Leukocyte trafficking and vascular integrity

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Annexin A2 limits neutrophil transendothelial migration by organizing the spatial distribution of ICAM-1.

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ABSTRACT

Intracellular adhesion molecule-1 (ICAM-1) is required for firm adhesion of leukocytes to the endothelium. However, how the spatial organization of endothelial ICAM-1 regulates leukocyte adhesion is not well understood. Here, we identified the calcium-effector protein annexin A2 as a novel binding partner for ICAM-1. ICAM-1 clustering promotes the ICAM-1-annexin A2 interaction and induces translocation of ICAM-1 into caveolin-1-rich membrane domains. Depletion of endothelial annexin A2 using RNA interference enhances ICAM-1 membrane mobility and prevents the translocation of ICAM-1 into caveolin-1-rich membrane domains. Surprisingly, this results in increased neutrophil adhesion and transendothelial migration under flow conditions and reduced crawling time, velocity and lateral migration distance of neutrophils on the endothelium. In conclusion, our data show that annexin A2 limits neutrophil transendothelial migration by organizing the spatial distribution of ICAM-1.
INTRODUCTION

ICAM-1 at the endothelial cell (EC) surface functions as an adhesive ligand for neutrophil β2-integrins supporting the rolling, arrest, crawling and transmigration of neutrophils in vivo (1-6). However, to what extent ICAM-1 is specifically involved and how ICAM-1 functionally facilitates each distinct step during neutrophil transmigration has been controversial. Depletion of the intracellular domain of ICAM-1 abolishes T-lymphocyte as well as neutrophil transmigration, but not adhesion (7-9). This work established the importance of the intracellular domain of ICAM-1 as signaling molecule in orchestrating leukocyte transmigration, but also showed the importance of the extracellular domain of ICAM-1 in leukocyte adhesion. Studies that used LFA-1 (αβ2,CD11a/CD18) and MAC-1 (αβ2,CD11b/CD18) deficient mice showed that neutrophil arrest depends mainly on LFA-1-ICAM-1 interactions whereas neutrophil crawling requires MAC-1-ICAM-1 interactions (2,3). Moreover, the binding site of ICAM-1 for MAC-1, but not LFA-1 is regulated by glycosylation which may provide a mechanistic explanation for sequential roles in ICAM-1-mediated arrest and crawling (10,11). In addition to LFA-1-ICAM-1 interactions in mediating neutrophil arrest, the LFA-1-ICAM-1 interaction has also been reported to be involved in neutrophil transmigration since it has been shown to be rapidly co-clustered into a ring-like-structure around transmigrating neutrophils (12,13). Since the intracellular tail of ICAM-1 contains no identified signaling domains, activation of intracellular signaling is thought to be mediated by receptor clustering (14,15).

Clustering of ICAM-1 using anti-ICAM-1 antibodies is commonly used to study leukocyte induced ICAM-1 signaling. It has been reported that antibody-induced clustering of ICAM-1 initiates a rise in intracellular calcium, activates small Rho GTPases, Protein kinase C, Src family protein kinases, eNOS and MAPK (7,15-18), and induces the recruitment of several membrane-actin binding proteins to the intracellular tail of ICAM-1 (14,19-22). Moreover, antibody-induced crosslinking of ICAM-1 has been shown to translocate ICAM-1 to F-actin- and caveolin-1-rich areas at the EC periphery (23). This translocation has been suggested to link the sequential steps of leukocyte adhesion and transcellular transmigration (23). Studies that examined ICAM-1 surface distribution showed that depletion or blockage of the intracellular tail of ICAM-1, using cell-penetratin-ICAM-1 peptides competitive for the RKIKK motif, induced a more homogeneous cell surface distribution and loss of ICAM-1-positive microvilli (24). ICAM-1 can form at least three different topologies on the cell surface through dimerization of domain 1 that does not interfere with LFA-1 or MAC-1 binding (25). Moreover, structural basis of ICAM-
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1 dimerization on the cell surface provides the existence of preformed ICAM-1 cis dimers on the cell membrane (26).

In line with this, Barreiro and colleagues showed that adhesive molecules such as ICAM-1 are physically organized and compartmentalized in preformed tetraspanin nanoplatforms (27,28). Those studies suggest the existence of multiple ICAM-1 complexes with different protein content, spatial organization or subcellular localization. However, little is known about how the spatial organization of ICAM-1 affects its adhesive function and what proteins regulate the distribution of ICAM-1 at the EC surface.

Here, we investigated the spatial organization of ICAM-1 at the EC surface and found annexin A2 as novel binding partner for ICAM-1. Our data show that annexin A2 negatively regulates the adhesion, crawling and subsequent transmigration of neutrophils across the endothelium by controlling the spatial organization of ICAM-1.

MATERIALS AND METHODS

DNA AND RNA CONSTRUCTS

YCam3.6 in a mammalian expression vector, based on a clontech-styl pEGFP-C1 backbone and driving expression from a CMV promoter was provided by Joachim Goedhart (Swammerdam Institute for Life Sciences, Amsterdam, the Netherlands). Briefly, the single chain Ycam3.6 biosensor consist of an enhanced (E)CFP, calmodulin E104Q, the calmodulin-binding peptide M13p, and a circular permutated (cp)-Venus. Binding of calcium makes calmodulin wrap around the M13 domain, increasing the FRET between the flanking FPs (47). shRNA in pLKO.1 targeting annexin A2 (A9) (TRCN 56145), annexin A2 (G5) (TRCN 56144) were purchased from sigma Aldrich mission library. ICAM-1-GFP was a kind gift from Dr. F. Sanchez-Madrid (University of Madrid, Madrid, Spain). pE AnnexinA2-GFP (N3) was purchased from Addgene.

