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Role of Janus Kinase 1 (JAK1) on neutrophils spreading and directed migration ability

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ABSTRACT

This study investigates the role of Janus Kinase 1 (JAK1) enzyme on neutrophils ability to spread and migrate towards chemoattractant. Neutrophils stimulated by the presence of chemoattractant can spread along the blood vessels wall and then migrate towards the chemical signal in a process known as chemotaxis. Isolated neutrophils from healthy volunteers were incubated with JAK inhibitor 1 in order to block JAK1 enzyme activity. The neutrophils ability to spread and undergo directed migration was assessed on glass coverslips and Dunn Chemotaxis chamber respectively. After stimulation by N-Formylmethionyl-leucyl-phenylalanine (fMLP), neutrophils treated with JAK inhibitor 1 showed the limited capability to spread. The reduced spreading area suggested that the spreading mechanism may have been disrupted. Neutrophils treated with JAK inhibitor 1 were recorded with shorter migration distance and appeared to have non-directed migration towards fMLP. The introduction of JAK inhibitor 1 has disturbed the spreading ability of neutrophils and limits their capacity to undergo migration towards the chemical signal site. The findings suggest that JAK1 enzyme may have a vital role to play in neutrophils ability to spread and undergo migration.

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INTRODUCTION

Neutrophils are the most abundant circulating white blood cells that facilitate the early stage of inflammatory reaction. In the circulation, the average diameter of mature neutrophils is about 7–10μm with segmented nucleus, and its cytoplasm is enriched with secretory vesicles and granules (Nathan, 2006; Borregaard, 2010). Neutrophils protect our body against foreign microorganisms, tissue injury or any inciting inflammatory signals. For neutrophils to arrive at the inflammation site, it has to undergo systemic activation mediated by molecules such as immune complexes, chemokines, cytokines, complement factors, opsonized particles and other biologically active mediators (Ramaiah and Jaeschke, 2007). In resting state, neutrophils are spherical in shape and poorly adherent (Lokuta *et al.,* 2003). In response to inflammatory stimuli, neutrophils rapidly changed their morphology and became polarized before subsequently migrating towards the inflammatory mediator. Cell spreading is one of the neutrophils main characteristic of the inflammatory reaction. The ability for neutrophil to spread is the focal point in determining the rate of neutrophils extravasation both physiologically and pathologically. Neutrophils flatten on the endothelial cells in response to chemical signals after rolling along the endothelium (Dewitt *et al.,* 2013). Transition of neutrophils morphology from the spherical shape is accompa-

nied by the rapid expansion of its plasma membrane. Neutrophils recruitment also requires migration through blood vessels walls that involves paracellular and transcellular migration (Woodfin *et al.,* 2011). Neutrophils directed migration or also known as chemotaxis towards the site of inflammation is regulated by a number of chemoattractants which includes bacterial by-product such as N-formyl-methionyl-leucyl-phenylalanine (fMLP), chemokines, and complement proteolytic fragment C5a (Zlotnik and Yoshie, 2000).

In neutrophils, activation of the cell-surface receptor leads to re-organization of the actin cytoskeleton that drives cell motility (Glogauer *et al.,* 2000). The ability to rearrange the actin cytoskeleton in the presence of inflammatory stimuli is an important functional response of activated neutrophils. Neutrophils released their granule components such as metal-binding proteins, peroxidases, and hydrolytic enzymes when spreading over biological surfaces or transmigrating into inflamed tissue. Selectins and their counter-receptors mediate the first contact of neutrophils with the endothelium followed by the cells rolling along the endothelial wall of postcapillary venules and integrin-mediated arrest (Zarbock and Ley, 2008). Janus kinase (JAK) is intracellular, non-receptor tyrosine kinases that transduce cytokine-mediated signals through the Janus kinase and signal transducers and activators of transcription (JAK-STAT) pathway (Yamaoka *et al.,* 2004). This JAK-STAT signalling pathway has been implicated in converting inactive epithelial cells to migratory cells. In mammals, responses for cells migration are activated by chemoattractant proteins including chemokines through binding to the seven-transmembrane G-protein-coupled receptors (GPCRs) and activation of the JAK-STAT pathway that triggers chemotactic responses. Inhibiting JAK or JAK kinase domain mutation causes defects in both responses. JAK plays a vital role in the signalling of numerous cytokines that have been implicated in the pathogenesis of inflammatory diseases and immune disorders (Soriano *et al.,* 2003). JAK inhibitor had been discovered and developed to inhibit the function of JAK enzyme in the immune system and inflammation disorders (Clark *et al.,* 2014). Although neutrophils play a beneficial role in our body, their improper activation may lead to tissue damage during an autoimmune or exaggerated inflammatory reaction (Németh and Mócsai, 2012). Therefore, JAK1 enzyme was selected as the target to study its role in neutrophils spreading and chemotaxis ability by introducing JAK inhibitor 1.

