCR2 and FPR have been created.^{77,92} These sublines can be useful for studying receptor signaling in more detail, or for testing selective receptor inhibitors. For example, targeting CXCR2 might alleviate lung injury induced by an excessive neutrophil influx.⁹³

Recently, Garner et al created an HL-60-derived cell line which expressed green fluorescent protein (GFP)-labeled β -actin, the most abundant actin isoform in neutrophils.⁹⁴ When differentiated with DMSO, this novel cell line has been shown to migrate towards fMLF with an efficiency approaching that of wild-type neutrophils, making it an interesting model to study actin dynamics during neutrophil chemotaxis.

HL-60 Cells for the Study of Phagocytosis Phagocytosis in Neutrophils – An Overview

Phagocytosis is a multi-step process that results in the engulfment of particles by a cell; to achieve this, distinct cellular mechanisms are required. First, the pathogen is recognized by a receptor on the surface of the neutrophil. This can be either an opsonic or a non-opsonic receptor. An example of non-opsonic receptors are C-type lectins, such as Dectin-1 (which recognizes β -glucan on fungal cells and yeast polysaccharide) and Mincle (which recognizes the trehalose dimycolate [TDM] motif on mycobacterial cell walls).⁹⁵ To recognize opsonized particles, neutrophils employ either Fc γ receptors (Fc γ R) (eg, CD32 or CD16), which bind the constant region of IgG antibodies, or complement receptors (eg, CR3 [CD11b/CD18] or CR4 [CD11c/CD18], both of which recognize proteolytic fragments of C3b).^{96,97} Upon binding, the receptors initiate intracellular signaling cascades, which differ for each receptor but have a similar outcome. Activation of small GTPases (eg, Rac2 in case of Fc γ R) or Rho in case of complement receptors) ultimately results in actin remodeling, leading to the uptake of the bound particle in a phagosome; this can either or not be mediated by the formation of pseudopodia.⁹⁵ In addition, the actin remodeling translocates azurophilic and specific granules closer to the phagosome. At the same time, an increase in intracellular calcium concentration induces the formation of pores in the phagosome, allowing fusion between the phagosome and the granules to take place.⁹⁸ This fusion leads to exposure of the pathogen to antimicrobial peptides (eg, defensins) and proteases (eg, NE); in case of specific

granules, this process is dependent on free cytosolic calcium. Finally, the recruitment of the NADPH oxidase complex to the phagosome enhances pathogen killing through generation of different reactive oxygen species (ROS), including hydrogen peroxide and hypochlorous acid.⁹⁹ Priming of neutrophils (eg, with TNF- α) has been shown to improve phagocytosis efficiency by increasing the number of phagocytic receptors on the plasma membrane and enhancing their affinity towards their ligand, as well as stimulating degranulation and superoxide production.¹⁰⁰

Phagocytic Receptors on (d)HL-60 Cells

In contrast to chemoattractant receptors, some phagocytic receptors are expressed on undifferentiated HL-60 cells, albeit at low levels (Table 3). Expression of C1qRp, CR1, CR3, CR4 and Fc γ RII increases upon differentiation, although the receptor levels usually do not reach those of peripheral blood neutrophils.^{85,101}

Phagocytosis Function in HL-60 Cells

Undifferentiated HL-60 cells have very low phagocytic ability, if any. uHL-60 were shown not to have cytotoxic activity towards opsonized chicken erythrocyte target cells; stimulation with IFN- γ induced some cell killing but this was not nearly as effective as compared to peripheral blood neutrophils.¹⁰⁷ Upon differentiation, HL-60 cells have been

shown to effectively phagocytose various particles, including latex beads, *Escherichia coli*, opsonized yeast and opsonized bacteria.^{33,87,111–116} Lerm et al investigated the phagocytic pathway in more detail by constitutively expressing Cdc42 in dHL-60 cells. Cdc42 is a small GTPase which has a role in actin polymerization; constitutively activated Cdc42 led to polarization of the cells in absence of stimulus and induced accumulation of actin filaments around phagosomes, preventing their fusion with azurophilic granules.¹¹⁷ Another group demonstrated that inhibition of this fusion can be employed by *Streptococcus pyogenes* which can survive inside both dHL-60 cells and peripheral blood neutrophils.⁸⁴

