

EDITORIAL

WHY ARE NEUTROPHILS POLYMORPHONUCLEAR?

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Most cells in the human body have a spherical or ovoid nucleus. The mature human neutrophil, unlike most other cells exhibits a distinctly non-spherical nucleus, which is segmented into three to five lobes. The possible mechanisms underlying this segmented nuclear shape have been explored. The structure of the nuclear envelope, composition of lamins and lamin-B receptor seems to have an important role in shaping the nucleus. Being the first line of defense, neutrophils migrate rapidly to the site of infection and destroy the invading pathogen. This requires negotiation through narrow capillaries, transmigration across the vessel wall and passage through tight tissue spaces. Segmented shape confers increased nuclear flexibility, thereby easing the migration of neutrophils through narrow channels. The segmented shape of the nucleus may also play a role in intranuclear chromatin organization and gene expression. The unique shape of the neutrophil nucleus seems to be an adaptation to facilitate its function.

Neutrophils are the predominant type of leukocytes in blood, constituting 40-75% of circulating leukocytes. A mature neutrophil exhibits a segmented nucleus with three to five distinct lobes which are connected by thin filaments. The multilobed nucleus of the neutrophil can assume a variety of shapes and is hence considered polymorphic, which means *many shaped*. Being the most abundant of the polymorphs, neutrophils are often referred to as polymorphonuclear leukocytes (PMN) or simply polymorphs (1). Neutrophils are highly motile cells and play an important role in infections, inflammatory conditions and autoimmune reactions. The relatively small, segmented nucleus of the neutrophil occupies about 21% of the cell volume. In contrast, the round nucleus of the lymphocyte occupies about 44% of the cell volume (1). In contrast to most other cells in the human body which have spherical or ovoid nuclei, neutrophils are characterized by a typically non-spherical nucleus.

The mechanism and purpose of nuclear lobulation are subjects of much speculation.

Neutrophil formation and lifespan

Neutrophils are formed in the bone marrow (BM) by a process termed Granulopoiesis, which takes approximately two weeks. It includes a mitotic phase and post-mitotic phase. Neutrophils arise from the common myeloid precursors that also give rise to other granulocytes and monocytes. Neutrophils differentiate along the granulocyte lineage. In the BM, the mitotic pool consists of myeloblasts, promyelocytes and myelocytes. The post-mitotic pool has metamyelocytes, band forms, segmented forms and mature neutrophils (2).

The myeloblast exhibits a large, spherical nucleus with two to five nucleoli. As it reaches the promyelocyte and myelocyte stages, there is increasing chromatin condensation beneath the nuclear membrane and disappearance of nucleoli.

Key words: neutrophil, nucleus, nuclear envelope, lamins

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The metamyelocyte is characterised by an indented or horseshoe shaped nucleus. In the Band stage, the nucleus assumes the shape of a coiled band. Subsequently, constrictions begin to appear, and progress thereby dividing the nucleus into lobes. The number of lobes a mature neutrophil develops appears to be determined in the band stage or earlier (2). Within each lobe, the dense heterochromatin occupies the peripheral areas and the open euchromatin occupies the central areas.

Once released from the BM, mature circulating neutrophils in the blood marginate to intravascular pools, notably in the lung, or transmigrate into tissues to become fully activated in local inflammatory reactions. Resting neutrophils have a very short lifespan of about 6-10 hours after which they die through a built-in apoptotic programme. Activated neutrophils can survive for several days at the sites of inflammation (3).

Plasticity of the multilobed neutrophil nucleus

The neutrophil nucleus consists of a chain of 3-5 distinct lobes. The number of lobes and the shape of individual lobes varies in different neutrophils. Campbell et al. observed nuclei of living, moving

human neutrophils *in vitro*. They found that the multilobed nuclear structure within each neutrophil is fixed. That is, the number, size and location of the lobes with respect to one another remain constant in a given neutrophil. The position of the lobes with respect to the length of the nucleus is also fixed (4).

Despite the constancy of nuclear lobe number and position, the neutrophil nucleus is a remarkably flexible and plastic structure. The individual lobes can temporarily deform in shape while negotiating through narrow capillaries. This flexibility of the neutrophil nucleus offers great advantage while passing through the microvessels and also while transmigration across the vessel wall. In such situations, a large round nucleus would be expected to offer considerably more resistance to movement (4).