MATERIALS AND METHODS

Blood collection

Human neutrophils were isolated from the blood of healthy volunteers as approved by UniKL Research Ethics Committee with the informed consent of participants. Butterfly needles were used in order to ensure that neutrophils are less or not activated during the collection process. The blood taken was then transferred into a universal container with 10µl of heparin to prevent coagulation.

Neutrophils isolation from whole blood

Heparinized blood (10ml) was mixed with 5ml Dextran (6% in Balanced Salt Solution (BSS)) and allowed to sediment for 30-45 min at room temperature. Dextran will cause the red blood cells (RBCs) to form aggregates and sediment at the bottom of the tube while the white blood cells remain suspended in solution (Heit *et al.,* 2008). After sedimentation, the white blood cells and plasma in the middle layer was carefully removed and centrifuged at 2000rpm for 1 min. Contaminating RBCs were removed by suspending the cells in 1ml of double distilled water for 20 sec. This step will cause hypotonic lysis, and the osmolarity of the neutrophils was immediately restored by adding 25ml BSS (pH 7.4) to the cells. The cells were then centrifuged at 2000rpm for 1 min. After that, the supernatant was removed and the pellet was resuspended in 5ml Hepes Buffered Krebs (HBK). The cells were centrifuged for the second time (2000rpm for 1 min) to remove any remaining RBCs, and the pellet containing neutrophils was washed and resuspended in 1ml of HBK with Bovine Serum Albumin (BSA) (Dewitt and Hallett, 2002).

Incubation of neutrophils with JAK inhibitor 1

JAK inhibitor 1 was purchased from Santa Cruz Biotechnology Inc. The cell-permeable JAK inhibitor 1 was introduced to the isolated neutrophils and labelled as treated group. 1µl of JAK inhibitor 1 was added to 1ml of isolated neutrophils (in HBK with BSA) and incubated at 37˚C for 30 min. The isolated neutrophils of the positive and negative groups were also incubated at the same temperature and duration accordingly.

Neutrophils spreading and data collection

Six wells multi-dish (Thermo Fisher Scientific) with a modified glass bottom were used to record the spreading capability of the neutrophils. Cell spreading is determined by observing the changes of cells shapes and morphology under the microscope. HoloMonitor™ M3 microscope was used to record neutrophils spreading in real time. 100µL of neutrophils were loaded in the six wells multi-dish and allowed to adhere onto the glass bottom for about 3 min. 10µL of chemoattractant (1mM fMLP diluted in DMSO) was added to the neutrophils.

The results were recorded in the form of continuous still images. ImageJ software was used to analyze and measure the cells spreading area.

Setting-up the cells migration chamber

Dunn Chemotaxis Chamber was used to record the migration capability of neutrophils towards chemoattractant (1mM fMLP). Isolated neutrophils were carefully seeded onto a clean thick glass covers slips. The glass covers slips were used to ensure dimensional stability. Then, both of the annular wells were filled with control medium and the cover slips loaded with neutrophils were inverted onto the chamber in an offset position, and leaving a narrow filling slit at one edge for entrance to the outer well. After firmly placing the glass cover slips on the face of the chamber and blotting up any excess medium, it was sealed in place using wax mixture (Vaseline:paraffin in a ratio of 1:1) around the edges except for the filling slit. In order to set up a chemotactic gradient, the medium was then drained from the outer well using a syringe and replaced with fMLP solution. The slit was finally sealed with the wax mixture.

Recording neutrophils directed migration behaviour

The Dunn Chemotaxis Chamber was placed under the microscope (40X magnification) for a time-lapse recording of neutrophils migration towards fMLP chemotactic gradient. The focus was positioned at the outer edge of the chamber's bridge to coincide with the recording field so that the direction of increasing chemoattractant concentration is outwards in the images. Continuous still images of migrating neutrophils were captured every 30 sec and recorded for 30 min. Images of the cells were examined and analysed using ibidi® Chemotaxis and Migration tool and ImageJ software.

