BACHELOR ASSIGNMENT

Through the Junction or Through the Body: the Choice of Neutrophils

Pathways of Trans-endothelial Transmigration of Neutrophils

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Contents

1. Abstract

Trans-endothelial migration is a process whereby neutrophils exit the bloodstream by crossing the layer of endothelial cells lining the blood vessel in order to respond to infections. There are two possible ways in which neutrophils can migrate through the wall of blood vessels, either going through the junction of bordering endothelial cells, the well accepted paracellular pathway, or right through the body of an endothelial cell, the transcellular pathway. This thesis will show that transcellular trans-endothelial migration does exist, it will discuss the mechanisms implicated in these processes are and how the decision to use which of the two modes is made. During paracellular trans-endothelial migration neutrophils appear to be guided to endothelial cell junctions by interactions of adhesion molecules. These interactions have also been implicated to alter junctional integrity, thus allowing passage of neutrophils. However, in response to different stimuli different adhesion molecules are used. During transcellular trans-endothelial transmigration neutrophils have been observed to extend protrusions to palpate the surface of endothelial cells to find a suitable location to migrate, if a suitable location is found they extend the protrusions to form a transcellular pore and migrate through the body. Because our knowledge regarding the details of this mechanism in neutrophils is still lacking, the question how neutrophils decide between the two routes cannot be answered yet. However to determine how neutrophils decide which mode of trans-cellular migration to use, more studies are necessary, especially to elucidate the dynamics of adhesion molecules in response to different types of stimuli for both paracellular and transcellular trans-endothelial transmigration.

2. Introduction

When a pathogen enters the body, or tissue damage is caused, the body will react to eliminate the pathogen or clear remove the damaged cells. The immune system is responsible for carrying out the elimination reaction to pathogens and damaged cells (Abbas *et al,* 2010, p. 3).

The immune system works in two phases. First, pathogens are recognized and eliminated by cells of the innate immune system. Secondly, recognition of a pathogen by the cells of the innate immune system will stimulate the adaptive immune system (Abbas *et al,* 2010, p. 5-9). The activated cells of the adaptive immune system will acquire adaptations that allows them to specifically recognize and destroy a certain pathogen in a much more efficient way than the innate immune system, whereas the innate immune system recognizes patterns of molecules that are associated with foreign organisms, substances or damage of its own tissues (Abbas *et al,* 2010, p. 20).

The innate immune system carries out the initial response to microbes that prevents controls and eliminates infection. The components that carry out these functions either serve a barrier function to prevent entrance of pathogens, or actively help to eliminate pathogens (Abbas *et al,* 2010, p. 27). The epithelial surfaces, such as the skin and mucosal surfaces of the gastrointestinal and respiratory tracts, serve a barrier function in the body. Phagocytes, cells that can identify and ingest pathogens, actively eliminate pathogens. The human body comprises several different types of phagocytes, including neutrophils, monocytes/macrophages and dendritic cells (Abbas *et al,* 2010, p. 27-30). All these cells rise from hematopoietic stem cells in the bone marrow (Abbas *et al,* 2010, p. 56-58). Apart from phagocytes and the epithelial surfaces, the components of the innate immune system include natural killer cells, which are involved in killing infected cells, and proteins that circulate the bloodstream, such as the complement system that serves to recognize pathogens and promotes destruction of these pathogens (Abbas *et al,* 2010, p. 37-45).

Neutrophils circulate the bloodstream after being formed in the bone marrow, to live for about six hours. In order to keep a sufficient number of neutrophils in the circulation, the adult body produces more than 10^{11} neutrophils per day (Abbas *et al,* 2010, p. 29). They contain a nucleus consisting of three to five lobules; hence they are sometimes called polymorphonuclear leukocytes. The cytoplasm of neutrophils contains two different types of granules, specific granules and azurophilic granules. Specific granules contain enzymes, such as lysozyme, collagenase and elastase. Azurophilic granules are lysosomes. They contain enzymes and microbicidal substances that help to destroy pathogens. Neutrophils are sometimes called the foot soldiers of the immune system, due to the fact that they are the first cells to migrate from the blood to the site of infection (Nathan, 2002). This ability of leukocytes to migrate out of the bloodstream and into the tissues to fight infections is of vital importance to immune system functioning (Schmidt *et al*, 2011).

Neutrophils are directed to the site of infection by a complicated and sophisticated cascade of signaling events. When a pathogen manages to enter the body's tissue, it is recognized by tissue resident macrophages (Abbas *et al*, 2010, p. 30-35). These cells start to produce the cytokines, TNF-α, IL-6 and IL-1β that diffuse away from the macrophage into the surrounding tissue. When cytokines reach the capillaries and venules that run through the tissues, they can activate the endothelial cells that form the wall of these blood vessels (Abbas *et al*, 2010, p. 30-35).

Endothelial cells are flattened cells with a large surface area that are tightly connected to each other. They are polar cells, their basal site growing on the basal membrane, their apical side facing into the lumen of the blood vessel. Hence, they connect the blood with the surrounding tissue (Daniel & van

Buul, 2012). When endothelial cells are activated by cytokines, they start to produce chemokines themselves which are expressed on the apical side of the cell (Schmidt *et al,* 2011). In response to cytokine induced activation, they start to express protein complexes on their apical membranes. These cues, that indicate an infection nearby in the tissue, can be recognized by neutrophils. In response, neutrophils will migrate to the site of infection (Abbas *et al,* 2010, p. 30-35).

In order to reach the site of infection, neutrophils must stop circulating in the blood, migrate out of the blood vessel by crossing the endothelium, then cross the basal membrane, and last, migrate through the tissue to the site of infection (Gambardella & Vermeren, 2013). The first part of this process, namely stopping within the circulation and crossing the endothelium is called trans-endothelial migration (Schmidt *et al,* 2013; Ley *et al*, 2013; Muller, 2011). This process starts with neutrophils accumulating and becoming adherent on the luminal the surface of the endothelium. This is a complex process called the neutrophil adhesion cascade (Abbas *et al*, 2010, p. 30-35). Over the past fifteen years, considerable insight has been gained in the molecular mechanisms that facilitate neutrophil recruitment into tissues. This has led to an expansion of the classical three step leukocyte adhesion cascade (Ley *et al,* 2007). Here, I will briefly review the evolved paradigm of the leukocyte adhesion cascade, now including: capture, rolling, slow rolling, arrest, adhesion strengthening, intraluminal crawling and ultimately trans-endothelial migration (Fig. 1).