MECHANISMS UNDERLYING THE SEGMENTED NUCLEAR SHAPE

Nuclear envelope (NE), lamins and Lamin-B receptor

Recent studies have clearly demonstrated that structure of the NE plays a major role in shaping the neutrophil nucleus. The NE of mature neutrophils

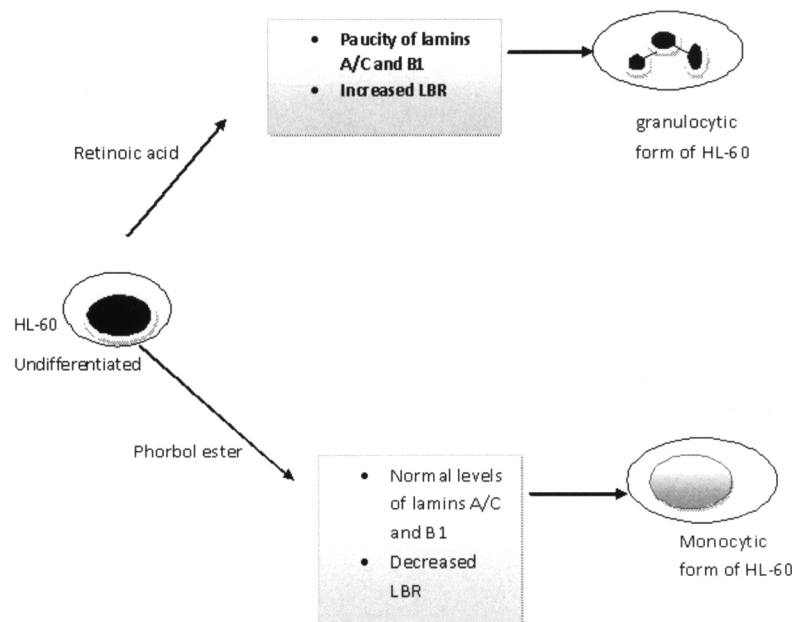


Fig. 1. Diagram depicting the behavior of undifferentiated form of HL-60 cells upon addition of retinoic acid and phorbol ester.

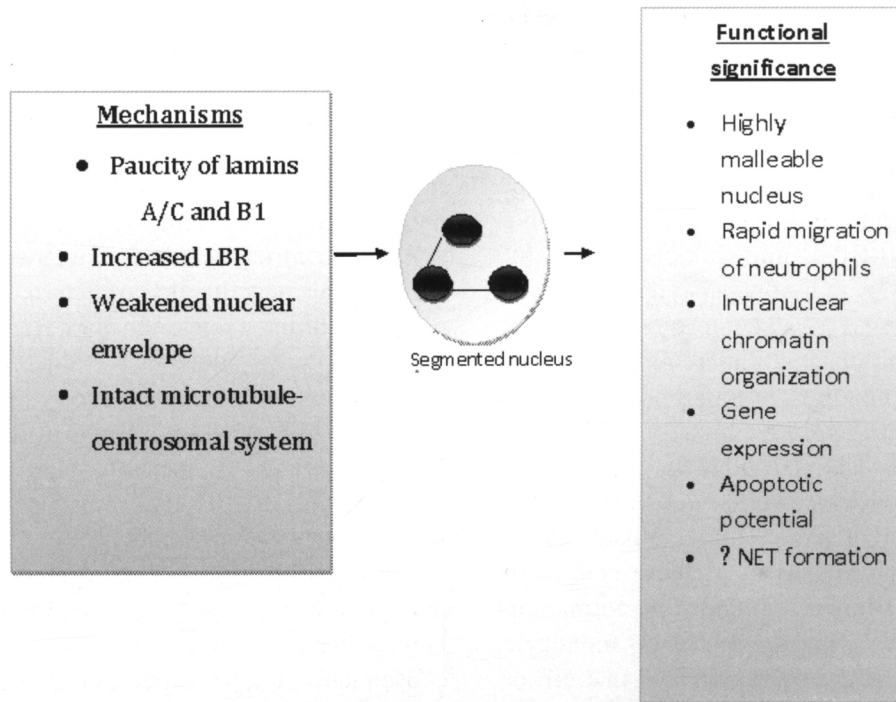


Fig. 2. Diagram summarizing the mechanisms underlying the segmented shape of the neutrophil nucleus and the possible functional significance of this unique nuclear shape.

consist of two parallel membranes – the outer nuclear membrane (ONM) facing the cytoplasm and the inner nuclear membrane (INM) facing the nucleoplasm. A network of proteins collectively called *nuclear lamina* underlies the inner nuclear membrane. Beneath the lamina lies the heterochromatin layer.

