Chemoattractants in neutrophil recruitment to target disease and its applications

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Date: 22-08-2018

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| --- | --- |
| Abbreviation | Explanation |
| CF | Cystic fibrosis |
| COPD | Chronic obstructive pulmonary disease |
| CpG | Cytosine phosphate guanosine motif |
| DAMPs | Damage-associated molecular pattern |
| ELR | Glutamic acid-leucine-arginine motif |
| fMLP | N-formyl-1-methionyl-1-leucyl-1-phenylalanine |
| ICAM | Intercellular adhesion molecule |
| IL | Interleukin |
| LFA | Lymphocyte function-associated antigen |
| LPS | Lipopolysaccharide |
| MAC-1 | Macrophage 1 antigen |
| MAPK | Mitogen-activated protein kinase |
| MMP | Matrix metalloproteinase |
| MPO | meyeloperoxidase |
| NETs | Neutrophil extracellular traps |
| PAMPs | Pathogen-associated molecular pattern |
| PEG | Polyethylene glycol |
| PI3K | Phosphatidylinositol 3 kinase |
| PIP2 | Phosphatidylinositol-4,5-bisphosphate |
| PIP3 | Phosphatidylinositol-3,4,5-triphosphate |
| PPR | Pattern recognition receptor |
| PSGL | P-selectin glycoprotein ligand |
| PTEN | Phosphatase and tensin homolog |
| TNF | Tumor necrosis factor |

# List of abbreviations

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# Abstract

The neutrophil is usually the first recruiter of the innate immune system to fight inflammation and possess several killing mechanisms to eliminate pathogens. The movement of the neutrophil consists of a multiple step recruitment cascade involving tethering, rolling, adhesion, crawling and transmigration and is regulated by chemotactic molecules. This process is unbalanced in some diseases where these chemotactic molecules are overproduced and thereby resulting in chronic inflammation. The central objective of this essay is how chemotaxis could be used as a target to inhibit neutrophil recruitment in pharmaceutical applications for chronic diseases where the innate immune system is in overdrive. It was found that chemokine receptors could be inhibited by specific antagonists and therefore blocking the neutrophil recruitment to inflammation. Additionally, other approaches using neutrophils could also be utilized for the treatment of disease. Unfortunately, there are still several obstacles that need to be overcome before this can be applied to fight disease.

# Introduction

Fighting inflammation is a vital part of the innate immune system. The innate immune system consists mainly of neutrophils which are the most abundant innate immune cells. They are typically the first immune cells to be recruited to an inflammatory site and possess multiple methods of eliminating pathogens. However, they must first move to the site of inflammation. The neutrophil recruitment includes a multiple step cascade which is regulated by chemotaxis. (1,2) Neutrophils can detect extracellular chemical gradients and move towards an increasing concentration of chemoattractants. Chemoattractants such as chemokines play an important role in regulating neutrophil trafficking in different phases of the neutrophil recruitment cascade. Chemokines are small molecules that act as proinflammatory mediators in the navigation of neutrophil recruitment. (3)

Some diseases, such as asthma, are of inflammatory nature where the innate immune system is in overdrive. In most chronic inflammation, growth factors and proinflammatory cytokines are overproduced and as a result the neutrophils and other immune cells are recruited. (4,5) To stop this reaction inhibitors can be designed to block the pathway of neutrophil recruitment.

The main objective of this report is how chemotaxis can be utilized in neutrophil recruitment to target these diseases. Firstly, the composition of the neutrophils will be explained and how they operate to eliminate pathogens. Secondly, the pathway of neutrophil recruitment to inflammation will be described. Thirdly, the role of chemoattractants in neutrophil recruitment will be discussed and how they can be targeted in chronic diseases, such as asthma in order to design a treatment. Lastly, the contribution of neutrophils in several pharmaceutical applications will be discussed.

