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Mechanisms of neutrophil transendothelial migration

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1. ABSTRACT

Neutrophil recruitment is an integral part of the immune response to infection as well as of inflammatory disorders. The process of neutrophil extravasation comprises a complex multistep cascade that is orchestrated by a tightly coordinated sequence of adhesive interactions with vessel wall endothelial cells. Adhesion receptors as well as signaling molecules in both neutrophils and endothelial cells regulate the recruitment of neutrophils into the site of inflammation or infection. The present review will focus on novel aspects with regards to the last step of neutrophil recruitment, namely the transmigration of neutrophils through endothelial cells.

2. INTRODUCTION

The short-lived neutrophils are the most abundant leukocytes in the blood and the first line of defense in innate immunity. Neutrophils can detect bacterial components such as LPS and fMLP via their specific receptors including Toll-like receptors or G-protein coupled receptors (1). This results in upregulation of the migratory activities of neutrophils, allowing them to accumulate at sites of acute inflammation within a few hours. Besides being essential for the defense against bacterial and fungal pathogens, neutrophils are also a hallmark of T helper type 1 (TH1)–associated inflammatory infiltrates (2). Activated recruited neutrophils release cytotoxic mediators that may

cause tissue damage, thereby contributing to autoimmunity and chronic inflammation. Neutrophil infiltration is a hallmark in rheumatoid arthritis, vasculitis, inflammatory bowel diseases and chronic lung diseases (3;4). Therefore, neutrophil extravasation to sites of inflammation, vascular injury or infection needs to be a tightly regulated process. Neutrophil recruitment requires a multistep cascade of adhesive and migratory events, which are mediated by three classes of adhesion receptors, the selectins, integrins and adhesion receptors of the immunoglobulin superfamily. These steps are (i) the initial selectin-mediated rolling, (ii) the chemokine-induced activation (iii) and the integrin-dependent firm adhesion and subsequent transendothelial migration (5-7). However, recent evidence has resulted in the expansion of the original three-step adhesion cascade, including new separate steps, such as integrin-mediated intravascular crawling following the firm arrest of neutrophils (6;8;9). Moreover, transendothelial migration (also designated as diapedesis) can take place in both a paracellular and a transcellular fashion (10). The further subendothelial interstitial migration of leukocytes in the inflamed tissue is predominantly mediated by beta1-integrin family members, such as the matrix binding alpha1beta1-, alpha2beta1- and alpha6beta1-integrins, as well as beta2-integrin family members, such as Mac-1 that binds to fibrinogen (11-13). This process is also facilitated by leukocyte-associated membrane-bound proteases or secreted proteases as well as glycosaminoglycan-degrading enzymes, which help the invasion of neutrophils through the extracellular matrix (11;14). After an introduction in the different steps of neutrophil endothelial interactions, the present review will focus on novel aspects regarding the last step of neutrophil recruitment, their transendothelial migration.

3. THE MULTISTEP PROCESS OF NEUTROPHIL RECRUITMENT

During rolling, neutrophils undergo a transient interaction with the endothelial cell surface that slows them down. Rolling interactions last seconds and are reversible, as they are mediated by weak binding between selectins, such as the E-, P- or L-selectin with their carbohydrate ligands, such as P-selectin glycoprotein-1 (PSGL-1) (15). The expression and the exposure of endothelial selectins, such as P- and E-selectin on the apical endothelial cell surface is a tightly regulated process. P-selectin is stored in Weibel-Palade bodies and can be rapidly translocated to the luminal surface upon endothelial cell activation with several stimuli. Contrastingly, E-selectin is constitutively not expressed, but is newly synthesized after a few hours (15). The P-selectin / PSGL-1 interaction is predominantly involved in the initial tethering (16), whereas slow and more stable rolling is mediated by E-selectin (17). There is evidence that the initial rolling and the subsequent firm adhesion are functionally interconnected at least at two levels: (i) Evidence exists that LFA-1 integrin, especially when it is in a low-affinity conformation, participates in slow rolling adhesions. The subsequent transition into the active conformation of LFA-1 promotes arrest from the rolling state (18;19). (ii) Second, besides functioning as a "brake" for the flowing neutrophils, rolling interactions allow neutrophils to sense chemokines, associated with the endothelial cell membrane via heparan sulphate proteoglycans. The chemokine-derived signals are essential for priming the firm adhesion step (20).