ANTIBODIES

Polyclonal rabbit antibodies against ICAM-1 for WB were purchased from Santa Cruz Biotechnology (Cat #SC-7891). Polyclonal rabbit antibody against annexin A2 (H50)(SC-9061) and The Alexa Fluor 405 monoclonal mouse antibody against ICAM-1 CD54 (15.2)(Sc-107 AF405) were purchased from Santa Cruz (Bio-Connect). Monoclonal ICAM-1 CD54 (BBIG-I1 / IIC81) antibodies used for ICAM-1 clustering were purchased from R&D systems (Cat #BBA9). Polyclonal Goat anti-mouse Fcγ IgG was purchased from Jackson (Cat #115-005-071). Monoclonal antibody against annexin A2 was a kind gift from Dr. Gerke (Institute of Medical
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Biochemistry ZMBE Center for Molecular Biology of Munich, Germany). Rabbit monoclonal annexin A2 (D11G2) was purchased from Cell Signaling (BIOKÉ) (8235). Monoclonal mouse antibody against Actin (AC-40) was purchased from Sigma (Cat# A3853). The Alexa Fluor 405 goat anti-rabbit IgG (Cat# A31556), Alexa Fluor 647 chicken anti-goat IgG (Cat# A21469), Alexa Fluor 488 chicken anti-rabbit IgG (Cat# A21441) and Texas red 568 Phalloidin (Cat #T7471) were purchased from Invitrogen. Alexa Fluor 405 Phalloidin was purchased from Promokine (Cat# PK-PF405-7-01). Polyclonal Rabbit antibody against caveolin-1 was purchased from BD Biosciences (Cat # 610059). Mouse monoclonal actin antibodies were purchased from Sigma-Aldrich (Cat # A3853). Mouse monoclonal human Ezrin (18) was purchased from Transduction Laboratories (BD) (Cat# 610602). Mouse monoclonal RhoGDI antibodies were purchased from Transduction Laboratories (Cat #610255), mouse monoclonal antibodies against VCAM-1 were purchased from R&D systems (Cat #2090), and mouse monoclonal Filamin A antibodies were purchased from Serotec (Cat #MCA464S). Secondary HRP-conjugated goat anti-mouse, swine anti-rabbit antibodies were purchased from Dako (Heverlee, Belgium). All antibodies were used according to manufacturer’s protocol.

CELL CULTURES, TREATMENT AND TRANSFECTIONS

Pooled Human umbilical vein ECs (HUVECs) purchased from Lonza (P938, Cat # C2519A), were cultured on fibronectin-coated dishes in EGM-2 medium, supplemented with singlequots (Lonza, Verviers, Belgium) HUVECs were cultured until passage 9. HEK-293T were maintained in Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen, Breda, The Netherlands) containing 10% (v/v) heat-inactivated fetal calf serum (Invitrogen, Breda, The Netherlands), 300 mg/ml L-glutamine, 100 U/ml penicillin and streptomycin and 1x sodium pyruvate (Invitrogen, Breda, The Netherlands). Cells were cultured at 37°C and 5% CO₂. HUVECs were pre-treated with 10 ng/ml recombinant Tumor-Necrosis-Factor (TNF)-α (PeproTech, Rocky Hill, NJ) 24 hours before each leukocyte TEM experiment, pre-treated with 1 µM ionomycin (Invitrogen, I-24222) for periods as indicated. Cells were transfected with the expression vectors according to the manufacturer’s protocol with Trans IT-LT1 (Myrus, Madison, WI, USA). Lentiviral constructs were packaged into lentivirus in Human embryonic kidney (HEK)-293T cells by means of third generation lentiviral packaging plasmids (Dull et al., 1998; Hope et al 1990). Lentivirus containing supernatant was harvested on day 2 and 3 after transfection. Lentivirus was concentrated by Lenti-X concentrator (Clontech, Cat# 631232). Transduced target cells were used for assays after 72 hours. ICAM-1-GFP was delivered into HUVECs by adenoviral transduction 48h before imaging.
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PULL-DOWN ASSAY
A synthetic, biotinylated peptides encoding the intracellular domains of human ICAM-1 and VCAM-1 were used in pull-down assays as previously described (Kanters et al. 2008). The following sequences were used:
ICAM-1: NH2-RQRKIKKYRLQQAQKGTPMKPNTQATPP-COOH;
VCAM-1: IIYFARKANMKGSYSLVEAQKSKV-COOH.

PULL-OUT ASSAY AND IMMUNOPRECIPITATION
1.2 mg/ml dynabeads goat-α-ms IgG (Dynal, Invitrogen) per condition were washed ones with 1 ml buffer 1 containing PBS + 2 mM EDTA and 0.1% BSA (Millipore) using a magnetic holder. Dynabeads were coated with 1.6 µg α-ICAM-1 CD54 (BBIG-11/IIC81, R&D systems (Cat #BBA9) antibodies per condition and incubated head-over-head at 4ºC for 45 min. The beads were washed twice using buffer 1 and resuspended in PBS++ containing 0.5 MgCl₂ and 1 mM CaCl₂. Overnight TNF-α treated (10 ng/ml) HUVEC (2-5 million cells) were pre-treated with 1 µM ionomycin (Invitrogen, I-24222) for 10 min. 1.2 mg/ml dynabeads per condition were used to cluster ICAM-1 for 10-30 minutes. Cells were washed once on ice using PBS++. Next, cells were lysed in 1 ml cold pH7.4 RIPA buffer containing 50 mM Tris, 100 mM NaCl, 10 mM MgCl₂, 1% NP40, 10% glycerol, 0.1% SDS, 1% DOC (Sigma-Aldrich), DNAse inhibitor and protease phosphatase inhibitor cocktail for 5 min. Cells were scraped together and lysates were transferred to a new tube. Then, ICAM-1 coated dynabeads were added to non-clustered-control cells. 50 µl whole cell lysate was taken from all conditions. Beads and cell-lysates were subsequently incubated head-over-head for 1-2h at 4ºC. Next cells were washed twice with RIPA buffer and three times with NP-40 lysis buffer. Beads were resuspended in 30 µl 2x SDS-sample buffer and assessed by Western blotting.