While the pathogen uptake mechanisms in dHL-60 cells seem to be mostly functional, the intracellular killing is much less efficient.^{112,116,118,119} This could be explained by the fact that HL-60 cells lack specific granules and do not acquire them upon differentiation.⁸⁴

HL-60 Cells to Study Neutrophil Respiratory Burst

Respiratory Burst in Neutrophils – An Overview

Another weapon in the arsenal of neutrophils is the respiratory burst, characterized by high production of ROS. These are highly reactive derivatives of oxygen molecules, capable of damaging pathogens both inside and outside the cell. Central to the ROS production is the

Table 3 Expression of Phagocytic Receptors on Undifferentiated (uHL-60) and Differentiated (dHL-60) Cells

Receptor	Examples of Ligands	Expression on uHL-60	Expression on dHL-60	References
C1qRp	C1q ^a	-	DMSO: - or + RA: -	[101,102]
CR1 (CD35)	C1q, C3b, C4b, MBL	-	DMSO: ++ RA: ++	[103–105]
CR3 (CD11b/CD18)	iC3b	+	DMSO: ++ RA: ++	[82,101,102,104,106]
CR4 (CD11c/CD18)	iC3b	-	DMSO: + RA: +	[85]
Fc γ RI	IgG1, IgG3, IgG4, IgG immune complexes	+ or ++	DMSO: ++ ATRA: ++ DMF: ++	[107–109]
Fc γ RII	IgG immune complexes	+ or ++	RA: ++	[107,108,110]
Fc γ RIII	IgG3, IgG immune complexes	-	unknown	[107,108]

Note: ^aThe question mark indicates that the affinity of C1qRp for C1q is still under debate.

Abbreviations: ATRA, all-trans retinoic acid; C1q, complement component 1q; C1qRp, C1q receptor; C3b, complement component 3b; C4b, complement component 4b; CR, complement receptor; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; Fc γ R, Fc γ receptor; IgG, immunoglobulin G; RA, retinoic acid; -, no expression; +, low expression; ++, moderate to high expression.

NADPH oxidase complex, which is called NOX2 in neutrophils.¹²⁰ NOX2 consists of a catalytic core (flavo-cytochrome b_{558} or cyt b_{558}) and a number of proteins which are required for its activation. Cyt b_{558} resides in the membrane surrounding the cell or the specific granules, and consists of the $p22^{phox}$ (α subunit) and the $gp91^{phox}$ (β subunit). $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ together form a complex that is located in the cytosol under resting conditions. Upon activation of the neutrophil, $p47^{phox}$ is phosphorylated at multiple sites by protein kinases, such as PKC, p38 MAPK or p21-activated kinase (PAK).¹²¹ This leads to the translocation of the $p40^{phox}/p47^{phox}/p67^{phox}$ complex to the membrane, where it associates with cyt b_{558} to form a NOX2 complex. Additional binding of the small GTPase Rac1 improves the binding between the NOX2 components necessary for activation.¹²⁰ The NADPH oxidase can then produce superoxide anions (O_2^-) by transferring electrons from the cytosolic NADPH to oxygen molecules on the other side of the membrane. The superoxide dismutates into hydrogen peroxide (H_2O_2), after which the enzyme MPO converts it to other oxygen species, such as hypochlorous acid.¹²² MPO is produced in large quantities at the promyelocytic stage of neutrophil development, upon which it is stored in azurophilic granules until neutrophil activation; in terminally differentiated cells, MPO mRNA is usually not detectable.¹²³

Respiratory Burst in dHL-60 Cells

Undifferentiated HL-60 cells have been shown to express both a functional NADPH oxidase and MPO, and thus to be capable of ROS production; however, the expression of $gp91^{phox}$, $p47^{phox}$ and $p67^{phox}$ RNA was found to be considerably higher in dHL-60 cells.^{123,124} Interestingly, the generation of ROS has been found to be critically important for the survival of the cells, as the addition of antioxidants significantly reduced their viability.¹²⁵ Stimulation of uHL-60 cells with compounds that normally induce ROS production (eg, phorbol 12-myristate 13-acetate [PMA], a direct activator of PKC) did not trigger an increased ROS production by uHL-60 cells.^{125,126} In contrast, dHL-60 cells responded to PMA and fMLF stimulation with a prompt peak in ROS production, accompanied by a decrease in mRNA expression of MPO.¹²⁶ The superoxide production by DMSO-dHL-60 cells in response to PMA and opsonized zymosan was comparable to that of peripheral blood neutrophils, but the total amount of

radicals was lower in dHL-60 cells, possibly indicating a reduced function of proteins downstream of the NOX2 complex, or an increased activity of anti-oxidative enzymes.¹²⁷ Of note, several studies reported that ATRA-dHL-60 produce a less powerful respiratory burst in response to fMLF as compared to DMSO-dHL-60.^{79,90} This is consistent with the observation that ATRA only induces the expression of fMLF receptors with a low ligand affinity.⁷⁹