Statistical analysis

The data was analyzed using One-way ANOVA and Student's t-test to evaluate significant differences among the different groups and expressed as mean±S.E.M. $p<0.05$ is considered as significantly different.

RESULTS

Neutrophils spreading area

Spreading area refers to the surface area of a neutrophil when the cell changes its shapes and becomes flat. The isolated neutrophils were divided into three different groups of; JAK inhibitor 1 treated group, positive control, and negative control. The spreading area of 100 randomly selected neutrophils from each group was measured. All results for the spreading area were expressed as mean±S.E.M. The spreading area for the negative control group was measured after neutrophils were completely attached to the glass cover slips, whereas the positive control and JAK inhibitor 1 treated group were measured after fMLP has been introduced to the attached neutrophils.

The positive control group (209.61μm^2) presented a bigger spreading area than the JAK inhibitor 1 treated group (129.48μm^2) (Figure 1), whereas the negative control group demonstrated the smallest spreading area (71.22μm^2). The morphological changes of neutrophils observed under the microscope happened after the chemoattractant (fMLP) has been introduced to the cells. Neutrophils in the negative control group mostly have the same morphology (Figure 2a and 2b) and its diameters were measured in the range of 8μm to 11μm. In some instances, some of the cells may have undergone spreading without adding chemoattractant, but the increase in its sizes was less than 0.5μm from its normal size. Adding fMLP caused neutrophils to change its shapes with visible lobes of granules. Increased in surface area were rapidly noticeable for neutrophils in the positive control group. Most of the neutrophils exhibited different morphological shapes during spreading (Figure 2c). The surface area of a spreading neutrophil was measured according to the X- and Y-axis. Some of the cells spread more paralleled to the X-axis and some cells spread more along the Y-axis. In order to determine the effect of JAK inhibitor 1 on neutrophils, the lipid soluble JAK inhibitor 1 was incubated with neutrophils and allowed to permeate. fMLP was introduced after the neutrophils treated with JAK inhibitor 1 has attached to the glass cover slip. The spreading area of the JAK inhibitor 1 treated group was evidently smaller as compared to the positive control group (Figure 2d). Neutrophils treated with JAK inhibitor 1 also required longer time to undergo spreading.

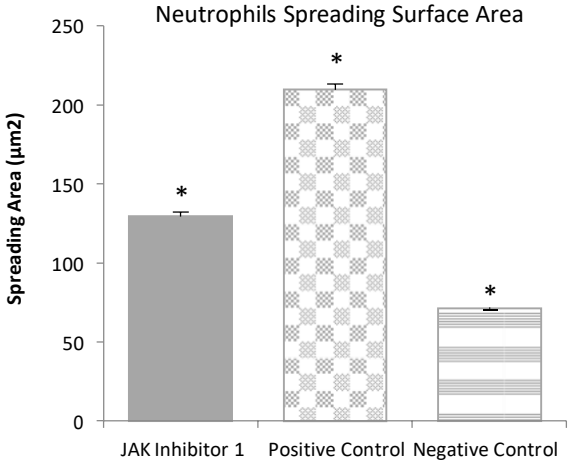

Figure 1: Measurement of neutrophils spreading areas after fMLP stimulation. The data is expressed as mean±S.E.M. (*p<0.05)

