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Review

Role of low density neutrophils in human health and disease

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ABSTRACT

Neutrophils, key cells of the innate immune system, are still regarded as a relatively homogeneous cell population with minimal discernible phenotypic and functional diversity. Amongst the reasons for this misconception is the fact that neutrophils are still largely defined by their morphology. In comparison, other immune cells, such as lymphocytes and macrophages, are now well known to form distinct subsets that differ morphologically and phenotypically. Recently, there has been a paradigm shift in the understanding of neutrophil diversity. The identification of low density neutrophils (LDN), a subpopulation of neutrophils initially described in patients with rheumatic diseases, has begun a new era in neutrophil biology research. The aim of this review paper is to describe LDNs in the context of a number of pathological and physiological conditions including systemic lupus erythematosus (SLE), cancer, human immunodeficiency virus (HIV), sepsis, asthma, and pregnancy. Within the context of each condition, this paper will discuss briefly the techniques used to isolate and characterize LDNs while focusing on the phenotype, function, proposed origin and physiological development of LDNs in each condition.

KEYWORDS: low density neutrophils, cancer, high density neutrophils, asthma, systemic lupus erythematosus, human immunodeficiency virus, sepsis, pregnancy

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INTRODUCTION

Historically, neutrophils (polymorphonuclear cells/ PMNs) have been viewed as phenotypically, functionally and morphologically uniform cells. Despite long-standing reports of neutrophil heterogeneity [1-3], neutrophils are still largely described as indiscriminate, suicidal, inflammatory first responders with minimal discernible phenotypic and functional diversity [4]. There has been a recent paradigm shift in the understanding of neutrophil diversity. A growing body of evidence is painting neutrophils as a plastic cell population with subsets capable of divergent physiological and pathological responses [5, 6]. Neutrophil variations have been identified under physiological conditions such as aging [7] and based on gender [8]. Furthermore, neutrophil subpopulations have been identified in a number of pathological conditions. For instance, in a murine cancer model, the presence of cancer was associated with specialized neutrophil subsets with a uniquely different transcriptional profile referred to as tumour associated neutrophils (TAN) [9]. Within the TAN family of neutrophils, two subsets were identified, namely N1 and N2 PMNs. N1 PMNs were created by the blockage of the transforming growth factor-β (TGB-β) and had an immune stimulatory and anti-tumour phenotype. N2 PMNs, which constituted the majority of TANs, were in turn immunosuppressive and pro-tumorigenic. Two unique neutrophil populations (PMN-I, PMN-II) have also been identified in a murine Methicillin-Resistant *Staphylococcus aureus* (MRSA) model [10]. PMN-I neutrophils are induced in MRSA-infected mice as a result of a mild secondary

systemic inflammatory response while PMN-II are induced in MRSA-infected mice as a result of a severe secondary inflammatory response. When compared to PMN-I neutrophils, PMN-II neutrophils have an immature nuclear morphology and express differing cytokines, chemokines, Toll like receptors (TLR) and adhesions molecules. These differences ultimately lead to impaired activation of macrophages resulting in inefficient anti-bacterial response and clearance of MRSA infection [10]. Neutrophil heterogeneity has also been identified in sepsis. Not surprisingly sepsis is associated with profound neutrophilia. However, despite the increase in circulatory neutrophils, septic patients are severely immune-compromised, prone to infections and often succumb to their illness [11]. Decreased immunity in the face of neutrophilia is suggestive of an altered function of circulating neutrophils during sepsis. In response to systemic endotoxins, a heterogeneous PMN population can be identified with an increase in the proportion of neutrophils characterized by high levels of cluster of differentiation (CD) markers, CD16, CD11b and CD11c and low levels of CD62L [12]. These cells displayed reduced interactions with opsonized bacteria, displayed high reactive oxygen species (ROS) production and were suppressive to T-cell proliferation [12].

Of all neutrophil subsets, the subpopulation garnering the most recent attention are low density neutrophils (LDN), interchangeably known as low density granulocytes (LDG). An important milestone in the identification and characterization of LDNs was a discovery made by Hacbrath *et al*. in 1986 [13]. In this study, the authors generated density gradient preparations of whole blood from patients with systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and acute rheumatoid fever (ARF). Upon examination of peripheral blood mononuclear cells (PBMC), the authors reported the presence of low buoyant neutrophil "contaminants" amongst the PBMC. To our knowledge this is the first known reference of LDNs in neutrophil biology. They attributed these findings to the presence of humoral factors in diseased patients' plasma that were capable of activating neutrophils resulting in degranulation, increase in cell volume and a subsequent decrease in cell density [13]. Since this initial report, LDNs have been identified in a variety of physiological and pathological conditions. Unfortunately, there is little consistency in the scientific literature regarding the isolation technique, source, function and origin of LDNs (Table 1). The aim of this review paper is to describe LDNs in the context of a number of pathological and physiological conditions including SLE, cancer, human immunodeficiency virus (HIV) infections, sepsis, asthma, and pregnancy. Also we discuss the current progress in isolation of LDNs, their phenotypic characterization and different proposed models of their physiological origin in the various disease states.

Neutrophil maturation and LDNs

According to the monophyletic theory of haematopoiesis, all hematopoietic cells including neutrophils originate in the bone marrow from a single pluripotent hematopoietic progenitor stem cell [14]. Neutrophil development and differentiation can be divided into six stages according to cell size, nuclear morphology, granule content and mitotic activity [15]. The most primitive neutrophil precursors are the myeloblasts. Myeloblasts are proliferative, mitotic, non-secretory and undifferentiated cells that account for only 2% of cells found in the bone marrow. Myeloblasts are characterized by their large loosely packed euchromatic nuclei, prominent nucleoli and scant basophilic cytoplasm packed with mitochondria but devoid of granules. The myeloblast stage is followed by two secretory stages, the promyelocyte and myelocyte stage. Promyelocytes, account for 5% of bone marrow cells and are the largest cells in the neutrophil development series (~15 um). Promyelocytes are mitotic, secretory cells characterized by their rounded nuclei and enhanced secretory machinery. The characteristic feature of promyelocytes is the production and presence of peroxidase-positive azurophil or primary granules. Myelocytes represent the last proliferative and secretory cell in the neutrophil development lineage and account for nearly 12% of all cells in the bone marrow. Myelocytes can be differentiated by their relative small size (~10 um), their indented nuclei and prominent Golgi complex. However, the most characteristic feature of myelocytes is the large number of granules that include the peroxidase-positive azurophil granules

Table 1. Summary of most of the literature regarding the isolation technique, source, function and origin of LDNs.