Several studies used RNA interference for better characterization of pathways involved in the respiratory burst. Thus, depletion of PKC β in dHL-60 cells resulted in decreased translocation of $p47^{phox}$ to the plasma membrane and subsequent reduction in superoxide production, whereas inhibition of PKC δ reduced superoxide production without affecting $p47^{phox}$ location.^{128,129} In addition, dHL-60 were shown to be sensitive to priming with TNF- α .¹³⁰

Finally, the reader is referred to an excellent paper by Seitz et al for a comparison of methods best suited to detect the respiratory burst in dHL-60 cells.¹³¹

HL-60 Cells to Study Neutrophil

Extracellular Traps NETosis – An Overview

NETosis, ie, cell death featuring expulsion of neutrophil extracellular traps (NETs), is a defense mechanism in which the neutrophil releases its DNA as decondensed chromatin mixed with histones and granule proteins. Currently, there is still much debate on the origin of the expelled DNA (ie, nuclear or mitochondrial) as well as whether the cell releasing a NET necessarily dies (suicidal NETosis) or remains viable and capable of exerting other functions in antimicrobial defense (vital NETosis).¹³² However, most studies agree that there are three central players in the process of NETosis: ROS, NE and protein arginase deiminase 4 (PAD4). ROS are produced upon assembly and activation of the NOX2 complex; during NETosis, they cause damage to the nuclear and granular membranes, allowing contact between nuclear and cytoplasmic content. NE and granular proteases, empowered by MPO, cleave histones to facilitate chromatin decondensation. PAD4 is a calcium-dependent enzyme which promotes chromatin decondensation by citrullinating the histones, thereby altering their charge and weakening their interaction with DNA.¹³³ The extended decondensation of chromatin leads to the rupture of the plasma

membrane and the release of NETs into the extracellular space.¹³⁴

Both physiological and artificial stimuli have been demonstrated to induce NETosis. Physiological stimuli include the bacterial lipopolysaccharide (LPS), CXCL8 and monosodium urate (MSU) crystals.^{135,136} Two of the artificial stimuli commonly used in NETosis research, are PMA and calcium ionophores (CIs).¹³⁷ PMA is a molecule that freely crosses the plasma membrane and directly activates PKC which on its turn promotes NOX2 activation.¹³⁸ CIs are a group of molecules which trigger calcium release into the cytoplasm from the endoplasmic reticulum stores.¹³⁹

NETosis in dHL-60 Cells

The first study into NET formation by HL-60 cells was performed in 2008 by Neeli et al, who demonstrated histone citrullination in ATRA-dHL-60 cells stimulated with a wide range of stimuli (eg, CIs, TNF- α , fMLF, LPS). The histone citrullination was completely independent of apoptosis, as no caspase activation was detected; vice versa, apoptosis-inducing compounds did not induce citrullination of histones. A comparison between dHL-60 and peripheral blood neutrophils showed that the extent and timespan of the histone citrullination were very similar between the two, but that dHL-60 cells were only triggered by much higher concentrations of the stimuli.¹⁴⁰ The data of Neeli et al were complemented by a later study, which showed that the expression of PAD4 increased upon differentiation of HL-60 cells with DMSO, and that stimulation with a CI led to expulsion of NETs.¹⁴¹ HL-60 cells deficient in PAD4 showed a delay in DNA decondensation and largely failed to expulse NETs due to defects in plasma membrane rupture.¹³⁴ Interestingly, NETs produced by dHL-60 cells in response to PMA and a CI had a different morphology from those produced by peripheral blood neutrophils: the strands of DNA were much shorter and remained close to the cells.¹⁴² Another study found that both DMSO- and ATRA-dHL-60 cells were capable of producing NETs in response to *Staphylococcus aureus*, albeit much less efficient than peripheral blood neutrophils.¹¹⁹