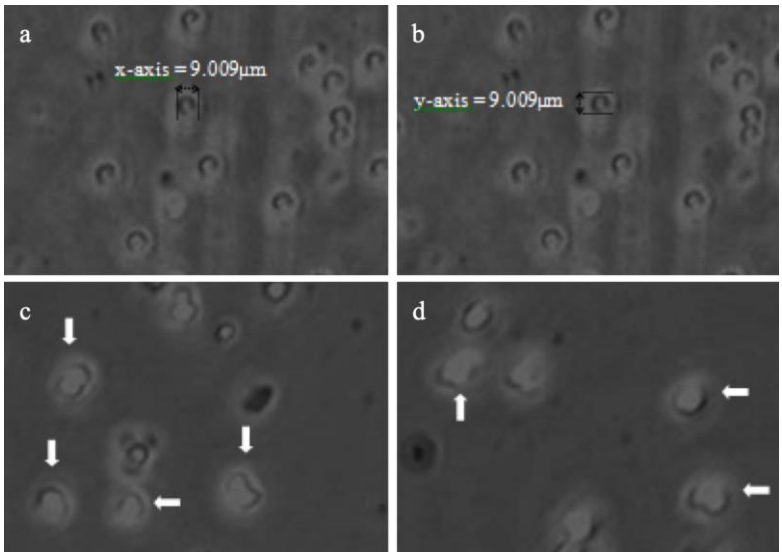

Figure 2: The size of neutrophils for (a & b) negative control group. (c) Positive control group and, (d) JAK inhibitor 1 treated group after fMLP stimulation

Accumulated distance of migrating neutrophils

Neutrophils from each of the tests group were observed under the microscope. The migrating ability of neutrophils treated with JAK inhibitor 1 was recorded and compared to the positive control group. fMLP was used as chemoattractant to track neutrophils movement and the data was analyzed using ImageJ Software for manual cell tracking. The mean accumulated distance of neutrophils treated with JAK inhibitor 1 was recorded at 4.25µm (Fig. 3). This distance was shorter than the accumulated distance of 27.52µm travelled by neutrophils in the positive control group. The migration of neutrophils was observed after the introduction of fMLP. As recorded, neutrophils either from JAK inhibitor 1 treated group or the positive control group still possessed the ability to migrate towards the fMLP gradient. However, the migration capability of JAK inhibitor 1 treated neutrophils has been relatively disrupted to a certain extent (Fig. 4a).

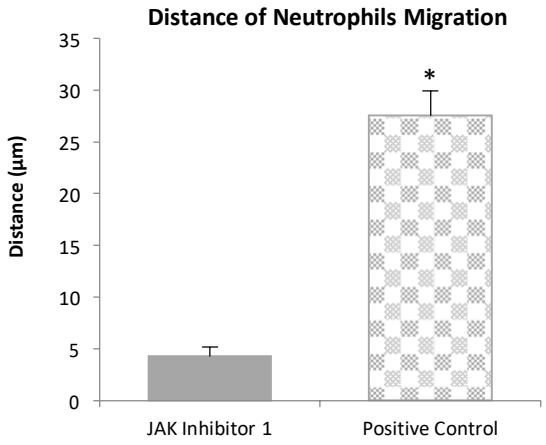

Figure 3: Average accumulated distance of neutrophils migration. The data are expressed as mean±S.E.M. (*, p<0.05)

Directed migration and centre of mass

Analysis using IBIDI Chemotaxis and Migration Tools Software showed that majority of neutrophils has migrated out from the centre of the migration plot (0, 0) (Fig. 5a and 5b). However, the distance of JAK inhibitor 1 treated neutrophils was obviously shorter than the positive control group. The total distance travelled by all JAK inhibitor 1 treated neutrophils and the positive control group are 18.94µm and 51.35µm, respectively.

In order to determine the directed migration of neutrophils, movements of the cells from the centre of mass was plotted. The centre of mass (Fig. 5a and 5b) represents the average point of cells endpoints after migration towards the fMLP source. The X and Y values indicate the direction in which the group of neutrophils primarily travelled. The centre of mass for neutrophils treated with JAK inhibitor 1 has only moved slightly away from the (0,0) coordinate, whereas neutrophils in the positive control group have moved further from the centre of the grid. This result implies that the migration ability of neutrophils treated with JAK inhibitor 1 has been compromised and less directed. This suggests a weak migration response to the fMLP source.

DISCUSSION

Activation of neutrophils by chemoattractant during inflammation leads to the cells leaving the blood vessel towards the site of inflammation. Physiological behaviour of neutrophils towards chemoattractant involves spreading as one of their main characteristics. Similar to IL-8, fMLP induces adherence and migration, degranulation, and toxic oxygen metabolites production in neutrophils (Stevenson *et al.,* 2004), and is also well known in inducing calcium flux and chemotaxis (Sogawa *et*