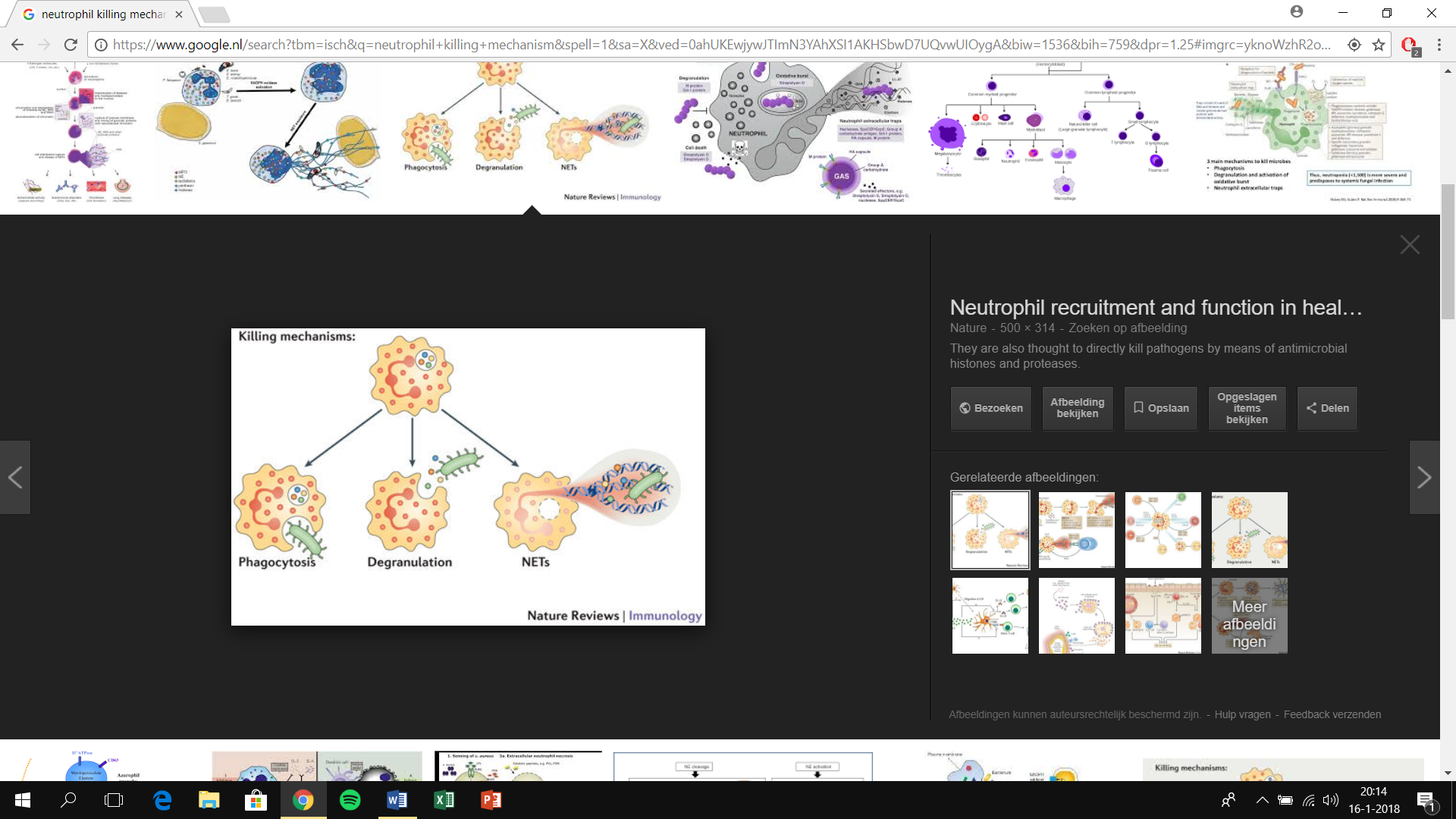
# Neutrophils

Neutrophils are the first responders of the innate immune response. Approximately 50-70% of the innate immune cells consists of neutrophils in humans. However, this number is different in other organisms, for example only 10-25% of the circulating leukocytes in mice are neutrophils. (6) The neutrophil can be characterized by several factors to differentiate from other immune cells. The characteristics of a neutrophil include a segmented nucleus and an enrichment of granules and secretory vesicles in the cytoplasm. During cell maturation, neutrophils can produce three types of granules which are loaded with pro-inflammatory proteins, such as azurophilic (primary) granules, specific (secondary) granules and gelatinase (tertiary) granules. The primary azurophilic granules contain peroxidase-positive components. These granules consists mainly of the peroxidase enzyme myeloperoxidase (MPO). This group can be further subdivided into more specific components: azurophilic granules can be differentiated into defensin-rich and defensin-poor granules. The heterogeneity of these granules can be explained by the different expression levels of granule proteins at different stages during maturation of the neutrophil and are therefore carrying different cargo of molecules. For example, the defensin-rich granule is produced later in the differentiation phase than defensin-poor granule and as a result the defensin-high granules have a higher secretory potential. (7) Defensins are antimicrobial peptides that can disrupt the structure of the outer membrane of many bacteria and therefore play an important role in eliminating pathogens. (8) Interestingly, these defensins are not expressed by neutrophils in mice, although several defensins are present in Paneth cells in the epithelium of the small intestines (6). The secondary specific granules are peroxidase-negative and contain mainly lactoferrin. Lactoferrin is an iron-binding glycoprotein that contains antimicrobial properties. It has been proposed that it can reduce inflammation by binding iron as it has been suggested that free iron could mediate inflammation. (9) Additionally, lactoferrin was also shown to have an ability to bind bacterial endotoxin lipopolysaccharide (LPS). (10) The tertiary gelatinase granules are the last type of granules and consist mainly of matrix metalloproteinase 9 (MMP9). Gelatinase is a proteolytic enzyme that is able to hydrolyse collagen and gelatine and is be able to penetrate through the endothelium membrane. When stimulated by inflammatory mediators, the gelatinase granules are faster secreted than the other types of granules and release their content. This fast exocytosis enables the transport of new adhesion proteins to the outer cell membrane that is needed for cell adhesion to the endothelium. (11) The multiple types of granules are created because several of the proteins cannot exist together in the innate form without digesting one another. These various molecules are therefore stored in compartments in the cytoplasm of the neutrophil. Additionally, neutrophils also carry secretory vesicles that can be easily mobilized to transport their cargo to the cell surface where proteins required for cell adhesion in neutrophil recruitment can be incorporated into the cellular membrane. (7)

## 3.1 Killing mechanisms of the neutrophil

When a neutrophil comes into contact with pathogens, they have several means of eliminating them. The first killing mechanism of neutrophils consists of phagocytosis of pathogens where they are encapsulated in phagosomes. The pathogens are disposed of using NADPH oxygenase-dependent mechanism (reactive oxygen species) or antibacterial proteins such as cathepsins, defensins, lactoferrin and lysozyme (figure 1A) which are released from the neutrophil granules into the phagosomes. The second killing mechanism includes the release of the aforementioned antibacterial proteins into the extracellular milieu (figure 1B) where it is brought into contact with the pathogens. The third way of eliminating pathogens is by releasing neutrophil extracellular traps (NETs) and only highly activated neutrophils are able to execute these NETs (figure 1C). NETs consist of a core DNA element with the attachment of histones, proteins (e.g. lactoferrin and cathepsins) and enzymes (e.g. MPO and neutrophil elastase) that are released from neutrophil granulates. NETs can immobilise pathogens and therefore prevent them from spreading. In addition to this, NETs can facilitate the encapsulation by phagocytosis of the captured microorganisms. It is also believed that these NETs can directly kill pathogens with the help of antimicrobial histones and proteases. (12,13)

Figure 1: The different killing mechanisms of a neutrophil. a: Phagocytosis: Pathogens can be engulfed and digested by the neutrophil; b: degranulation: the neutrophil releases the antibacterial proteins from its granules; c: NETs: core DNA, histones, proteins and enzymes are released to immobilize pathogens (1)



a.

b.

c.

## 3.2 Timing of the killing mechanisms of the neutrophil

The neutrophil can utilise these various killing mechanisms. It is therefore an interesting question how the neutrophil decides which mechanism it uses to eliminate pathogens. More specifically, can a single neutrophil eliminate pathogens by means of multiple killing mechanism at once and are neutrophils capable of coordinating together to eliminate pathogens? Whether the neutrophil utilizes multiple killing mechanisms could be explained by the timing of these events. It has been established that neutrophils can eliminate pathogens by means of phagocytosis within minutes of exposure *ex vivo*. The rate of degranulation depends on the content of the granules; secretory vesicles are initially released within 10 minutes after stimulation, after that the gelatinase (tertiary) granules, the specific (secondary) granules and the azurophilic (primary) granules are released. (14) Yet, NET formation is a more extensive process, usually taking between 2-3 hours. (15) In contrast to phagocytosis of pathogens, the neutrophil does not survive the attack using NET formation. Accordingly, a single neutrophil could perform all three killing mechanisms if they occur in the right order; the neutrophil has to perform phagocytosis first, followed by degranulation and at last NET formation. (13) This has been shown using in vitro studies with *S. aureus* where the neutrophil eliminated this bacteria first using phagocytosis and only at later time points utilising the NET formation. (15) It is therefore possible for a single neutrophil to perform several killing strategies. However, the question about the decision making abilities of the neutrophil still remain unclear.

# Neutrophil recruitment to inflammation

In the event of an immune response, neutrophils are recruited to sites of tissue damage or infection and need to migrate from the blood vessels to reach the site of inflammation. It is helpful to have an understanding of the recruitment mechanism before the pathway can be utilized as a target for pharmaceutical applications. The recruitment of neutrophils consists of a multiple step cascade that involves tethering, rolling, adhesion, crawling and finally transmigration of neutrophils (figure 2). Neutrophil recruitment is initiated when tissue-resident sentinel cells, such as macrophages and mast cells, come in contact with pathogen-associated molecular pattern (PAMPs) molecules and damage-associated molecular pattern (DAMPs) molecules and release inflammatory mediators (e.g. interleukin (IL)-1β and tumor necrosis factor (TNF)) and neutrophil-active chemoattractants (e.g. chemokines and lipid mediators) resulting in the activation of the endothelium cells. Alternatively, endothelial cells can also be directly activated by the detection of pathogen with the pattern-recognition receptor (PRR). (1,2)

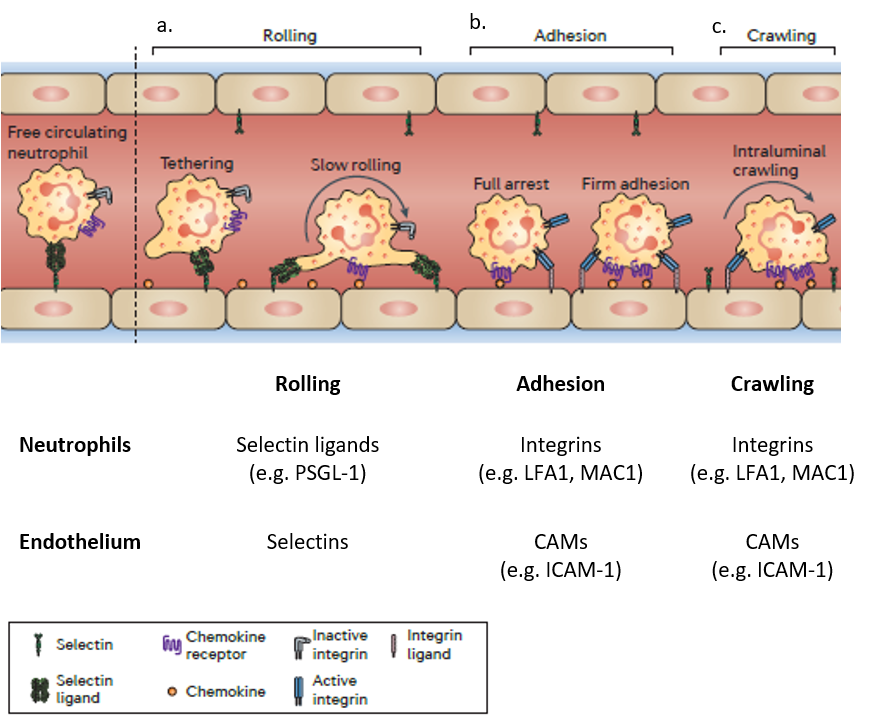


Figure 2: Schematic representation of the multiple step cascade of neutrophil recruitment. a: Tethering and rolling of the neutrophil. Selectins on the endothelium are upregulated and bind to selectin ligands located on the neutrophil surface; b: Adhesion of the neutrophil to the endothelial cells. Integrins on the neutrophils are activated by chemokines and can then bind to CAMs located on the endothelium; c: Crawling of the neutrophil. Neutrophils seek suitable locations for transmigration. (1)

