Leukocyte trafficking and vascular integrity

Heemskerk, N.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: https://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
SUMMARY AND CONCLUDING REMARKS
SUMMARY AND CONCLUDING REMARKS

Dynamic remodeling of endothelial cell (EC) junctions is essential for proper function of the vascular system. Disruptive stressors such as thrombin, histamine, adherent and transmigrating leukocytes, and effects of blood pressure or temperature, daily impact the integrity of EC. The constant cycle of endothelial junctional disassembly and assembly enables EC to cope with this dynamic vascular environment. In this thesis, we aimed to examine which endothelial proteins orchestrate junctional remodeling in response to common endothelial activators such as thrombin or transmigrating leukocytes.

TRIO-MEDIATED RAC1 ACTIVITY LOCALLY RESTORES JUNCTIONAL STABILITY

The regulation of the F-actin cytoskeleton by RhoGTPases is indispensable for proper junctional dynamics. However, little is known about which GEFs and GAPs regulate local RhoGTPase cycling to mediate transient remodeling of EC junctions. In chapter 5 we show that TRIO, a GEF for Rac1, regulates stabilization of VE-cadherin-based junctions through its first GEF domain. Cell-cell junctions between Trio-deficient EC were unstable and showed continuous dis- and re-assembly. Mechanistically, we show that homophilic ligation of VE-cadherin in nascent junctions recruits Trio to VE-cadherin. Next, Trio can activate Rac1 in close proximity of the VE-cadherin-catenin complex enabling the formation of cortical actin bundles along the junction at the expense of radial actin bundles perpendicular to the junction. The subsequent connection of the VE-cadherin-catenin complex to these cortical actin bundles increases junctional stability and endothelial monolayer resistance (Fig. 1). In contrast to our model, Rac1 cycling has also been described to be required for VE-cadherin endocytosis and the formation of reactive oxygen species (ROS), which both mediate junctional instability. These contradictory roles for Rac1 in EC are not in conflict with each other since its spatiotemporal activity is tightly regulated by numerous pathway-specific GEFs and GAPs. For instance, Trio deficiency did not prevent Rac1-induced membrane protrusive activity during formation of initial cell-cell contact. Thus, prior to stabilization of the nascent cell-cell contact induced by local signaling through the VE-cadherin-Trio-Rac1 axis, other Rac-GEFs likely contribute to promote initial cell-cell contact formation for example by junction-associated lamellipodia (JAILs). Still, many details of the mechanisms driving spatiotemporal Rac1 activation at distinct stages of junction formation remain to be uncovered. The recently published DORA Rac1 biosensor will be a good tool to dissect the consecutive involvement of distinct GEFs in Rac1-mediated junctional stabilization.
Summary and concluding remarks

Figure 1. Trio-mediated Rac1 activation stabilizes VE-cadherin based cell-cell contacts. The process of adherens junctions (AJ) formation can be segmented into three phases: first, membrane protrusions limit the gap between two cells to mediate initial contact; second, VE-cadherin molecules engage in homophilic interactions and form clusters; and third, VE-cadherin clusters trigger the transition from radial to cortical actin bundles along the junction. Subsequent connection of the VE-cadherin-catenin complex to cortical actin bundles increases junctional stability and endothelial monolayer resistance. Trio-mediated rac1 activity plays a crucial role during the third stage of the AJ formation process.

Annexin A2 controls neutrophil transendothelial migration by regulating the spatial organization of ICAM-1 into caveolae

The endothelial β2-integrin ligand ICAM-1 facilitates effective neutrophil trafficking in and out of the vasculature. Being involved in rolling, arrest, crawling and transmigration of neutrophils in vivo, ICAM-1 has been postulated to reside in various membrane complexes with different protein content, spatial organization and subcellular localization, to regulate leukocyte behavior in each distinctive step. However, little is known about how the spatial organization of endothelial ICAM-1 affects ICAM-1-mediated leukocyte adhesion and how the cell surface distribution of ICAM-1 between various membrane domains is regulated. In Chapter 3 we report on annexin A2, an ICAM-1-binding protein that regulates the spatial distribution of ICAM-1 at the plasma membrane during the adhesion phase of leukocyte TEM. We established that clustered ICAM-1 translocates from ezrin-rich membrane domains to caveolin-1-rich membrane domains, a step mediated by annexin A2 (Fig. 2). Endothelial annexin A2 depletion increased neutrophil adhesion and transmigration, whereas neutrophil crawling distance and velocity was reduced. These findings suggest that the redistribution of ICAM-1 into caveolae has a
limiting effect on ICAM-1-mediated leukocyte adhesion, likely by limiting
the number of accessible ICAM-1 molecules at the endothelial surface.
ICAM-1 in caveolae has also been reported to be rapidly transported to
the basolateral surface of ECs by a process named transcytosis \(^\text{12}\). The
amount of apically exposed ICAM-1 and ICAM-1 transcytosis events have
been proposed to be part of a mechanism for transcellular migration, but
not paracellular migration of T cells and neutrophils \(^\text{12,16,17}\). Future studies
investigating the role of endothelial signaling in leukocyte TEM should
include biosensors to investigate at what stage a particular protein is
participating in the process. Notably, addressing spatial and temporal
differences of ICAM-1-adaptor-GTPase-effector complexes would be an
interesting line of future research. Important questions to study are: when
does ICAM-1 clustering occur? And for which particular step would this be
required; do neutrophils induce clustering during the crawling phase? The
generation of an ICAM-1 FRET-based biosensor to allow imaging of spatial
and temporal ICAM-1 clustering in real-time during TEM of leukocytes
may help to address this intriguing topic.