An extensive study by Manda-Handzlik et al compared the NET-forming capacity of HL-60 cells after differentiation with various compounds (DMSO, ATRA, DMF). Surprisingly, the results indicated differences between the differentiation agents: ATRA-dHL-60 cells only released

NETs in response to PMA; DMSO-dHL-60 cells only in response to a CI, and DMF-dHL-60 cells in response to both.¹⁴² However, these data have been contradicted by other studies, which demonstrated that ATRA-dHL-60 cells can also react to CIs and DMSO-dHL-60 cells also to PMA.^{143,144}

Importantly, Takishita et al found that the DNA expelled by dHL-60 cells was genomic rather than mitochondrial, as removal of mitochondrial DNA had no effect on PMA-induced NET release.¹⁴³

HL-60 Cells to Study Immunosuppressive Function of Neutrophils

Recently, Zhang et al described the generation of MDSC-like neutrophils from HL-60 cells.¹⁴⁵ To this end, the cells were differentiated with DMSO and treated with a combination of granulocyte-monocyte colony-stimulating factor (GM-CSF) and IL-6. The resulting cells suppressed cytokine production and apoptosis of T lymphocytes. In addition, MDSC-like dHL-60 cells had a gene expression signature that was very similar to that of natural MDSC cells. This is a promising model for studying neutrophil immunosuppressive function, eg, in cancer and autoimmunity research.

Cell Lines Less Commonly Used as Neutrophil Model

PLB-985 Cell Line

PLB-985 cells were introduced in 1987 as a novel cell line; however, later research revealed PLB-985 to be a subline of HL-60, having nearly identical DNA and very similar gene expression.¹⁴⁹⁻¹⁵¹

NB4 Cell Line

NB4 is a cell line originally established in 1991 from a female patient with relapsed acute promyelocytic leukemia. It is cultured in RPMI-1640 medium with serum supplementation, but without any other additives.¹⁵² The cell line has mainly attracted attention as a model to study cell differentiation in leukemia, but NB4 cells treated with ATRA display many features of terminally differentiated neutrophils and are therefore perfectly suitable for studying neutrophil function as well.^{152,153} In contrast to HL-60 cells, NB4 cells express a fusion protein that is formed between the retinoid receptor RAR α and the tumor-suppressor protein promyelocytic leukemia (PML).¹⁵⁴ This fusion protein, PML-RAR α , binds to transcriptional

targets of ATRA but does not initiate transcription. Addition of substantial amounts of ATRA displaces the fusion protein from the DNA and allows normal transcription of ATRA target genes.¹⁵⁵ Differentiated NB4 cells are capable of producing ROS and possess functional azurophilic granules but do not contain specific or gelatinase granules; while the presence of both lactoferrin and MMP-9 has been demonstrated, they are not contained in granules.^{153,156–158} A study by Barber et al compared the changes in expression of CD markers between NB4 and HL-60 cells upon ATRA differentiation (Table 4).¹⁵⁹ The authors suggest that the difference in marker expression can be explained by the actions of PML-RAR α in NB4 cells, whose function may differ from wildtype RAR α even after ATRA binding.

Kasumi-1 Cell Line

Kasumi-1 is a cell line established in 1991 from a male pediatric patient with acute myeloblastic leukemia. Kasumi-1 cells proliferate with a doubling time of 40–45 hours, but require the presence of stimulatory cytokines (eg, granulocyte colony-stimulating factor [G-CSF], IL-6) in the culture medium.¹⁶⁰ Whereas Kasumi-1 have mostly been of interest for leukemia rather than neutrophil research, recently Schoenherr et al found that silencing of the RUNX1-ETO oncogene induced features of neutrophil differentiation, including reduced proliferation and

higher expression of NE and cathepsin G, markers of azurophilic granules.¹⁶¹ As Kasumi-1 cells are in a very early stage of myeloid differentiation, this model might provide research opportunities for studying (early) neutrophil development.