## 4.1 Neutrophil tethering and rolling

Once the endothelium cells are activated, the expression of adhesion molecules are upregulated that sets the first step in motion; free circulating neutrophils are tethered to the endothelium cell wall (figure 2A). This process is mediated by two types of selectins; P-selectin and E-selectin. P-selectin is pre-stored in the storage granules of the endothelium (Weibel-Palade bodies) and is released to the surface of the endothelium. E-selectin has to be synthesized *de novo* and is produced within 90 minutes. (16) When these selectins are present on the surface of the endothelium they can bind to glycosylated ligands; P-selectin glycoprotein ligand 1 (PSGL1) that is present on the neutrophil aiding the tethering of free circulating neutrophils. This proceeds with rolling of the neutrophil in the direction of the blood flow. (1) Another type of selectin (L-selectin) is expressed on cell surface of the neutrophil and can facilitate the secondary tethering of another neutrophil to an already rolling neutrophil. (17)

When the neutrophil is trapped to the endothelium by the selectin-PSGL1 bond, it can roll under a shear stress that requires the balance of dissociation of the P-selectin-PSGL1 bond at the rear of the neutrophil and association of new bonds at the front. This involves a formation of a long membrane tether at the rear of the cell that can rotate the cell forward. (18) The neutrophil tether is covered with lymphocyte function-associated antigen 1 (LFA1) that can bind to intercellular adhesion molecule 1 (ICAM1) and ICAM2 located on the endothelium cell wall slowing down the rolling neutrophil coming to its arrest. (19)

## 4.2 Neutrophil adhesion

The rolling of the neutrophil on the endothelium ensures contact with certain chemoattractants (e.g. chemokines) located on the endothelium which are needed in order to activate the next step: adhesion of neutrophils to the endothelium (figure 2B). How the chemoattractants can regulate neutrophil recruitment will be discussed in more detail in paragraph 6. Binding of LFA1 to ICAM1 is essential for firm adhesion. (20) The chemoattractants trigger the activation of G-protein-coupled chemokine receptors on neutrophils which induces conformational changes of the cell-surface-expressed integrins (e.g. LFA1 and macrophage-1 antigen (MAC1)) that lead to a higher affinity to their ligands on the endothelium (e.g. ICAM1 and ICAM2). G-protein-coupled chemokine receptor is a transmembrane receptor that can facilitate inside-out signalling. (21) These conformational changes of integrins are strictly regulated and can be gradual or immediate. Chemoattractants are also responsible for re-localisation of the intracellular stores of MAC1 to the cell surface. When the integrins are activated, the cytoskeletal protein talin 1 binds to the β subunit of the integrin cytoplasmic tail and induces the extension of the integrin LFA1. This extension of LFA1 leads to a conformational change that has direct affinity to its ligand ICAM1 and stimulating slow rolling of neutrophils on the endothelium. However, if talin 1 is simultaneously activated with kindlin 3, which is a protein that can also bind to the β subunit of the integrin cytoplasmic tail, it induces a conformational change of LFA1 that promotes neutrophil arrest on the endothelium. (22–24) The binding of integrins to their ligands activates signalling pathways inside the neutrophil (outside-in signalling) which stabilizes adhesion of the neutrophil to the endothelium.