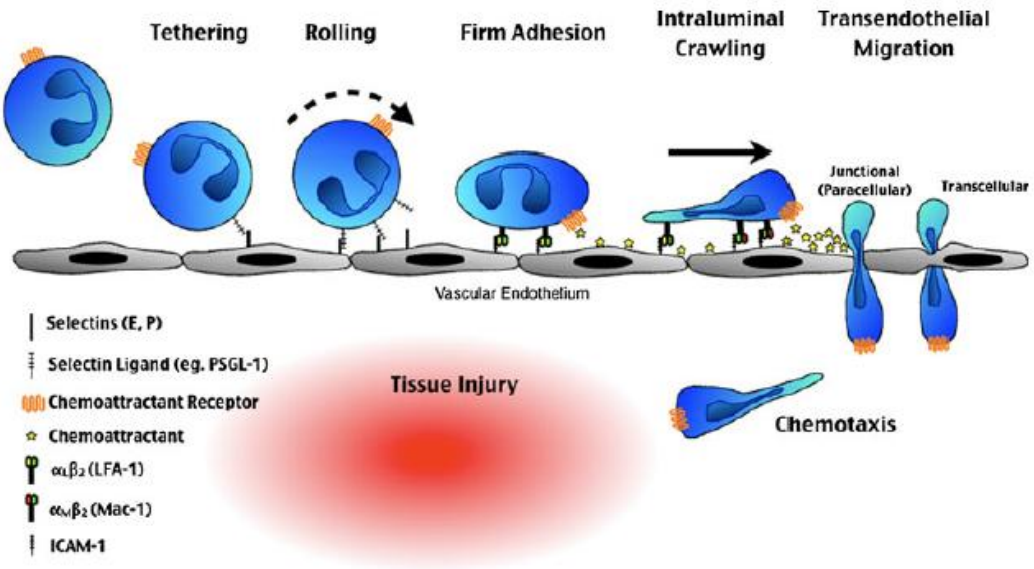

Figure 1. Schematic representation of the steps of the neutrophil recruitment cascade. This figure includes the adhesion molecules that are involved. (McDonald & Kubes, 2011)

The first step in the leukocyte recruitment cascade is tethering of the neutrophil to the endothelial cells, subsequently followed by rolling (Ley *et al*, 2007). The release of proinflammatory cytokines induces the expression of E and P-selectin on the apical surface of the endothelial cells. Selectins transiently bind their ligands which are continuously expressed on the neutrophil's cell membrane (Ley *et al*, 2007). The transient binding enables the neutrophil to 'stick' the endothelium and gently reduce its speed in a rolling fashion within the high shear stress conditions of blood flow (Voisin & Nourshargh, 2013, Ley *et al,* 2007).

Slow rolling mediates the switch from selectin-mediated rolling to integrin dependent firm adhesion on the endothelium (Ley *et al*, 2007). During slow rolling, interaction of selectins with their ligands causes β-integrin, LFA-1 (Leukocyte Function Associated Antigen 1), to become activated on the surface of neutrophils. Activated integrins bind stronger to their ligand, ICAM-1 (Intercellular

Adhesion Molecule 1), on endothelial cells than selectins do, thus causing the rolling to slow down and strengthen contacts between neutrophils and the endothelial cells. Enhanced contacts allow neutrophils to detect chemoattractants on the endothelial cell surface which elicit signalling events that mediate full arrest of neutrophils through complex regulation of integrin-affinity. This leads to multiple interactions of high-affinity integrins with their ligands (Voisin & Nourshargh, 2013; Woodfin *et al,* 2010). Together, high-affinity integrin interaction and chemoattractant sensing, lead to termination of rolling of the neutrophil. After neutrophils have come to a full stop they undergo a series of morphological changes. These morphological changes are characterized by polarization and spread caused by actin cytoskeletal rearrangements which results in extension of an invasive lamellopodium at the leading edge and a uropod at the trailing edge. (Voisin & Nourshargh, 2013; Schmidt *et al,* 2011).

When neutrophils come to a complete stand still, the process of intraluminal crawling starts (Ley *et al*, 2007). During intraluminal crawling, neutrophils migrate towards a preferred site of trans-endothelial migration. Research by Phillipson and colleagues (2006) indicates that neutrophils exhibit intraluminal crawling in a β2-integrin dependent fashion utilizing neutrophil Mac-1 and endothelial integrin ligand ICAM-1. A mechanism by which neutrophils transition from LFA-1 dependent firm arrest to β-integrin Mac-1 dependent intraluminal crawling is still unknown (Voisin & Nourshargh, 2013). Neutrophils can cover distances up to 60µm before finding a suitable location for trans-endothelial migration (Phillipson *et al,* 2006). When a suitable location is found, the formation of transmigratory cups is observed to precede trans-endothelial migration. These structures are ICAM-1, VCAM-1 (vascular cell adhesion molecule 1) and actin enriched domains at the contact surface of the neutrophil with the endothelial cell(s) that protrude from the endothelial surface to embrace the adherent neutrophil (Carman & Springer, 2004; Barreiro *et al,* 2002).

After coming to a halt on the surface of the endothelium, neutrophils have to take another crucial barrier: they have to cross the endothelial cell layer in order to reach the site of infection. This process is termed trans-endothelial migration (Schmidt *et al,* 2013; Muller, 2011; Woodfin *et al,* 2010). There are two possible modes in which this process could be carried out, either by squeezing through the junction of bordering endothelial cells (the paracellular pathway), or by passing through the body of an endothelial cell (the transcellular pathway) (Carman, 2009).

The aim of this thesis is to review if, how and when these two alternative pathways are used by neutrophils. For this I will first explain the mechanism of the paracellular pathway of trans-endothelial transmigration. Secondly, I will discuss the evidence that transcellular trans-endothelial migration does occur and describe several of the proposed mechanisms to this mode of trans-endothelial migration. At last, I will discuss the relative contributions of both modes to the trans-endothelial migration process and how the decision between the two modes is made.