Induced Pluripotent Stem Cells as a Model for Human Neutrophils

Another emerging alternative for peripheral blood neutrophils is the use of induced pluripotent stem cells (iPSCs). iPSCs can be derived from any somatic cell by “erasing” its differentiation memory and thus reverting it to a pluripotent state. This can be done by the addition of several transcription factors crucial for maintaining the pluripotent phenotype.¹⁶² A commonly used combination of transcription factors is OCT4, SOX2, KLF4 and c-MYC. These factors can be added to terminally differentiated cells, eg, through plasmid transfection or retroviral transduction.¹⁶² Other reprogramming factors are used less often, and are described in detail by Xiao et al¹⁶³.

Upon their generation, iPSCs can be expanded and/or cryopreserved.¹⁶⁴ Subsequently, the pluripotent stem cells can be differentiated into a whole range of cell types, including hematopoietic cells and specifically neutrophils.^{165,166} Differentiation is performed by culturing the iPSCs in the presence of a combination of specific growth factors and cytokines, including stem cell factor (SCF), IL-6 and G-CSF. A detailed protocol for neutrophilic differentiation of iPSCs has been described by Sweeney et al.¹⁶⁷ A simpler method for the generation of iPSC-derived neutrophils has been proposed by Lachmann et al in 2015; this protocol relies on the addition of only two cytokines: IL-3 and G-CSF.¹⁶⁸ However, neutrophils obtained with this method have been shown to be less fully differentiated and less capable of ROS production and bacterial killing.^{168,169}

The use of iPSCs has both advantages and disadvantages compared to other models for human neutrophils. One obvious advantage is the close resemblance of iPSC-derived neutrophils to neutrophils found in peripheral blood, as shown by the presence of all neutrophil-specific granules. In addition, ROS production, phagocytic activity and chemotaxis capacity of iPSCs are very similar to that of peripheral blood neutrophils.¹⁶⁶ Another advantage is the possibility to create disease-specific cell lines by generating iPSCs from patients with different mutations. This has been elegantly illustrated by Brault et al, who

Table 4 Changes in Expression of CD Markers on HL-60 and NB4 Cells Upon ATRA Differentiation^a, as Described by Barber et al¹⁵⁹

Marker	HL-60	NB4	Marker	HL-60	NB4
CD9	-	↓	CD45	↑	-
CD11a	-	↑	CD45RO	↑	↓
CD11b	↑	↑	CD53	↑	↑
CD11c	↑	↑	CD54	↑	↑
CD13	-	↓	CD64	↑	↑
CD14	-	↑	CD65	-	↑
CD29	↓	↓	CD66c	↑	↑
CD31	-	↓	CD95	↓	-
CD32	-	↑	CD117	↓	-
CD36	↑	-	CD126	-	↑
CD38	↑	↓	CD138	-	↑
CD43	↑	↓	TCR α/β	-	↑
CD44	↓	↓			

Notes: ^aThe table only contains markers that were detectable on undifferentiated cells. A change is defined as an increase or reduction of two-fold or more.

Abbreviations: CD, cluster of differentiation; TCR, T cell receptor; -, no change in expression; ↑, expression increased after differentiation; ↓, expression reduced after differentiation.

established iPSC cell lines from patients with chronic granulomatous disease (CGD), a genetic disorder characterized by dysfunctional neutrophils which are unable to produce ROS.¹⁷⁰

Disadvantages associated with the use of iPSCs for neutrophil research are mostly practical. The generation of iPSC-derived neutrophils takes at least a month even if the iPSCs themselves are already available.¹⁶⁷ In addition, the process is expensive due to the large amounts of cytokines required for the differentiation. Therefore, extra care is advised when planning the experiments.

Neutrophil Function in vitro – Effect of Environmental Factors

Glucose Concentration

Neutrophils can only be kept alive within a short time frame, but different buffer solutions are suitable, ranging from phosphate-buffered saline (PBS) to glucose-supplemented culturing media, such as RPMI-1640 (glucose concentration 11.1 mM) or DMEM (glucose concentration 5.6 or 25 mM).^{171,172} For comparison, the fasting blood glucose levels in healthy individuals range from 3.5 to 5.5 mM.¹⁷³ Studies addressing the effect of glucose on neutrophil function have shown that pre-incubation of neutrophils in medium containing more than 5 mM glucose led to reduced chemotaxis, phagocytosis and bactericidal capacity.^{174,175} Above 11.1 mM, there was also a reduction in respiratory burst, actin polymerization and neutrophil adhesiveness.^{174,176,177} Menegazzo et al reported that at 25 mM, glucose promoted the formation of NETs; however, this was contradicted by the group of Joshi et al, who stated that high glucose actually impaired NET formation.^{178,179}