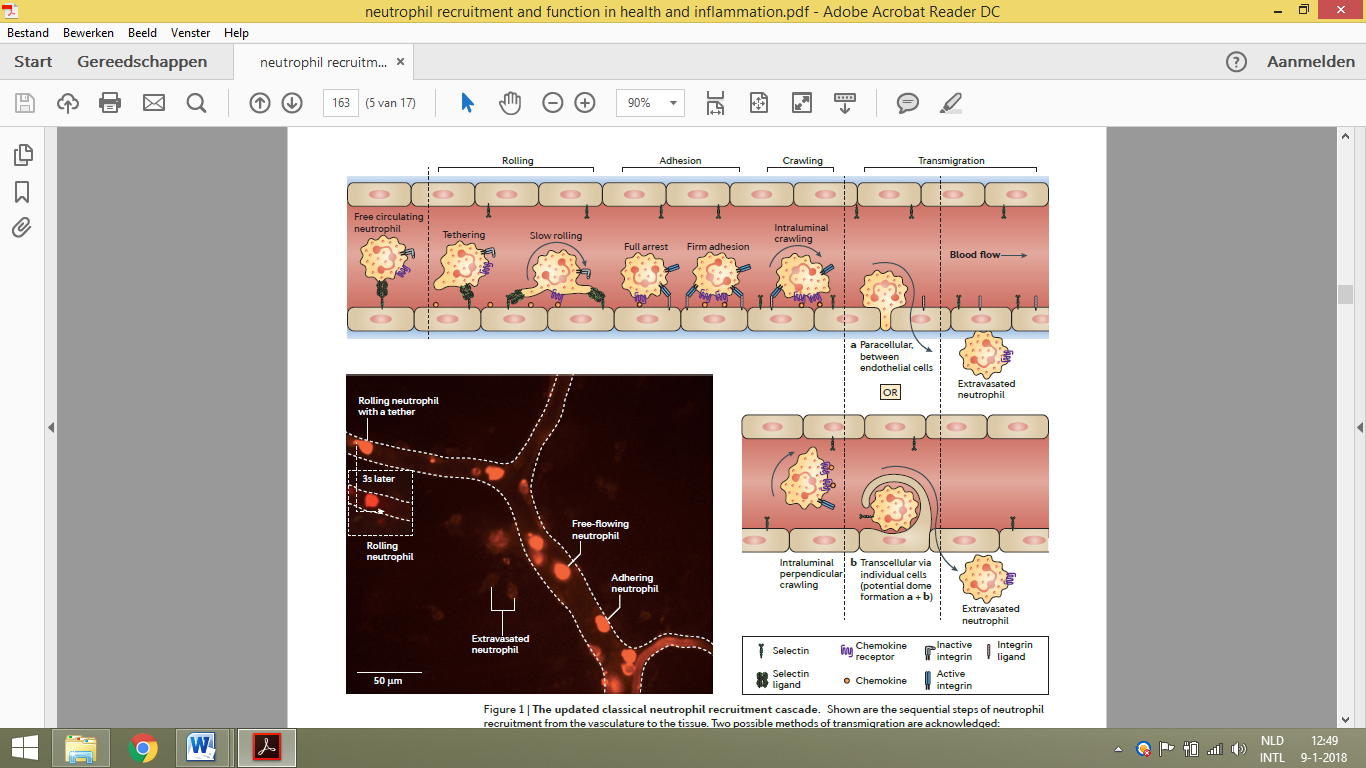
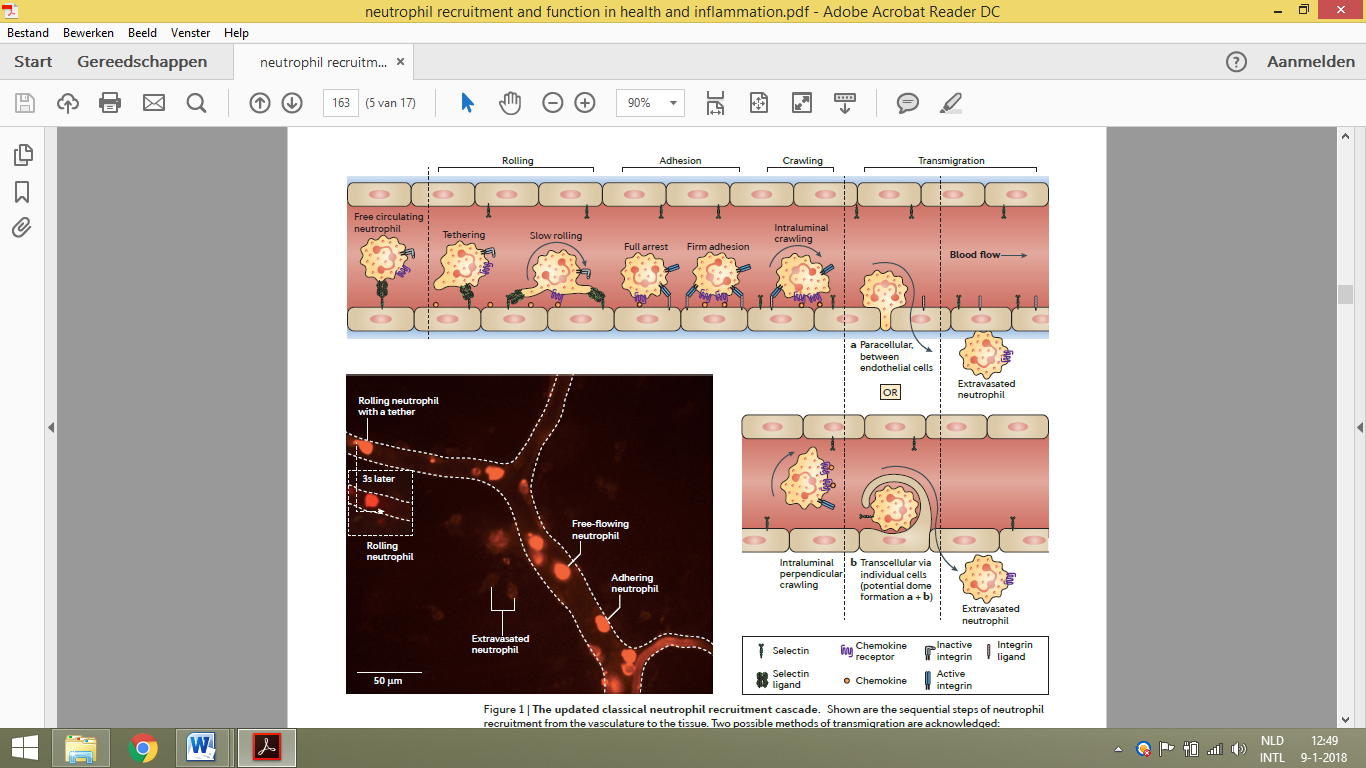
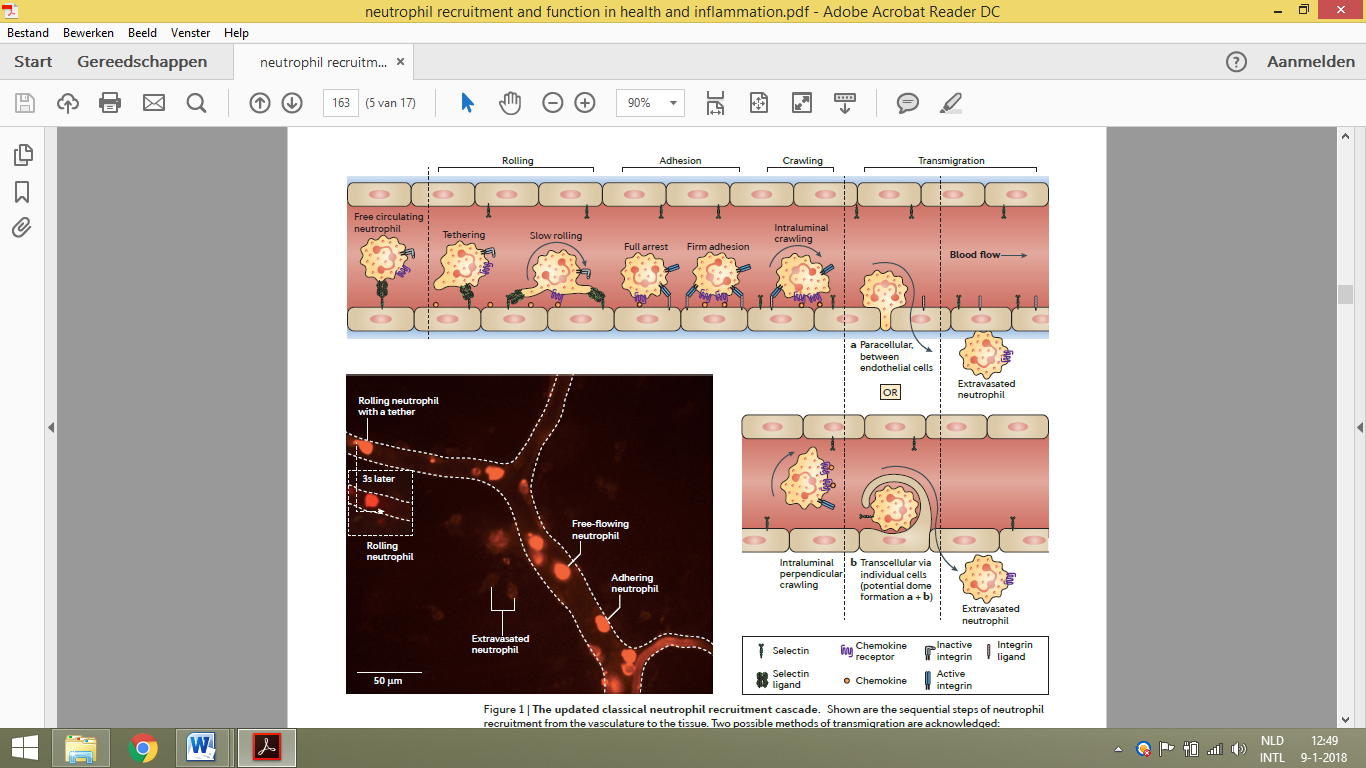
## 4.3 Neutrophil crawling

After neutrophils are firmly attached to the endothelium, the neutrophil needs to prepare for transmigration by crawling (figure 2C). The neutrophil sends out pseudopods to actively scan and explore the endothelium environment for a suitable position for transmigration while still being tightly attached to one point on the endothelium. It actively crawls to the endothelial cell to cell junctions where it is easier for neutrophils to transmigrate. This active crawling depends on the interaction between neutrophil-expressed MAC1 and endothelial cell-expressed ICAM1. (20) Neutrophils were shown to crawl in the direction of a chemotactic gradient. (25)

## 4.4 Neutrophil transmigration

Once the neutrophil has crawled to a suitable location, it still has to navigate through multiple barriers (the inner layer of endothelium, basement membrane and pericytes) in order to transmigrate. Transmigration across the endothelium can happen in two different ways; neutrophils can either pass between endothelial cells (paracellular; figure3A) or through an endothelial cell (transcellular; figure 3B). (26) It was seen *in vitro* that paracellular transmigration usually occurs usually at tricellular corners of the endothelium although this is not confirmed *ex vivo.* This process requires the interaction with the neutrophil expressed MAC1 and endothelial expressed ICAM1 and the continuous basement membrane has to be partially digested by proteases released by the neutrophil. Ideally the neutrophil moves through parts of the membrane with low expression of extracellular matrix components such as collagen. (27) Neutrophils usually favour the paracellular way although they can also transmigration in a transcellular way. Yet, this process is much less efficient. The endothelial cells have to form a transmigratory cup which consists of microvilli-like projections that have to engulf the neutrophil. (28)

Figure 3: Schematic representation of the transmigration of neutrophils. a: paracellular transmigration: the neutrophil can pass between the endothelial cells; b: transcellular transmigration: the neutrophil passes through the endothelial cell. (1)



Paracellular transmigration

Transcellular transmigration

a.

b.

# Reverse neutrophil migration

It was believed that once the neutrophil has left the vasculature and reached the site of inflammation, most neutrophils die in the tissue during inflammation and are disposed of by macrophages. Yet, other observations suggests that neutrophils can re-enter the vasculature in mouse models where it was reported that reverse transmigration occurred. (29) Additionally, it was shown that extravasated neutrophils could return to the vasculature in zebrafish embryos under sterile inflammation. (30) These reverse transmigration neutrophils were rescued from apoptosis when re-entered in to the vasculature and were shown to be distinct from neutrophils that not undergone reverse transmigration. Indeed, it appears a small percentage of neutrophils contain a distinct phenotype that consists of a specific profile of cell-surface receptors (CD54high and CXC chemokine receptor 1 low (CXCR1low)) that is able to transmigrate back to the vasculature. In patients with chronic inflammation the amount of peripheral blood neutrophils expressing this reverse transmigration phenotype is far higher than in healthy donors, approximately 1-2% in comparison to approximately 0.25% of the population, respectively. (31) The implication of reverse transmigration is not fully understood and it is not clear why these two distinct phenotypes exist. It is possible that this occurs to preserve neutrophils so that they have a longer lifespan. A possible complication of reverse transmigration of neutrophils that have been in contact with pathogens is the spreading of inflammation to other regions and reach other organs and thereby possibly facilitate chronic inflammation. An interesting question is if reverse transmigration is contributing to chronic inflammatory diseases. Unfortunately a direct answer is not available. However, this information could be helpful if these distinct neutrophils could be targeted as they are more abundant in patients with chronic inflammation.