3. Paracellular trans-endothelial transmigration

The process of paracellular trans-endothelial transmigration was the first of the two modes of trans-endothelial migration to be identified and has been investigated extensively (Carman, 2009). The mechanism of paracellular trans-endothelial transmigration is based on interactions of neutrophils with the endothelial surface. This activates intracellular signaling cascades which alter the behavior of both endothelial cells and neutrophils to allow passage through the endothelial cell junctions. This cross-talk between endothelium and neutrophils leads to cytoskeletal rearrangement alters neutrophil movement and junctional integrity (Schmidt *et al*, 2011). This chapter will first discuss the role of adhesion molecule interactions in guiding the neutrophil through the junction during paracellular trans-endothelial migration. Secondly, the role of junctional complexes in paracellular trans-endothelial migration will be discussed.

3.1 The role of Adhesion complexes in paracellular trans-endothelial migration

To date, numerous adhesion complexes within the endothelial cell junctions have been identified and many of them have been implicated to play a role in paracellular trans-endothelial migration. Here I will discuss several molecules that play important roles in para-cellular trans-endothelial migration, including JAM-A, ICAM-2 and PECAM-1. Although these molecules are important, the composition of the endothelial junction is much more complex, also including other adhesion complexes, such as Endothelial cell-selective adhesion molecule (ESAM), CD99, JAM-B and JAM-C, but will not be discussed here (Daniel & van Buul, 2013).

During intraluminal crawling, neutrophils are thought to seek for a suitable location to migrate through the endothelium. ICAM-2, JAM-A and PECAM-1 have been implicated in playing an important role in the process of trans-endothelial migration. Both PECAM-1 and JAM-A expressed in the endothelial cell junction undergo homophilic interactions with PECAM-1 and JAM-A expressed on neutrophils. ICAM-2 on the other hand binds integrin ligand LFA-1 on the neutrophil surface (Schmidt *et al,* 2011).

Woodfin and colleagues (2009) have proposed a mechanism whereby junctional molecules ICAM-2, PECAM-1 and JAM-A support trans-endothelial migration in a sequential fashion. Activation of the endothelium by two different stimulants, TNF-α or IL-1β, resulted in different effects of ICAM-2, PECAM-1 and JAM-A on the transmigratory response. While blocking ICAM-2, PECAM-1 and JAM-A with monoclonal antibodies they found no reduction in the fraction of transmigrating neutrophils when they measured the fraction of transmigrating wild type neutrophils in response to TNF-α. In contrast, when they measured fraction of transmigration of neutrophils in response to IL-1β, when blocking ICAM-2, PECAM-1 or JAM-A using monoclonal antibodies, they found a strong reduction of transmigration. No additive effect was observed when blocking all three adhesion molecules instead of only one. These findings suggest ICAM-2, PECAM-1 and JAM-A act to mediate different stages of the transmigratory process (Woodfin *et al,* 2009).

To further investigate and determine the location of arrest they used PECAM-1, ICAM-2 and JAM-A deficient mice (Woodfin *et al,* 2009). These mice were transfected with TNF-α receptor deficient neutrophils stimulated with IL-1β in their cremasteric venules. They used immunofluorescence and confocal microscopy to analyze the site of arrest of neutrophils on the endothelial junction.

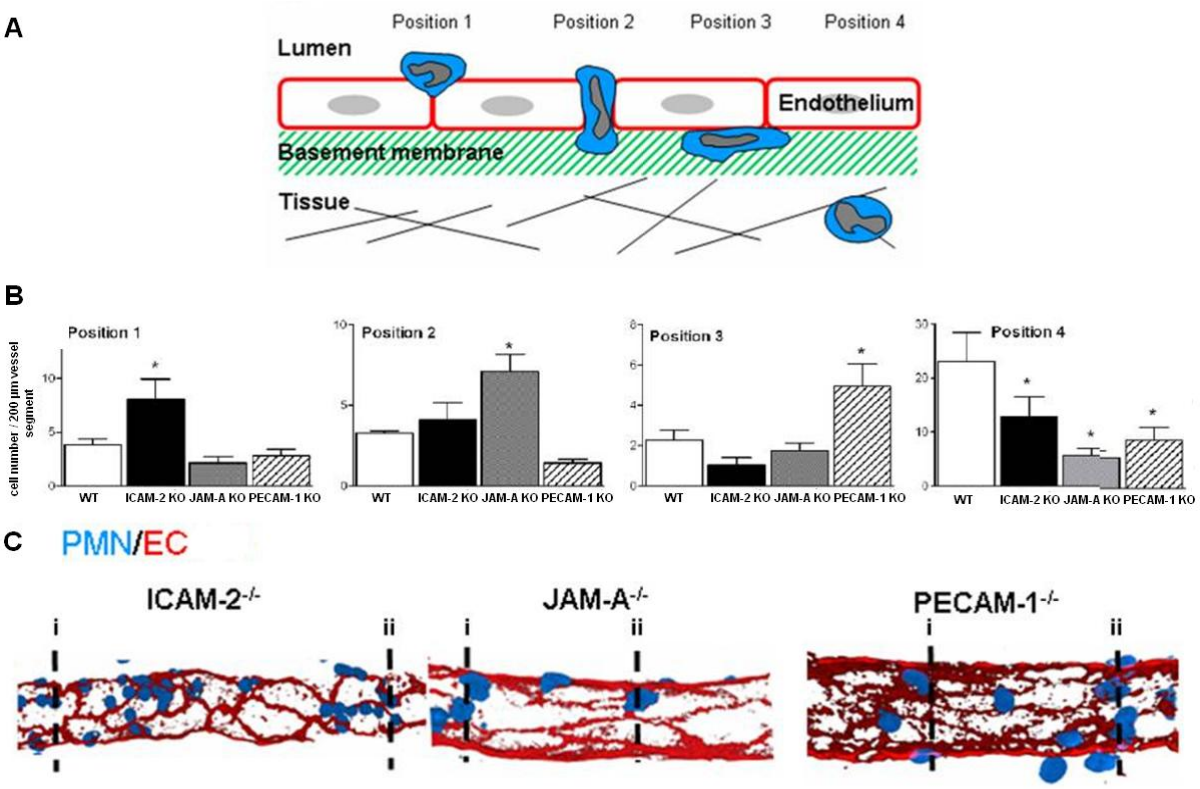

Figure 2. (A) Schematic diagram illustrating the quantification criteria to analyze the position of arrest of neutrophils in cremasteric mouse venules. (B) Mouse cremasteric venule endothelial cells deficient in the adhesion molecules ICAM-2, JAM-A or PECAM-1 were transfected with neutrophils. The graphs are showing the quantification of confocal microscopy data indicating the number of neutrophils that arrested at each position for each adhesion molecule deletion in the endothelial cells. (C) 3D confocal microscopy images of representative longitudinal mouse cremasteric venules stained for endothelial cell junctions (red) and Neutrophils (blue) showing the position of arrest of neutrophils in response to each endothelial cell adhesion molecule deficiency. (Adapted from Woodfin *et al,* 2009