Author	Samples	Density centrifugation medium	Antibody selection	LDN density (g/mL)	LDN %	LDN features	LDN induction	LDN origin
Hacbarth *et al.* 1986	RA SLE ARF	Ficoll/Hypaque	No	1.076	N.R.	N.R.	Diseased humoural factors	Activation, Degranulation
Morqbel *et al.* 1987	Healthy	Ficoll/Hypaque + MTZ	No	1.1012-1.1067	N.R.	↑ CR1 ↑ CR3/CD11b	fMLP (1^{-8}M)	Activation, Degranulation
Morisaki *et al.* 1992	Sepsis, Healthy	Percoll	No	1.07-1.08	4-40%	↓ Chemotaxis ↓ β-glucuronidase activity ↓ CD10	Circulating activating factors	Activation, Degranulation
Berends *et al.* 1994	Healthy	Percoll + Dextran sedimentation	$CD16^{+ve}$	< 1.090	17-90%	↑ CD11b ↑ Lactoferrin release	Erythrocyte dextran sedimentation,	Activation, Degranulation
Schmielau *et al.* 2001	Cancer, Healthy	ICN	$CD15^{+ve}$	N.R.	N.R.	N.R.	Cancer, fMLP (10^{-8}M - 10^{-6}M)	Activation
Bennet *et al.* 2003	Paediatric SLE, JCA, Healthy	No	$CD14^{-ve}$	N.R.	N.R.	N.R.	Active SLE, IFN-γ	Granulopoesis, Immature neutrophils
Denny *et al.* 2010	SLE, Healthy	Ficoll/Hypaque	$CD10^{+ve}$ $CD15^{+ve}$ $CD16^{+ve}$ $CD14^{-ve}$ $CD33^{-ve}$ $CD66b^{+ve}$ $CD11b^{+ve}$	N.R.	17%	Endothelial cytotoxicity, ↑ Type I IFN & TNF-α, secretion, ↓ Phagocytosis	SLE	Unique neutrophil subset
Brandau *et al.* 2011	HNC, LC, BUC	LSM	$CD66^{+ve}$ $CD15^{+ve}$ $CD11b^{+ve}$ $CD33^{+ve}$ $CD14^{-ve}$ $CD15^{-ve}$ $CD125^{-ve}$	N.R.	2-10%	↓ Apoptosis, ↑ Survival ↓ Oxidative burst, ↓ Chemotaxis	Cancer	Immature neutrophils

Table 1 continued..

Cloke *et al.* 2012	HIV	Histopaque 1077	$CD3^{-ve}$ $CD14^{-ve}$ $CD15^{+ve}$ $CD11b^{high}$ $CD66b^{high}$ $CD33^{high}$ $CD16^{low}$ $Arginase^{low}$	< 1.077	N.R.	N.R.	HIV	Activated mature neutrophils
Ssemaganda *et al.* 2014	Pregnancy	Histopaque 1077	$CD15^{high}$ $CD66b^{high}$ $CD15^{high}$ $CD63^{high}$ $CD33^{high}$ $CD16^{low}$ $Arginase^{low}$	< 1.077	N.R.	↓ Arginase 1 ↑ CD66b ↑ CD15 ↑ CD63 ↑ CD33 ↓ CD16	Pregnancy	Activated mature neutrophils
Fu *et al.* 2014	Asthma	LSM	$CD15^{+ve}$ $CD16^{+ve}$ $CD66b^{+ve}$ $CD11b^{+ve}$ $CD33^{-ve/low}$ $CD14^{low}$	N.R.	7.5%	↓ CD14 ↓ CD33 ↑ CD66b ↑ CD11b ↓ CD16	Severe asthma	Activated mature neutrophils
Sagiv *et al.* 2015	Cancer	Histopaque	$CD11b^{+ve}$ $CD66b^{+ve}$	< 1.077	18.8%	↑ CD11b ↓ CD66	Cancer	Activated mature neutrophils, Plasticity

RA = Rhematoid Arthritis, SLE = Systemic Lupus Erythematous, ARF = Acute Rheumatic Fever, N.R. = Not Reported, MTZ = Discontinous Metrizamide, fMLP = formyl-methionyl-leucyl-phenylalanine, CR = Complement Receptor, LCN = Lymphocytes Seperation Medium, JCA = Juvenile, Chronic Arthritis, HNC = Head and Neck Cancer, LC = Lung Cancer, BUC = Bladder or Ureter Cancer, LSM = Lymphocyte Seperation Medium.
CD66b – respiratory burst, adhesion, specific granule marker; CD15 – cell-cell interaction, phagocytosis, degranulation, respiratory burst, PMN marker; CD63 – azurophilic granule marker; CD33 – adhesion, immature PMN marker; CD16 – degranulation, marker of mature PMNs; CD14 – monocyte marker, some PMNs have low CD14 expression.

noted in the promyelocytes as well as newly produced smaller peroxidase-negative, specific or secondary granules. The presence of the prominent secretory complex is what differentiates the secretory neutrophil precursors, namely promyelocytes and myelocytes from the later non-secretory progeny namely metamyelocytes, band and segmented neutrophils. Metamyelotcytes represent the first stage at which neutrophils can be morphologically differentiated from basophils and eosinophils. Metamyelocyte are characterized by a cytoplasm packed with hundreds of granules, the majority of which are specific granules as opposed to azurophilic (2:1 ratio), and a dense nucleus with a distinctive kidney-bean indentation. The metamyelocyte stage is followed by the band and segmented stages. Band neutrophils are characterized by an elongated, horseshoe-shaped nucleus. As maturation continues, the nuclear constriction continues, ultimately leading to the formation of two to four nuclear lobes resulting in the formation of a mature, segmented polymorphonuclear neutrophil. The post mitotic phase of neutrophil maturation spans from metamyelocyte stage to the segmented neutrophil, lasts approximately 7 days and accounts for ~22% of all bone marrow cells [14-16]. Normally only mature PMNs are released into circulation from the bone marrow (Figure 1). As a result, under healthy physiological conditions, band and other immature neutrophil phenotypes represents less than 3% of circulatory neutrophils [16-20]. Neutrophilia, induced by acute inflammation accelerates neutrophil recruitment from the bone marrow, resulting in the depletion of basal PMN reserves in the bone marrow. To compensate for the increase in demand, the post-mitotic maturation time is decreased from 7 days to 4 days with an increase in the release of functionally competent yet morphologically immature neutrophils [21]. In the face of chronic systemic conditions such as SLE and cancer, the increased inflammatory load could necessitate the need for emergency granulopoiesis, thus resulting in a relatively increase proportion of immature neutrophils founds in circulation (Figure 1).