The lamina is composed of a dense network of type V intermediate filament proteins called lamins, which are thought to convey structural stability to the nuclear envelope (5). Two main types of lamins are described, which are type-A and type-B. Type-A lamins include Lamin A and Lamin C, which are produced by alternate splicing of LMNA gene. Type-A lamins are reported to be the main contributors to nuclear stiffness. Type B lamins include Lamins B1 and B2, each encoded by its own gene (6-7). The nuclear lamina acts as a stiff load-bearing element necessary for the structural integrity of the nucleus.

Lamin-B receptor (LBR), an integral membrane protein of the INM has an important role in segmentation of neutrophil nucleus. The N-terminal end of LBR is located in the nucleoplasm, binding

to lamin-B and heterochromatin. The C-terminal end of LBR resides within the INM. LBR is viewed as *stitching together* the INM and heterochromatin layers (8). LBR is said to play an essential role in determining the nuclear shape.

It has been observed that the nuclear shape of granulocytes has changed along the evolutionary line. The mature granulocytes of most non-vertebrates and a few reptiles, like turtles and snakes, have round nuclei. Many fish, birds like eel and chicken, and elephants possess hyposegmented nuclei. Rats and mice exhibit ring-shaped neutrophil nuclei (3, 9). Most mammals possess lobulated nuclei, comparable to humans. Some mammals like camel, hyena, guinea pig and rabbit exhibit hypersegmented granulocytes. The evolutionary changes in nuclear shape roughly correlate with the changes in structure of lamin B receptor. This supports the evidence that LBR has a role in determining the shape of the neutrophil nucleus (3).

The pivotal role of LBR in determining granulocyte nuclear shape was demonstrated with

the Pelger-Huet anomaly (PHA). Pelger-Huet anomaly is an autosomal dominant disorder linked to the mutations in the gene encoding LBR. PHA is characterized by hypolobulated nuclei in blood granulocytes, most evident in the neutrophils. Hoffmann et al. observed that expression of LBR affects nuclear shape in a dose dependent manner (10-11). Heterozygous individuals having half the normal amount of LBR exhibit bilobed neutrophil nucleus. Homozygous individuals having only trace amount of LBR possess ovoid neutrophil nuclei with no lobulation. These observations re-emphasize the role of LBR in shaping the neutrophil nucleus.

In-vitro granulopoiesis in HL-60 cells

The human myeloid leukemic cell line HL-60, furnishes a convenient tissue culture model for *in-vitro* granulopoiesis. Treatment of these cells with Retinoic acid (RA) induces granulocyte formation, and Phorbol ester (TPA) induces monocyte formation (Fig. 1). It has been observed that NE of granulocytic forms of HL-60 exhibit a paucity of lamins A/C and B1. The monoytic forms of HL-60 reveal a corresponding increase in lamins A/C and B1 (3, 12). Consistent with these findings, mature circulating neutrophils show paucity of lamins A/C and B1. The circulating monocytes and other cells with ovoid nuclei have normal quantities of lamins A/C and B1. This change in the composition of lamins is assumed to be responsible for the high deformability of the granulocyte NE compared to that of other cells. It is suggested that deformability of the NE is an important factor in determining the shape of the neutrophil nucleus (3, 12).

Hoffmann et al. (3) observed low levels of LBR in undifferentiated forms of HL-60 cells. As these cells were induced to form granulocytes, the nuclear segmentation was associated with a rapid increase in the LBR levels. In the absence of sufficient LBR during *in-vitro* granulopoiesis, the granulocytic forms of HL-60 developed ovoid nuclei and heterochromatin redistributed towards the centre of the nucleus (8). Therefore, elevated levels of LBR seem to be necessary during *in-vitro* and *in-vivo* granulopoiesis. Supporting this notion, recent studies have demonstrated that the highest level of LBR gene expression appears to be the BM, the site of granulopoiesis (5). It appears possible that

these LBR bridges severely distort and weaken the neutrophil NE, thereby facilitating changes in the shape of the nucleus.

Elevated levels of LBR in neutrophil NE are believed to 'tie together' the INM-lamin-heterochromatin layers. LBR is said to have a role in sequestering the heterochromatic regions to the periphery of the nuclear lobes. The current view is that LBR contributes to the segmented nuclear shape and compartmentalization of heterochromatin to the periphery of the nuclear lobes (5, 8).

