Docking onto the endothelium: Trio directs leukocyte extravasation
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Citation for published version (APA):
van Rijssel, J. (2011). Docking onto the endothelium: Trio directs leukocyte extravasation

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The endothelium that lines the vasculature is an essential barrier that permits the extravasation of leukocytes in a tightly controlled way. Inflammatory stimuli induce the endothelium to upregulate adhesion molecules, such as E-selectin, VCAM-1 and ICAM-1 (Pober and Sessa, 2007). Leukocyte adhesion and subsequent clustering of these adhesion molecules induces several signaling pathways that mediate morphological changes in the endothelium, which support the leukocyte diapedesis process (Nourshargh et al., 2010). In addition, the endothelium forms large membrane protrusions around adherent leukocytes. These structures are called endothelial docking structures (Barreiro et al., 2002; Carman et al., 2003). Although it is not yet completely resolved whether these structures support leukocyte adhesion, docking structure formation clearly correlates with leukocyte transendothelial migration (TEM) (Carman and Springer, 2004; Wolburg et al., 2005; van Buul et al., 2007; Phillipson et al., 2008; Petri et al., 2011).

Small GTPases of the Rho family are known to be key regulators of cytoskeletal membrane dynamics (Etienne-Manneville and Hall, 2002). Rho family GTPases have been shown to play a central role in regulating the morphological changes induced by engagement and clustering of ICAM-1.

Small GTPases are enzymes that can bind GDP or GTP, which determines their inactive or active state, respectively (Etienne-Manneville and Hall, 2002) (Figure 2). The exchange of GDP for GTP is catalyzed by so-called guanine nucleotide exchange factors (GEFs) (Rossman et al., 2005). Upon stimulation of GEF activity, the GEF will bind the GTPase, which results in the dissociation of GDP. A conformational change within the GTPase subsequently allows the binding of GTP, which replaces the GEF. In its GTP-bound state the GTPase is now able to bind its specific effector molecules and induce signaling. GTPase signaling is eventually terminated by hydrolysis of the bound GTP to GDP, which is stimulated by binding of GTPase-activating proteins.

**Figure 1.** Overview of the formation of docking structures around adhered leukocytes. Docking structures form either on the apical side of the endothelial cell or at a cell-cell junction, with ICAM-1/VCAM-1 indicated in green and VE-cadherin in blue. Scanning electron microscopy image (right) showing an adhered leukocyte on activated endothelium. Arrows indicate endothelial membrane-protrusions, forming a docking structure. Asterisk indicates leukocyte. Bar, 1 μm. Image is taken from Van Buul et al., Journal of Cell Biology, 2007.
(GAPs). This binding promotes the intrinsic hydrolase activity the GTPase. Inactive GTPases are retained in the cytosol by binding to Guanine nucleotide dissociation inhibitors (GDIs) (Garcia-Mata et al., 2011).

The small GTPase RhoA becomes activated upon ICAM-1 clustering and mediates the formation of F-actin stress fibers and local endothelial barrier permeability (Etienne et al., 1998; Adamson et al., 1999; Wojciak-Stothard et al., 1999; Etienne-Manneville et al., 2000; Thompson et al., 2002; van Buul et al., 2007) (Figure 3). The GTPase RhoG also becomes activated after ICAM-1 clustering and controls the formation of endothelial docking structures (van Buul et al., 2007). In addition to RhoA and RhoG, the GTPase Rac1 becomes activated following ICAM-1 clustering (van Buul et al., 2007). However, its precise contribution to docking structure formation and leukocyte TEM is thus far unclear.

Compared to its extracellular domain, ICAM-1 has a relatively small intracellular domain, comprising only 28 amino acids. Since this intracellular domain does not possess enzymatic activity, signaling is likely relayed via adapter proteins. Adapter proteins that interact with the intracellular domain of ICAM-1 (Figure 4) may scaffold downstream signaling proteins, such as Rho GTPases and their GEFs, and allow local activation of signaling by ICAM-1.
Figure 3. Signaling that regulates endothelial docking structure formation.

SCOPE OF THIS THESIS

In this thesis, I wish to define the role of the endothelium during leukocyte extravasation. I have focused my research on the surface adhesion molecules in the endothelium, ICAM-1 and VCAM-1. Moreover, I studied the consequences of the clustering of these molecules, as happens upon leukocyte binding, on intracellular signaling in endothelial cells.

In addition, my research was particular concentrated on the role of the GEF Trio that can exchange GDP for GTP on at least three different GTPases. Figure 5 shows schematically the signaling pathways that are covered in this thesis.

In chapter 1 we provide an overview of the different signaling pathways induced by ICAM-1 that regulate endothelial docking structure formation and leukocyte TEM. To gain more insight in the proximal signaling events that control ICAM-1-induced endothelial docking structure formation, we searched for novel binding partners of the intracellular domain of ICAM-1.

In chapter 2 we identify the F-actin crosslinker protein filamin B and its homologue filamin A as novel binding partners of the intracellular domain of ICAM-1 in endothelial cells. We show that filamin B regulates the lateral mobility of ICAM-1 in the plasma membrane and that filamin B regulates both leukocyte adhesion and TEM under physiological flow conditions. These results show that filamin is an important adapter protein for ICAM-1-mediate leukocyte TEM.
Chapter 3 describes that clustering of ICAM-1 induces the co-recruitment of VCAM-1 and vice versa. This co-recruitment was found independent of integrin engagement and is partially dependent on lipid rafts and F-actin polymerization. Co-recruitment of ICAM-1 and VCAM-1 promotes adhesion of leukocytes to ICAM-1 or VCAM-1 expressing cells, showing the functional significance of this phenomenon.

Chapter 4 shows that upon ICAM-1 clustering, ICAM-1 translocates to an immobile fraction within the plasma membrane. This ICAM-1 translocation requires both filamin
A and F-actin polymerization. Moreover, we demonstrate that ICAM-1 lateral mobility in the plasma membrane requires the motor protein myosin II and the GTPase Rac1, which thereby support the adhesive function of ICAM-1.

In Chapter 5 we demonstrate that activation of the GTPases Rac1 and RhoG following ICAM-1 clustering requires filamin and the filamin-binding GEF Trio. Filamin is required for the activation of Trio upon ICAM-1 clustering, suggesting that filamin may scaffold ICAM-1-mediated Trio activation. We show that the activity of the N-terminal GEF domain of Trio is required for Rac1 and RhoG activation after ICAM-1 clustering and for subsequent docking structure formation and neutrophil diapedesis under physiological flow conditions.

In Chapter 6 we show that Trio becomes upregulated in the endothelium under inflammatory conditions in vitro and in vivo. We demonstrate that Trio in turn regulates cytokine-induced expression of the adhesion receptors ICAM-1, VCAM-1 and E-selectin. We show that Trio mediates TNF-α-induced Rac1 activation through its N-terminal GEF domain. Although Trio does not regulate ICAM-1 and VCAM-1 through the established Rac1 targets NF-κB and the MAPKs Jnk and p38, we demonstrate that Trio is required for TNF-α-induced transactivation of the VCAM-1 promoter.

Finally, in chapter 7 we show that Rac1 and RhoG become activated by Trio independently from each other. Furthermore, we show that Trio controls fibronectin-mediated cell spreading and lamellipodia dynamics by activating Rac1, but not RhoG.