Activation of mature neutrophils as indicated phenotypically by an increase in the expression of CD11b and CD66, results in sequential release of neutrophil granules. Neutrophil granules serve as intracellular stores of bactericidal and proteolytic proteins and enzymes. During neutrophil development, granules first appear in the transition from myeloblast to promyelocyte stage and granulation continues until neutrophil maturation is complete [22]. Structurally, four granule subsets, including azurophil (or primary), specific (or secondary), gelatinase (or tertiary) and secretory vesicles have been described [23]. Azurophil granules are the lysosomes of neutrophils and contain myeloperoxidase (MPO), defensins and acid hydrolases. Specific granules are smaller and more numerous than the azurophil granules. Specific granule proteins are only synthesized at the myelocyte stage and consist of bacteriostatic and bactericidal agents including proteolytic enzymes and complement activators. As their name suggests, gelatinase granules are packed with gelatinases, phosphates and matrix metalloproteinases (MMPs). Gelatinase granule contents are formed at the metamylelocyte stage and neutrophils use their targeted release to facilitate tissue migration. Secretory vesicles are the last neutrophil granules to be formed by endocytosis [24]. Neutrophils have been shown to display granule heterogeneity with respect to function and propensity of exocytosis [24]. Following an exocytotic trigger, neutrophils do not indiscriminately and simultaneously release all granule contents. It's more prudent for neutrophils to grade their response according to the situation. In response to a mild inflammatory stimulus, neutrophil degranulation is marked by the release of gelatinase and secretory granules. Only a sustained exocytotic stimulation allows for the release of the tissue destructive specific and azurophil granules [24, 25]. The release of the intracellular granules following neutrophil activation naturally results in the decrease in neutrophil density, thus resulting in the co-purification of PMNs with PBMCs (Figure 1).

Techniques for isolation and identification of LDNs

Density centrifugation

Since the defining property of LDNs is a reduced buoyant density compared to conventional high density neutrophils (HDNs) or normal density

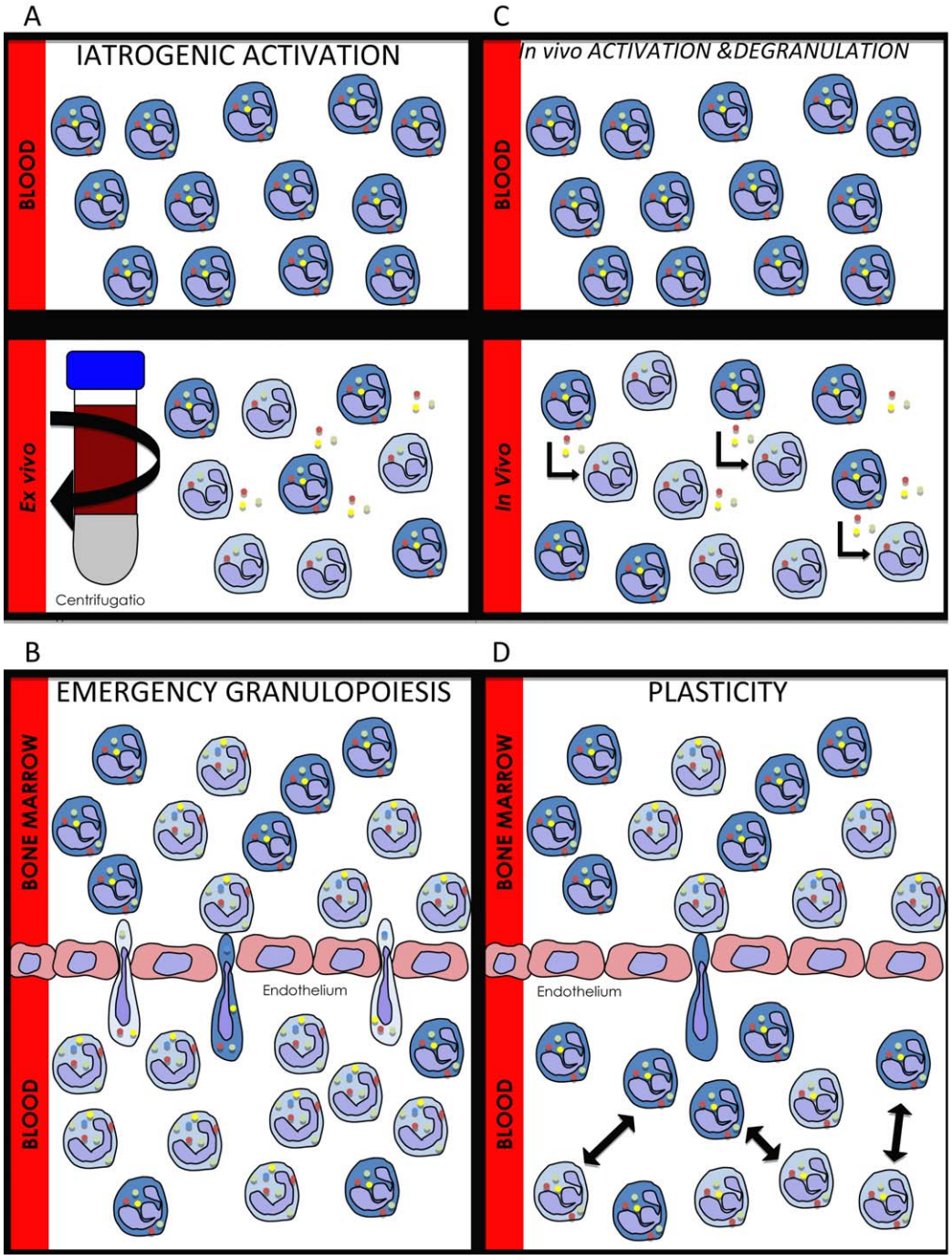

Figure 1. A) LDNs can be induced *in vitro* simply as a consequence of handling and purification procedures. Sequential centrifugation and washes have been shown to activate HDNs, resulting in degranulation and generation of LDNs. **B)** In circumstances with increased demand for circulatory neutrophils immature neutrophils can be recruited into circulation prior to granule and nuclear maturation resulting in a relative increase in LDN proportion. **C)** LDNs can also be generated *in vivo* as a result of activation of HDNs while in circulation in response to humoral activating factors. Degranulation of circulatory HDNs will result in a relative increase in LDNs. **D)** It is possible that under certain conditions such as cancer, there may be a real time plasticity of HDNs into LDNs *via* degranulation and LDN conversion into HDNs *via de novo* granule formation. This concept has only been shown in murine cancer model and has yet to be demonstrated in humans.