# Chemoattractants

The next step in a better understanding of neutrophil recruitment and applying the neutrophil recruitment to a pharmaceutical target is to understand how neutrophils are regulated by chemotaxis. The movement of the neutrophil can be controlled by several chemoattractant signals derived from pathogens or damaged tissues that can lead the neutrophil to the site of inflammation. Neutrophils can prioritise chemoattractant signals and respond to them in a hierarchical manner. These chemoattractant molecules can be divided into two groups; intermediate chemoattractants and end-target chemoattractants. Intermediate chemoattractants include chemokines and lipid mediators. They can signal through the phosphatidylinositol 3 kinase (PI3K) and phosphatase and tensin homolog (PTEN) pathway (figure 4). It was thought that the PI3K pathway was the only pathway available for chemotaxis in neutrophils. The PI3K pathway can phosphorylate phosphatidylinositol-4,5‑bisphos­phate (PIP2) into phosphatidylinositol-3,4,5-trispho­sphate (PIP3) initiating pseudopod formation at the leading edge of the neutrophil. (32) The end-target chemoattractants include N-formylated peptides and complement 5a. N-formylated peptides such as N-formyl-1-methionyl-1-leucyl-1-phenylalanine (fMLP) and complement 5a are generated by bacteria in the event of an infection. Neutrophils react to them more strongly and can dominate the intermediate chemoattractants, thus creating a hierarchy of intracellular signaling pathways. The end-target chemoattractants can signal through the p38 mitogen-activated protein kinase (p38 MAPK) pathway. (33,34)

Furthermore, it is proposed that neutrophil migration is regulated by nucleotides. ATP is released when neutrophils come into contact with chemoattractants and can promote cell migration. The nucleotides do not act as a chemoattractant but it can promote chemokinesis. The exact role of ATP and other nucleotides is still not fully understood and need to be further investigated. (35)

