Signaling in vascular biology: The role of GTPases and GEFs in endothelial cell function
Kroon, J.

Citation for published version (APA):
MYOSIN-X IS REQUIRED FOR THE FORMATION OF ICAM-1-RICH FILOPODIA THROUGH THE SMALL GTPASE CDC42 TO SUPPORT LEUKOCYTE ADHESION

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Manuscript in preparation
ABSTRACT

Inflammation has emerged as a crucial force driving the initiation and progression of atherosclerotic lesions. Therefore, it is critical to understand the details of leukocyte migration across the endothelial barrier, a key event in inflammation. Upon binding of the leukocyte integrins LFA-1 and Mac-1 to endothelial ICAM-1, ICAM-1 is clustered and induces membrane sheets that protrude from the apical membrane of the endothelium around the adherent leukocyte. The formation of these membrane sheets is believed to originate from small microvilli-like structures that are present on the luminal surface of inflamed endothelium. However, how these structures are formed is not clear. We used ICAM-1-antibody coated beads to mimic leukocyte adhesion and showed, using scanning electron microscopy, that microvilli-like structures initially surround the adhered beads, followed by extensions of larger membrane sheets. The small GTPase Cdc42 and Myosin-X, but not Rac1, are involved in the formation of these microvilli-like structures. In fact, clustering of ICAM-1 recruits Myosin-X in a Cdc42-dependent manner. Since Myosin-X is one of the characteristic markers of filopodia, we qualified these microvilli-like structures as filopodia. Together, our work suggests a prominent role for Cdc42 and Myosin-X in the formation of ICAM-1-rich filopodia that control leukocyte adhesion to the endothelium.
INTRODUCTION

Leukocyte transendothelial migration (TEM) is a multistep process that can be divided into different steps. First, leukocytes tether and roll over the activated endothelial monolayer. Next, they firmly adhere to the endothelium and finally, they penetrate through the endothelial cell monolayer, either through cell-cell junctions, called paracellular migration, or through the endothelial cell body, called transcellular migration (Nourshargh et al., 2010; Heemskerk et al., 2014). All these individual steps require specific proteins and adhesion receptors. This was already postulated by Butcher in the early nineties (Butcher, 1991). The adhesion phase involves $\beta_1$- and $\beta_2$-integrins (VLA-4/LFA-1/Mac-1) on the leukocytes and Ig-like adhesion molecules, e.g. ICAM-1/CD54 and VCAM-1/CD106 on the activated endothelium (Hordijk and Van Buul, 2009).

In particular ICAM-1 becomes highly upregulated under inflammatory conditions (Van Rijssel et al., 2013) and is essential for efficient leukocyte TEM. Loss of the intracellular tail of ICAM-1 severely perturbs leukocyte transendothelial migration (Adamson et al., 1999; Lyck et al., 2003). Moreover, upon binding of leukocytes, ICAM-1 is clustered in a ring-like structure around the adherent leukocyte (van Buul et al., 2010). As a consequence, the endothelial membrane protrudes apically into the lumen and surrounds the adherent leukocyte. These structures have been identified as “docking structures” (Barreiro et al., 2002a) or “transmigratory cups” (Carman and Springer, 2004). The nomenclature may suggest involvement of these structures in leukocyte docking to the endothelium, also known as firm adhesion. Besides this, these structures could also be important for supporting leukocyte transmigration, known as diapedesis. However, it is still unclear how these structures are initiated.

To avoid any confusion, we will use the term “docking structure” throughout this manuscript. Using ICAM-1-antibody coated beads, we set out to test the initial formation of these docking structures in detail.