Surprisingly, Olins et al. discovered that the NE of circulating mature neutrophils have reduced levels of LBR, when compared to the granulocytic forms of HL-60. It has also been noted that the highest level of LBR gene expression occurs in the BM, the site of granulopoiesis.

Considering these conflicting facts, it is speculated that early in the post-mitotic granulopoietic phase, lamins decline, while LBR levels increase. As a consequence, the NE is tied tightly to the underlying heterochromatin by the LBR bridges. Later along the timeline, the amount of LBR decreases. From this stage, the peripheral heterochromatin maintains the segmented nuclear shape (5). Therefore, increased levels of LBR and decreased levels of lamins A/C and B1 in the NE are important mechanisms facilitating nuclear lobulation (3, 13).

The persistence of microtubule-centrosomal system in the post-mitotic phase is also said to have a role in nuclear lobulation. The mechanical forces exerted by the microtubule-centrosomal system distorts the NE, thereby facilitating nuclear shape changes (4). The current hypothesis is that nuclear shape change involves the following factors (5, 13) (Fig. 2).

1. Reduced levels of lamin A/C and B1, which increase the flexibility of the NE. Deformability of the NE is an important factor in determining the shape of the nucleus.

2. Increased levels of LBR during granulopoiesis, which augments the connections between NE and the underlying heterochromatin. This distorts the NE, thereby facilitating changes in nuclear shape. The dense heterochromatin also plays a role in maintaining the nuclear shape.

3. Intact microtubule-centrosomal system which create tension in the NE, thereby promoting

nuclear distortions.

The theory emphasizing the role of lamins and LBR in determining the nuclear shape of the neutrophil is questionable in many ways. Can normally ovoid nuclei (eg HeLa cells) be transformed into lobulated forms by elevating LBR levels and reducing lamins A and B1? Is the neutrophil hypersegmentation seen in Vit B12 and folate deficiency associated with a corresponding increase in LBR or further loss of lamins A/C? (3). These questions remain to be answered.

FUNCTIONAL SIGNIFICANCE OF THE SEGMENTED NUCLEAR SHAPE

Passive deformation

Normal circulating neutrophils have a short lifespan of 6-10 hours after which they die by apoptosis. During their short lifespan neutrophils are subjected to various mechanical stresses. To enter the peripheral circulation, neutrophils have to squeeze through narrow migration channels located in the wall of the marrow sinuses. Subsequently, they must negotiate through microvascular beds of muscle, kidney, brain, heart and lungs during which they repetitively deform. However, neutrophils recover their spherical shape once they pass into larger vessels (14).

Hogg et al. (15) have studied the behavior of neutrophils in pulmonary circulation and observed that neutrophil transit through the lung is normally delayed by the narrow pulmonary capillaries. The pulmonary microcirculation has a unique arrangement consisting of 50-100 sequential capillary segments with an average length of $14.4 \pm 5.8 \mu$ and a diameter of about $2-15 \mu$. The diameter of neutrophils is $6-8 \mu$ (14, 16). While negotiating through capillaries narrower than their size, neutrophils have to deform. This deformation which occurs when neutrophils are subjected to mechanical stress is referred to as *passive deformation* (14). It may be noted that in this situation, the neutrophil deforms under the influence of external stresses. Deformability of neutrophils is one of the principle factors regulating their movements through narrow capillaries (17).

Kaleridis et al. investigated the role of the nucleus in deformability of neutrophils. They performed deformation tests on the neutrophil and its nucleus

using micropipette under low aspiration pressure. They observed that the nucleus plays a significant role in the mechanical and rheological behavior of the neutrophils, especially while passing through openings much smaller than their size (18). Using video observation simultaneously illuminated for fluorescence and phase contrast microscopy, Campbell et al. (4) analyzed the nuclear movements of moving neutrophils *in vitro*. Their observations indicate that the neutrophil nucleus is a remarkably flexible structure. Individual lobes can temporarily deform and the interconnecting filaments are able to stretch extensively to accommodate their movements. The high deformability of the neutrophil nucleus is thought to depend on the amount and composition of lamins in the NE. Lesser the lamins, more pliable the NE (12).