WESTERN BLOTTING
Cells were washed twice with PBS, and lysed with 95°C SDS-sample buffer containing 4% β-mecapto-ethanol. Samples were boiled at 95°C for 4 minutes to denature proteins. Proteins were separated on 4-12% NuPAGE Novex Bis-Tris gels (Invitrogen), transferred to Immobilon-PVDF transfer membranes (Millipore Corp., Billerica, MA) and subsequently blocked with 2.5% (w/v) bovine serum albumin (BSA) in Tris-buffered saline with Tween 20 (TBST) for 60 minutes. The immunoblots were analyzed using primary antibodies incubated overnight at 4°C and secondary antibodies linked to horseradish peroxidase (HRP) (GE Healthcare, UK), after each step immunoblots were washed 6x with TBST. Signals were visualized by enhanced chemiluminescence and light sensitive films (GE Healthcare, UK).
**FRET measurements: Microscopy, image acquisition, and analyses**

We use a Zeiss Observer Z1 microscope with 40x NA 1.3 oil immersion objective, a HXP 120 V excitation light source, a Chroma 510 DCSP dichroic splitter, and two Hamamatsu ORCA-R2 digital CCD cameras for simultaneous monitoring of ECFP and Venus emissions. Image acquisition was performed using Zeiss Zen 2011 microscope software. The lowest achievable HXP excitation power, through a FRET filter cube (Exciter ET 436/20x, and 455 DCLP dichroic mirror (Chroma), the emission filter is removed) was used to excite the ECFP donor. The emission is directed to the left side port by a 100% mirror, to an attached dual camera adaptor (Zeiss) controlling a 510 DCSP dichroic mirror. Emission wavelengths between 455-510 nm are directed to an emission filter (ET 480/40, Chroma) and then captured by the ‘straight’ Hamamatsu ORCA-R2 camera resulting in ECFP image acquisition. The Emission wavelength 510 nm and higher are directed to a six positions LEP filter wheel (Ludl Electronic Products) placed in front of the second ‘rear’ Hamamatsu ORCA-R2 camera. Position 1 in the emission filter wheel is equipped with an ET 540/40m used for Venus image acquisition. Position 2 is left empty to allow mCherry image acquisition (EX BP 572/25, BS FT 590, EM BP 629/62, Zeiss). The LEP filter wheel is controlled by the MAC 6000 controller system (Ludl Electronic Products). Exposure time of DIC image was set to 76 ms and exposure time of simultaneous ECFP and Venus acquisition was set to +/-800 ms, images were subsequently recorded every 5 seconds by two 1344 (H) x 1024 (V) Hamamatsu ORCA-R2 digital CCD cameras (2x2 binning) for periods as indicated. Offline ratio analysis between ECFP and Venus images were processed utilizing the MBF ImageJ collection (Tony Collins). The raw ECFP and Venus image stacks were background (BG) corrected using the plug-in ‘ROI, BG subtraction from ROI’. Then, the ECFP and Venus stacks were aligned using the registration plug-in ‘Registration, MultiStackReg’. A smooth filter was applied to both image stacks to improve image quality by reducing the noise. Next, both image stacks were converted to a 32-bit image format, required for subsequent masking. A threshold was applied exclusively to the Venus image stack, converting the background pixels to ‘not a number’ (NaN). It allows elimination of artifacts in ratio image stemming from the background noise. Finally the Venus/ECFP ratio was calculated using the plug-in ‘Ratio Plus’, and a custom lookup table was applied to generate a color-coded image illustrating the high ‘red’ and low ‘blue’ activities. Note that some of the plug-ins, namely MultiStackReg, and Ratio Plus are not included in the basic MBF ImageJ collection and should be downloaded from the plug-in page in the ImageJ website (http://rsb.info.nih.gov/ij/plugins/index.html). Normalized intensity graphs; the intensity of an ROI of interest and
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The BG of the raw ECFP and Venus image stacks were measured using the plug-in ROI, Multi Measure in ImageJ. The raw ECFP and Venus intensities were BG subtracted using equation ECFP = (ECFP raw - BG), subsequently Venus only was corrected for bleed through using the equation VenusC = (Venus) - (0.5748*(ECFP)), and normalized using the equation ECFPNorm = ECFP/Average (ECFP 3-13), and finally the Venus/ECFP ratio was calculated using the equation Venus/ECFP = VenusNorm/ECFPNorm using Excel.

**IMMUNOFLUORESCENCE STAINING**

HUVECs were cultured on FN-coated glass bottom (14-30 mm) until confluency. After treatment, cells were washed with cold PBS, containing 1 mM CaCl$_2$ and 0.5 mM MgCl$_2$, and fixed in 4% (v/v) formaldehyde for 10 minutes. After fixation, cells were permeabilized in PBS supplemented with 0.5% (v/v) Triton X-100 for 10 minutes followed by a blocking step in PBS supplemented with 2.5% (w/v) BSA. Cells were incubated with primary and secondary antibodies and after each step washed with PBS and mounted on microscope glasses using Mowiol. Image acquisition was performed on a confocal laser scanning microscope (LSM510/Meta; Carl Zeiss Micro-Imaging) using a voxel size of 0.06x0.06x0.48 µm and a 63x NA 1.4 oil immersion objective. Line profile plots were generated in ImageJ using the plot profile plug-in.

**DETERGENT-FREE MEMBRANE FRACTIONATION**

HUVEC were transduced with control or annexin A2 shRNA and left for 72 hours. Anti-ICAM-1 antibodies were allowed to bind to 4h TNF-α-stimulated (10 ng/ml) HUVEC monolayer for 30 min. ICAM-1 was subsequently cross-linked using secondary goat anti-mouse IgG antibodies for another 30 min. Next procedures were all carried out on ice. Two ten cm$^2$ confluent HUVEC were washed and scraped into base buffer (20 mM Tris-HCL, pH 7.8, 250 mM sucrose) to which had been added 1mM CaCl$_2$ and 1 mM MgCl$_2$. Cells were pelleted by centrifugation for 2 min at 250 $g$ and resuspended in 1 ml of base buffer containing 1 mM CaCl$_2$ and 1 mM MgCl$_2$ and protease inhibitors (Invitrogen). The cells were then lysed by passage through a 22 g x 3” needle 20x. Lysates were centrifuged at 1,000 x$g$ for 10 min. The resulting post nuclear supernatant was collected and transferred to a separate tube. The pellet was again lysed by the addition of 1 ml base buffer, followed by sheering 20x through a needle and syringe. After centrifugation at 1,000 x$g$ for 10 min, the second post nuclear supernatant was combined with the first. An equal volume (2 ml) of base buffer containing 50% OptiPrep (Sigma-Aldrich St. Louis, MO) was added to the combined post nuclear supernatants and placed in the bottom of a 12 ml centrifuge tube. An 8
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A ml gradient of 0% to 18% OptiPrep in base buffer was poured on top of the lysate, which was now 25% in OptiPrep. Gradients were centrifuged for 90 minutes at 52,000 x g using an SW-41 rotor in a Beckman ultracentrifuge. After centrifugation, gradients were fractionated into 0.8 ml fractions, and the distribution of various proteins was assessed by Western blotting. This method was developed by Macdonald and Pike (36).