During the activation step, chemokines including interleukin-8, and chemoattractants including complement C5a, leukotriene LTB4, platelet activating factor and bacteria-derived formylated peptides, induce rapid neutrophil adhesion, by converting the low-affinity, selectin-mediated interaction into the high-affinity, integrin-mediated firm adhesion (6;20;21). Neutrophil adhesion to endothelial cells is mediated by interactions between integrins, VLA-4 (alpha4beta1), Mac-1 (alphaMbeta2) and LFA-1 (alphaLbeta2), present on neutrophils, and members of the immunoglobulin superfamily, such as ICAM-1, ICAM-2, VCAM-1, or the receptor for advanced glycation endproducts (RAGE) on the endothelial surface (22). These endothelial counter-receptors are constitutively expressed (ICAM-1, ICAM-2, RAGE) or further up-regulated (ICAM-1, RAGE) or are induced (VCAM-1) (22;23). The importance of integrin-mediated adhesion for neutrophil extravasation and the immune response is evidenced by several studies engaging mice deficient in one or more leukocyte integrins (24-27), or by the leukocyte adhesion deficiency syndrome (LAD I) in men lacking beta2-integrins (22;28).

During extravasation, the activity of leukocyte integrins is predominantly regulated by conformational changes and clustering, whereas integrin expression on leukocytes is marginally affected. The activation of integrins through "inside-out" signaling, predominantly mediated by chemokines, involves several pathways, including small GTPases (29), as well as interactions between the cytoplasmic tail of the integrin and actin-binding proteins, that induce integrin conformational changes (30). The small GTPase RAP1 (31) regulates LFA-1 affinity (32-35). Guanine-nucleotide-exchange factors (GEFs), such as CALDAG-GEFI (calcium- and diacylglycerolregulated GEFI) (36;37), or VAV1 (38) that activate RAP1 participate in the modulation of leukocyte integrin affinity. CALDAG-GEFI-deficiency resulted in reduced chemokine-induced neutrophil arrest in humans and mice (36;38). In addition, actin-binding proteins such as talin (30;39) or alpha-actinin (40) as well as 14-3-3 proteins (41) have been shown to activate the integrins by targeting the beta-chain. Talin is a cytoskeletal protein consisting of two domains, a globular head and a rod-like domain. The head of talin is thought to induce the separation of the alphaL- and beta2-cytoplasmic tails of LFA-1 resulting in the induction of LFA-1 high-affinity conformation (42;43).

Neutrophil adhesion to the endothelial cells can be further strengthened by integrin-mediated outside-in signaling that takes place as a result of integrin clustering and conformational changes as a result of integrin ligation. Two Src-like protein tyrosine kinases can mediate the LFA-1- and Mac-1-induced stabilization of adhesion after the initial firm arrest, as their inhibition accelerates the detachment of adherent neutrophils under flow conditions

(44). Similar functions have been ascribed to the GEFs VAV1 and VAV3 (38). In addition, Wiskott-Aldrich syndrome protein (WASp)-deficient neutrophils display defects in the actin cytoskeleton and integrin clustering following the initial arrest. The WASp-deficiency phenotype became visible only under flow conditions (45). Another factor that has been implicated in leukocyte post-adhesion strengthening events is paxillin that interacts with the cytoplasmic domain of the alpha4-integrin chain (46).