In the nucleus of a mature neutrophil, the thin filaments interconnecting the lobes may also play a role in its migratory capacity. While passing through narrow channels, these filaments create 'tension free' or 'resistance free' zones, thereby easing the passage of the segmented nucleus. Once each lobe passes out, there is a sudden 'fall' in frictional force which could facilitate the passage of the subsequent lobe. In such a situation, a spherical nucleus or an elongated nucleus with uniform breadth would offer constant resistance throughout its passage through the narrow channel. A suitable experimental model needs to be designed to test this hypothesis. Frictional forces generated during the passage of cells with segmented and non-segmented nuclei, through narrow openings need to be measured. This necessitates close co-ordination between cell biologists and bio-physicists.

Earlier studies had assumed that throughout the process of mechanical deformation, neutrophils remain passive without undergoing any functional changes. However, recent studies have questioned this view. Yapp and Kamm have observed that above a threshold stimulus, mechanical deformation can result in neutrophil activation and pseudopod projection (14). Kitagawa et al. (19) have observed that neutrophils can sense the mechanical stimulus and transmit the signal downstream resulting in breakdown of cytoskeleton and reduction in cell stiffness (14, 19). The process of converting physical forces into biochemical signals and integrating these

signals into the cellular responses is referred to as mechanotransduction (14). Mechanical deformation of neutrophils in the narrow pulmonary capillaries is shown to enhance adhesiveness to ICAM-1 through upregulation of adhesion molecules CD11b/CD18, cytoskeletal remodeling and increase in free intracellular Ca^{2+} (19). These observations clearly demonstrate that mechanical deformation above a certain threshold is capable of activating the neutrophils and therefore, cannot be considered a totally passive process.

Active deformation

Neutrophils are highly motile cells. Their locomotive capacity was appreciated a century ago by Von Recklinghausen and Conheim. They referred to neutrophils as 'amoeboid cells' because of their amoeba-like movement (2). Being the first line of defense, neutrophils rapidly migrate to the site of infection and destroy the invading pathogen. This necessitates active movement of neutrophils. In response to chemotactic factors, neutrophils adhere to the vascular endothelium, rapidly transmigrate across the vessel wall, crawl through the tight tissue spaces and reach the focus of infection. Upon reaching the focus of infection, neutrophils destroy the pathogen by means of phagocytosis, followed by intracellular release of hydrolytic enzymes and generation of oxygen free radicals (1, 20).

Neutrophils exit the circulation via two routes - *intercellular* (in between the endothelial cells) and *transcellular* (through the endothelial cell). Transcellular channels, are about one micron in diameter (2) and negotiation through these narrow channels necessitates an extreme degree of deformation of the neutrophil and its nucleus. Serial section electron microscopy (EM) has demonstrated significant stretching and elongation of the neutrophil and its nucleus during transendothelial migration (21). Neutrophils move in a crawling fashion, projecting a pseudopod in the direction of movement. The portion of nucleus near an extending pseudopod is swept into the pseudopod, dragging the rest of the nucleus along with it (4). These changes in shape of neutrophils which occur during directed migration towards a chemotactic agent is termed 'active deformation'. The energy required for active deformation is produced by the neutrophils

themselves (2, 20-21).

Deformability of immature neutrophils

Van Eeden (22) studied the behavior of immature polymorphs in the rabbit lung by using an experimental model of streptococcal pneumonia. In response to infection, there was an accelerated release of polymorphs from the BM. The transit time through the post mitotic pool of granulopoiesis was shortened, thereby releasing younger, immature polymorphs into circulation (23). In that study, the dividing polymorphs in the BM were labeled using the thymidine analog 5-bromo-2- deoxyuridine (P^{BrdU}). As the mature circulating polymorphs were unlabelled, the P^{BrdU} labeled polymorphs (P^{BrdU}) represented the newly-released polymorphs. It was observed that P^{BrdU} preferentially sequestered in the lung capillaries and were slow to migrate into the infected alveolar spaces.

Doerschuk et al. segregated the newly released labeled polymorphs (P^{BrdU}) and tested their *in-vitro* deformability using five micron filters (16). It was observed that these immature polymorphs released from the BM maturation pool were less deformable compared to the mature circulating forms. Immature neutrophils lack nuclear segmentation and are referred to as 'stab forms' or 'band forms', because their nuclei resemble a coiled band. It seems reasonable to speculate that the unsegmented nuclei of these immature band forms may not offer the same degree of deformability as compared to the mature segmented nuclei.