ICAM-1 MOBILITY ASSAYS
Live-cell imaging was conducted with a ZEISS LSM510 confocal microscope (Carl Zeiss MicroImaging, USA) using ZEN 2007 (Carl Zeiss Micro Imaging) image acquisition software with appropriate filter settings. To assess the mobility of ICAM-1 on the plasma membrane, we bleached a small fraction of ICAM-1-GFP positive plasma membrane and measured the fluorescent recovery of ICAM-1-GFP into this region for 200 seconds. Data was further analyzed in ImageJ. Intensities were normalized with the following equation: $(\text{intensity}_{\text{pre-bleech}} - \text{intensity}_{\text{After-bleech}}) / (\text{intensity}_{\text{pre-bleech}} - \text{intensity}_{\text{After-bleech}}) \times 100$. To examine ICAM-1 mobility TNF-α treated ECs were incubated with 10 µm size polystyrene beads (Polysciences, Inc) that were coated with a monoclonal antibody against ICAM-1 CD54 (BBIG-I1/IIC81, R&D systems (Cat #BBA9) for 30 minutes. Percentage ICAM-1-GFP positive rings were quantified as the number of ICAM-1-GFP positive rings divided by the number of beads per ECs.

NEUTROPHIL ISOLATION
Neutrophils were isolated from whole blood derived from healthy donors. Whole blood was diluted (1:1) with 5% (v/v) TNC in PBS. Diluted whole blood was pipetted carefully on 12.5 ml Percoll (room temperature) 1.076 g/ml. Tubes were centrifuged (Rotanta 96R) at 2000 rpm, slow start, low brake for 20 minutes. Ring fraction containing lymphocytes and monocytes was discarded. After erythrocyte lysis in an ice-cold isotonic lysis buffer (155 mM NH$_4$Cl, 10 mM KHCO$_3$, 0.1 mM EDTA, pH7.4 in Milli-Q(Millipore), neutrophils were centrifuged at 1500 rpm for five minutes at 4°C, incubated once with lysis buffer for 5 minutes on ice, centrifuged again at 1500 rpm for five minutes at 4°C, washed once with PBS, centrifuged again at 1500 rpm for five minutes at 4°C and resuspended in HEPES medium (20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM CaCl$_2$, 1 mM MgSO$_4$, 1.2 mM K$_2$HPO$_4$, 5 mM glucose (all from Sigma-Aldrich), and 0.4 % (w/v) human serum albumin (Sanquin Reagents), pH7.4) and kept at room temperature for not longer than four hours until use. Neutrophil counts were determined by cell counter (Casey).
**Neutrophil TEM under physiological flow**

HUVECs were cultured to 70% confluence in FN-coated 6 well plate, and transduced with lentivirus containing shAnxA2 and shCtrl for 24h. HUVECs were cultured in a FN-coated ibidi µ-slide VI⁰.⁴ (ibidi, Munich, Germany) the day before the experiment was executed and stimulated overnight with TNF-α (10 ng/ml). Freshly isolated neutrophils were resuspended at 1x10⁶ cells per ml in HEPES medium and were incubated for 30 minutes at 37ºC. Cultured HUVECs in ibidi flow chambers were connected to a perfusion system and exposed to 0.5 ml/minute HEPES shear flow for 10 minutes (0.8 dyne/cm²). Neutrophils were subsequently injected into the perfusion system and real-time leukocyte-endothelial interactions were recorded for 20 minutes by a Zeiss Observer Z1 microscope using a 40x NA 1.3 oil immersion objective. All live imaging was performed at 37°C in the presence of 5% CO₂. Transmigrated neutrophils were distinguished from those adhering to the apical surface of the endothelium by their transition from bright to phase-dark morphology. Percentage adherent or transmigrated neutrophils were manually quantified using the ImageJ plug-in Cell Counter (type 1, adherent cells, type 2, transmigrated cells). Neutrophil rolling velocity, crawling Time, crawling velocity and crawling distance were quantified using the ImageJ plug-in manual tracking.

**Statistics**

Filamin and annexin A2 binding to anti-ICAM-1-coated beads was tested using a one-way ANOVA assuming no matching or pairing, comparing the mean of each column with the mean of every other column, that were corrected for multiple comparisons by Tukey multiple comparisons test, with a single pooled variance. The student T-test performed statistical comparison between experimental groups. A two-tailed p-value of < 0.05 was considered significant. Unless otherwise stated, a representative experiment out of at least three independent experiments is shown.

**Results**

**The intracellular tail of ICAM-1 interacts with annexin A2**

To identify new proteins involved in the regulation of ICAM-1-mediated leukocyte transendothelial migration (TEM), we performed a pull down assay using biotinylated peptides encoding the intracellular tail of ICAM-1. Peptide-bound proteins were subsequently separated by SDS-PAGE, visualized by silver staining and specific protein bands were analyzed by mass spectrometry. A protein band interacting with the ICAM-1 peptide that migrated at a molecular mass of 36 kDa was identified by mass
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spectrometry as the calcium effector protein annexin A2. To corroborate the interaction of annexin A2 with the intracellular domain of ICAM-1, biochemical pull down assays from cell lysates using peptides were repeated, analyzed by Western blot and confirmed the binding of annexin A2 to the intracellular tail of ICAM-1, and not to the intracellular domain of VCAM-1 or streptavidin beads (Fig. 1a). Filamin A, previously reported to interact with the intracellular domain of ICAM-1 (14), was used as a positive control (Fig. 1a). To investigate the endogenous interaction between annexin A2 and ICAM-1, we immunoprecipitated endogenous ICAM-1 using magnetic beads coated with anti-ICAM-1 antibodies that induced the clustering of ICAM-1 (14). Anti-VCAM-1 or anti-IgG isotype control antibody-coated beads were used as controls. Using this method endogenous ICAM-1 and VCAM-1 were effectively precipitated from TNF-α-treated endothelial cells (Fig. 1b). Analysis of the binding of annexin A2 and Filamin A showed that these proteins interacted with ICAM-1 using anti-ICAM-1 antibody-coated beads (Fig. 1b). In contrast to annexin A2 that was only co-precipitated with anti-ICAM-1 antibody-coated beads, a low amount of Filamin A did also co-precipitate with anti-VCAM-1 antibody-