In ICAM-2 deficient endothelium, neutrophils arrested at a very early stage, on the interphase between the vascular lumen and junctions, even before penetrating the junction. In contrast, in JAM-A deficient endothelium most neutrophils were found arrested within the endothelial junction. Furthermore, in PECAM-1 deficient endothelium, neutrophils were found to get stuck just underneath the cell junction, not able to penetrate the basal membrane. Collectively, these finding provide evidence for a sequential role for ICAM-2, JAM-A and PECAM-1 in regulating trans-endothelial migration in response to IL-1β. In this mechanism ICAM-2 appears to provides a gradient to guide neutrophils to the endothelial junction, JAM-A facilitates completion of passage through the junction and PECAM-1 serves to facilitate passage through the basal membrane (Woodfin et al, 2009).

A recently discovered cellular component, the lateral border recycling compartment (LBRC), might play a role in regulating the localization of adhesion receptors to facilitate trans-endothelial transmigration (Muller, 2011; Mamdouh *et al,* 2008). The LBCR is composed of a series of interconnected vesicles and tubules which is located close to the junctional plasma membrane. The LBRC membrane translocates to the location of transmigration by use of kinesin motor molecules along microtubules. Fluorescence imaging studies by Sullivan and colleagues (2013) showed that the LBRC recycles PECAM and JAM-A to the junctional plasma membrane by constant delivery and uptake of PECAM and JAM-A containing vesicles. The purpose of PECAM and JAM-A cycling is not yet understood. However, it might help to create gradients of adhesion complexes, thus facilitating a chemotactic response of leukocytes (Muller, 2011). The LBCR is essential for both paracellular and

transcellular trans-endothelial migration, as blocking the kinesin motor molecules blocks translocation of the LBCR to the location of transmigration (Mamdouh *et al,* 2008).

In addition to the sequential roles of ICAM-2, JAM-A and PECAM-1 described above by Woodfin (2009), JAM-A is also implicated to play a role in attraction of neutrophils toward the endothelial junction (Corada *et al*, 2005). JAM-A is expressed strongly in the endothelial junctions and on the surface of neutrophils and undergoes both homophilic interactions and integration with β2 integrin LFA-1. Their study, using both JAM-A deficient mice and JAM-A blocking monoclonal antibodies, revealed that by blocking JAM-A a reduced the fraction of neutrophils underwent extravasation of mesenteric post capillary venules *in vivo* in mice with inflammatory peringitis and ischemia-reperfusion injury (Corada *et al*, 2005). Microscopic examination of the venules showed that neutrophils were trapped on the surface of the endothelium. When analysing the effect of JAM-A deficiency *in vitro* by seeding neutrophils on fibronectin coated coverslips and inducing a chemotactic response, they observed a less efficient chemotactic response toward a chemotactic stimulus. Immunofluorescence analyses showed a reduction of actin polymerization at the leading edge of the neutrophil, inability to retract the uropod and defective polarization of neutrophils. This was accompanied by increased adhesion to matrix proteins preventing normal movement. According to the authors, these data suggest that JAM-A may direct the movement of neutrophils toward the endothelial cell junction (Corada *et al,* 2005). Other studies have suggested the same function for JAM-A (Woodfin *et al,* 2007; Nourshargh *et al,* 2006) whether endothelial JAM-A or neutrophil JAM-A is responsible for movement towards the cell junction is unclear. Studies using transfection of JAM-A deficient neutrophils to normal mice endothelium, and vice versa, find both endothelial JAM-A as neutrophil JAM-A to be essential for directed movement toward the junction (Woodfin *et al,* 2007; Nourshargh *et al*, 2006; Corada *et al,* 2005).

In conclusion, endothelial cells appear to utilize adhesion molecules in different ways to different stimuli. The effect of different proinflammatory cytokines on the expression and location of adhesion molecules seems to alter the mechanism by which neutrophils cross the vessel walls. In addition, different mechanism could be used in different types of endothelium or different locations within the body (Schmidt *et al,* 2011).

3.2 Breaking the interaction of Junctional molecules

While moving through the junction, neutrophils must employ a mechanism to break interactions of junctional molecules. Junctional sites contain a diverse array of multiprotein complexes that serve to maintain endothelial integrity as well as regulate permeability to macromolecules (Schmidt *et al*, 2011). In addition to the previously named adhesion molecules, proteins form connective complexes to maintain junctional integrity. These include VE-cadherins, Occludin and Claudin complexes (Fig. 3) (Daniel & van Buul, 2012). Neutrophils employ physical disruption of junctions, they force their way through, as well as molecular mechanisms to weaken the junction (Schmidt *et al,* 2011). Although our knowledge regarding the regulation of junctional complexes is limited, metalloproteinases and protein tyrosine kinases and phosphatases have been implicated to play a role in regulation of endothelial junctions (Daniel & van Buul, 2012). Here I will discuss a proposed mechanism which involves protein tyrosine phosphatases and causes short disruption of VE-cadherin bonds

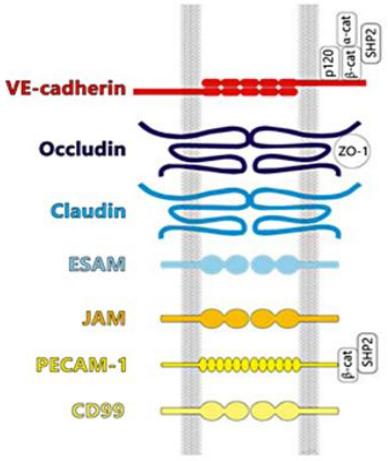

Figure 4. Schematic representation of the main multiprotein complexes that form the edothelial junction (not drawn to scale). (Adapted from Daniel & van Buul, 2012)

during trans-endothelial transmigration.