Here we show that initial ICAM-1 clustering induces endothelial microvilli-like structures that surround the beads, followed by the induction of full membrane sheets to finally form a docking structure. ICAM-1-rich microvilli-like structures are positive for F-actin and require Cdc42 and Myosin-X motor activity. Furthermore, using ICAM-1-antibody coated beads, we show that ICAM-1 clustering depends on Cdc42 and Myosin-X. Based on its presence and the role of Myosin-X in the formation of microvilli-like structures, we identify these structures as filopodia. This work uncovers a novel function for Myosin-X and Cdc42 in ICAM-1-mediated adhesion of leukocytes to the endothelial cell surface.
RESULTS

Upon leukocyte adhesion, TNFα-stimulated HUVECs show membrane protrusions that surround the adhering leukocyte, known as docking structures and visualized by scanning electron microscopy (SEM) (Figure 1A-a). This technique allows detailed analysis of morphological changes of the endothelium upon leukocyte adhesion. To study the involvement of the endothelium only, ICAM-1-antibody coated beads mimicking adherent leukocytes were used (van Buul et al., 2010). Similar to leukocytes, these beads efficiently induced apical endothelial membrane protrusions (Figure 1A-b). To study the formation of these structures in more detail, we performed a time-course of anti-ICAM-1-antibody coated beads adhesion to the endothelium. After already 2 minutes, small microvilli-like structures were formed that crawled up against the adhered beads (Figure 1B). After approximately 10 minutes, membrane sheets were observed surrounding the adhered beads. Importantly, non-coated or IgG-coated beads did not induce any changes in the morphology of the endothelial cells (Figure S1A). Using SEM, multiple microvilli-like structures were detected on the apical surface of TNFα-stimulated HUVECs (Figure 1C). Quantification of the number of microvilli-like structures showed that after 10 minutes, about 50% of all adhered ICAM-1 antibody-coated beads were fully surrounded with microvilli-like structures (Figure 1D). Finally, ex vivo imaging of the lumen of an isolated aorta that was stimulated overnight with TNFα showed the presence of similar microvilli-like structures around adherent ICAM-1 antibody-coated beads (Figure S1B).