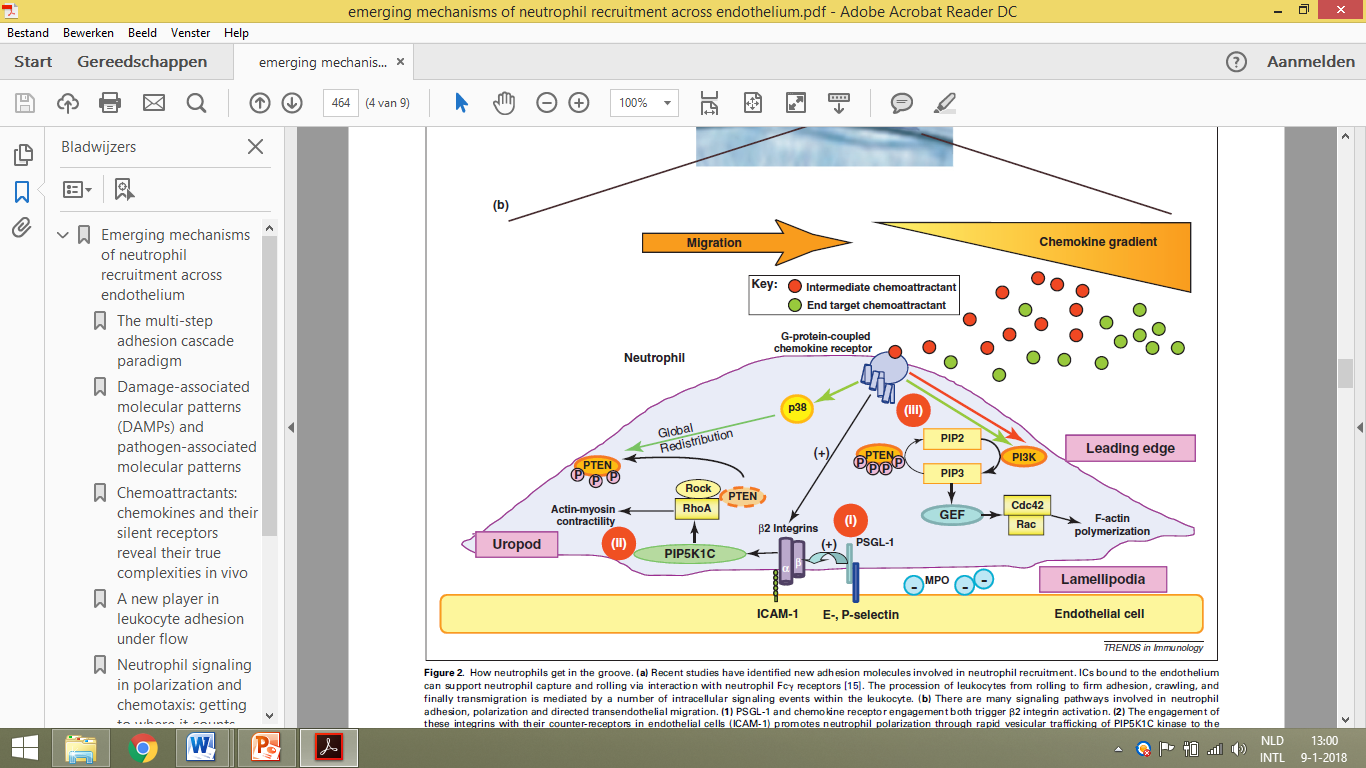
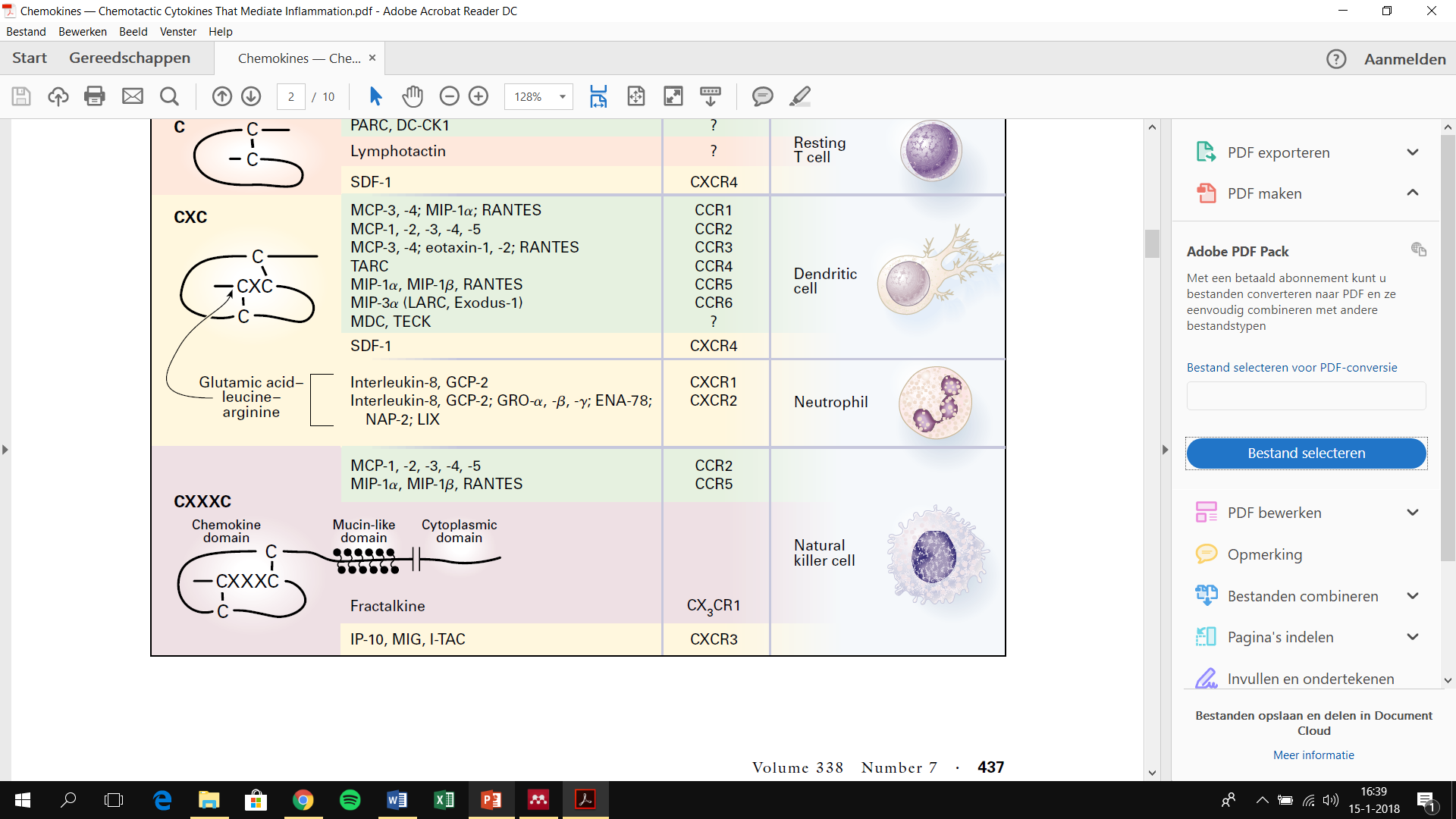
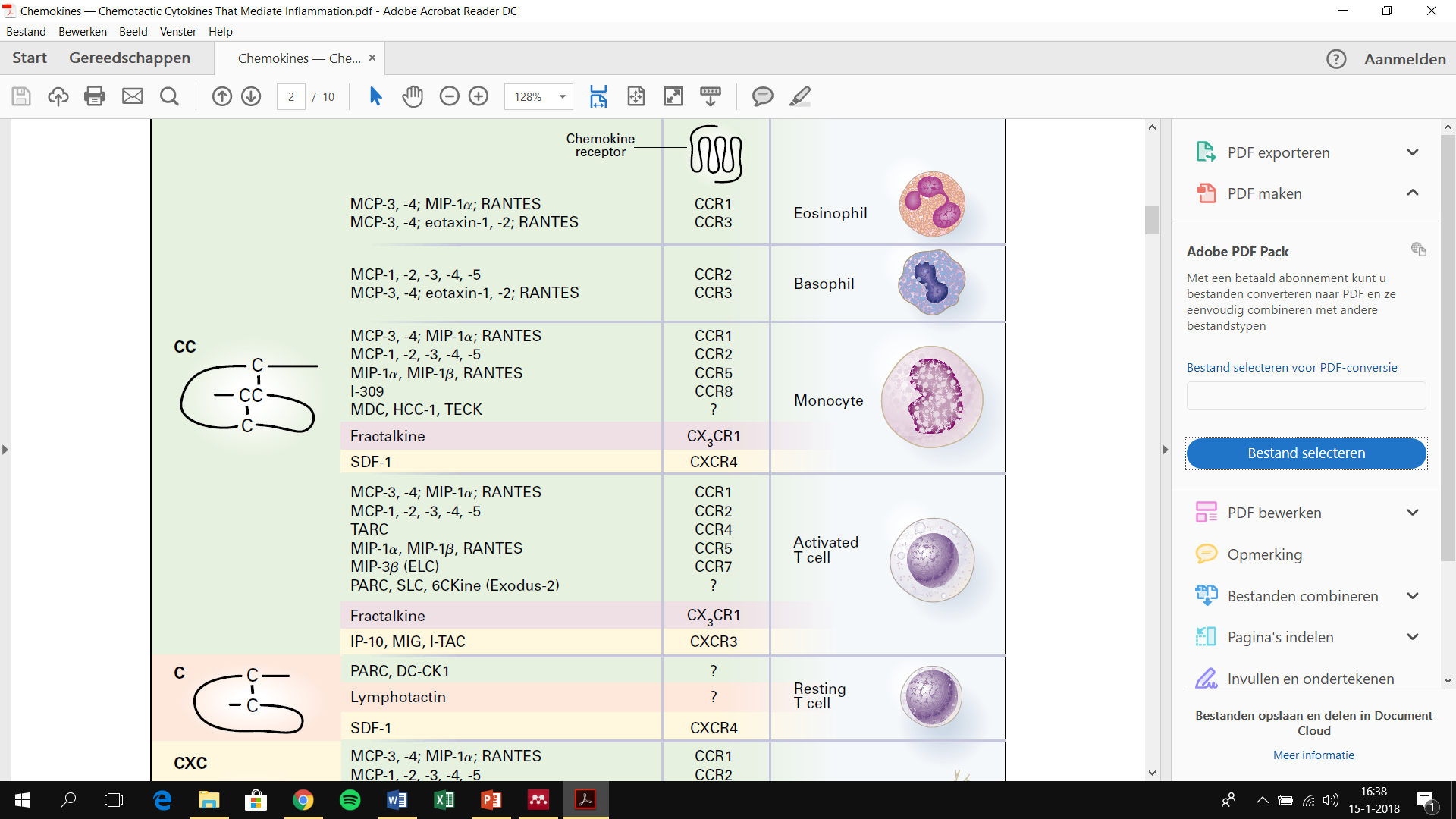