Figure 2. A model of annexin A2 controlling ICAM-1-mediated neutrophil transendothelial
migration. In endothelial cells unengaged ICAM-1 resides in ezrin-rich membrane
domains. Upon LFA-1 induced ICAM-1 clustering, ICAM-1 is redistributed to caveolin-1-rich
membrane domains in an annexin A2 dependent manner. Subsequent ICAM-1 endocytosis
or transcytosis limits the number of free accessible ICAM-1 molecules at the endothelial
surface, thereby negatively regulating ICAM-1-mediated neutrophil adhesion.
A RhoA mediated contractile ring prevents plasma leakage during leukocyte diapedesis

Every day, billions of leukocytes cross the endothelial barrier to fulfill protective immune functions against pathogens, cancerous cells and foreign material. Despite numerous crossings, immune cell traffic seems to have minor impact on endothelial integrity and permeability 18–22. Already in 1988, researchers showed that intimate contact between neutrophils and ECs was maintained during the entire TEM process. This intimate contact could explain transmigration of leukocytes without vascular damage 23. However, a mechanism was lacking. In chapter 4 we show that local RhoA-mediated F-actin rings contribute to endothelial pore confinement which maintains endothelial barrier integrity during leukocyte diapedesis 24. Neutrophil diapedesis initiates the clustering of ICAM-1 which in turn recruits the RhoA GEFs LARG and Ect2. RhoA activation by LARG and Ect2 results in local Rock2b-mediated Myosin II activation near the endothelial pore. Subsequent tightening of F-actin-rich membrane around transmigrating neutrophils creates intimate neutrophil-endothelial contact that prevents plasma leakage during the entire TEM process (Fig. 3a, b). Our study has been focused particularly on the diapedesis of neutrophils and monocytes during inflammation-driven leukocyte recruitment. Perhaps, endothelial pore confinement could also maintain endothelial integrity during immune surveillance or adaptive immune responses. For example during TEM of B cells, T-lymphocytes or dendritic cells through high endothelial venules in lymph nodes. However, endothelial pore confinement is not the only mechanism used by endothelial cells to cope with the mechanical disruptive strains induced by transmigrating leukocytes. Some studies showed that ECs reseal their endothelial barrier prior to or during neutrophil penetration of the basal lamina by the formation of endothelial dome structures or ventral lamellipodia 25–27. Our data indicates that these alternative pathways are likely relevant downstream of endothelial pore confinement (Fig. 3a).

Exploring RhoA cycling dynamics using a RhoA FRET sensor led to an unexpected finding; RhoA was not activated during the opening of endothelial junctions upon leukocyte breaching, despite the fact that this has been proposed for years due to fact that thrombin-mediated RhoA activation results in contraction-mediated opening of endothelial cell-cell junctions 28,29. We detected RhoA activation after junctional opening, i.e. during the mid-diapedesis step, when a leukocyte was already half way through. This suggests that ECs are equipped with mechanisms that initiate the opening of endothelial junctions independently of RhoA signalling. Moreover, we found that many F-actin-rich contractile rings comprise apical membrane protrusions. These Rac1-mediated
projections, also known as ‘docking structures’ or ‘transmigratory cups’ \cite{30-32}, have been suggested to mediate apical projection-guidance. Which means the guidance of leukocytes along adhesion molecule-enriched apical membrane protrusions on the endothelial apical surface. \cite{12,31,33-36}. Two common principles are emerging; apical projections give directional guidance to leukocytes whereas the basolateral F-actin ring prevents vascular leakage during leukocyte crossing.