Disruption of VE-cadherin bonds weakens the junction and thus allows neutrophils to pass. Vascular endothelial protein tyrosine phosphatase (VE-PTP) is a component of the VE-cadherin complex and plays an essential role in maintaining endothelial cell junctional integrity (Nottebaum *et al,* 2008). Through fluorescent imaging studies, Nottebaum and colleagues (2008) found that VE-PTP specifically associates with VE-cadherin in the endothelial junction. Knocking out VE-PTP expression by RNA interference resulted in increased extravasation of neutrophils. When they allowed neutrophils to bind to endothelial cells, that were activated with TNF-α, before doing immunoprecipitation experiments, they observed an 80% reduction of VE-cadherin coprecipitating with VE-PTP. Furthermore, they propose that interaction of neutrophils with endothelial cell's ICAM-1 induces dissociation of VE-PTP. These combined results suggest that weakening of VE-cadherin bonds might be induced by a mechanism that is activated by ICAM stimulation. Interaction of neutrophils to ICAM-1 on activated endothelial cells might lead to dissociation of VE-PTP and subsequently loss integrity of VE-cadherin interactions Nottebaum *et al*, 2008). This process could allow neutrophils to migrate across the endothelial junctions by breaking VE-cadherin interactions.

Although the process of paracellular trans-endothelial transmigration is well-studied and well accepted, still no specific mechanism for this process in neutrophils has been identified. However in general, adhesion molecules seem guide and direct neutrophils toward the endothelial junctions. Moreover, interactions between neutrophils and endothelial cells, which might also be through adhesion molecules, weaken the endothelial cell junction to allow neutrophils to pass.

4. Transcellular trans-endothelial migration

In contrast to the well-studied pathway of paracellular trans-endothelial migration, the occurrence of pathway transcellular trans-endothelial migration is not that well accepted. The first person to hypothesize that cells might also pass right through the body of another cell was Adami. He intuitively challenged that 'leukocytes might pass directly through endothelial cells as one soap bubble may pass through another', thereby making way for a possible transcellular route (Carman & Springer, 2008).

4.1 Does Transcellular trans-endothelial migration exist

The first studies to investigate the mechanisms for transcellular endothelial migration demonstrated evidence for both the paracellular and the transcellular route as early as in the 1961. By using electron microscopy on serial sections of pancreatic microvasculature from dogs, Williamson and Grisham (1961) were able to visualize leukocytes, predominantly neutrophils, that were both completely and partially enclosed by endothelial cytoplasm as well as leukocytes migrating through the endothelial junctional areas (Fig. 4). However, the use of solely transmission electron microscopy does not provide definite proof for transcellular trans-endothelial migration (Muller, 2011).

With the development of techniques for the isolation and culture of primary endothelial monolayers in the 1970, it became possible to study transmigration of leukocytes *in vitro* (Gimbrone *et al*, 1974; Jaffe *et al,* 1973). At first, the lack of *in vitro* proof for the transcellular route in endothelial models has prevented widespread acceptance of the concept as well as mechanistic investigation (Furie *et al,* 1987; Feng *et al,* 1998; Muller *et al*, 2003).

However a decade ago, the first in vitro studies have shown unambiguous observations of transcellular trans-endothelial migration. For example, Carman et al (2004) reported observations of transcellular trans-endothelial migration in human umbilical vein endothelial cell (HUVEC) monolayers by monocytes, neutrophils and lymphocytes. They activated the HUVEC monolayers with TNFα and washed them with a monocyte, neutrophil or lymphocyte specific chemoattractant before incubation with one of the three cell types. After several different incubation times, the monolayers

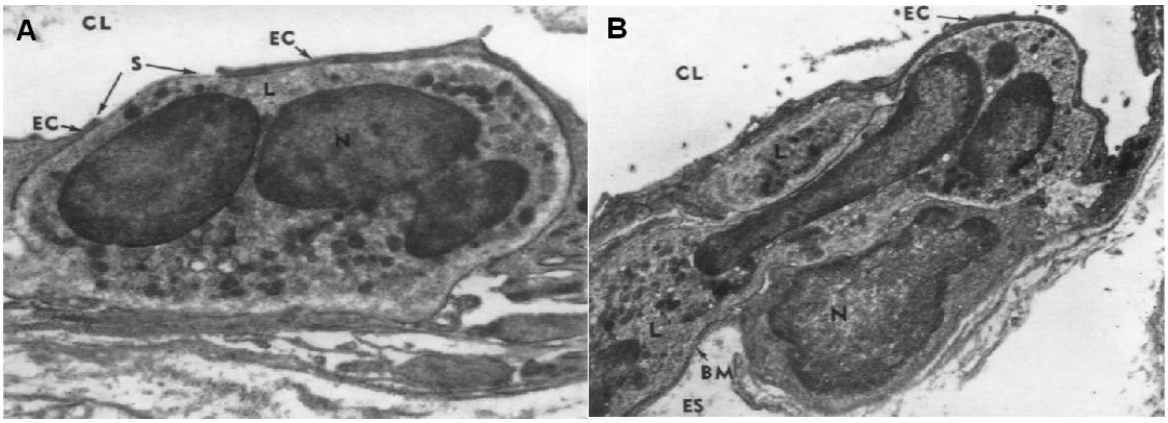

Figure 4 (A) Shows an electron microscopy image of a leukocyte which is almost completely enclosed by the endothelial membrane of an endothelial cell. (B)Shows an electron microscopy image of a leukocyte which is completely enclosed by endothelial membrane of an endothelial cell. Abbreviations indicating structures in the cell are: Endothelial cell, EC, Leukocyte, L, Nucleus, N, Basal membrane, BM, Extravascular space, ES, Capillary lumen, CL, and the narrow segment which is not completely enclosed in endothelial cytoplasm, S. (Adapted from Williamson & Grisham, 1961)

were stained with antibodies against integrin LFA-1, it's ligand ICAM-1 and VE-cadherin, a marker for endothelial junctions, and analyzed using high resolution serial-sectioning confocal microscopy. By using the VE-cadherin marker they could unambiguously differentiate between paracellular and transcellular trans-endothelial migration. A small fraction (5%) of neutrophils was unambiguously observed to use the transcellular route of trans-endothelial migration. The real fraction of neutrophils taking the transcellular route might even be larger, as cells transmigrating close to a junction site were scored as taking the paracellular route. Furthermore, research by Cinamon and colleagues recorded transcellular migration by neutrophils by using video microscopy (videos) (Cinamon *et al*, 2004).

With this accumulating evidence, it is now clear that neutrophils are able to transmigrate over the endothelial wall by two modes, paracellular and transcellular (Woodfin *et al,* 2010; Muller, 2013). As intensive research has already partially uncovered the mechanisms for paracellular trans-endothelial migration, an additional goal will be to elucidate the mechanisms involved in transcellular trans-endothelial migration.