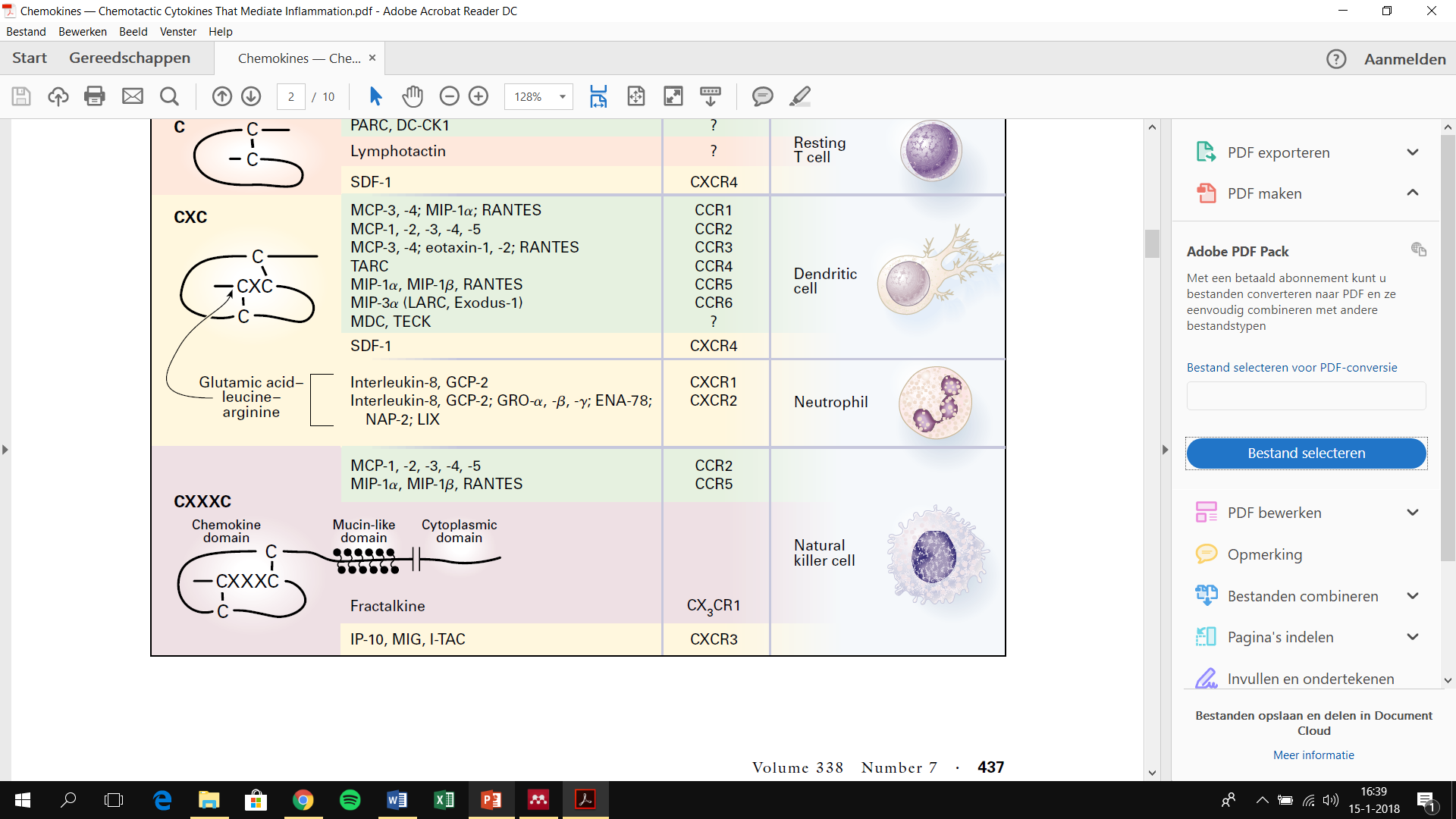
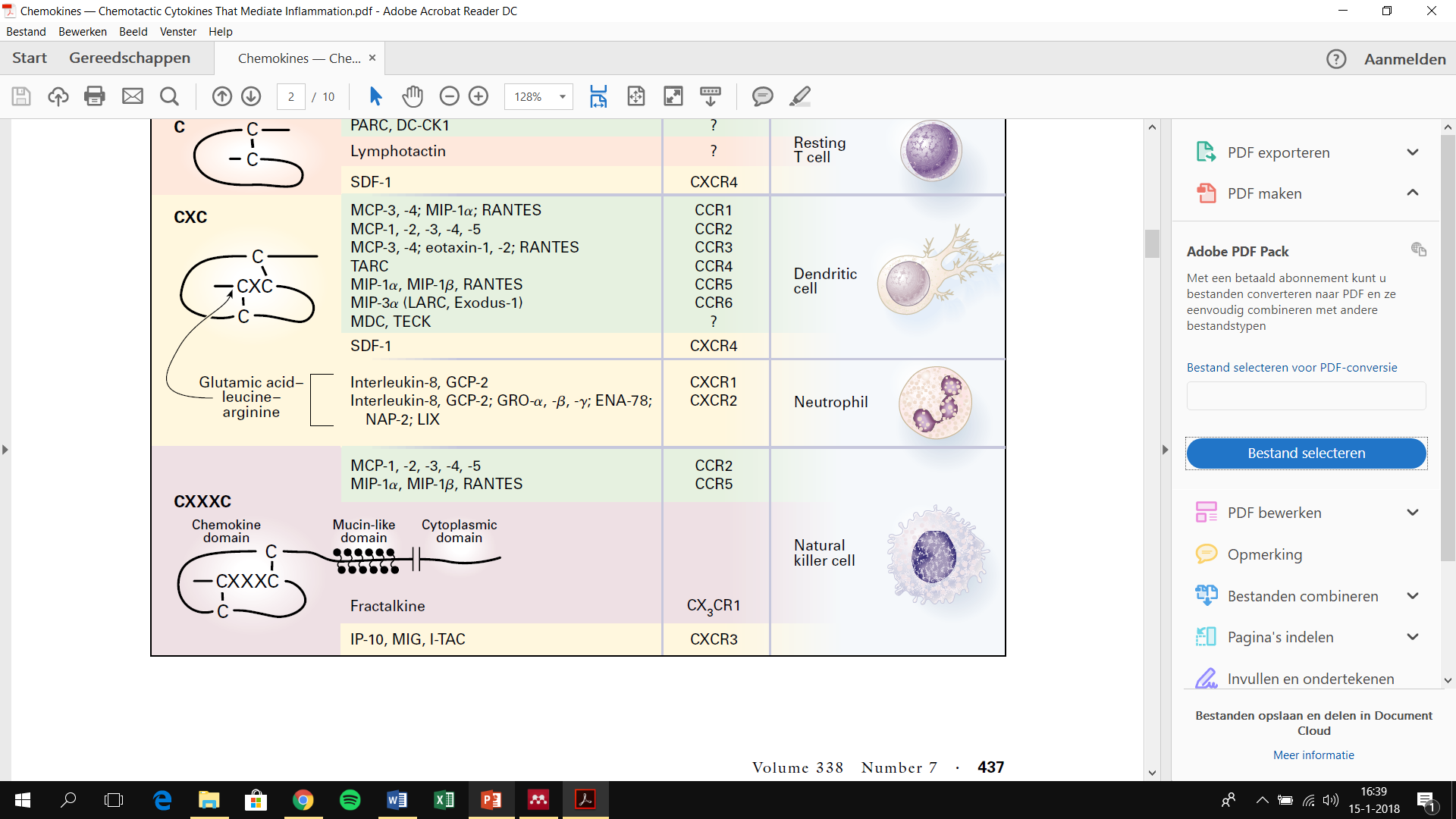
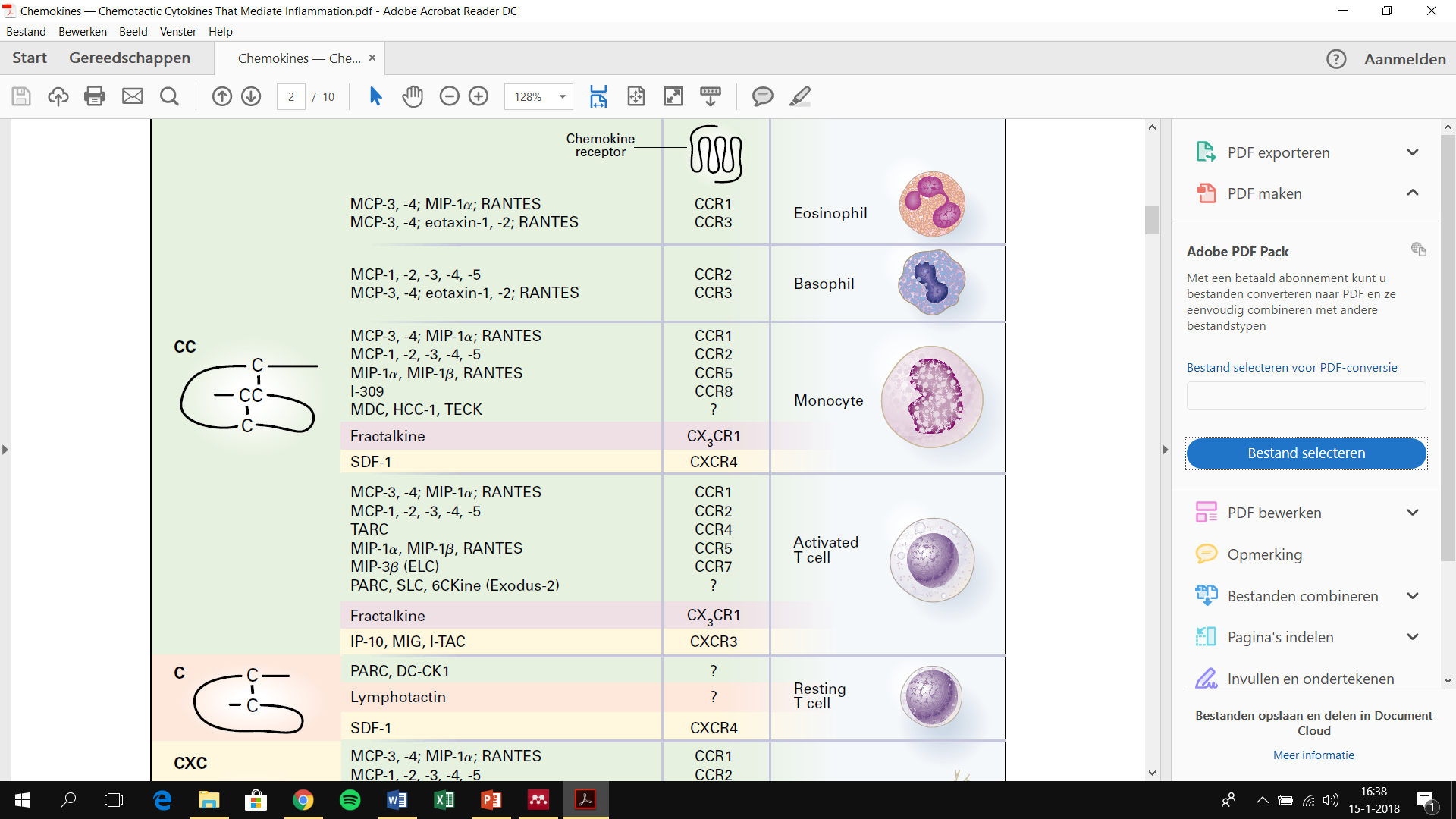