neutrophils (NDNs), the use of centrifugation on discontinuous density gradients is fundamental to their isolation and characterization (Figure 2). Density centrifugation can be used in multiple configurations to isolate a variety of cell types. The basis of density centrifugation is the use of a medium with a range of banding densities. Once cells of varying inherent densities are loaded and centrifuged within a centrifugation medium, cells will localize to a point where their density precisely coincides with the density of the surrounding solution. Commonly used mediums in density centrifugation include Percoll, Ficoll and Histopaque [25]. These media are generally used due to their biocompatibility and densities that are ideal for isolation of leukocytes, monocytes and granulocytes. Layering solutions of different densities generates discontinuous gradient solutions, which can be been used to isolate neutrophils and PBMCs. For instance, when a dichotomous gradient composed of 57% Percoll v/v in PBS (density of 1.075 g/mL) layered over 67% Percoll v/v in PBS (density of 1.088 g/mL) is used to isolate PMNs, PMNs will localize to a band between the 57% and 67% Percoll layers, while the lower density PBMCs will converge above the 57% Percoll layer but below the plasma band [25]. Percoll solutions have also been used to separate different PMN subpopulations. Morisaki *et al*. (1992) used Percoll centrifugation to isolate PMN subtypes in patients experiencing a bacterial infection. Using this technique, PMNs with varying densities could be separated into three subpopulations: high-density subpopulations (1.09-1.10 g/mL), intermediate-density subpopulations (1.08-1.09 g/mL) and low-density subpopulations (1.07-1.08 g/mL). Even though the variance between densities appears to be minimal, different neutrophil subpopulations seem to have significant differences regarding their functions in the body [18]. Few other authors differentiate between low and intermediate density neutrophils [3]. As a general rule, neutrophils with a density of less than 1.090 g/mL are considered to be LDNs, while those with a density of greater than 1.090 g/mL are considered normal or HDNs [26] (Figure 2). Another gradient solution commonly used in density centrifugation is Ficoll-Hypaque, prepared by the mixing of Ficoll 400 and isopaque to create a solution with a density of 1.077 g/ml [25]. Similarly to Percoll, centrifugation of whole blood on a Ficoll-Hypaque gradient isolates PBMCs and platelets from granulocytes and erythrocytes by virtue of their lower density. PBMCs are identified as a layer of cells located at the interface of the Ficoll-Hypaque gradient while all other leukocytes, including neutrophils, sediment with erythrocytes. Although routinely used for leukocyte isolation, reports in the literature indicate that Ficoll may activate native neutrophils [27]. Haslett *et al*. (1985) experimentally demonstrated that Ficoll purification altered neutrophil morphology, increased oxidative activity and reduced chemotactic response due to the resemblance of Ficoll to bacterial LPS [28].

CD markers

A group of antibody clones that all recognize the same cell surface protein can be said to cluster together. A CD marker is defined as a specific cohort of antibody clones that define a single cell surface protein. The CD marker system allows for unambiguous identification of a specific cell type based on the unique set of cell surface proteins that are involved in signalling, adhesion, and metabolism [29]. Often CD markers are expressed in more than one cell type. The analysis of a heterogeneous cell population with the goal of discerning the phenotypes and subpopulations that are present is called immunophenotyping [30]. Through the use of several monoclonal antibodies with different fluorophores along with different gating strategies it has become possible to accurately detect specific cell populations and subpopulations [31]. A number of different CD markers have been used to classify the developmental stages of immune cells in humans [32]. The identification of purified populations of neutrophils and its precursors can rely on cell size and granularity, as well as on the expression profile of CD13, CD15, CD11b and CD16 surface markers. CD13 shows dynamic changes in expression during granulocytic differentiation. In combination with CD11b and CD16, these changes define the sequential stages of granulopoiesis. CD13 expression is up-regulated on myeloblasts and promyelocytes and then down-regulated on myelocytes. CD13 expression is gradually up-regulated again as neutrophils reach their final stages of differentiation and develop into segmented neutrophils. On the other hand, CD11b and CD16, which are initially