**Figure 1.** The intracellular tail of ICAM-1, but not VCAM-1 interacts with annexin A2. (a) Immunoblot analysis of protein extracts prepared from TNF-α-stimulated EC that were incubated with peptides encoding the intracellular tail (PD: C-term) of ICAM-1 or VCAM-1. Extracts were probed with antibodies directed against annexin A2 and filamin A. Streptavidin beads were used as a negative control. WCL: whole cell lysate. (b) Immunoprecipitation of ICAM-1 using magnetic beads coated with anti-ICAM-1 antibodies. Anti-VCAM-1 or anti-IgG isotype control antibody-coated beads were used as controls. Beads were incubated 30 minutes at the cell surface of TNF-α-stimulated EC before lysis. Extracts were probed with antibodies directed against ICAM-1, VCAM-1, filamin A and annexin A2. Data are from three experiments (a, b).
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**Figure 2.** Rolling neutrophils transiently increase intracellular calcium levels in EC driving annexin A2 to associate with the plasma membrane and ICAM-1. (a) Schematic illustration of the Ycam3.6 calcium sensor design containing enhanced (E)CFP (blue), calmodulin E104Q (grey), the calmodulin-binding peptide M13p (white), and a circular permuted (cp)-Venus (yellow). (b) Time-lapse Venus/CFP ratio images of Ycam3.6 calcium biosensor simultaneously recorded with an epifluorescent microscope showing spatiotemporal calcium measurements during the onset of flow (0.9 dyne per cm²) starting after 30 seconds, and upon neutrophil rolling, adhesion and transmigration (arrow). Calibration bar shows the increase in intracellular calcium concentrations (red) relative to basal calcium concentration (blue). Scale bar, 150µm. (c) Quantification of temporal intracellular calcium concentration in multiple EC during the onset of flow, and neutrophil rolling, adhesion and transmigration. Data
coated beads (Fig. 1b). Annexin A2 and Filamin A were not co-precipitated with anti-IgG isotype control antibody-coated beads (Fig. 1b). Thus, our findings show that annexin A2 binds to the intracellular tail of ICAM-1, but not to the intracellular tail of VCAM-1.

**Rolling neutrophils transiently increase intracellular calcium levels in EC driving annexin A2 to associate with the plasma membrane and ICAM-1**

Annexin A2 is a calcium effector protein comprising five calcium-binding domains that facilitate the binding of annexin A2 to the plasma membrane upon release of intracellular calcium (29). To investigate calcium signalling during leukocyte diapedesis under shear stress conditions, we used the fluorescence resonance energy transfer (FRET)-based genetically encoded calcium indicator Ycam3.6 (30) (Fig. 2a). The single chain Ycam3.6 biosensor consist of an enhanced (E)CFP, calmodulin E104Q, the calmodulin-binding peptide M13p, and a circular permutated (cp)-Venus. Binding of calcium makes calmodulin wrap around the M13 domain, increasing the FRET between the flanking FPs (30). EC transfected with Ycam3.6 biosensor were grown to confluence in FN-coated perfusion chambers, stimulated with TNF-α overnight and subsequently exposed to shear stress (0.8 Dyne/cm²). Intracellular changes in calcium levels were measured by donor/acceptor ratio imaging and showed that the initial onset of flow rapidly triggered the transient influx of calcium, as was also shown by others (31-33) (Fig. S1). The levels of calcium influx normalized within 2 minutes. To exclude any flow-induced calcium signals, we perfused the neutrophils after at least 20 minutes of initial flow onset. The results showed that initial neutrophil contact during the rolling stage rapidly induced a transient increase in intracellular calcium concentration in EC, preceding the firm adhesion step (Fig. 2b and Supplementary Video 1). Interestingly, firm adhesion of neutrophils to the endothelium was observed directly after the temporary burst of released intracellular calcium (Fig. 2b and Supplementary Video 1). We measured represents mean and s.e.m of three experiments including at least 12 cells per experiment. (d) Confocal imaging of cytosolic annexin A2-GFP translocation to the plasma membrane after ionomycin stimulation. Scale bar, 20µm. (e) Immunoblot analysis of protein extracts prepared from TNF-α-stimulated EC that were incubated with anti-ICAM-1-coated beads for 30 minutes pre lysis (meaning clustered ICAM-1) or post lysis (meaning non-clustered ICAM-1 conditions). EC were stimulated with ionomycin for 2 minutes to induce high intracellular calcium levels. Extracts were probed with antibodies directed against filamin A, ICAM-1 and annexin A2. Quantification of annexin A2 and filamin A binding, presented as ‘quantum level’ (sum of grayness of each pixel in blot) minus background (QL-Bg), normalized to that of ICAM-1. * P < 0.05 AnxA2 versus AnxA2 + ionomycin (ANOVA). Data represents mean and s.e.m of seven experiments (b,c) five experiments (d) three experiments (e).
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A 5-fold increase in sensor ratio almost instantly when the neutrophil touched the endothelium, in line with previous findings by Ziegelstein and colleagues (34). Surprisingly however, only 20-30% of the sensor-expressing endothelial cells showed an increase in calcium signal upon rolling neutrophils. This may reflect the heterogenic expression of the adhesion molecules like selectin and ICAM-1 between mutual endothelial cells. Quantification of the biosensor activity showed the significance of the transient and rapid intracellular calcium changes preceding neutrophil adhesion (Fig. 2c). To test if the supernatant of the neutrophils may increase intracellular calcium levels, we treated endothelial cells with supernatant of PMA-stimulated neutrophils and did not observe any calcium influx (Fig. S2). This excludes that secreted molecules from the neutrophils induced the calcium influx in the endothelium.