4. NEUTROPHIL TRANSENDOTHELIAL MIGRATION

After their firm adhesion, neutrophils crawl over the endothelial cell surface to the nearest junction using their integrins Mac-1 and LFA-1, a process called locomotion or crawling (8). Recently, *in vivo* evidence about neutrophil crawling was presented (9). Transendothelial migration primarily takes place in a paracellular manner, i.e. at the intercellular junctions. In particular, tricellular junctions (47), as well as endothelial junctions positioned above areas where the basement membrane expresses lower levels of matrix proteins (48), have been proposed as preferential areas of extravasation for transmigrating neutrophils *in vivo*. Moreover, recent evidence indicated that neutrophils and other leukocytes may enter the extravascular tissue in a transcellular manner, i.e. through the endothelial cells (12;49;50). In fact, the transcellular pathway may become more prominent when intravascular crawling is disabled *in vivo* (9). *In vitro* findings, pointed to the predominance of the paracellular route for neutrophils, however, 5% of neutrophils were found to use the transcellular route (49). Interestingly, longer TNF-alpha pretreatment or ICAM-1 overexpression resulted in an increase of the relative contribution of the transcellular pathway in neutrophil transmigration (51).

For the paracellular pathway, endothelial junctions represent the major barrier for the transmigrating neutrophil. At least two types of junctions are involved in transmigration: (i) Adherens junctions (zonula adherens) formed by VE-cadherins that promotes calcium-dependent, homophilic cell-cell contacts. The link between cell-membrane-associated VE-cadherin and the actin cytoskeleton is mediated by intracellular catenins. (ii) Tight junctions (zonula occludens), the most apical junctions, form a close intercellular adhesive contact and consist of three types of transmembrane proteins, occludin, claudins and junctional adhesion molecules (JAM), which are linked intracellularly to cytoskelettal signaling molecules such as zonula occludens-1 (ZO-1) (52).

4.1. VE-cadherin

VE-cadherin acts as a gatekeeper for the passage of leukocytes, since antibodies against VE-cadherin increase the permeability of endothelial-cell monolayers and the rate of neutrophil extravasation *in vivo* (53), whereas *in vitro* studies indicate that VE-cadherin gaps form transiently during diapedesis (54). However, it is still unclear, whether the disappearance of VE-cadherin from the junction at the site of neutrophil transmigration is a prerequisite for the process or a consequence thereof. A potential explanation is that neutrophil adhesion to endothelial cells results in a decrease in VE-cadherin-mediated adhesion partially regulated by VE-cadherin phosphorylation. The phosphorylation of tyrosines 658 and 731 in the cytoplasmic tail of VE-cadherin as well as of Ser 665 has been correlated with the barrier function of VE-cadherin (55;56). More recently ICAM-1-mediated neutrophil adhesion to endothelial cells was shown to induce VE-cadherin tyrosine phosphorylation promoting neutrophil transmigration (57). Other pathways including the GTPase Rac1, reactive oxygen species and the ROS-activated proline-rich tyrosine kinase 2 that can phosphorylate beta-catenin have also been implicated as signaling intermediates (58;59).

Interestingly, neutrophil-derived elastase is able to degrade the extracellular part of VE-cadherin. Thus, neutrophils may engage this mechanism to facilitate their migration through endothelial cell-cell junctions (60;61). However, the importance of proteolytic cleavage of VE-cadherin by neutrophil proteases is unclear, since elastase-deficient or MMP-9-deficient neutrophils had no defect in transendothelial migration under flow (62).

4.2. JAMs and related molecules

JAMs are immunoglobulin superfamily members, consisting of two extracellular Ig-like domains (63;64) that are found in tight junctions of endothelial and epithelial cells most likely due to their class-II PDZ domain-binding motif at their final carboxy-terminus, which predisposes them to interact with junction-associated PDZ-domain-containing molecules (63;64). JAM-A is also found on different circulating blood cells including platelets, neutrophils, monocytes, and lymphocytes (65), whereas the expression pattern of JAM-B and JAM-C is more restricted with JAM-C being also present on platelets and a B-cell subpopulation (66-69). Besides their propensity to interact in a homophilic fashion through a conserved motif in their membrane-distal domain (70-72), JAMs are also engaged as counter-receptors for leukocyte integrins: In particular, JAM-A binds to LFA-1 (73;74), JAM-B associates with VLA-4 (75) and JAM-C interacts with Mac-1 (68).