To investigate these structures in more detail we transfected cells with ICAM-GFP. ICAM-1-rich microvilli-like structures were rich for F-actin, indicating an active involvement of the actin cytoskeleton in the formation of these structures at the apical surface (Figure 2A). Besides this, these structures appear to originate from the basolateral plane of endothelial cells. The small GTPase Cdc42 is important for the induction of microvilli-like structures (Nobes and Hall, 1995; Bohil et al., 2006). To study if Cdc42 is required for ICAM-1-rich microvilli-like structures, we expressed Myc-tagged dominant active (Q61L) and negative (T17N) mutants of Cdc42 into endothelial cells, together with GFP-tagged ICAM-1 constructs. We found that dominant negative Cdc42 prevented the formation of ICAM-1-rich microvilli-like structures whereas the active form of Cdc42 promoted the presence of ICAM-1-rich microvilli-like structures (Figure 2B). Interestingly, expressing a tailless ICAM-1 mutant together with the active Cdc42 mutant did not drive ICAM-1 into microvilli-like structures, but rather induced extreme long extensions expressing both Cdc42 and tailless ICAM-1. Importantly we found that, Rac1, a small GTPase known to be involved in docking structure formation (van Rijssel et al., 2012), is not required for the induction of ICAM-1-rich microvilli-like structures, since dominant negative mutants of Rac1 (T17N) showed no defect in the formation of ICAM-1-rich microvilli-like structures (Figure 2C). In addition to this, expression of the dominant active mutant (Q61L) of Rac1 appears not to result in an increase in microvilli (Figure 2C). Thus, ICAM-1 requires its intracellular tail to be properly distributed to Cdc42-mediated microvilli-like structures.
Figure 1. Microvilli-like structures are induced upon ICAM-1 clustering. (A) a. Scanning electron microscopy (SEM) recordings from a differentiated HL-60 cell that is adhered to an activated HUVECs. Arrowheads show membrane sheets, referred to as “docking structures” around adherent cell. Bar, 1μm. b. ICAM-1 antibody coated bead was allowed to adhere for 30 minutes to activated endothelium. Arrowheads show membrane sheets that arise from the endothelial cell surface alongside the bead. 1μm. (B) ICAM-1-antibody coated beads were allowed to adhere to the activated endothelium as indicated. After 2 minutes, microvilli-like structures arise from the endothelial dorsal plane, indicated by the arrowheads. After 10 minutes, membrane sheets are observed. Bar, 10μm. (C) SEM shows dorsal surface of endothelial cells that are transfected with ICAM-1-GFP constructs and treated with 10ng/mL TNFα, showing the induction of microvilli-like structures (D). Quantification of the number of beads surrounded by microvilli-like structures after the indicated time (in minutes) of incubation on activated endothelium. Data are the mean of three independent experiments in duplicate. *p<0.05.
Figure 2. Filopodia are rich for ICAM-1 and actin and require Cdc42. (A) Endothelial cells are treated with TNFα and transfected with ICAM-1-GFP. Region of interest (ROI) showed detailed Z-stack confocal recordings with expression of ICAM-1 (green) as well as F-actin (red) in the dorsal microvilli-like structures (arrows). Upper panel shows apical- and lower panel shows baso-lateral focal plane. (B) HUVECs are transfected with ICAM-1-GFP full length or tailless (DC) mutant (green) or Myc-tagged Cdc42 constitutively active (Q61L) or dominant negative (T17N) in red. F-actin is in grayscale. ICAM-1 requires its C-terminal domain and active Cdc42 to induce ICAM-1-rich filopodia. (C) HUVECs are transfected with ICAM-1-V5 (red) and GFP-tagged constitutively active Rac1 (Q61L) or dominant negative Rac1 (T17N) in green. The asterisks indicate cells that are double transfected. In both conditions, ICAM-1-rich filopodia in red were present (arrowheads). F-actin is depicted in white. Bar, 20 μm.
The motor protein Myosin-X (MyoX) acts downstream from Cdc42 in the formation of filopodia (Bohil et al., 2006). Since the microvilli-like structures showed many morphological similarities to filopodia, we tested if MyoX plays a role in the formation of ICAM-1-rich microvilli-like structures. Using Western blotting we checked the expression of MyoX in several cell types, including endothelial cells (Figure 3A). MyoX was abundantly expressed in endothelial cells as well as in CHO and melanoma A7 cells, whereas its expression was limited or even absent in HeLa, M2 and Cos7 cells. To study MyoX localization, we used GFP-tagged constructs of MyoX, since a proper antibody to stain endogenous MyoX for immunofluorescent imaging is not available. These data showed that GFP-MyoX co-localized to some extent with ICAM-1 in filopodia structures. Upon clustering induced by ICAM-1-antibody coated beads, recruitment of MyoX to ICAM-1 was observed (Figure 3B). Previous research showed that the motor domain of MyoX is responsible for the induction of filopodia (Bohil et al., 2006; Tokuo et al., 2007). Therefore we tested if the motor function of MyoX was required to induce ICAM-1-rich microvilli-like structures. Co-expression of a MyoX mutant (MyoX-headless) that lacked the motor function, with mCherry-tagged ICAM-1 full length constructs showed that ICAM-1-rich microvilli-like structures require MyoX including its motor function (Figure 3C). Based on these data, we decided to term the ICAM-1-rich microvilli-like structures filopodia.

We next assessed if MyoX is physically recruited to ICAM-1 upon clustering. Therefore we performed a biochemical pull down experiment using magnetic beads that are coated with ICAM-1 antibodies. The beads were allowed to adhere to the endothelium for 30 minutes. Next, cells were lysed and analyzed for MyoX binding using Western blotting. As a control, ICAM-1 antibody-coated beads were added to endothelial cell lysates, preventing possible ICAM-1 clustering, but able to efficiently precipitate ICAM-1. The data revealed that under non-clustered ICAM-1 conditions, a basal interaction of MyoX with ICAM-1 is detected (Figure 3D). However, clustering of ICAM-1 increased the interaction of MyoX with ICAM-1 (Figure 3D). These data indicate that MyoX is actively involved in ICAM-1 clustering.