4.2 Mechanism of transcellular trans-endothelial migration

Whereas the location for paracellular trans-endothelial migration is intuitively logic, namely the endothelial junction regions, there is none for transcellular trans-endothelial migration (Carman, 2009). The first obvious step for migration across the endothelium must be determining a location to do so. Secondly, obstructive cell components and organelles must be displaced to allow the transmigrating neutrophil to pass through the endothelial cell. Finally, the neutrophil has to pass through the body of the endothelial cell, which requires fusion of the apical and basal plasma membranes (Carman *et al,* 2009). The regulation of this process could be regulated by either the leukocyte, the endothelial cell or both could be involved. A few processes have been proposed to play a role in transcellular trans-endothelial migration. In this part I will discuss the formation of protrusions and formation of the transcellular pore as steps in the mechanism of transcellular trans-endothelial migration.

The first step to start extravasation after adhesion to the endothelium of the vessel wall is to find the right spot. Neutrophils are thought to use a mechanism called lateral migration or intraluminal crawling whereby they extend invadosome like protrusions (ILPs) to find the right spot to cross the endothelium (Carman *et al,* 2008; Phillipson *et al*, 2006). As stated before, the process of intraluminal crawling is also observed in paracellular trans-endothelial migration. The occurrence of this process is generally interpreted as a mechanism to allow migration toward the junctional regions of endothelial cells (Phillipson *et al*, 2006; Schenkel *et al,* 2004). However, Carman and colleagues also observed the process of lateral migration to precede transcellular extravasation of lymphocytes *in vitro*. These results could indicate that lateral migration serves as a mechanism to place leukocytes in the optimal position for extravasation (Carman *et al*, 2007).

However, the process of intraluminal crawling is observed in the context of transcellular trans-endothelial migration, it is not essential for the transcellular pathway. Research by Philipson (2006) observed that intraluminal crawling no longer occurs in Mac-1, a β2 integrin, deficient leukocytes. Moreover, a larger fraction of leukocytes was observed to use the transcellular route in response to Mac-1 deficiency (Phillipson *et al*, 2006).

4.2.1 Invadosome-like-protrusions

During intraluminal crawling, neutrophils are thought seek out sites permissive for trans-endothelial migration. A possible mechanism that allows them to find these sites is proposed by Carman and colleagues (2007). Through life-cell fluorescent imaging combined with differential interference

contrast modalities they were able to visualize leukocyte protrusions that form invaginations in the endothelial cell layer during intraluminal crawling. The observed structures showed similarity to both podosomes and invadopodia. podosomes and invadopodia are defined as actin dependent adhesive and/or protrusive structures. Due to the similarity of the observed protrusions but also the undefined nature of the leukocytes protrusions they were named invadosome-like-protrusions (ILPs) (Carman, 2009). The formation of ILPs seems to be a highly dynamic process as they form rapidly in a clustered fashion. The invaginations in the endothelium cell surface, caused by ILPs, were observed to displace and distort the cytoplasm and underlying structures, like actin filaments, microtubules and endoplasmic reticulum (Fig. 5a). When encountering an endothelial cell's nucleus, the ILPs were observed to only penetrate shallowly (Fig. 5b, c) and to retract quickly. This observation suggests that leukocytes have no a priori knowledge whether they can or cannot extravagate at the location of the nucleus but rather use their palpating behaviour to discover through trial and error where to extravagate. Furthermore, in nonnuclear areas the protrusions were only shallow and retracted quickly. These observations combined made the authors hypothesize that the efficiency for transcellular trans-endothelial migration is regulated by the sum local resistance provided by the endothelial organelles. They propose that palpation by ILPs serves as a stochastic mechanism to identify locations of relatively low endothelial resistance, thus leukocytes would take the path of least resistance. This idea is also supported by two studies in which lifespan and density of podosomes was measured while palpating polyacrylamide collagen-coated substrates of defined rigidity. They demonstrated that lifespan and density of fibroblast podosomes is dependent on membrane flexibility (Collin *et al,* 2008; Collin *et al,* 2006).

The previously described study (Carman *et al,* 2007) deals with lymphocytes and monocytes, but not neutrophils. To my knowledge, the functional aspects of this mechanism have not yet been described in neutrophils. However, similar protrusions were described by a study using transmission electron microscopy on neutrophils (Fig. 6) (Cinamon *et al,* 2004).