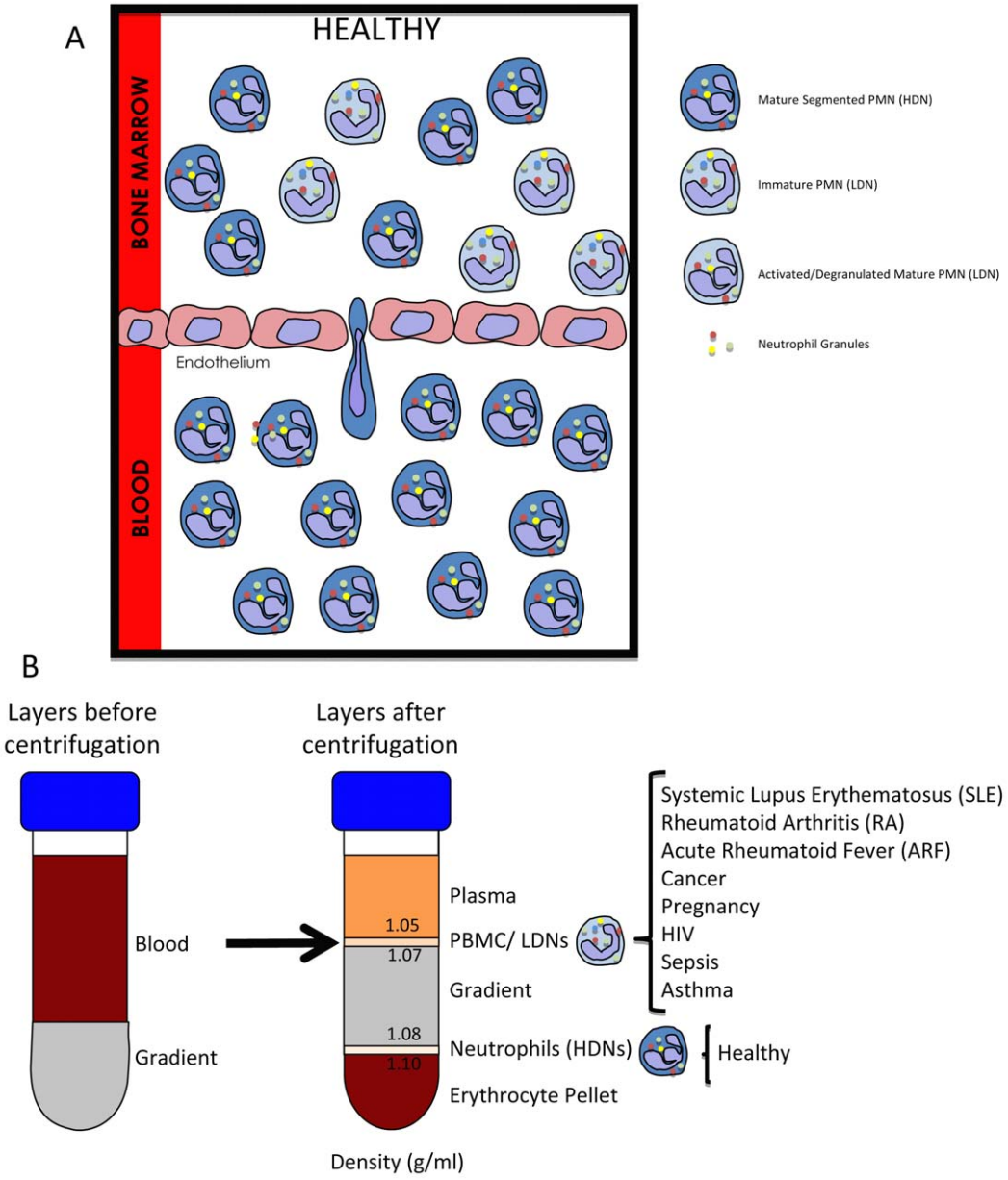

Figure 2. Density, phenotype and origin of LDNs. A) Representative schematic of neutrophil proportion in the bone marrow and blood in health. Under physiological conditions the bone marrow is populated by neutrophils at various stages of development with varying granule content and nuclear morphology. In health only mature neutrophils, characterized by a full complement of granules and segmented nuclear morphology are deployed out of the bone marrow into the blood stream. Due to a lack of activating humoral factors and absence of chronic inflammation or acute infections, activated mature neutrophils and immature neutrophils (or LDNs) are rarely observed in circulations. **B)** Schematic representing the effect of density centrifugation in high and low density neutrophils. When peripheral blood is layered on top of a density gradient medium and centrifuged, cells of varying densities will rest in bands corresponding to their inherent densities. The higher density cells will be found lower along the column while lower densities cells will be found higher along the column. In health, most neutrophils are characterized as high density (or HDNs), as most neutrophils will be localized to a band above the erythrocyte pellet but below the gradient band, corresponding to a density of > 1.09 g/ml. Under various pathological conditions, the proportion of neutrophils that co-purify with the lower density PBMCs (or LDNs) increases. LDNs are characterized by a density of < 1.09 g/ml.

expressed at low levels, show progressively increased expression during the developmental process, particularly in the later stages of neutrophil differentiation [32-34].

A study by Kuijpers *et al*. (1991) showed that expression of PMN surface molecules could be influenced by cell purification procedures (Figure 2) [35]. Also, surface expression of several antigens that were expressed on circulating neutrophils increased significantly after density-gradient centrifugation. The isolation method caused increased expression of CD13, CD16, CD18, CD45, and CD67 but did not alter CD32 (FcRII), CD54 (ICAM-I), CD58 (LFA-3), Leu-8 and human leukocyte antigen (HLA) class I antigen expression [35]. Upregulation of antigens was determined by stimulation of purified neutrophils. Upregulation of CD63 was an excellent marker for release from azurophil granules [35].

The role of LDNs in disease

Systemic lupus erythematosus

SLE is a systemic autoimmune disease resulting in widespread chronic inflammation that clinically manifests in multiple tissues including the skin, vascular system, central nervous system and renal system. SLE is characterized by the accumulation of auto-antibodies against double stranded DNA, histones, nucleosomes and other nuclear components that ultimately results in the activation of the complement system, the innate immune system, the adaptive immune systems and development of auto-immunity [36]. Despite a truly enormous body of literature on SLE dating back to the 1950s, we still do not fully understand the pathogenesis of the auto-immunity in SLE nor do we understand all the clinical manifestations of SLE. This lack of knowledge has prevented the development of clear diagnostic criteria and adequate interventions. Neutrophils have been implicated in the pathogenesis of SLE [37], and LDNs were identified in SLE patients as early as 1986 [13]. The early studies on LDNs in SLE attributed the presence of LDNs to the effects of diseased blood humoral factors that activate mature normal density neutrophils resulting in degranulation, increase in cell volume and a subsequent decrease in cell density [13]. In a 2003 study, Bennett *et al*. showed that the gene expression pattern of PBMCs of patients with active SLE is characterized by the over-expression of interferon (IFN)-regulated and granulopoiesis-specific genes. Further, this unique genomic signature was independent of age, gender and ethnicity and was lost after remission or following intervention with high doses of intravenous steroids. The authors also identified a concurrent increase in LDNs in the PBMC fraction of SLE patients. The presence of low-density yet highly granular contaminants of the PBMCs was attributed to the predominance of immature PMNs [38]. This is the first report of immature PMNs in SLE and is in stark contrast to earlier reports by Hacbarth *et al*. (1986) who attributed the origin of LDNs to the activation and degranulation of normal density neutrophils (Table 1). This variation may be due to the different patient populations used in the study. The Bennet study was performed on an ethnically heterogeneous patient population in Dallas, Texas with an average age of 13 years. Hacbarth study was performed in Brazil but patient demographics were not reported. Further, the isolation of LDNs differed significantly between the two studies. While Hacbarth *et al*. relied solely on Ficoll separation to isolated LDNs, Bennet *et al*. relied on CD markers and flow cytometry to identify LDNs [13, 38].

Starting in 2010 the Kaplan group from the University of Michigan's Division of Rheumatology released several comprehensive reports on SLE that greatly enhanced our understanding of SLE LDNs. Through these publications the authors described a novel negative selection protocol for LDN isolation and provided a detailed examination of SLE LDN morphology, function and origin [19, 39]. The Michigan group's LDN isolation protocol combined density centrifugation and the use of specific surface markers with flow cytometry. The purification protocol begins with the isolation of peripheral blood and density centrifugation on a Ficoll/Hypaque column, allowing for the isolation of PBMCs from granulocytes and erythrocytes. PBMCs are incubated with an LDN isolation antibody cocktail consisting of CD3, CD7, CD19, CD79b, CD56, major histocompatibility complex II (MHCII), CD86 and CD235a. This antibody cocktail contains specific surface markers unique