Of note, reduced chemotaxis and ROS production were also reported when neutrophils were pre-incubated in glucose-free medium, indicating that there is an optimal glucose concentration at which neutrophil function is maximal.^{175,180} Interestingly, glucose had no effect on neutrophil viability.^{174,180}

pH

In healthy individuals, the pH of blood lies between 7.35 and 7.45, but at sites of inflammation, the pH often drops to 7.0 or lower.^{181,182} Low pH can greatly influence the function of neutrophils. In a more acidic environment, neutrophils will produce less ROS and release fewer NETs.^{183–186} Neutrophil chemotaxis is optimal around pH 7.2–7.5 and is inhibited by pH values outside that

range.^{187,188} In addition, pH values under 7.2 have been shown to inhibit phagocytosis.¹⁸⁹ In contrast, other neutrophil functions, such as release of specific granules and adhesion to endothelium, can be promoted in an acidic environment.^{189,190} In addition, Geffner et al observed an increased cytotoxic capacity of neutrophils under low pH, despite earlier reports of an inhibited respiratory burst in acidic conditions.¹⁹¹ The authors hypothesize that while the production of some ROS species is inhibited, the production of others may be increased, contributing to neutrophil cytotoxicity; however, more research is needed to test this hypothesis.

Oxygen Levels

Atmospheric oxygen tension equals 21 kPa, but in the circulation this value drops to 5–13 kPa.¹⁹² In tissues, especially during inflammation, hypoxia can occur as the oxygen tension can drop below 2.5 kPa.¹⁹² Examples of factors that contribute to this low oxygen tension are high oxygen consumption by neutrophils (fueling the respiratory burst) and depletion of oxygen by the invading pathogen.^{193,194} Thus, in the human body, neutrophils often exert their function under hypoxia. This is important to realize, since most experiments on human neutrophils are routinely performed under atmospheric oxygen pressure, and the results of these experiments may not necessarily reflect the situation in vivo.

Hypoxia has two major effects on neutrophil function. Firstly, the low availability of oxygen molecules restricts the production of reactive oxygen species.^{195–200} Depending on the extent of hypoxia, this can have an inhibitory effect on the formation of NETs and bactericidal activity.^{201–203} Vice versa, exposing neutrophils to hyperoxia leads to increased respiratory burst.²⁰⁴

Secondly, hypoxia significantly delays neutrophil apoptosis, a feature which is dependent on Hypoxia-Inducible Factor 1 α (HIF-1 α).^{195–200} HIF-1 α forms a heterodimer with HIF-1 β , constituting the transcription factor HIF-1.²⁰⁵ Under normoxic conditions, the proline residues of the HIF-1 α protein are hydroxylated by prolyl hydroxylases.²⁰⁶ This modification promotes the interaction of HIF-1 α with the von Hippel-Lindau tumor-suppressor protein (VHL), inducing ubiquitylation and consequent proteasomal degradation of HIF-1 α .²⁰⁷ As prolyl hydroxylases require the presence of oxygen as a co-factor, their function is inhibited under hypoxic conditions, resulting in the preservation of HIF-1 α .²⁰⁸ HIF-1 α can then migrate to the nucleus, associate with HIF-1 β and initiate the transcription of genes involved

in various processes, including glycolysis, angiogenesis and cell migration.^{209–211} It has been suggested that HIF-1 α inhibits neutrophil apoptosis by upregulating NF- κ B signaling, which can stimulate the transcription of anti-apoptotic factors.^{199,212}

In addition to these two hypoxia-driven effects, a few studies have shown that low oxygen tension stimulates degranulation, which was found to be independent of de novo protein synthesis.^{202,213}

Most studies found no effect of hypoxia on the expression of surface receptors, including receptors for CXCL8, fMLF and GM-CSF, and very little effect on the release of inflammatory mediators, such as CXCL8, vascular-endothelial growth factor (VEGF), IL-17 and IL-6.^{195,203,214–217} In addition, separate studies have suggested that hypoxia might promote phagocytosis and render neutrophils less responsive to glucocorticoids and inflammatory mediators such as TNF- α and CXCL8, the latter being linked to dysfunction of the PLC/DAG signaling pathway downstream of the GPCR receptor.^{215,218,219} As the PLC/DAG pathway is involved in the activation of integrins (vide supra), it is not surprising that some studies report an inhibitory effect of hypoxia on the upregulation of CD11b/CD18 and neutrophil migration.^{220,221} Another study reports no difference in chemotaxis between neutrophils under normoxic and hypoxic conditions.²⁰³

