The Rho-GEF Trio regulates a novel pro-inflammatory pathway through the transcription factor Ets2

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Summary

Inflammation is characterized by endothelium that highly expresses numerous adhesion molecules to trigger leukocyte extravasation. Central to this event is increased gene transcription. Small Rho-GTPases not only control the actin cytoskeleton, but are also implicated in gene regulation. However, in inflammation, it is not clear how this is regulated. Here, we show that the guanine-nucleotide exchange factor Trio expression is increased upon inflammatory stimuli in endothelium. Additionally, increased Trio expression was found in the vessel wall of rheumatoid arthritis patients. Trio silencing impaired VCAM-1 expression. Finally, we excluded that Trio-controlled VCAM-1 expression used the classical NFκB or MAP-kinase pathways, but rather acts on the transcriptional level by increasing phosphorylation and nuclear translocalization of Ets2. These data implicate Trio in regulating inflammation and provide novel targets for therapeutic purposes to treat inflammatory diseases such as rheumatoid arthritis.

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Introduction

At the interface between the circulation and the underlying tissue, the endothelium plays a key role in controlling leukocyte recruitment during inflammatory diseases such as rheumatoid arthritis (RA) and atherosclerosis. Under inflammatory conditions as induced by cytokines such as TNF-α and IL1β, the endothelium undergoes both dramatic functional and morphological changes (Bradley, 2008; Pober and Sessa, 2007). From a quiescent anti-thrombotic, non-adhesive state, it changes into a more permeable and pro-adhesive surface mediating leukocyte adhesion and transmigration. Increased expression of the adhesion molecules E-selectin (CD62E), Vascular Cell Adhesion Molecule-1 (VCAM-1/CD106) and Intercellular Cell Adhesion Molecule-1 (ICAM-1/CD54) is responsible for both the initial and firm adhesion of the leukocytes to the endothelium and the subsequent transendothelial migration (TEM) (Noursargh et al., 2010). Besides adhesion molecule up-regulation, the endothelium also undergoes shape changes through remodeling of the actin cytoskeleton and down-regulation of junctional molecules. This ultimately leads to decreased barrier function of the endothelium (Cain et al., 2010; McKenzie and Ridley, 2007).

Among the TNF-α-induced regulatory pathways that stimulate the expression of the adhesion molecules E-selectin, VCAM-1 and ICAM-1, the transcription factor NF-κB and the mitogen-activated protein kinase (MAPK) pathway have been most intensively studied (Denk et al., 2001; Pietersma et al., 1997; Read et al., 1997; Viemann et al., 2004). In addition, transcription factors such as Interferon Regulatory Factor-1 (IRF-1), GATA and Sp1 have also been implicated in TNF-α-mediated up-regulation of adhesion molecules on the endothelium (Neish et al., 1995a; Neish et al., 1995b; Tsyo et al., 2010; Umetani et al., 2001). Although NF-κB is evidently one of main regulators of endothelial adhesion molecule expression, the contributions of other transcription factors and their roles in regulating the individual adhesion molecules is relatively poorly understood.

The changes in endothelial morphology and the remodeling of the actin cytoskeleton, induced by TNF-α have been proposed to be at least partly regulated by the activity of the small GTPase Rac1 (Cain et al., 2010; Wójciak-Stothard et al., 1998). Rac1 is one of the best studied members of the Rho family of GTPases and also plays crucial roles in controlling endothelial cell motility, barrier function and leukocyte TEM (Garrett et al., 2007; Lampugnani et al., 2002; Tan et al., 2008). Interestingly, besides regulating these morphological processes, Rac1 has also
been implicated in regulating TNF-α-induced expression of endothelial adhesion molecules (Ahmad et al., 2002; Chen et al., 2003; Deshpande et al., 2000; Min and Pober, 1997; Papaharalambus et al., 2005). However, the upstream signaling proteins that locally activate Rac1 upon TNF-α stimulation are still unknown.

Local small GTPase activation and downstream effector protein binding is known to critically depend on the exchange of GDP for GTP by guanine nucleotide exchange factors (GEFs) (Rossman et al., 2005). To identify Rac1-GEFs that mediate Rac1 activation induced by TNF-α, we performed an expression screen of Rac1-GEFs in endothelial cells and found that the expression of the GEF Trio was up-regulated under inflammatory conditions both in vitro and in vivo. We demonstrate that silencing Trio expression interferes with TNF-α-mediated up-regulation of adhesion molecules VCAM-1 and ICAM-1 mRNA and protein expression. Additionally, VCAM-1 expression is specifically mediated through a pathway involving Rac1 activation via the terminal GEF-domain of Trio. Finally, Trio increased VCAM-1 expression by enhancing transcriptional activity at the VCAM-1 promoter through phosphorylation and nuclear translocation of the transcription factor Ets-2, independently of NF-κB or JNK/p38 MAPKs.