to B and T lymphocytes, monocytes, natural killer (NK) cells, and remnant erythrocytes. Magnetic beads are then used to select for antibody-coated cells thus allowing for the negative selection of granulocytes. This unique approach is significantly more accurate at eliminating cellular contaminants with densities similar to LDNs. In addition, by utilizing a combination of flow cytometry and surface antigen expression profile, the researchers were able to further characterize the phenotype of LDNs in SLE. Using this more stringent isolation protocol, the authors once again confirmed the presence of LDNs in the PBMC fraction of adult patients suffering from SLE. LDNs contributed to less than 5% of PBMCs in healthy individuals and increased to 17% in SLE with a range between 1.2-54%. SLE patients with LDN proportions similar to healthy individuals (< 5%) lacked clinical manifestations such as vasculitis and synovitis, while over 80% patients with elevated LDN counts exhibited clinical symptoms. Morphologically, 60% of SLE LDNs had a polymorphonuclear morphology characteristic of mature PMNs. The remaining 40% of LDNs had a premature morphology with band, lobular or myelocyte-like nuclei, had abundant granules and lacked evidence of vacuolization or apoptosis [39]. In 2011, the Michigan group also presented quantitative polymerase chain reaction (qPCR) data showing that SLE LDNs expressed elevated mRNA levels of bactericidal proteins typically present in neutrophil primary granules that are most highly expressed during the early promyelocytic stages of neutrophil differentiation in the bone marrow [40]. These results support the Benett *et al*. (2003) hypothesis that SLE LDNs are neutrophils with an immature phenotype [38]. Paradoxically however, the Michigan group also found that SLE LDNs display a surface antigen pattern consistent with mature neutrophils characterized by high expression levels of CD15, CD16, CD10, CD31, CD11c, CD114 and low expression levels of CD115, CD123, the monocyte marker CD14 and the immature PMN marker CD33. SLE LDNs also displayed elevated levels of neutrophil activation markers CD66b and CD11b [19]. Taken together, these findings support the Hacbarth *et al*. (1986) hypothesis that LDNs represent a subpopulation of *in vivo*-activated mature PMNs [13]. Functionally, the authors found LDNs to be pro-inflammatory neutrophils characterized by increased IFN-γ expression, increased IFN-γ and tumor necrosis factor (TNF-α synthesis), increased endothelial cell toxicity, reduced phagocytic capacity and unaltered levels of intracellular bactericidal activity and MPO expression. Further, SLE LDNs displayed significantly enhanced NETosis [40, 41], while the serum of SLE patients are characterized by impaired NET clearance caused by a dysfunction in DNase I activity. LDNs with an increased propensity for NETosis and secretion of immunoactive cytokines in combination with impaired NET clearance in SLE patients may contribute to the clinical outcomes of SLE and prolonged autoimmune events. This series of studies provided evidence to support both the hypotheses of SLE LDN origin. Together these findings probably suggest that SLE LDNs are most likely neither purely activated nor purely immature PMNs, but rather represent a heterogeneous subset of neutrophils with unique phenotype and function [37].

HIV

To date, the only report on the role of LDNs in HIV is produced by a single group from the United Kingdom [42-44]. The work into LDNs by Kropf's group was initially prompted by findings of increased arginase activity in PBMCs of HIV-infected individuals with low $CD4^{+ve}$ T cell counts [44]. Centrifugation of peripheral blood of seropositive HIV patients on Histopaque 1077 yielded a PBMC fraction that contained LDNs. The relative percentage of LDNs in the PBMC fraction was positively correlated to viral load and inversely correlated to $CD4^{+ve}$ T cell counts, suggesting that LDN proportion increases with disease progression and severity [42]. Further, NDNs but not LDNs became progressively activated with disease progression as measured by viral load and $CD4^{+ve}$ T cell count [43]. Morphological analysis of HIV LDNs using light microscopy revealed cells with a mature phenotype consisting of segmented neutrophils similar to NDNs. HIV LDNs had higher surface expression levels of CD11b, CD15, CD33 and CD66b but lower surface expression levels of CD16 compared to autologous NDNs. It is difficult to draw conclusions regarding the origin

of HIV LDNs based on the reported expression profile. The elevated CD33 and decreased CD16 expression are suggestive of an immature phenotype, while increased CD11b (a marker of secretory vesicles), CD15 and CD66b (a marker of specific granules) are suggestive of an activated mature phenotype. However, HIV LDNs displayed increased CD63 surface expression and decreased arginase expression. Arginase is stored intracellularly in azurophilic granules that can be distinguished from other granules based on the expression of CD63 [45]. Upon activation, neutrophils degranulate and release their contents to the extracellular space. Although the possibility of a heterogeneous population of immature and activated mature LDNs cannot be fully accounted, the combination of the segmented nuclear morphology of HIV LDNs as well as the concurrent increase in CD63 expression with a decrease in arginase expression provides compelling evidence that HIV LDNs are activated mature PMNs [46].

Cancer

Research into the role of PMNs in cancer progression and resolution has been predominated by murine studies which have established that neutrophils can have both protumor [47, 48] and antitumor or anti-metastatic functions [9, 49]. Our understanding of the role of PMNs in the progression and resolution of human cancer is limited. Schmielau *et al*. (2001) published the first report of LDNs in the field of human cancer [50]. Although the report never explicitly identified LDNs as a unique population of cells, they did describe activated granulocytes of low density that co-purified with PBMCs. When examining the peripheral blood samples of patients with metastatic adenocarcinomas of colon and breast, the researchers noticed significant contamination (up to 50%) of PBMCs with $CD15^{+ve}$ LDNs. Under normal conditions, the blood of healthy patients was devoid of such contaminants. However, *in vitro* incubation of healthy donor blood with *f*MLP (10^{-8}-10^{-6} M) was capable of inducing the aberrant neutrophil co-purification in a dose-dependent manner. The authors attributed the presence of neutrophils in the PBMC fraction to the *in vivo* activation of neutrophils as indicated by elevated plasma levels of an oxidative stress marker. In a large multicentre study from Germany, Brandau *et al*. (2011) described a subset of granulocytes that co-purified with PBMC in density gradients in the peripheral blood of patients suffering from head and neck cancer, lung cancer or cancers of the bladder and ureter [51]. In addition to the co-purification with PBMCs, LDNs were further characterized by the expression of the activation marker CD66b [52], as well as the immature marker CD33. Morphologically, cancer LDNs resembled immature cells with a nuclear morphology consistent with various stages of neutrophil maturation. Further, $CD66b^{+ve}/CD33^{+ve}$ cancer LDNs consisted of three cohorts with varying CD16 and CD11b expression ($CD16^{+ve}/CD11b^{+ve}$, $CD16^{-ve}/CD11b^{+ve}$ and $CD16^{-ve}/CD11b^{-ve}$). Together, these findings suggest that cancer LDNs are comprised of immature neutrophils at different stages of neutrophil maturation. In this study, cancer LDNs constituted 2-10% of all PBMCs in the peripheral blood of all patients. LDNs were virtually absent in the peripheral blood of healthy donors. Cancer LDNs displayed reduced apoptosis, chemotaxis, oxidative burst activity and cytokine secretion, a finding consistent with their immature phenotype. Further, these cells were capable of suppressing T-cell proliferation and IFN-γ production, and thus may be contributing to the peripheral immunosuppression typically noted in cancer patients [51].

Perhaps the biggest contribution towards deciphering the role of LDNs in human cancer was recently published by Sagiv *et al*. (2015). In this study, the authors confirmed the presence and accumulation of LDNs during transient inflammation induced in mice as well as cancer bearing mice and humans [53]. The authors were able to demonstrate that LDNs are composed of two morphologically distinct subsets that are distinctly regulated. Further, the authors showed that with tumour progression NDNs, which typically display an antitumor phenotype and function, are pushed towards a more pro-tumor phenotype and function typical of LDNs, in a TGF-ß-dependent manner. LDNs were virtually absent in the peripheral blood of healthy patients but were significantly enriched (up to 50%, average 18.8%) in the peripheral blood of patients with advanced stage IV lung or breast cancer.