Figure 4: Signalling pathway in a neutrophil in the presence of a chemokine gradient. (26)

## 6.1 Chemokines

One of the chemoattractants that can regulate neutrophil recruitment are chemokines. Chemokines are positively charged molecules that are a part of a large family of small cytokines consisting mainly of a low molecular weight ranging from 7 – 15 kDa and are able to regulate the residence and migration of most immune cells. (36) There are four types of chemokines and can be discriminated by different structure regards to the sulphide bonds (figure 5): C, CC CXC and CXXXC chemokines. The types of chemokines can activate several immune cells, although only the CXC chemokines have a crucial role in the activation of neutrophils. CXC chemokines containing a glutamate-leucine-arginine (ELR) motive immediately before the CXC motif at the amino-terminal are essential in neutrophil activation. (37) The most important ELR-CXC chemokines involved in neutrophil activation are CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7 and CXCL8. These CXC chemokines can activate neutrophils via G-protein-coupled chemokine receptor and CXC chemokine receptors (CXCR1 and CXCR2) located on the neutrophil surface and stimulate the adhesion of the neutrophil to the endothelium. The chemokines are immobilized on the endothelium by binding with the negatively charged heparan sulphates that serves as an anchor to prevent washing these molecules away by the shear forces and they subsequently form an intravascular chemotactic gradient. (25)

Figure 5: Schematic representation of the structure of the different chemokines at the amino-terminus. C represents the amino acid residue cysteine and X can be any amino acid residue. Lines between the C residues are sulphide bridges. (38)



# The type of inflammation on neutrophil recruitment

Another interesting question is whether a neutrophil can discriminate between an infection and sterile injury. Inflammation can be differentiated by sterile inflammation and infection. Sterile inflammation includes injuries such as hypoxia, burn or chemical injury where DAMPs are generated. In the event of an infection, PAMPs are released which include bacterial and viral products (LPS and unmethylated cytosine phosphate guanosine motifs (CpG). (39) The origin of the inflammation could be important information as it could be useful for determining the course of the pathway of neutrophils to inflammation. Little is known about this and although different initiating signals (DAMPs vs. PAMPs) are activating the same receptors (PPRs), it cannot be excluded that different intracellular pathways are activated by DAMPs and PAMPs. For example, there was shown to be a difference in the adherence step of neutrophil recruitment between sterile inflammation and infection in liver sinusoids (40). During sterile inflammation, the interaction between MAC1 and ICAM1 is required for adhesion. However, during an infection the integrins do not interact, instead another interaction occurs between the neutrophil expressed CD44 and endothelium expressed hyaluronan.

# Pharmaceutical applications

## 8.1 Targeting neutrophils to inhibit their inflammatory response in chronic diseases

A better understanding of the neutrophil recruiting cascade can be helpful in order to use the mechanism to inhibit inflammation in certain chronic diseases. This includes the understanding of the behaviour of chemoattractants on the recruitment of neutrophils and can be used for the development of new therapies. It can be possible to target specific elements in the chemoattractant pathway to inhibit an inflammatory response. For example, asthma is a chronic inflammatory disease of the lungs. Palmqvist et al reviewed to target the chemokines and their receptors involved in neutrophil recruitment in order to find a treatment for this disease (table 1). (41) This was also discussed for chronic kidney disease where there is a desire to block chemokines and their receptors to stop neutrophil recruitment to reduce renal damage. (42) Chemokine receptors can be inhibited either by using a modified chemokine or a small molecule that acts as an antagonist or by directly blocking the receptor with a neutralizing antibody. However, several issues are raised when trying to block

|  |  |  |
| --- | --- | --- |
| Disease | Target | References |
| Airway diseases (e.g. asthma) | Chemokines and their receptors to inhibit neutrophil recruitment to prevent an asthmatic reaction. For example, an antagonists for the chemokine receptor CXCR2 can prevent binding of the chemokine CXCL8 | (41,43) |
| Kidney disease | Inhibit chemokines and their receptors (e.g. CXCR2) using an antagonist to prevent renal damage | (42) |
| Chronic obstructive airway diseases (e.g. COPD and CF) | Lung resident neutrophils. Nanoparticles with anti-inflammatory drugs can reach lung resident neutrophils with an antibody. The neutrophils can then transfer the drug to the site of inflammation | (44) |

Table 1: Examples of targeting parts of the neutrophil recruitment system for the treatment of specific diseases

chemokines and their receptors to reduce neutrophil trafficking. Firstly, it is important to determine which chemokines or chemokine receptor must be inhibited for a significant reduction of neutrophil recruitment. This is related to the fact that often multiple chemokines are involved in activating neutrophil recruitment. Therefore, a specific combination of chemokines has to be inhibited for the treatment of each disease. Secondly, selective inhibition is required as chemokines can bind to several receptors. Thirdly, it is not known if a continuous inhibition of chemokines have negative effects on other neutrophil recruitment events, for example an acute inflammation caused by an infection. It seems that targeting chemokines and chemokine receptors is very complex and raises several new complications. A whole other approach is to utilize neutrophils as a carrier. Vij et al tried to utilise nanoparticles as drug carriers that can target (lung resident) neutrophils which is especially applicable for patients with chronic obstructive airway diseases such as chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF). The nanoparticles carry anti-inflammatory drugs (e.g. ibuprofen) that have only shown potential in treatment of these chronic lung diseases at high doses that can cause significant side effects. The drugs can be delivered by these nanoparticles as they are covalently bound to antibodies to target specific cell types, such as neutrophils. Once they are bound to a neutrophil, the neutrophil is then the vehicle to deliver the drug to the scene of inflammation. The advantage of this method is that high doses of this drug are not required anymore as the drugs are locally released. The problem is that the nanoparticles must be able to penetrate through the mucus layer that is extensively present in these lung diseases. The researchers found that only nanoparticles that are coated with a muco-inert polymer such as polyethylene glycol (PEG) is able to cross through the mucus barrier. (44)

## 8.2 Using neutrophils for a cell based drug delivery system

Although using the chemoattractant pathway as a way of targeting neutrophils in inflammation, it is also possible to utilize neutrophils in other ways to battle disease. For example, Wendel et al utilise neutrophils as a cell based drug delivery system to eliminate bacterial infections. A new drug delivery approach is urgently needed as there is a severe increase of microbial resistance against antibiotics (45). They opted for using neutrophils with their natural active defence mechanism as a transporting device to target the infection site instead of an earlier developed liposomal delivery system where the targeting efficiency is very limited because of the passive targeting mechanism (46). The researchers successfully loaded neutrophil granulocytes with a deactivated non-pathogenic bacteria carrying a broad-band antibacterial drugs. This system was functioning *in vitro* with the complete elimination of *Escherichia coli* as well as the gram-negative bacterium *Fusobacterium necrophorum* that causes liver abscesses in cattle that is fed high grain diets. In mouse models this drug delivery system proved to be promising where the infection by *F. necrophorum* in the liver was targeted and was significantly reduced. (47) This cell based drug delivery system is a new pharmaceutical application that is very promising and need to be further developed to defeat the growing problem of the multidrug resistant bacteria. The advantage of this system is that very small quantities of drugs are required as it is only delivered to the specific area and healthy tissues and cells are shielded from the drug enabling the use of more aggressive antibiotics that otherwise have a high toxicity. However, the side effects of this drug delivery system have not yet been investigated. It assumes that the bacterium carrying the antibiotics is not harmful and does not have any negative effect. It is not clear how the drug is released from the neutrophil and it cannot be excluded that the drugs are released in the present of healthy tissue. This method of delivering drug to specific areas of inflammation has only been performed on mice-models. It is important to keep in mind that the quantity of neutrophils in blood can vary in different organisms; 10-25% in mice compared to 50-70% in human. This is important when experiment are performed in mice-models and there is a desire to translate this to human applications. It is not known how the human body will react to this method.

# Conclusion

The neutrophil recruitment cascade is very complex and many factors are involved in this system. Neutrophils have multiple options of eliminating pathogens, however they first have to arrive to the scene of inflammation before they can act. There are numerous ways of targeting this pathway for a possible treatment of chronic inflammation. The neutrophil proved to be very versatile in using it for pharmaceutical applications. The inhibition of chemokines is a complicated process and there are still many obstacles before any of these methods can be set into clinical trials. For each disease a unique method has to be developed as there are too many components of the neutrophil recruitment cascade and its regulating factors that it is nearly impossible to design a universal methods that can be applied to every disease. Furthermore, other approaches also proved to be successful where the neutrophil is utilized for delivering drugs to specific sites of inflammation, although they still need to be optimized.

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