To further check the latter hypothesis, we used ICAM-1 antibody-coated beads to study the role of MyoX and Cdc42 on the functional consequences of ICAM-1 clustering. Endothelial cells were transfected with fluorescently tagged ICAM-1 together with dominant negative Cdc42, expressed in an IRES-GFP vector or GFP-tagged headless MyoX mutant constructs. The IRES-GFP vector expressed the Cdc42 mutants in the cytosol whereas the GFP is expressed in the nucleus. This prevented possible interference with the mCherry tag of the ICAM-1 construct. We first tested the adhesion function of ICAM-1 and counted the number of beads that adhere to a transfected cell that was stimulated with TNFα. The data revealed that blocking either Cdc42 or MyoX significantly reduced the number of ICAM-1 antibody-coated beads that adhered to ICAM-1-mCherry-positive cells (Figure 4A). When focusing on ICAM-1 recruitment, GFP- or mCherry-tagged ICAM-1 constructs were transfected into TNFα-stimulated endothelial cells and ICAM-1 antibody-coated beads were allowed to adhere to the cells
Figure 3. Myosin-X associates to clustered ICAM-1. (A) Western blot analysis showed expression of endogenous Myosin-X. Right lane is lysate of cells that express Myosin-X wt construct as positive control. Moesin is shown as loading control. (B) Co-expression of Myosin-X wild type (in green) and ICAM-1-V5 (in red) showed co-localization of both proteins at the tips of microvilli-like structures, see arrowheads in ROI. Clustering of ICAM-1 recruited Myosin-X to sites of beads adhesion, see arrowheads in ROI. Bar, 20μm. Bar ROI, 5μm. (C) Expression of headless Myosin-X mutants (lacking motor function; in red) perturbed ICAM-1-rich filopodia (ICAM-1 was stained in green). Asterisks show Myosin-X headless-expressing cells, lacking ICAM-1-rich filopodia and arrowheads show cells that were only transfected with ICAM-1 and expressing filopodia. Bar, 20μm. (D) ICAM-1 clustering showed that Myosin-X recruitment to ICAM-1 was enhanced as compared to the non-clustered condition. ICAM-1. Actin was shown as loading control of the total cell lysates.
for 30 minutes. As a result, ICAM-1 was successfully recruited around approximately 50% of all beads that adhered (Figure 4B and 4C). To check if the motor function of MyoX is involved in ICAM-1 recruitment, we used the MyoX-headless mutant. Interestingly, overexpression of MyoX-headless significantly reduced the number of beads that recruited ICAM-1 compared to control conditions within 30 minutes (Figure 4B and 4C). Moreover, expressing a dominant negative IRES-GFP mutant of Cdc42 together with ICAM-1 drastically reduced the number of adherent beads and completely abolished ICAM-1 recruitment within 30 minutes (Figure 4B and 4C). In contrast, the constitutively active IRES-GFP Cdc42 mutant showed a potent recruitment of ICAM-1 (Figure 4C). These data indicate that functional ICAM-1-rich filopodia require Cdc42 and MyoX to mediate ICAM-1 clustering and adhesion.
Figure 4. Myosin-X and Cdc42 are required for optimal ICAM-1 clustering. (A) Number of ICAM-1 antibody-coated beads per field of view that adhere to cells that express next to ICAM-1-mCherry, GFP (CTRL), Cdc42-T17N-IRES-GFP or GFP-Myosin-X headless mutant. Data are the mean of three independent experiments in duplicate. *p<0.05. (B) ICAM-1 antibody-coated beads were allowed to adhere to the cells for 30 minutes and ICAM-1 (in green) recruitment (arrowheads) was recorded. DIC image shows localization of beads. Expression of headless GFP-Myosin-X mutant (red) or Cdc42-T17N-IRES-GFP induced a reduction of ICAM-1 recruitment efficiency. Bar, 20μm. (C) Quantification showed that efficient ICAM-1 recruitment requires Cdc42 and Myosin-X. Data are the mean of three independent experiments in duplicate. *p<0.05.
DISCUSSION

In this study, we explore the role of Cdc42 and Myosin-X in the formation and function of ICAM-1-rich apical filopodia. We showed that ICAM-1-rich filopodia require active Cdc42 and the motor function of Myosin-X. Expression of dominant negative Cdc42 or motor-activity deficient Myosin-X mutants perturbed the formation of ICAM-1-positive filopodia. Moreover, under those conditions, ICAM-1 function and clustering were limited. From these findings, we conclude that Cdc42 and Myosin-X are required for optimal formation and function of ICAM-1-rich filopodia.