Cancer LDNs also had elevated activation markers as noted by increased expression of CD11b and CD66b. Similar to previous reports in other pathological systems, morphologically, cancer LDNs consisted of both mature and immature (band nuclear morphology) neutrophils. LDNs consists of both immature myeloid-derived suppressor cells (MDSCs) and mature cells that are derived from HDNs in a TGF-ß dependent mechanism [53]. What sets this publication apart from other LDN publications is that the authors attempted to decipher the origin of the heterogeneous LDN populations instead of simply describing them. The authors performed murine bromodeoxyuridine (BrdU) pulse chase experiment that allowed them to quantify the rate of newly generated LDNs and HDNs. In this murine cancer model, within 24 hours nearly 50% of all new LDNs were $BrdU^{+ve}$ while only 3% of HDNs were $BrdU^{+ve}$. By 48 hours, nearly 50% of HDNs were $BrdU^{+ve}$. This suggests that when challenged by cancer, immature LDNs quickly leave the bone marrow prior to complete maturation, while HDNs require an additional day to reach complete maturation before being released from the bone marrow. Also, the authors injected BrdU-labelled LDNs and HDNs into label-free tumour-bearing mice and found that within 3 hours 40% of HDNs transferred into LDNs while 10% of LDNs transferred into HDNs, providing the first evidence of neutrophil plasticity in cancer [53]. While these results are cause for great advancement in the field of neutrophil and cancer biology, further studies are necessary to determine the role of LDNs in human cancer.

Sepsis

Neutrophil dysfunction and morphological alterations have been noted in septic patients and the severity of the functional deficits has been correlated to the severity of infection [54]. LDNs have also been reported in septic patients. Morisaki *et al*. (1992) prepared Percoll density gradient from the peripheral blood of 12 patients suffering from severe acute sepsis, seven with moderate infections and ten systemically healthy patients. Density centrifugation yielded three distinct neutrophil subpopulations: HDNs (density 1.09-1.10 g/mL), intermediate density neutrophils (IDN) (density 1.08-1.09 g/mL) and LDNs (density < 1.08 g/mL). In healthy patients, 76 ± 9% of all neutrophils were HDNs while LDNs constituted only 4 ± 3% of all neutrophils. Conversely those patients with severe infections only had 8 ± 6% HDNs while 40 ± 10% of neutrophils were LDNs. Unfractionated neutrophils isolated from patients with severe infection displayed reduced β-glucuronidase activity as well as reduced chemotactic response towards bacterial factor and activated serum. These findings correlated with decreased intracellular enzymatic activity and chemotactic ability of LDNs when compared to HDNs. In addition, the noticeably fewer intracellular granules and multiple vacuoles that characterize LDNs isolated from septic patients is suggestive of an active or "spent" phenotype [18]. Lastly, significantly fewer $CD10^{+ve}$ neutrophils were isolated in patients with severe infections when compared to healthy individuals while the percentage of $CD10^{+ve}$ neutrophils gradually increased with sepsis treatment. CD10 expression is acquired in the maturation phase of neutrophils in the bone marrow [55-57] and is reported to be involved in down regulating the inflammatory response [58]. The low basal level of $CD10^{-ve}$ in healthy individuals is likely necessary to allow the body to mount an effective and immediate immune response to bacteremia and infections. The increased number of $CD10^{-ve}$ neutrophils in burn patients can be attributed to the increased demand for neutrophils resulting in insufficient maturation time in the bone marrow. Also, decreased expression of CD10 may be a negative feedback or compensatory mechanisms to prevent uninhibited inflammatory reactions. Consistent with this finding, McCormack *et al*. (1987) identified a population of $CD10^{-ve}$ neutrophils that constituted 5% of all neutrophils in healthy individuals [55] and enriched in thermal burn patients [57]. Paradoxically, and unlike $CD10^{-ve}$ LDNs of septic patients, $CD10^{-ve}$ neutrophils in healthy patients displayed enhanced chemotaxis in response to activated complement [55]. Unfortunately, McCormack *et al*. did not perform density centrifugation on healthy $CD10^{-ve}$ cells, thus making a direct comparison to septic LDNs difficult. All together, these findings suggests that the defect associated with neutrophil dysfunction in septic patients is due to the relative increase in

blood LDNs. Proportional increase in the LDN fraction during sepsis is likely caused by the activation and degranulation of HDNs in response to circulating factors such as complement products and bacterial endotoxins, as well as a concurrent increase in the demand for neutrophils from the bone marrow resulting in an increase in immature circulating neutrophils [59] (Figure 1).

Asthma

Neutrophils have been implicated in the pathophysiology of asthma and the presence of neutrophils in nasal sputum can be diagnostic of severe asthma [60]. However, to date only a single study has reported and examined LDNs in human subjects with persistent asthma [61]. Fu *et al*. noted an increase in the numbers of $CD16^{+ve}$ granulocytes in the PBMC fraction of asthmatic patients following centrifugation of peripheral blood of patients on lymphocyte separation medium (LSM). Asthma LDNs displayed high expression levels of CD15, CD16, CD11b, CD66b and low expression of CD14 and CD33. LDNs displayed significantly increased CD15, CD11b and CD66b expression when compared to NDNs isolated from the same patients. No difference in CD33 and CD14 expression was noted among LDNs and NDNs from asthmatic patients. Together these findings are suggestive of activated and mature phenotype. Despite the lack of increase in CD33 expression, a combination of immature neutrophils with band nuclear morphology as well as segmented neutrophils were noted upon microscopic evaluation. The authors did not perform further experimentation to rule out the possibility of a heterogeneous LDN population. LDNs constituted less than 2% of all PBMCs of healthy controls. No increase in LDNs was noted in patients with mild asthma. Similar to other pathological conditions such as SLE, HIV and sepsis, with increasing disease severity the LDN proportion increased to as high as 39% with an average of 7.4% in individuals with moderate to severe asthma. Unlike the LDN number, which increases with disease severity, the authors failed to show a similar trend with CD11b and CD66b expression, suggesting that only LDN levels and not LDN phenotype/ activation change as a function of asthma severity. Due to the paucity of robust clinical data it is difficult to demonstrate a functional or causal role of LDNs in the pathogenesis and prognosis of asthma.