A first evidence for a putative role of JAM-A in neutrophil diapedesis derived from experiments demonstrating inhibition of neutrophil recruitment in experimental meningitis with an antibody to JAM-A (76). JAM-A-deficient mice displayed reduced neutrophil recruitment in a peritonitis model, however, in this model, it was exclusively JAM-A on the myeloid cells / neutrophils that was necessary for transmigration, whereas JAM-A on endothelial cells was found to not be required (77;78). Contrastingly, endothelial JAM-A mediated neutrophil extravasation in a liver model for ischemia-reperfusion injury model (78).

The heterophilic binding of JAM-C to Mac-1 was found to mediate a firm platelet-neutrophil interaction especially under low-shear rate (68). In addition, soluble JAM-C blocked neutrophil transmigration through endothelial and epithelial cells (79-81). Neutrophil

accumulation in a model of lung inflammation was inhibited by blocking antibodies against JAM-C and was enhanced by endothelial-specific overexpression of JAM-C (81). However, whether these functions of JAM-C can be corroborated with JAM-C deficient mice remains controversial (82;83). In addition to binding to Mac-1, JAM-B efficiently binds to JAM-B (84), and an antibody to JAM-C dissociated the JAM-C/JAM-B heterodimers at the endothelial cell-cell contacts, thereby rendering JAM-C accessible for the integrin Mac-1 (85). We recently demonstrated an unexpected function of JAM-C to mediate an increase in endothelial permeability. Knockdown of JAM-C increased activity of the small GTPase RAP1 in endothelial cells and promoted VE-cadherin-mediated interendothelial contacts, representing the first functional interplay between tight junctions and adherens junctions in endothelial cells (72;86). In contrast, JAM-A may promote the activity of RAP1 in epithelial cells (87). Together, the underlying mechanism for the role of JAM-C in neutrophil transmigration is rather complex. It remains to be determined which of the above outlined pathways is operative in mediating the effect of JAM-C in neutrophil extravasation *in vivo*.

The JAM-related endothelial selective adhesion molecule (ESAM) differs from JAMs by having a longer cytoplasmic tail ending in a type-I PDZ-domain binding motif (63). In a recent study, ESAM-deficient mice displayed reduced neutrophil diapedesis and thereby delayed neutrophil accumulation in thioglycollate-induced peritonitis, as well as upon TNF-alpha or IL-1beta stimulation (88). A potential explanation could be that ESAM expression regulates the activity of RhoA GTPase, which is involved in regulation of the endothelial barrier and neutrophil transmigration (89;90). It is intriguing to hypothesize that JAMs and related molecules such as ESAM regulate the activity of small GTPases in endothelial cells and thereby participate in neutrophil transmigration. However the underlying mechanistic details are not yet defined and merit further investigation.

4.3. Platelet endothelial cell adhesion molecule-1 (PECAM-1) and CD99

PECAM-1 is a member of the immunoglobulin superfamily consisting of six Ig domains and is expressed at the intercellular borders of endothelial cells as well on platelets, neutrophils, monocytes and some T cells (91;92). The two amino terminal Ig domains of PECAM-1 are involved in a homophilic interaction which is considered to operate during neutrophil transendothelial migration. Blocking the homophilic interaction of PECAM-1 inhibits transendothelial migration *in vitro* and *in vivo* (93-97), and leukocytes blocked at the PECAM-1-dependent step remain adherent on the apical surface of the endothelial cells. Endothelial PECAM-1 recycles between the junctions and the subjunctional plasmalemma, and is targeted to the zone of active leukocyte transmigration (97). PECAM-1 knockout mice in the mouse strain C57Bl/6 did not show a reduction in neutrophil recruitment (98), however, breeding these mice into different mouse strains did result in a decrease in the recruitment of neutrophils into inflamed peritoneum (99). Interestingly, PECAM-1 deficiency was found to affect preferentially interleukin (IL)-1beta- but not TNF-alpha-induced inflammation (100).