In addition, increased levels of intracellular calcium drives the majority of annexin A2 to the plasma membrane as is indicated by the rapid translocation of cytosolic annexin A2-GFP to the plasma membrane after ionomycin treatment (Fig. 2d and Supplementary Video 2). Also endogenous stimuli like histamine induced annexin A2 translocation to the plasma membrane (Fig. S3). To study if the interaction between endogenous ICAM-1 and annexin A2 is regulated by high calcium, we immunoprecipitated ICAM-1 using magnetic beads coated with anti-ICAM-1 antibodies on intact ECs treated with ionomycin (pre-lysis). The co-precipitation of annexin A2 with anti-ICAM-1 antibody-coated beads was enhanced upon high intracellular calcium conditions (Fig 2e). Quantification showed that pre-treating EC with ionomycin significantly promoted the co-precipitation of annexin A2 with α-ICAM-1 antibody-coated beads, whereas filamin A levels were not significantly altered (Fig 2e). Note that addition of anti-ICAM-1 antibody-coated beads to an EC lysate (post-lysis) did not result in the co-precipitation of annexin A2 or filamin A, indicating that the interaction with endogenous annexin A2 required intact membranes to promote the interaction between annexin A2 and ICAM-1 upon ICAM-1 clustering. Together, these findings showed that rolling neutrophils transiently increase intracellular calcium levels in EC that may transiently drive annexin A2 to associate with the plasma membrane and ICAM-1.

ICAM-1 is localized in F-actin- and annexin A2-rich microvilli

To study the spatial organization of ICAM-1 at the EC surface under non-ligand-bound conditions, we co-stained TNF-α-stimulated ECs for endogenous ICAM-1, F-actin and annexin A2. Overnight TNF-α-stimulated ECs showed heterogeneous distribution of ICAM-1 on the endothelial surface (Fig. 3a). Moreover, ICAM-1 is particularly enriched in F-actin-rich
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Figure 3. ICAM-1 at the EC surface is localized in microvilli rich in F-actin and annexin A2. (a) ECs show heterogeneous ICAM-1 surface expression after TNF-α stimulation. Immunofluorescence analyses of ICAM-1 and F-actin in TNF-α-stimulated ECs. Open and filled arrows indicate low and high endothelial surface expression of ICAM-1, respectively. (b) Immunofluorescence analyses of ICAM-1 and F-actin in TNF-α-stimulated ECs. Filled arrows indicate ICAM-1 localization at the plasma membrane in microvilli. (c) Immunofluorescence analyses of ICAM-1, F-actin and annexin A2 in TNF-α-stimulated ECs. Annexin A2 is localized in nanometer-scale clusters at the plasma membrane (d) Line profiles of ROI marked in Fig. 3 c. Line profile 1 indicates co-localization between ICAM-1, F-actin and annexin A2 in microvilli. Line profile 2 indicates annexin A2 membrane domains outside microvilli that does not co-localize with ICAM-1. Line profile 3 indicates membrane domains that are rich in ICAM-1, annexin A2 and F-actin and domains that only contain annexin A2 or F-actin.
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**Figure 4.** Annexin A2 regulates the spatial distribution of ICAM-1 at the EC surface and is required for ICAM-1 translocation to caveolin-1-rich membrane domains upon ICAM-1 clustering. (a) Schematic representation of ICAM-1 crosslinking prior membrane fractionation. ICAM-1 at the EC surface is clustered by incubation with the BBIG-I1 mAb for 30 minutes followed incubation with a secondary goat anti-mouse antibody for 30 minutes. (b) Immunoblot analysis of protein extracts that were separated on density by OptiPrep gradient centrifugation to visualize the spatial distribution of proteins at the plasma membrane in non-clustered and clustered ICAM-1 conditions. Extracts used for membrane fractionation were prepared 72 hours after transduction with control (b, c) or annexin A2 shRNA (d, e). Extracts were probed with antibodies directed against ICAM-1, caveolin-1, annexin A2, RhoGDI and Ezrin. Lane 1-5 indicate the cytosolic protein fraction. Lane 6-10 indicate high density membrane fractions rich in caveolin-1. Lane 11-14 indicate low density membrane fractions rich in Ezrin and Annexin A2. (f) Quantification of the spatial distribution of ICAM-1 at the plasma membrane in non-clustered and clustered ICAM-1 conditions. RhoGDI indicates the cytosolic fraction (g) Quantification of spatial distribution of ICAM-1 in control and annexin A2 depleted ECs after ICAM-1 clustering. (h) Quantification of the spatial distribution of caveolin-1 in control and annexin A2 depleted ECs after ICAM-1 clustering. (i) Quantification of the spatial distribution of ICAM-1 and caveolin-1 at the plasma membrane in non-clustered and clustered ICAM-1 conditions. RhoGDI indicates the cytosolic fraction.
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extensions called microvilli that protrude out of the EC apical surface (Fig. 3b) (24,35). Immunofluorescent analysis of annexin A2 and ICAM-1 showed enrichment of both proteins in microvilli (Fig. 3c,d). In contrast to ICAM-1, annexin A2 is also enriched in membrane domains other than F-actin-rich microvilli (Fig. 3c,d). Thus, unengaged ICAM-1 is distributed at microvilli where it co-localized with F-actin and annexin A2.