Figure 3. Mechanisms that prevent vascular leakage during leukocyte diapedesis. (a) Electron microscopy studies in the late 80’s showed that leukocytes and endothelial cells maintain intimate contact during the entire diapedesis process. Transmigration occurred without vascular damage or leakage. In the years that followed several research groups searched for a mechanism that could explain this remarkable observation. Vascular leakage during leukocyte diapedesis is prevented by: I domes, that fully encapsulate migrating leukocytes, II RhoA-mediated endothelial pore confinement, contractile F-actin rings in the endothelium that form a natural crimp ring preventing plasma leakage into the underlying tissue. III Ventral lammelipodia (VL), Rac1-mediated F-actin-rich membrane protrusions at the basal surface of the endothelium that seal and restore the endothelial monolayer after leukocyte breeching. VE-cadherin complex (green), EC (brown) leukocytes (purple). (b) Mechanistically endothelial pore confinement is triggered by ICAM-1 clustering that activates RhoA through the RhoA GEFs LARG and Ect2. RhoA activation occurs during the mid-phase of diapedesis i.e. when the neutrophil partly breeched the endothelium. RhoA-mediated Myosin II activation through the kinase Rock2b induces contraction of F-actin-rich membrane around transmigrating neutrophils forcing tight neutrophil-endothelial contact that prevents plasma leakage during TEM. F-actin (red), neutrophil (yellow), EC (brown).
CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Could some of the findings in this thesis be translated into new treatments for patients that suffer from spontaneous leukocyte induce hemorrhages or endothelial barrier dysfunction in general?

Under some conditions such as disturbed flow and prolonged endothelial injury, the adherence of neutrophils to activated platelets could lead to a chain reaction. ROS produced by leukocyte-platelet aggregates could inflict more vascular damage, extracellular matrix exposure platelet activation and prolonged inflammation. Such an destructive feed forward loop may accelerate disease progression in for instance, thromboembolism or in a chronically expanding atherosclerotic lesion \(^{37,38}\) where a blood clot or stenosis obstructs the blood flow to a tissue inflicting hypoxia, ischemia, infarction and tissue death. Interestingly, platelet-matrix interactions require an increase in microvascular permeability \(^{39}\). Therefore, prevention of vascular leakage by directly targeting the endothelial junctions may be a successful manner of intervention to prevent prolonged vascular permeability and tissue damage during acute inflammation. Several potential targets could be further explored for this specific type of intervention. For instance Y685 on VE-cadherin that specifically blocks vascular permeability \(^{20}\) or endothelial specific GEFs such as Trio or EPAC, that are involved in stabilizing the endothelial barrier \(^{40,41}\). In addition, we show that leukocyte diapedesis and vascular permeability are each regulated by different unique exchange factors (chapter 6). Once their specific involvement in junctional regulation is validated, each of these proteins might be interesting as a therapeutic target to accompany the family of barrier strengthening agents. Moreover, several clinical trials aim to specifically inhibit vascular leakage by targeting Rho/ROCK signaling (www.clinicaltrials.gov). Fasudil is a FDA-approved Rho-kinase inhibitor showing protection against anaphylaxis in a murine sepsis model. However, as an unwanted side-effect, blocking Rho-kinase may additionally promote permeability under TEM conditions. Therefore, blocking both events separately at the same time is likely to increase therapy efficiency.

In other diseases such as immune thrombocytopenia, it is the lack of platelets that causes serious tissue damage and spontaneous hemorrhages \(^{42}\). Blocking neutrophil diapedesis by mutating Y731 in the cytoplasmic tail of VE-cadherin attenuates these spontaneous organ hemorrhages. Therefore, specifically targeting Y731 may present a novel therapeutic target to prevent spontaneous bleedings in these patients. Alternatively, some of these patients may benefit from drugs that are designed to strengthening endothelial junctions around transmigrating leukocytes.
We anticipate that future research and validation of the GEFs and GAPs involved in endothelial pore strengthening and confinement (chapter 6) may open therapeutic options for this type of drug development. Recently, a genetic study unravels that ECs express a distinctive gene profile encoding for an unique combination of transcription factors, chemokines, adhesion receptors and angiocrine growth factors. Understanding what molecules regulate tissue-specific transmigration of each immune cell subtype would have a great impact on for example cancer immunotherapy.

The key principles of leukocyte diapedesis, the path of least resistance, chemotaxis, durotaxis, haptotaxis and shear stress are evidently involved in the modulation of diapedesis into distinct tissues. These principles together with the unique tissue-specific gene profile may open a whole new avenue for cancer drug development. Although our findings do not have a direct benefit for patients, the advances made in this thesis greatly improves our general understanding about transendothelial migration and vascular integrity as a whole and increase the potential to find useful drugs that benefit cardiovascular diseases, cancer, acute inflammation and chronic inflammation.

In conclusion, this body of work shows that the endothelium has several mechanisms to cope with disruptive strains that challenge its barrier during inflammation and leukocyte diapedesis. ICAM-1 regulates both leukocyte adhesion and endothelial barrier strengthening during inflammation-driven leukocyte diapedesis. Increasing evidence points out that dynamic remodeling of EC junctions to increase vascular permeability or inflammation-driven leukocyte recruitment are independent processes that are likely occur in parallel. EC barrier maintenance during inflammation and leukocyte diapedesis require unique exchange factors which can be further explored to investigate where these closely associated pathways uncouple.
REFERENCES