Small finger-like membrane projections that stick out of the plasma membrane have been carefully studied and termed microvilli or filopodia (Chhabra and Higgs, 2007). The distinction between these two structures is based on phenotypic characteristics. Filopodia adhere to the substratum whereas microvilli do not and point into the lumen, although also filopodia that do not adhere to the substratum have been described (Bohil et al., 2006). More specifically, the protein content in the tip of these structures is distinctive. Filopodia tips show clear localization of Myosin-X and the Ena/Vasp protein complex, whereas microvilli tips do not have such specific protein distribution (Chhabra and Higgs, 2007). Our data revealed that the tips of ICAM-1-rich microvilli-like structures show, next to F-actin, clear Myosin-X localization. Based on this phenotype, we identified the ICAM-1-rich microvilli-like structures as filopodia. The formation of filopodia requires Cdc42 activity, since Cdc42-deficient cells do not express any filopodia (Bohil et al., 2006). Also Myosin-X plays a prominent role in the formation of these structures through the use of its motor function (Bohil et al., 2006). Myosin-X mutants that lack the head domain, responsible for the motor function, show impaired formation of filopodia. Our data support a role for both Cdc42 and Myosin-X in the formation of ICAM-1-rich filopodia.

ICAM-1 is one of the major adhesion molecules on activated endothelium that mediates the firm adhesion of leukocytes, in particular neutrophils, to the endothelium. Several groups reported that ICAM-1 is found in small finger-like and actin-rich structures that protrude up from the endothelial cell surface into the lumen of the blood vessel, which we here identify as filopodia (Barreiro et al., 2002b; Carman et al., 2003; van Buul et al., 2010). However, whether ICAM-1 adhesive function depends on the presence of ICAM-1-rich filopodia is not clear. Also, how ICAM-1-rich filopodia are induced is not fully elucidated. Carman and colleagues indicated that microvilli-like structures form the basis of larger cup-structures that are rich for actin (Carman et al., 2003). Oh and co-workers showed an important role for one of the ERM proteins, ezrin, in the induction of filopodia, or what they referred to as microvilli-like structures (Oh et al., 2007). They show that, when overexpressed, ezrin directly binds to the basic region of the intracellular tail of ICAM-1, although it needs further research to formally proof that the endogenous proteins ezrin and ICAM-1 directly interact (Romero et al., 2002). Interestingly, they showed that depletion of the filopodia, by perturbing the interaction between ICAM-1 and ezrin, led to decreased leukocyte adhesion. Furthermore it has
been shown that expression of the constitutively active form of Cdc42 increases dorsal filopodia (Bohil et al., 2006). We underscore these findings and add to that the functional importance of Cdc42 and Myosin-X in ICAM-1-mediated adhesion. Our study suggests that filopodia formation is important for the maturation of “docking structures”. These structures are involved in efficient leukocyte transendothelial migration. The mechanism that is responsible for the formation of filopodia may serve as a new target for therapies that aim to manipulate leukocyte extravasation under pathophysiological conditions.
MATERIALS AND METHODS

Reagents and Abs - Monoclonal antibodies (mAbs) against ICAM-1 was from R&D Systems (Minneapolis, MN); Polyclonal Abs against ICAM-1 (for WB) and b-catenin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The GFP and Myc (clone 9E10) mAb were purchased from Invitrogen (Carlsbad, CA).

Expression Vectors – ICAM-1-GFP was a kind gift from Dr. Olga Barrero and Dr. Francisco Sanchez-Madrid (Madrid, Spain). ICAM-1-ΔC-GFP mutant was generated by deleting the last 27 C-terminal amino-acids. ICAM-1-wt and C-terminal deletion mutant cDNA were sub-cloned into the pAdCMV-V5-DEST vector using the Gateway expression system (Invitrogen) or subcloned into the cDNA mCherry vector. GFP-tagged Myosin-X wild type and headless mutant were a kind gift from Dr. Richard Cheney (UNC, NC, USA). The myc-tagged Cdc42 mutants were used to generate IRES-GFP vectors containing dominant negative (T17N) or constitutively active (Q61L) mutants.