Neutrophils in Pelger-Huet anomaly (PHA) and laminopathies

Hoffmann et al. (3) evaluated the function of neutrophils from five heterozygous PHA individuals. The chemotactic function *in vitro* was evaluated through millipore filters, and *in vivo* through skin windows. It was observed that neutrophils in PHA migrated significantly more slowly through these structural barriers compared to control granulocytes. The hypolobulated neutrophils in PHA were less deformable compared to the controls.

The only functional defect to be identified in PHA is its association with soft tissue infections, which is thought to be due to defective transmigration of the neutrophils. It is suggested that altered

nuclear morphology renders them less capable of migrating across the vascular endothelium (11). These observations suggest that a segmented nucleus is essential for malleability of neutrophils and also their migration through narrow channels (5). Laminopathies are a group of disorders caused by mutations in the LMNA gene, usually manifesting as muscular dystrophies. There is a functional loss of lamins in the cells of the affected connective tissues, which results in abnormal nuclear shapes and increased nuclear fragility. This causes early cell death in the affected tissues. Studies on laminopathies have clearly established that lamins are necessary for the structural integrity of the nucleus and viability of cells (7).

It is evident that the NE of normal neutrophils is deficient in lamins. However, in the context of the neutrophils, this deficiency of lamins is of an advantage as it enhances nuclear flexibility. Similar deficiency of lamins in the NE of connective tissue cells causes cell death. What is most remarkable about the neutrophil is that the normal state is characterized by a deficiency of lamins, a condition that might be expected to produce pathologies or cell death in other tissue types. Considering the fact that neutrophils are destined to apoptotic death within few hours, it has been speculated that loss of lamins may actually facilitate their early death (5).

Nuclear shape, gene expression and apoptosis

Martinelli et al. observed that immature neutrophils showed preferential expression of genes involved in protein biosynthesis, metabolism and transcriptional control. In contrast, mature circulating neutrophils showed expression of genes that regulate signal transduction, inflammatory responses, transcription and apoptosis (24). This could explain the apoptotic potential of mature neutrophils.

Several studies have demonstrated that neutrophil nuclear segmentation is correlated with gene organization and gene expression. Within the nuclear lobes of a mature neutrophil, the densely packed, inactive heterochromatin occupies the peripheral areas. The gene rich, transcriptionally active euchromatin occupies the central areas. Shape of the nucleus and structure of the nuclear lamina are thought to contribute to this type of intranuclear chromatin organization (6). It seems that the nuclear

envelope furnishes a framework for the attachment of inactive heterochromatin (25). This intranuclear compartmentalization may increase the accessibility of genes necessary for differentiation or conversely reduce the accessibility of unnecessary genes to transcription factors (6, 26).

Neutrophil apoptosis involves nuclear condensation and pyknosis. Importantly, the cell remains intact and does not release its potentially toxic contents before being phagocytosed by macrophages. In many types of cells apoptosis is characterized by nuclear condensation and invagination, an event that occurs in neutrophils during their maturation in the bone marrow. This is also evidence that once released into circulation, neutrophils undergo apoptosis spontaneously without the need for external stimuli (27). External stimuli prolong the lifespan of neutrophils, thereby delaying apoptosis. Therefore, in some respects, has the mature neutrophil already entered the route towards apoptosis?

Neutrophil extracellular traps (NETs) are a potent bacterial killing mechanism exhibited by the dying neutrophils. First described by Brinkmann et al. in 2004 (28), NETs are extracellular meshes made up of chromatin strands and coated with bactericidal agents. It is suggested that NET formation is a method to immobilize the pathogens, thereby limiting the spread of infection. NET formation occurs when the fragile NE breaks causing a sudden explosion of chromatin. Whether the unique shape of the neutrophil facilitates NET formation is an interesting concept which needs to be explored.

CONCLUSIONS

Neutrophils are highly motile cells which migrate rapidly to the site of infection and destroy the invading pathogen. To accomplish this task, neutrophils have to pass through narrow capillaries, transmigrate across the vascular endothelium and squeeze through tight tissue spaces. In this context, the segmented nuclear shape confers great degree of flexibility, thereby facilitating rapid motility of the neutrophils. In such situations a large, round nucleus is likely to offer more resistance to movement of the cell. Possible mechanisms underlying the segmented nuclear shape include structure of the nuclear

envelope, composition of lamins and increased levels of LBR. The unique shape of the nucleus may also play a role in intranuclear chromatin organization, gene expression and apoptotic potential. Nuclear segmentation can be considered an adaptation to facilitate optimal functioning of the neutrophils.

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