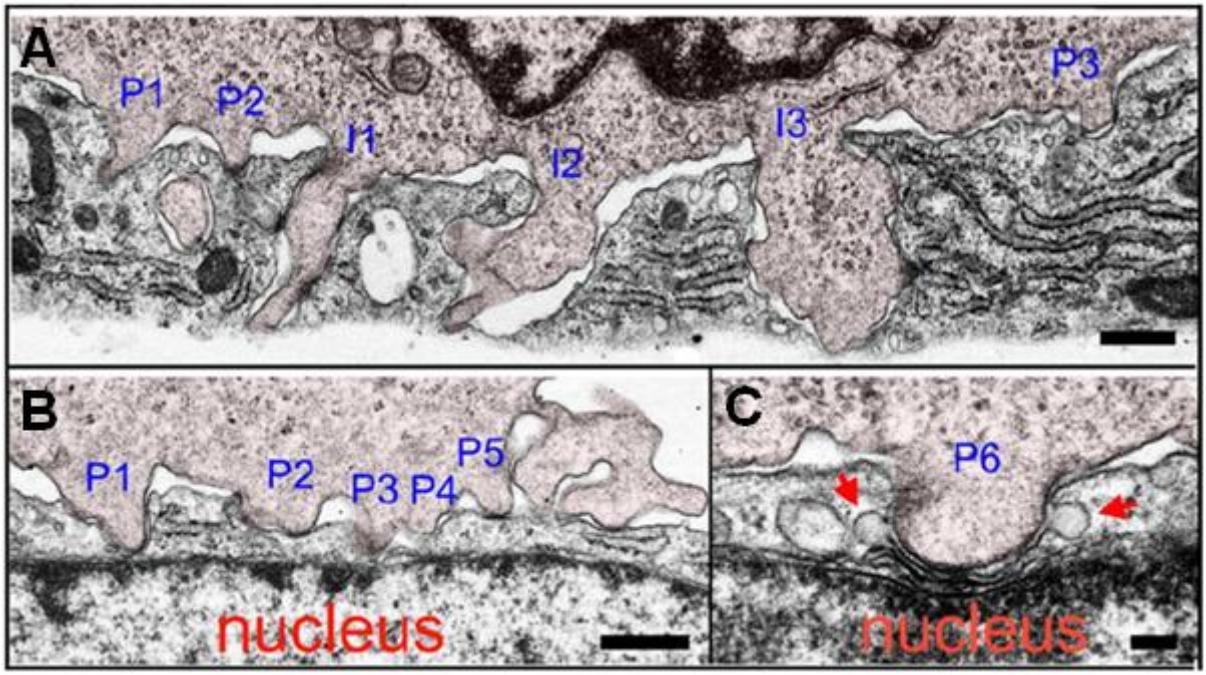

Figure 5. Transmission electron microscopy images of lymphocytes migrating on TNF-α activated human dermal microvascular endothelial cells. (A) The lymphocyte extends three shallow ILPs (P1-3) and three longer extending invasive podosomes (I1-3). (B) Shallow lymphocyte ILPs which have formed directly above the nucleus. (C) Lymphocyte podosome shows a mild indentation, but not invagination, above the nucleus. (Adapted from Carman *et al*, 2007)

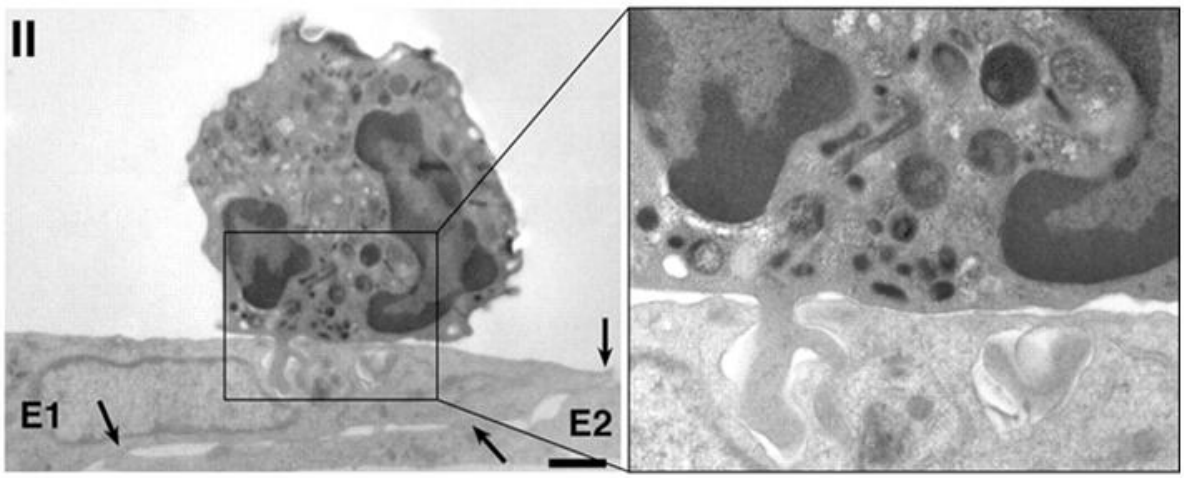

Figure 6. Transmission electron microscopy image of ultrathin sections of a neutrophil extending multiple invaginations into the apical side of human umbilical vein endothelial cells. E1 and E2, endothelial cell 1 and 2, the arrows indicate the endothelial junction. (Adapted from Cinamon *et al*, 2004)

4.2.2 Transcellular pore formation

When a proper location for transmigration across the endothelium is selected, neutrophils must utilize a mechanism that allows them to cross the endothelial cell body. Palpating ILPs have been found to play a role in transcellular pore formation by progressive extension. Extension of ILPs causes the apical and basal membrane of the endothelial cell to close proximity. The extended ILPs are now called invasive podosomes (Fig. 5a) and can reach all the way to the basal endothelial surface and is thought to facilitate formation of a transcellular pore through which neutrophils can transmigrate (Carman *et al,* 2007).

In addition to processes carried out by neutrophils, also the endothelial cells themselves are thought to actively contribute to the formation of the transcellular pores (Carman *et al*, 2007). *In vitro* and *in vivo* ultrastructure analyses revealed an enrichment of endothelial vesicles and vesiculo-vacuolar organelles (VVOs) adjacent to the site of invasive podosome penetration by lymphocytes. Quantitative analyses showed a fourfold increase of density of these vesicles in endothelial cells penetrated by invasive podosomes compared to endothelial cells with adherent lymphocytes which did not extend invasive podosomes. These results indicate that invasive podosomes trigger recruitment and fusion of endothelial vesicles with the endothelial plasma membrane (Carman *et al,* 2007). When analysing the properties of the observed vesicles by live-cell fluorescence imaging they found the membrane areas where invasive podosomes protruded were enriched with the fusogenic SNARE proteins VAMP2 and VAMP3, which play a role in fusion of intracellular vesicles with the cell membrane. When blocking the function of the SNARE complex, they observed an 87% reduction of transcellular pore formation in lymphocytes (Carman *et al,* 2007). Thus, the fusion of vesicles to the endothelial membrane appears to be essential for transcellular pore formation. The authors hypothesize that the protrusion of podosomes triggers signals in the endothelial cell that causes the vesicles to fuse with the membrane surrounding the podosome, thus providing additional membrane to promote pore formation and ultimately allowing the leukocyte to cross.

To date and as described above, we found multiple lines of unambiguous evidence for the existence of the transcellular trans-endothelial migration pathway. Furthermore, a possible mechanism is proposed. However, most of the details concerning transcellular trans-endothelial migration remain unknown. For example, there is no coherent view on the molecules that are involved in this process. Also, research specifically aimed to elucidate the mechanism of transcellular trans-endothelial migration has, to my knowledge, not been done yet.

5. Relative contribution of transcellular trans-endothelial migration

A number of *in vivo* studies have assessed the relative contribution of trans- and para-cellular trans-endothelial migration of neutrophils in several different types of settings. However, compared to lymphocytes, relatively few studies have focused on the relative contributions of both modes of neutrophil extravasation (Sage & Carman, 2011).

Experiments which were using a model of peripheral inflammation in the skin have resulted in findings of predominant use of the transcellular pathway, in rodents and humans, with relative contributions ranging between 83 and 100% (Hoshi *et al,* 1999; Feng *et al*, 1998; Shubert *et al,* 1989). Furthermore, studies using a model of peripheral inflammation in mice's cremasteric venules have reported a relatively large contribution of the transcellular mode, with relative contributions ranging between 14 to 70% (Phillipson *et al*, 2008; Phillipson *et al*, 2006).