**Annexin A2 regulates the spatial organization of ICAM-1 at the plasma membrane**

To examine the spatial organization of ICAM-1 at the plasma membrane during ICAM-1-mediated neutrophil TEM, we used a detergent-free membrane fractionation (36). Neutrophil-mediated ICAM-1 clustering was mimicked using antibody crosslinking, according to previous studies (37-39) (Fig. 4a). Without antibody-mediated clustering, ICAM-1 co-sedimented with ezrin and annexin A2 in fraction 10-12 outside of caveolin-1-rich membrane domains (fraction 6-10) (Fig. 4b). Antibody-mediated crosslinking of ICAM-1 drives ICAM-1 to the EC poles, regions that are rich in caveolin-1 (23). In line with these findings, we found that antibody-mediated clustering of ICAM-1 increased co-sedimentation of ICAM-1 with caveolin-1 to fractions 8-10, but also induced the dissociation of ezrin with the plasma membrane (Fig. 4c). Note that the membrane distribution of annexin A2 was not affected by ICAM-1 clustering (Fig. 4b,c). To investigate if annexin A2 regulated the spatial organization of ICAM-1 at the plasma membrane, we depleted annexin A2 using short hairpin RNA (shRNA). Annexin A2 depletion shifted non-clustered ICAM-1 to less dense fractions 11-13 (Fig. 4d). Strikingly, crosslinking ICAM-1 under these conditions prevented the co-sedimentation of ICAM-1 with caveolin-1, whereas annexin A2 depletion did not alter the distribution of caveolin-1 or ezrin dissociation from the plasma membrane upon ICAM-1 clustering (Fig. 4e). These findings showed that annexin A2 regulates the spatial ICAM-1 distribution at the EC surface and that annexin A2 is required for the translocation of ICAM-1 to caveolin-1-rich membrane domains upon ICAM-1 clustering.

**Annexin A2 negatively regulates ICAM-1 mobility at the EC surface**

To investigate whether annexin A2 affects ICAM-1 dynamics at the plasma membrane, we examined ICAM-1 mobility using FRAP. ECs were transiently transfected with ICAM-1-GFP, treated with TNF-α and subsequently a region of interest was bleached (Fig. 5a). The recovery of ICAM-1-GFP in the bleached region was measured using confocal laser scanning microscopy and showed that the mobile fraction of ICAM-1 was around 40% in control conditions (Fig. 5b). However, in annexin A2-
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**Figure 5.** Annexin A2 regulates the lateral mobility of ICAM-1 at the EC surface. (a) Time-lapse confocal images showing FRAP of ICAM-1-GFP indicative for the lateral mobility of ICAM-1 at the plasma membrane and in microvilli. White box indicates the ICAM-1-GFP positive membrane part that has been bleached. Scale bar Scale bar, 10µm. (b) Quantification of ICAM-1-GFP recovery kinetics after photo bleaching showing the mobile fraction of ICAM-1-GFP at the EC surface in control (green line) and annexin A2 depleted (red line) ECs. Bar graph shows quantification of the mobile fraction of ICAM-1 after 150 seconds. (c) Endothelial annexin A2 depletion by shRNA. Immunoblot analysis of protein extracts prepared from HUVEC expressing ICAM-1-GFP. Extracts were prepared 72 hours after transduction with control or
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Deficient ECs, the mobile fraction of ICAM-1 was significantly increased to more than 55% (Fig. 5b). Subsequently, annexin A2, F-actin, endogenous ICAM-1 and ICAM-1-GFP, levels were analysed by Western blot. Importantly, Annexin A2-deficient ECs did not affect endogenous ICAM-1 as well as ICAM-1-GFP protein levels in TNF-α-treated ECs (Fig. 5c). Moreover, using anti-ICAM-1 antibody-coated beads, we found that ICAM-1-GFP was more rapidly and more frequently recruited to beads when annexin A2 was depleted than under control conditions (Fig. 5d,e and Supplementary Video 3). Quantification showed that the kinetics of ICAM-1-GFP recruitment to anti-ICAM-1 antibody-coated beads was significantly faster in annexin A2-deficient ECs than in control ECs (Fig. 5f). Also the number of ICAM-1-GFP-positive rings was increased in annexin A2-deficient ECs compared to the control (Fig. 5f). Thus, annexin A2 negatively regulates ICAM-1 mobility at the EC surface.

Down regulation of endothelial annexin A2 increases neutrophil adhesion to TNF-α stimulated ECs.

ICAM-1 at the EC surface has been described to support the rolling, arrest, crawling and transmigration of neutrophils (1-6). To investigate how altered ICAM-1 membrane distribution and dynamics in annexin A2-deficient ECs affect neutrophil TEM under physiological flow conditions, we simultaneously measured neutrophil TEM through control and annexin A2-deficient ECs in a parallel flow set-up (Fig. 6a). This set-up allowed us to use primary human neutrophils from the same donor. Neutrophils were simultaneously perfused over TNF-α-stimulated ECs under physiological shear stress conditions (0.8 Dyne/cm²). Neutrophil rolling velocity, adhesion, crawling and transmigration was recorded by a wide-field microscope and subsequently quantified using ImageJ software. Annexin A2 depletion in ECs did not affect neutrophil rolling velocity (Fig. 6b). However, the adhesion of neutrophils to annexin A2-deficient ECs was significantly increased compared to control ECs (Fig 6c and Supplementary...
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Video 4). Quantification of the number of adherent neutrophils showed a 2-3 fold increase in neutrophil adhesion when annexin A2 was silenced using two distinct short hairpins targeting annexin A2 (Fig. 6d). Surprisingly, neutrophil crawling distance on the EC surface as well as their velocity was significantly reduced in annexin A2-deficient primary ECs (Fig. 7 a-c). Despite slower apical movement, neutrophils spend less time crawling over the EC cell surface before transmigration (Fig. 7d). In