Besides the cation-independent Ig domain 1 and 2-dependent homophilic binding of PECAM-1, a cation-dependent PECAM-1 heterophilic interaction mediated by Ig domain 6 of the molecule has been reported to participate at a late step of diapedesis, namely the migration through the basement membrane (94). In this context, we recently identified a member of Ly-6 family, CD177, as a novel heterophilic binding partner of PECAM-1. CD177 is a 58- to 64-kDa glycosyl-phosphatidyl-inositol (GPI)-anchored glycoprotein, which is expressed exclusively on neutrophils but not on other blood cells. In contrast to the homophilic PECAM-1 interaction, the heterophilic interaction between CD177 and PECAM-1 could be blocked by antibodies to Ig domain 6 of PECAM-1. In addition, we demonstrated that this heterophilic interaction functions in neutrophil transendothelial migration (101).

Another interesting observation is that PECAM-1 homophilic ligation results in an upregulation of the laminin receptor alpha6beta1-integrin on transmigrating neutrophils thereby enhancing the subsequent penetration of the basement membrane by neutrophils. Consequently, neutrophils treated with antibodies to alpha6beta1-integrin are trapped between endothelium and the basal lamina (102). These findings also point to the fact that PECAM-1 acts a signaling receptor. PECAM-1 contains two immunoreceptor tyrosine-based inhibitory motifs, which are involved in signals mediated by Src-homology-2 containing phosphatases, such as SHP-1 and SHP-2 (103-105). In addition, antibody crosslinking of PECAM-1 has been shown to upregulate the activity of beta1- and beta2-integrins on neutrophils and other leukocytes (106;107).

CD99 is a highly *O*-glycosylated molecule expressed on both neutrophils and other leukocytes and at the interendothelial junctions that acts in a homophilic manner to mediate transmigration (108). It controls a step in diapedesis that is distinct from and distal to the step mediated by PECAM-1, as cells blocked at the CD99-dependent step are arrested halfway across the endothelial junction (108;109). Mouse CD99 was recently cloned and antibodies to mouse CD99 inhibited the recruitment of antigen-specific T cells into inflamed areas of the skin and edema formation (110). In addition, endothelial CD99 was found to participate in neutrophil diapedesis *in vivo* (110). CD99L2 is a recently identified molecule expressed on both leukocytes and endothelial cells that shares 32% amino acid identity with CD99. CD99L2 was shown to specifically participate in neutrophil but not lymphocyte diapedesis *in vivo* and blocking CD99L2 inhibited neutrophil transmigration through the vessel wall at the level of the perivascular basement membrane (110).

4.4. ICAM-1

Besides the well established evidence that the LFA-1 interaction with ICAM-1 mediates firm adhesion of neutrophils to the endothelium, recent evidence pointed to the importance of this interaction for transendothelial migration as well. During neutrophil transmigration, LFA-1

rapidly redistributes to form a ring-like cluster at the neutrophil-endothelial junctional interface through which transmigration occurs. Endothelial ICAM-1 colocalizes with this ringlike LFA-1 cluster (50). Moreover, a "cuplike" transmigratory structure comprising of ICAM-1-enriched microvilli-like projections was shown to surround transmigrating neutrophils during diapedesis (49). Neutrophil attachment on endothelial cells and ICAM-1 ligation is thought to induce cytoskeletal remodeling associated with ICAM-1 clustering, in a manner dependent on cortactin (111). Cortactin and its tyrosine phosphorylation are required for the clustering of ICAM-1 around transmigrating neutrophils (112).

Recently, an involvement of ICAM-2 in neutrophil recruitment *in vivo* was demonstrated by engaging antibodies to ICAM-2 and ICAM-2-deficient mice. Intravital microscopy suggested an involvement of ICAM-2 in neutrophil diapedesis as opposed to rolling and adhesion (113).

5. CONCLUSIONS

Despite the significant progress in our understanding of the process of transendothelial migration in the recent years, there is obviously still a lot to learn. In particular, the relative importance of each of the molecules *in vivo* needs to be established. Experiments comparing more than one of the adhesion pathways should be designed. In addition, it is conceivable that the different adhesion pathways involved in transmigration may cooperate at different levels and that a hierarchy may exist amongst them. However, these concepts require further investigations. Future experiments need also to focus on the tissue-, vascular bed- and inflammatory stimulus-specificity of each of the above described pathways. Understanding the molecular contributors of neutrophil and leukocyte transmigration in detail will provide the platform for the design of specific therapeutic approaches in inflammatory diseases.

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