One study by Faustmann (1985) describes the transmigratory mode used by neutrophils in acute inflammation of the brain's microvasculature of cats reported predominant use of the transcellular pathway.

In contrast to the relatively high contribution of the transcellular mode of trans-endothelial migration in certain specific tissues, *in vitro* studies in HUVEC endothelial models have reported a very small contribution of the transcellular mode, which is 5% (Riethmuller *et al,* 2008; Cinamon *et al,* 2004*;* Carman *et al,* 2004).

In summary, the relative contribution of the transcellular pathway for extravasation seems to depend strongly on the inflammatory stimuli, tissues and whether the study was done *in vivo* or *in vitro.*

6. Conclusions and discussion

To date, we do have a general idea about the mechanisms that facilitate paracellular trans-endothelial migration: neutrophils are attracted towards endothelial junctions by interactions between the neutrophil's and endothelial adhesion molecules. Then, intricate interplay between endothelial cells and the neutrophil forms a trans migratory cup. And finally, neutrophils are thought to migrate through the junction by both actin rich protrusions, i.e. 'muscle' their way through, and are pulled through by interactions with adhesion molecules expressed in the endothelial junctions. Furthermore, interaction between neutrophils and endothelial cells is thought to weaken cellular junctional molecule interactions

The specific adhesion molecules that are employed during different stages of paracellular trans-endothelial migration and the functions they carry out seem to differ in response to different stimulatory factors. Furthermore, different types of endothelia might also employ different sets of adhesion molecules to facilitate trans-endothelial migration.

Systematic research to specify the different subsets of adhesion molecules that are employed by different types of endothelia or in response to different cytokines is still required before we will understand the details of this highly dynamic and complex process. This knowledge will be required for application in, for example, drug development.

As the amount of evidence, supporting the existence of the transcellular pathway of trans-endothelial migration, grew and methods to study trans-endothelial migration became more accurate, the occurrence of this process became more and more accepted. By now, the first studies have attempted to elucidate the mechanisms by which neutrophils transmigrate via the transcellular pathway. Their observations indicate that neutrophils exhibit intraluminal crawling prior to, however not essential for, transcellular trans-endothelial migration. During intraluminal crawling, neutrophils have been observed to extend protrusions that palpate the endothelial surface, possibly searching for an area with sufficiently little resistance to allow passage. When such an area is located, neutrophils can extend their protrusions, bringing the apical and basal membrane in close proximity, thus facilitating formation of a transcellular pore serving to cross the endothelial cell. Endothelial cells are thought to actively facilitate passage of neutrophils by fusing vesicles to the cell membrane, thus providing additional membrane to allow transmigration.

Our knowledge of the transcellular process is by far not as detailed as what we know about paracellular process. Furthermore, only several different locations and settings have been studied regarding the occurrence, let alone relative contributions, of transcellular trans-endothelial transmigration. Future research should focus on systematically identifying the adhesion complexes and their functions that are used in the process of transcellular trans-endothelial migration. Furthermore, additional studies regarding the relative contributions of both modes of trans-endothelial migration of neutrophils, in different settings and tissues, should be carried out to further unravel the mechanisms involved.

Several theories have been proposed on how neutrophils decide to take the paracellular or the transcellular route of trans-endothelial migration. One proposed by Lossinksky and Shivers (2004), argues that leukocytes take 'the route of least resistance' to cross the endothelial barrier. Simplified, this idea suggests that the relative ease of carrying out a paracellular crossing or forming a transcellular pore determines the route of trans-endothelial migration.

Formation of a transcellular pore requires bending or indentation of the endothelial cell membrane, which is dependent on the local resistance (Carman *et al*, 2007). The local resistance is determined by the cellular structures and organelles that lay below the cell membrane. In order to form a cellular pore, these must be displaced (Carman *et al*, 2007). Then, membrane must be recruited and has to fuse to allow passage of the neutrophil (Carman *et al*, 2007). To carry out a paracellular crossing, adhesion complexes must be rearranged and interactions of junctional molecules, like cadherin, occludin and claudin complexes, must be disrupted. In some locations, for example the blood brain barrier, there will be a higher degree of organization and stronger barrier function (Sage & Carman, 2011). As both routes of trans-endothelial migration will require energy, according to Lossinsky and Shivers theory, the decision to use one or the other pathway will be decided by which process requires less energy. Thus, the decision would rely on endothelial activity and phenotype (Sage & Carman, 2011)

Endothelial cells are subject to the complex environment they live in. Cues from that environment, for example inflammatory and vasoactive stimuli, stimuli from bordering cells and the composition of the basal membrane could, according to Lossinsky and Shivers theory, have a strong impact on the relative contribution of each route of trans-endothelial migration. As *in vitro* studies usually do not include those distinct environmental cues, they also cannot influence endothelial junction and cytoskeletal organization. This could explain the small contribution of the transcellular pathway in *in vitro* models (Sage & Carman, 2011)

In addition to the role of the endothelium in decision making, the activation state of the neutrophil may play a role as well (Muller, 2011). As explained before, the contribution of transcellular trans-endothelial migration in *in vitro* HUVEC models is relatively low. However, when directly stimulating the neutrophils and monocytes that were used in this study with chemokine or chemoattractant to the apical side of the HUVEC monolayer, in order not to create a chemotactic gradient; they observed an increase of the use of the transcellular pathway of up to 30% (Mamdouh *et al,* 2009). These results indicate that the activation state of the neutrophil plays a role in the decision making process

The mechanism for paracellular trans-endothelial transmigration is well-studied and well accepted, however, our knowledge regarding the details of this mechanism in neutrophils is still lacking. The process of transcellular trans-endothelial transmigration, which occurrence was only recently accepted, is therefore much less studied. Hence, our understanding of this process is also still incomplete. The question how neutrophils decide which route to take is therefore difficult to answer. Factors that might play a role have been identified. These include properties of the endothelial cells and the activation state of the neutrophil and the environment in which the endothelial cell and neutrophil carry out trans-endothelial migration. However to answer this question, more studies are necessary, especially to elucidate the dynamics of adhesion molecules in response to different types of stimuli for both paracellular and transcellular trans-endothelial transmigration.

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