Temperature

While normal body temperature is around 37°C, purification of neutrophils from peripheral blood is commonly done at room temperature. It is highly advisable not to perform

Table 6 Effect of Environmental Factors on Neutrophil Function

Neutrophil Function	High Glucose	Low pH	Hypoxia	Low Temperature
Chemotaxis	↓	↓	–	↓
Phagocytosis	↓	↓	↑	↓
ROS production	↓	↓/↑	↓	↓
NETosis	↓/↑	↓	↓	?
Bacterial killing	↓	↑	↓	↓
Degranulation	?	↑	↑	?
Survival	–	?	↑	?

Abbreviations: ROS, reactive oxygen species; ↑, function enhanced by environmental factor; ↓, function inhibited by environmental factor; –, no change in function; ?, change in function unknown; ↓/↑, contradictory results reported.

functional tests at room temperature, as neutrophil phagocytosis, bacterial killing, chemotaxis, adhesion and ROS production are all significantly decreased at room temperature as compared to 37°C.^{184,187,222–227} In addition, it has been demonstrated that several receptors and proton channels have a reduced activity at room temperature.^{228–231} Reassuringly, re-warming the cells to 37°C after incubating them at room temperature or even at 4°C has been shown to restore neutrophil functionality.^{227,232}

At inflammatory sites, the temperature can be higher than 37°C; therefore, the functionality of neutrophils at higher temperatures might also be of interest.²³³ The majority of studies show that neutrophils are in general less responsive to activating stimuli at temperatures above 41°C.^{222–224,234}

Concluding Remarks

Neutrophils are an essential part of the innate immune system, and since 1979 several granulocytic cell lines

Table 5 Advantages and Limitations of Different Cell Models for Neutrophil Research

Cell Model	Advantages	Limitations
HL-60	<ul style="list-style-type: none"> - Most commonly used, many protocols available - Suitable for genetic editing - NETosis described - Easy to culture - Immunosuppressive differentiation described 	<ul style="list-style-type: none"> - Differentiation mechanism not entirely clear - Bacterial killing inefficient - No specific/gelatinase granules
PLB-985*	Same as HL-60	Same as HL-60
NB4	<ul style="list-style-type: none"> - Clear differentiation mechanism - Easy to culture 	<ul style="list-style-type: none"> - No specific/gelatinase granules - Few protocols available
Kasumi-1	<ul style="list-style-type: none"> - Suitable for studying early neutrophil differentiation 	<ul style="list-style-type: none"> - Few protocols available - Expensive to culture
Induced pluripotent stem cells	<ul style="list-style-type: none"> - Closely resembling wildtype neutrophils - Patient-specific cell lines can be created 	<ul style="list-style-type: none"> - Expensive to culture - Protocol time-consuming

Note: *Subline of HL-60 cells.

have been established and characterized. Especially the HL-60 cell line has received attention over the past decades. While many functional assays have been performed with differentiated HL-60 cells, no consensus has so far been reached on which differentiation agent produces cells that are phenotypically and functionally closest to neutrophils. Perhaps future research will shed more light on this, as well as provide a more thorough comparison between HL-60 and other granulocytic cell lines. Of course, cell lines provide an alternative for the isolation of fresh neutrophils from blood and can be easily transfected, but cannot completely replace those cells. Novel findings will always have to be confirmed in final experiments with the primary cells. A summary of the advantages and limitations of different cell lines is displayed in [Table 5](#).

Induced pluripotent cell lines provide another alternative for the use of donor neutrophils. Although lengthy and expensive for now, the procedure for creating iPSC-derived neutrophils will hopefully become simpler in the future, allowing for a model highly resembling human neutrophils from peripheral blood.

Environmental factors, such as temperature, pH and oxygen and glucose levels, have proven to have a strong influence on the function of neutrophils isolated from the blood. Variations in these factors are not only important for laboratory work, but are also found in physiological conditions, eg, during inflammation. [Table 6](#) summarizes the effect of changes in environment on neutrophil function. Depending on the context of the research, these factors can be adjusted to create an environment as close to the human body as possible.