**Figure 6.** Annexin A2 depletion in EC enhanced the adhesive capacity of ICAM-1 to bind neutrophils β2-integrins (a) Schematic representation of the experimental set-up used to simultaneously measure two conditions with same donor neutrophils. (b) Quantification of neutrophil rolling velocities over TNF-α-stimulated control and annexin A2 deficient primary ECs. (c) Epi-fluorescent live-cell imaging of neutrophil TEM through TNF-α-stimulated control and annexin A2 deficient primary ECs under physiological flow conditions (0.9Dyne/cm²). (d) Quantification of adherent neutrophils through TNF-α treated ECs under physiological flow conditions after 72 hours transduction with control shRNA (open bar), annexin A2 shRNA A9 (black bar) or annexin A2 shRNA G5 (grey bar). * P < 0.05 and ** P < 0.01 control versus AnxA2 (ANOVA) (d). Data are representative of five independent experiments with > 5 rolling events per group (b) five experiments with > 150 events per group (d) (error bars (b,d), s.e.m).
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line with this, we observed that most neutrophils crawled in a direct line to the EC junctions where they breeched the endothelial monolayer (data not shown). Consequently, the absolute number of neutrophils that finished the final step, i.e. crossing the endothelial barrier, was significantly increased when annexin A2 was depleted (Fig. 7e). When calculating the number of transmigrated neutrophils relative to the number of adhering neutrophils, there was no difference in percentage of neutrophils that

Figure 7. Neutrophil crawling distance and velocity was significantly reduced in annexin A2 deficient ECs, but neutrophil diapedesis was not affected. (a) Graphical representation of neutrophil crawling tracks over TNF-α-stimulated control and annexin A2 deficient primary ECs. (b) Quantification of neutrophil crawling distance, crawling velocity (c) and crawling time (d) over TNF-α-stimulated control and annexin A2 deficient primary ECs. (e) Quantification of transmigrated neutrophils through TNF-α treated ECs under physiological flow conditions after 72 hours transduction with control shRNA (open bar), annexin A2 shRNA A9 (black bar) or annexin A2 shRNA G5 (grey bar). (f) Quantification of percentage of adherent cells that completed transmigration after 20 minutes. **** \( P < 0.0001 \) control versus AnxA2 (Student’s t-test) (b). * \( P < 0.05 \) control versus AnxA2 (Student’s t-test) (c,d). * \( P < 0.05 \) control versus AnxA2 (ANOVA) (e). Data are representative of five independent experiments with > 5 crawling events per group (a-d) five experiments with > 150 events per group (e,f) (error bars (a-f), s.e.m).
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crossed the endothelium in the absence or presence of annexin A2 (Fig. 7f). Altogether, our results showed that annexin A2 regulates the adhesion, crawling and subsequent transmigration of neutrophils across the endothelium by controlling ICAM-1 membrane distribution and dynamics that in turn mediates the efficiency of neutrophil TEM.

**DISCUSSION**

ICAM-1 at the EC surface functions as an adhesive ligand for neutrophil \( \beta_2 \)-integrins required for effective neutrophil TEM. However, little is known how cell-surface distribution of ICAM-1 is regulated and how this spatial organization controls the adhesive function of ICAM-1. It has been hypothesized that multiple ICAM-1 complexes with different protein content, spatial organization and subcellular localization regulate leukocyte behavior at different stages of leukocyte TEM (21,23,28). In agreement with this hypothesis, we found that altered ICAM-1 membrane distribution significantly alters neutrophil TEM efficiency. We found that clustered ICAM-1 translocates from ezrin-rich membrane domains to caveolin-1-rich membrane domains, which was impaired in annexin A2-depleted ECs. This altered ICAM-1 membrane distribution enhanced the lateral mobility and adhesive capacity of ICAM-1 to bind neutrophils. These findings suggest that the translocation of ICAM-1 into caveolae has a limiting effect on ICAM-1-mediated leukocyte adhesion. A previous study showed that ICAM-1 in caveolae is rapidly transcytosed and transported to the basolateral surface of ECs (23). This event together with the expression level of ICAM-1 on the cell surface has been suggested to regulate transcellular migration, but not paracellular migration of T cells and neutrophils (23,40,41).

Depletion of the intracellular domain of ICAM-1 impairs leukocyte transmigration, but not adhesion (8,9,42). Partly in line with these studies, we found that annexin A2-mediated alteration of ICAM-1 distribution affects neutrophil adhesion but not the percentage of transmigrating neutrophils. This suggests that the spatial organization of ICAM-1 at the endothelial surface is of primary importance for the adhesion, and less for the transmigration or diapedesis of leukocytes. Studies using LFA-1 (\( \alpha_L \beta_2, \)CD11a/CD18) and MAC-1 (\( \alpha_M \beta_2, \)CD11b/CD18) deficient mice show that neutrophil firm arrest depends mainly on LFA-1-ICAM-1 interactions, whereas neutrophil crawling requires MAC-1-ICAM-1 interactions (2,3). Interestingly, depletion of annexin A2 increased neutrophil adhesion efficiency, but reduced the time, velocity and distance of crawling neutrophils. Possibly, the increased ability of ICAM-1 to move through the
membrane may result in an increased efficiency to interact with neutrophil integrins. As a consequence, neutrophils adhere more rapidly to the endothelium. Why neutrophil crawling depends on the spatial distribution of ICAM-1 needs further investigation. Interestingly, overexpression of annexin A2 did not result in decreased leukocyte adhesion or TEM (data not shown). For annexin A2 to properly localize at the plasma membrane, it requires binding to the small molecule S100A10. However, if the expression of S100A2 is limiting, this may prevent the translocation of annexin A2 to the membrane and consequently its functionality in regulating ICAM-1.

Calcium signalling in EC has been implicated in all steps of leukocyte TEM (17,34,43-45). In fact, the major role for calcium signalling is believed to be involved in MLCK activation and subsequent actomyosin contraction to induce transient openings in the EC junctions allowing leukocyte transmigration (46-49). In contrast to multiple transient calcium spikes at each stage of diapedesis, we found that EC intracellular calcium was released upon initial neutrophil-EC contact prior neutrophil adhesion under flow conditions. As an alternative model for calcium being involved in junctional opening, we show that the calcium-effector annexin A2 is recruited to the plasma membrane upon release of intracellular calcium. Increased annexin A2 at the plasma membrane may modulate ICAM-1 function and thereby affect neutrophil TEM efficiency.

In conclusion, our work discovers that annexin A2 regulates ICAM-1 function through its spatial distribution on the endothelial surface, which limits neutrophil TEM efficiency. These findings may provide new targets for drug therapy to treat inflammatory-based diseases.

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Abbreviations:
FRAP, Fluorescent Recovery After Photobleaching;
TEM, transendothelial migration;
ECs endothelial cells.

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