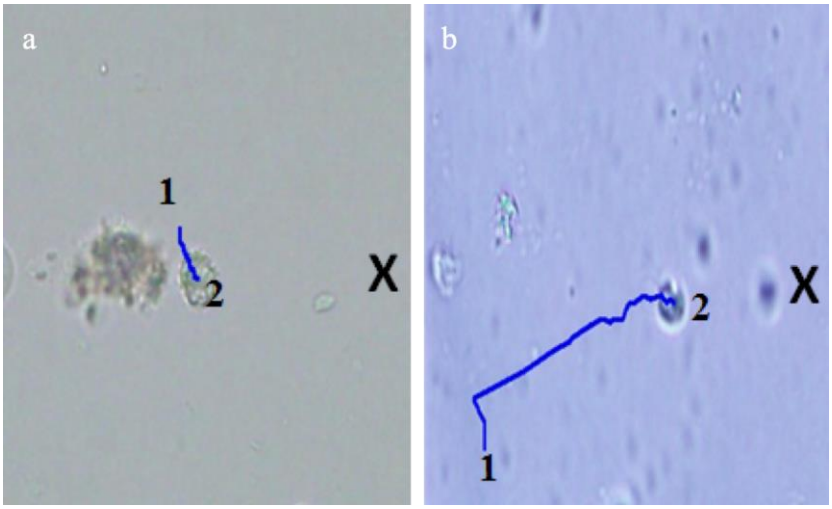

Figure 4: Neutrophils migration towards fMLP source (labelled X). (a) Distance travelled by neutrophils treated with JAK inhibitor 1, and (b) distance for positive control group

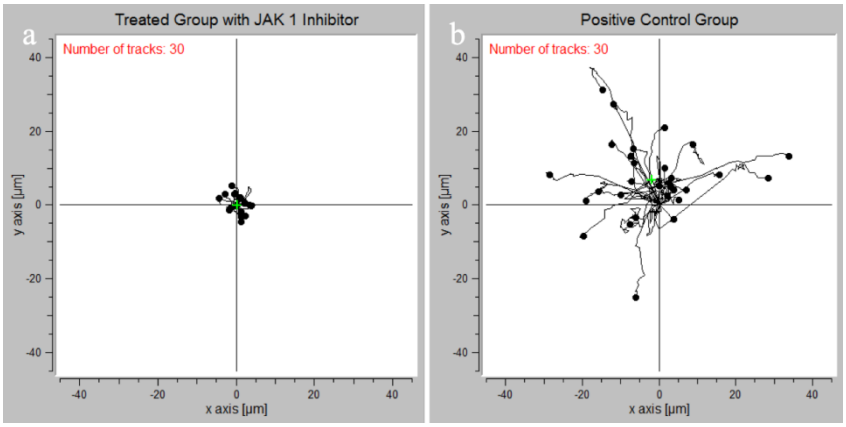

Figure 5: Neutrophils migration plots and the centre of mass. The (0,0) coordinate in a plot (a) and (b) indicates the centre of the plot

al., 2011). Neutrophils secrete adhesive molecules that help it to adhere to and spread on glass (Bertram *et al.,* 2012).

Adding chemoattractant (fMLP) activates the cells to spread to its maximum capacity. However, most of the negative control neutrophils were able to maintain its original spherical shape without the presence of chemoattractant. Neutrophils are circular-spherical before any shape or morphological changes, and unstimulated neutrophils were proven to be in spherical shape (Selz, 2011). Resting non-adherent neutrophils are circular cells of about 7µm to 9µm in diameter. Neutrophils demonstrated limited ability to spread along the X- and Y-axis after the introduction of JAK inhibitor 1. Spreading neutrophils have bigger cells surface area as compared to the cells that did not spread. Ca^{2+} influx will initiate the spreading mechanism in neutrophils (Dewitt *et al.,* 2013). With the present of chemoattractant that binds and activates GPCRs, dissociation of the GPCR-specific Gα subunit from the shared Gβγ dimer will occur. This action will continue with the hydrolysis of PIP_2 into IP_3 and DAG by PLCβ. IP_3 was involved in releasing Ca^{2+} from ER (Hillson *et al.,* 2006; Ishak, 2012). In the presence of Ca^{2+}, the cytoskeleton system of cells will begin with the spreading process.