Pregnancy

Unlike all other conditions discussed to this point, pregnancy is a normal physiological condition as opposed to a pathological condition. During pregnancy, women experience altered immunity characterized by increased systemic inflammation, neutrophilia, decreased peripheral NK cells and increased number of regulatory T cells. Together these changes induce an immunosuppressive state that allows for the successful implantation and development of the fetus [62]. Much like in HIV, an increase in arginase has been noted in maternal peripheral blood. This increase has been attributed to the presence of arginase secretion by LDNs that co-purify with PBMCs following density centrifugation [63]. Compared to NDNs isolated from maternal and cord blood, LDNs expressed higher levels of CD63, CD33 CD15 and CD66b as well as lower arginase levels. Together this is suggestive of an activated and degranulated phenotype. Interestingly, the blood isolated from the placentae only held LDNs. They also showed that the LDNs in cord and maternal blood showed different levels of maturation, activation, and degranulation markers, suggesting that there are multiple LDN phenotypes.

CONCLUSION

The concept of the low density neutrophil is not novel. LDNs were first reported in the literature nearly three decades ago [13]. An increase in research into neutrophil heterogeneity has led to a recent surge of interest in LDNs as indicated by a near three-fold increase in the number of publications in the last 15 years. Despite the recent interest, the LDN phenomenon is still poorly understood. The confusion regarding the nature, purpose and function of LDNs stems from the fact that LDNs have only been studied in the context of specific conditions. Much of what we know about LDNs is derived from studies in patients with SLE, HIV and cancer [13, 33, 43]. This focused approach does not allow for broad generalizations and assumptions. Further, there is significant variation in the methodologies of

published studies and divergent outcomes in terms of LDN morphology, phenotype, flow cytometric analysis and function. Nevertheless, evidence indicates that LDNs are not a uniform cell type in the different patient populations that have been studied [6]. Rather, LDNs represent a population of cells with multiple phenotypes that vary greatly in each condition (summarized in table 1, figure 2).

LDNs by definition display a reduced cellular density, with reports ranging from 1.077 g/ml to 1.1067 g/ml [13]. This variation is a result of variation in the methodologies and centrifugation media used to isolate LDNs. That being said, neutrophils with densities of less that 1.090 g/ml can be considered LDNs [16]. Morphologically, LDNs have been reported as either a homogenous population of mature neutrophils or as a heterogeneous population of mature segmented neutrophils and immature neutrophils with a banded nuclear morphology [6]. This variation is further corroborated by variation in phenotype [41, 43]. Generally, LDNs express high levels of the classic neutrophil markers CD15 and CD16 as well as high levels of the neutrophil activation marker CD11b and CD66b while lacking the expression of other leukocytes and monocyte markers. However, in some conditions such as SLE and asthma, LDNs lack the immature neutrophil marker CD33 [5, 5]. Conversely, in cancer, HIV and during pregnancy, LDNs are characterized by increased expression of CD33 [33, 43, 54]. Therefore, there is evidence to suggest that LDNs are simply activated mature neutrophils that have degranulated, as well as conflicting evidence to suggest that LDNs represent neutrophils that have been deployed from the bone marrow prior to complete maturation as a result of disruptions in neutrophil development [22]. This discrepancy brings into question the true origin of LDNs.

There are four possible sources of LDNs: methodology, activation, immaturity and plasticity (Figure 1). One confounding variable, which calls into question the validity of some LDN studies, is the possibility that they are simply a by-product of the methodology and therefore an artifact introduced during experimentation. The validity of early reports of LDNs in conditions such as allergic rhinitis and RA [64] have been called into question by reports suggesting that LDNs may have been artificially induced due to the handling and isolation techniques [16, 65]. One possible source of LDN is the activation of HDNs [3]. Thus factors that could activate and promote neutrophil degranulation during sampling, transport and isolation may artificially create LDNs. For instance the use of glass tubes, polyethylene tubes, non-sterile instruments and buffers or endotoxin-containing reagents can prime and activate PMNs [14]. In addition, variations in the pH and osmolarity of the centrifugation medium have been reported to affect the proportion and activity of low-density cells [66]. Berends *et al*. (1994) best illustrated the effects of methodology on leukocyte density by demonstrating the effects of erythrocyte dextran sedimentation, a common technique used in the isolation of leukocytes from whole blood, on leukocyte density [16]. Without dextran sedimentation, LDNs comprised 17.7 ± 9% of total neutrophils. Dextran sedimentation increased LDN proportion by 40%, while further sample manipulation through additional washes increased LDN proportion to as high as 90% [16]. The increase in LDNs was attributed to increased neutrophil activation and degranulation as measured by elevated CD11b expression and increased lactoferrin release. A similar increase was not noted in neutrophils that were directly fixed prior to manipulation. This report clearly demonstrates that following isolation from peripheral blood, neutrophils are not inert and will continue to interact and be affected by their surroundings. There is an ever-increasing body of evidence to suggest the presence of LDNs in various physiological and pathological conditions and it seems very unlikely that all LDNs are iatrogenically induced.

In 2015, Sagiv *et al*. reported plasticity among low and high-density neutrophils in a murine cancer model, but unfortunately this phenomenon that has yet to be confirmed in humans. Mature HDNs can conceivably decrease their density *via* degranulation to become LDNs. However, the transition from LDN to HDN would require *de novo* granule synthesis. Although unconfirmed, one study gives credence to this idea by showing that neutrophils are able to form gelatinase granules once they have already left the bone marrow [67].

A deeper understanding and characterization of LDNs and their role in disease pathogenesis, as well as a greater understanding of how these

cells interact with other aspects of the immune response, is necessary in order to determine the potential of these cells as therapeutic agents and prognostic indicators in inflammatory diseases.

CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to disclose.

ABBREVIATIONS

ARF	:	Acute Rheumatoid Fever
BrdU	:	Bromodeoxyuridine
CD	:	Cluster of Differentiation
*f*MLP	:	N-Formylmethionine-leucyl-phenyalanine
HDN	:	High Density Neutrophils
HIV	:	Human Immunodeficiency Virus
HLA	:	Human Leukocyte Antigen
IDN	:	Intermediate Density Neutrophils
IFN	:	Interferon
LDG	:	Low Density Granulocytes
LDN	:	Low Density Neutrophils
LPS	:	Lipopolysaccharides
LSM	:	Lymphocyte Separation Medium
MHC	:	Major Histocompatibility Complex
MMP	:	Metalloproteinases
MPO	:	Myloperoxidase
MRSA	:	Methicillin-Resistant *Staphylococcus aureus*
NDN	:	Normal Density Neutrophils
NK	:	Natural Killer
PBMC	:	Peripheral Blood Mononuclear Cells
PMN	:	Polymorphonuclear cells, Neutrophils
RA	:	Rheumatoid Arthritis
ROS	:	Reactive Oxygen Species
SLE	:	Systemic Lupus Erythematosus
TAN	:	Tumour Associated Neutrophils
TGF- ß	:	Transforming Growth Factor-ß
TLR	:	Toll Like Receptor
TNF	:	Tumour Necrosis Factor

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