Cell cultures, treatments and transfections - HUVECs were obtained from Cambrex (East Rutherford, NJ) and cultured as described previously (van Buul et al., 2010). Endothelial cells were activated with 10ng/ml TNFα (R&D systems) overnight to mimic inflammation. All cell lines were cultured or incubated at 37°C at 5% CO2. Hela cells were maintained in growth medium (DMEM with 10% fetal calf serum; Sigma). Cells were transiently transfected with the expression vectors indicated in each experiment according to the manufacturer’s protocol using LipofectAMINE PLUS (Invitrogen) or Fugene6 (Roche, Basel, Swiss). The HL60 pro-myelocytic cell line was obtained from UNC-LCCC Tissue Culture Facility and grown in Optimem plus 5% fetal bovine serum. In all experiments described, differentiated HL60 cells were used, unless indicated differently. Differentiation to a neutrophil-like lineage was achieved by adding 1.3% DMSO for 3–5 days and resulted in increased expression of the LFA-1 and Mac-1 receptors (Van Buul et al., 2007).

Scanning electron microscopy - Transfected cells were grown on glass coverslips, fixed in 2.5% glutaraldehyde/PBS for 30 min at room temperature, and processed for scanning EM as described previously (van Buul et al., 2010). Cells were examined on a scanning electron microscope (Philips SEM 525 with Orion frame grabber) at 15 kV.

Antibody-coated beads - 3- or 10-μm polystyrene beads (Polysciences, Inc.) were pretreated with 8% glutaraldehyde overnight, washed five times with PBS, and were incubated with 300 μg/ml ICAM-1 mAb (R&D systems) according to the manufacturer’s protocol.

Immunofluorescence (IF) – Cells were cultured on glass cover slips, fixed and immunostained with indicated primary Abs as described previously (van Wetering et al., 2002). Subsequent visualization was performed with Alexa-conjugated secondary Abs (Invitrogen). F-actin was visualized with fluorescently-labeled Phalloidin (Invitrogen). Images were recorded with a ZEISS LSM510 confocal microscope. Crosstalk between the different channels was avoided by use of sequential scanning.

Bead adhesion assay - For IF or scanning EM, 1μg/mL of Ab-containing-beads were washed and re-suspended in assay medium (DMEM, supplemented with 10% fetal bovine serum). 1μg/mL of Ab-coated beads was incubated in wells of 24-well dishes containing glass cover slips, on which TNF-α-pretreated HUVECs or Cos7 cells were cultured. After the appropriate time, unbound beads were removed and cover slips were put on ice, gently washed 3 times with ice-cold PBS containing 1mM Ca2+/Mg2+ and subsequently processed for IF. For biochemistry, 10μg/mL of Ab-coated beads were incubated on the cells after which cells were washed as described above and subsequently lysed and processed as described.

Statistics – Student-T test was used to calculate statistical significance.
Acknowledgements
We wish to thank Dr. Olga Barrerio and Dr. Francisco Sanchez-Madrid (Madrid, Spain) for the ICAM-1 cDNA and Dr. Richard Cheney for his kind gift of the Myosin-X cDNA. Prof. dr. Peter Hordijk is gratefully acknowledged for reading the paper. JK is financially supported by the DHF Dekker grant (#2005T3901).

Author Contributions
Conceived and designed experiments: JK JvB. Performed the experiments: JK JvB FvA MH KH JvB. Analyzed the data JvB. Wrote the paper JK JvB.

Disclosure
The authors declare to have no financial interest.
REFERENCES


MYOX-DRIVEN DOCKING STRUCTURES AS CARGO TRANSPORTERS
Figure S1. SEM of control beads. (A) Beads coated with isotype control IgG antibodies or left untreated were allowed to adhere to the endothelium for 30 minutes and imaged using the scanning EM. No specific membrane structures were observed. (B) ICAM-1 antibody-coated beads were able to induce large membrane sheets around a group of beads. Also, the beads induced membrane protrusions on isolated descending aorta that were pretreated with TNFα. Images were taken with SEM.

Video 1. ICAM-1-GFP recruitment in time to ICAM-1 antibody-coated beads. Endothelial cells were transfected with ICAM-1-GFP and stimulated with TNFα. ICAM-1 antibody-coated beads were added and images were recorded every 30 seconds for 50 minutes using a confocal microscope. The focal plane was set 5μm above the basolateral surface of the endothelium, to properly image the formation of ICAM-1-rich structures.