In neutrophils, one of the main cytoskeleton systems involved in shape changes is actin cytoskeleton. In order for neutrophils to migrate, the interaction between calcium ion and actin cytoskeleton is compulsory. Actin cytoskeleton is required for cell crawling, which is the nature of cell motility in multicellular organisms. The crawling motion of cells or migration is a highly synchronized process involving the cytoskeleton contraction, F-actin polymerization, and cellular adhesion to the surrounding tissue (Chodniewicz and Zhelev, 2003). These processes are signalled by GPCR. The findings implicate that activation of tyrosine kinase by chemoattractant receptors may possibly provide an alternative signalling pathway for F-actin polymerization.

JAK1 enzyme has been classified as a member of protein-tyrosine kinases (PTK) and is characterized by the presence of a second phosphotransferase-related domain immediately N-terminal to the PTK domain (Ren *et al.,* 2013). Prior studies indicated that tyrosine kinases could be responsible

for GPCR signalling in neutrophils. Neutrophils express three members of the Src tyrosine kinase family which are Fgr, Hck, and Lyn (Lukashova *et al.,* 2003). Deficiency in Src-family kinases may reduce the fMLP-induced activation of the Janus Kinase and p38 MAP kinases. It is believed that JAK1 has a significant role in the JAK-STAT signal transduction process for cellular migration (Igaz *et al.,* 2001; O'Shea *et al.,* 2002). It is expected that JAK inhibitor 1 disrupts the activity of JAK1 enzyme thereby interfering with the JAK-STAT signalling pathway. Activation of Src-family kinases in neutrophils by G-protein-coupled receptors are likely to occur parallel to the PI3Kγ and PLCβ pathways (Futosi *et al.,* 2013), and this is possibly mediated through the interaction of Src-family kinases with either G-protein-coupled receptors or G-protein subunits. The deficiency of Src-family kinases indicates Janus Kinase involvement in GPCR signalling and points towards JAK1 as one of the tyrosine kinase proteins involves in spreading capability of neutrophils.

The shorter average distance of migration suggests that JAK inhibitor 1 did not inhibits neutrophils migration completely, but rather limits the distance. JAK1 was involved in the migration of neutrophils when chemoattractant induced the JAK-STAT signalling pathway that is critical for G-protein stimulation (Soriano *et al.,* 2003). This will then resulted in triggering cascade of protein activation that leads to instigation and clustering of integrin inside the lipid raft where conformational changes occur in the extracellular domain of activated integrin. Interaction with ICAM protein on endothelial cells happened with the activated integrin causing neutrophils to adhere and migrate (Woodfin *et al.,* 2011). The rise in Ca^{2+} causes membrane up-regulation and upshift in affinity of β2-integrin MAC-1 which is known to participate in both arrest and migration of neutrophils (Solovjov *et al.,* 2005). The migration of neutrophils involves intracellular integrin trafficking and uropod detachment, both of which are calcium dependent (Eddy *et al.,* 2000).

In addition, the JAK-STAT signalling pathway also helps in cells polarisation. It has been suggested that the presence of chemotactic stimulus will activate PI3K along the region of the cells facing the chemoattractant and bring about the production of PIP3 that leads to directed migration of cells (Wong *et al.,* 2010). A synchronization of events along the pathway such as cells rolling to arrest and the polarization is achieved by the influx of Ca^{2+} initiated by chemokine signalling that is transduced by the JAK-STAT signalling pathway. This leads to integrin binding that orchestrates the localized release of calcium and arrest, which triggers a global increase in calcium and migration. Within this region, high affinity integrin and stress activated calcium channels may function together as effective force transducers that guide the direction of the cytoskeletal contractile force (Schaff *et al.,* 2008). It is proposed that in fMLP-stimulated neutrophils, PI3K produces PIP3 at the leading edge that causes redistribution of cellular proteins such as F-actin and provides direction for cells movement. The PIP3 acts as leukocyte navigation and the localized production of PIP3 at the leading edges are apparently vital for directed and persistent movement (Karunarathne *et al.,* 2013).

CONCLUSION

In this current work, it can be strongly implicated that JAK inhibitor 1 may have disrupted the JAK-STAT signal transduction process which acts as the first translator that take the signal from the cells surface, and starts moving it down the chain of command, and causing cell to spread and subsequently migrate. However, the restricted capabilities of neutrophils to either change its shape and spread, and undergo migration towards chemoattractant suggested the existence of alternative mechanisms for signalling pathway or the involvement of other enzymes. This finding is valuable in understanding neutrophils behaviour during the inflammation process and as a potential cellular target to fight inflammatory disorders.

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