Abbreviations

ATRA, All-trans retinoic acid; BLT, Leukotriene B4 receptor; C1q, Complement component 1q; C1qRp, C1q receptor; C3a, Complement component 3a; C3aR, C3a receptor; C5a, Complement component 5a; C5aR, C5a receptor; CD, Cluster of differentiation; Cdc42, Cell division control protein 42 homolog; CGD, Chronic granulomatous disease; CI, Calcium ionophore; CR, Complement receptor; CXCL, CXC motif chemokine ligand; CXCR, CXC chemokine receptor; Cyt b558, Flavocytochrome b558; DAG, Diacylglycerol; dbcAMP, Dibutyryl cyclic AMP; DMEM, Dulbecco's modified Eagle's medium; DMF, Dimethylformamide; DMSO, Dimethyl sulfoxide; FcγR, Fcγ receptor; fMLF, N-formyl-Methionine-Leucyl-Phenylalanine; FPR, Formyl peptide receptor; GAG, Glycosaminoglycan; G-CSF, Granulocyte colony-stimulating factor; GDP,

Guanosine diphosphate; GFP, Green fluorescent protein; GM-CSF, Granulocyte-monocyte colony-stimulating factor; GMPs, Granulocyte-monocyte progenitor cells; GPCR, G protein-coupled receptor; GTP, Guanosine triphosphate; HIF, Hypoxia-inducible factor; HL-60, Human leukemia-60 cell line; iC3b, inactivated C3b; ICAM-1, Intercellular adhesion molecule 1; IFN-β, Interferon β; IL, Interleukin; IgG, Immunoglobulin G; iPSC, Induced pluripotent stem cell; KLF4, Kruppel-like factor 4; LDN, Low-density neutrophil; LOX-1, Lectin-type oxidized LDL receptor 1; LPS, Lipopolysaccharide; LTB4, Leukotriene B4; Mac-1, Macrophage-1 antigen; MAPK, Mitogen-activated protein kinase; MBL, Mannose-binding lectin; MDSC, Myeloid-derived suppressor cell; M-MDSC, Monocytic MDSC; MMP-9, Matrix metalloproteinase 9; MPO, Myeloperoxidase; MSU, Monosodium urate; NADPH, Nicotinamide adenine dinucleotide phosphate (reduced form); NDN, Normal-density neutrophil; NE, Neutrophil elastase; NET, Neutrophil extracellular trap; NF-κB, Nuclear factor kappa-light-chain enhancer of activated B cells; NOX2, NADPH oxidase 2; OCT4, Octamer-binding transcription factor 4; OLFM4, Olfactomedin 4; PAD4, protein arginase deiminase 4; PAF, Platelet-activating factor; PAK, p21-activated kinase; PBMC, Peripheral blood mononuclear cell; PBS, Phosphate-buffered saline; PI3K, Phosphoinositide 3 kinase; PIP₃, Phosphatidylinositol(3,4,5)-phosphate; PKC, Protein kinase C; PLC, Phospholipase C; PMA, Phorbol 12-myristate 13-acetate; PML, Promyelocytic leukemia; PMN-MDSC, granulocytic MDSC; PSGL-1, P-selectin glycoprotein ligand 1; RA, Retinoic acid; Rac, Ras-related C3 botulinum toxin substrate; Rap1, Ras-related protein 1; RARα, Retinoic acid receptor A; Rho, Ras homolog; ROCK, Rho-associated coiled-coil-containing protein kinase; ROS, Reactive oxygen species; RPMI-1640, Roswell Park Memorial Institute 1640; RUNX1, Runt-related transcription factor; SCF, Stem cell factor; SLE, Systemic lupus erythematosus; SOX2, Sex-determining region Y box-2; SPARC, Secreted protein acidic and rich in cysteine; TDM, Trehalose dimycolate; TGF-β, Transforming growth factor β; TNF-α, Tumor necrosis factor α; VE-cadherin, Vascular-endothelial cadherin; VEGF, Vascular-endothelial growth factor; VHL, Von Hippel-Lindau tumor-suppressor protein.

Author Contributions

MB, MG and SS wrote and revised the manuscript. All authors made substantial contributions to conception and design, analysis and interpretation of available literature;

took part in drafting the article or revising it critically; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no competing interests in this work.

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