Materials and Methods

Cell culture

Primary human umbilical vein endothelial cells (HUVEC) were purchased from Lonza (Baltimore, MD, USA) and cultured during regular passaging following fibronectin (10 μg/ml) coating of the tissue culture flasks (TPP, Trasadingen, Switzerland) or glass slides in EGM2-containing singlequots (Lonza). Endothelial cells were cultured until passage 9. Endothelial cells were treated with 10 ng/ml TNF-α, 10 ng/ml IL-1β, 100 ng/ml IFN-γ or 0.5 μg/ml lipopolysaccharides (LPS) for periods as indicated.

Luciferase reporter assays

Adenoviral 4×NF-κB-MLP-Luc and VCAM-WT-Luc luciferase reporter vectors were generated similarly to previously reported vector (Mottershead et al., 2008). The pGL3-VCAM-1-luc construct was a kind gift from Dr T. Minami, University of Tokyo, Japan. HUVEC were co-transduced with a reporter vector and a control β-gal vector at multiplicity of infection (MOI) of 300 and 30, respectively. 48 h after transduction, cells were treated with TNF-α for indicated times. Subsequently, cells were lysed in a 1× Passive Lysis Buffer (Promega, Madison, WI, USA) and luciferase activity was measured in a Luciferase Assay reagent (Promega) with a microplate reader (GENios Plus, Tecan Group Ltd., Männedorf, Switzerland), while normalizing the data for β-gal activity (Korchynskyi and ten Dijke, 2002).

Monocyte adhesion and TEM under flow

HUVECs were cultured in a fibronectin-coated Ibi∆-slide VI1×4 (IbiDI, München, Germany) for 2–3 days until confluency and stimulated overnight with TNF-α (10 ng/ml). Freshly isolated monocytes (0.4×10^6 cells/ml) were perfused over HUVEC monolayers at 0.5 ml/min (shear stress of 1 dyne/cm²). During this time leukocyte–endothelial interactions were recorded in six random fields with a Zeiss Axiovert-200 microscope (Plan-apochromat 10×/0.45 M27 Zeiss-objective) equipped with a motorized stage. Transmigrated monocytes were distinguished from adhering to the apical surface of the endothelium by their transition from bright to phase-dark morphology.

Antibodies and reagents

Antibodies directed against Trio (clone D20 and clone H120), DOCK180 (clone H-4), Vav2 (clone H-200), Sox1 (clone A-9), ICAM-1 (clone H-108), VCAM-1 (clone C-19), Vascular Endothelial-Cadherin (VE-Cadherin) (F8), RhoA (clone 26C4), ERK1/2, p-ERK1/2, p65 (clone α) and p50 (clone E-10) were purchased from Tecbu-Bio (Heemstegaard, Netherlands). Ets-2 antibody was purchased from Acris Antibodies GmbH (Herford, Germany). Antibodies against actin (clone AC-40), as well as MG-132, cyclohexamide and actinomycin D, were purchased from Sigma-Aldrich (Zwijndrecht, Netherlands). β-Pix (AB-3829) antibody was purchased from Millipore (Leiden, Netherlands). E-selectin (BBA-18) antibody and IFN-γ were purchased from R&D systems (Abingdon, UK). mAb against Trio (clone D20) and CDC42 (clone 44) were purchased from BD biosciences (Breda, Netherlands). Antibodies against iκB, p-IκBα, p53, p38 and p-Jnk, p-p38, p-ATF2 were from Cell Signaling (Danvers, MA, USA). mAb RhG~ was purchased from Millipore. Secondary HRP-conjugated goat anti-mouse, swine anti-rabbit and rabbit anti-goat antibodies were purchased from Dako (Heverlee, Belgium). Monoclonal GFP (IL-8) antibody, secondary goat anti-rabbit IR 680, goat anti-mouse IR 800 and donkey anti-goat IR 800 antibodies were purchased from LI-COR (Westbur, Leiden, Netherlands). Texas Red- and alexa-633-conjugated phallidin were from Invitrogen (Breda, Netherlands). TNF-α and IL-1β were purchased from Peprotech EC (London, UK). NIS23766 was purchased from Merck (Nottingham, UK). ITX3 was purchased from Chembridge (San Diego, CA, USA).

Adenovirus production

Adenovirus GFP-TrioD1 (1233–1685), GFP-TrioD2 (1849–2218), GFP-TrioN (1–1685) were obtained by PCR amplification, with pEGFP-TrioD1, pEGFP-TrioD2 and pEGFP-TrioN as template and primer pairs JR22F (GAGAATTCCTATGCGGCATGTTAAGTCAAGT) and JR4R (GAGAATTCCTATGCGGCATGTTAAGTCAAGT) or JR2E22F (GAGAATTCCTATGCGGCATGTTAAGTCAAGT) and JR4R (GAGAATTCCTATGCGGCATGTTAAGTCAAGT). The PCR product was cloned as a Sall/EcoRI (TrioD1/D2) or MfeI/SaFl (TrioN) fragment into pENTR1A (Invitrogen) with Sall/EcoRI (TrioD1/D2) or EcoRI/SaFl (TrioN) and recombined into the pAd/CMV/V5-DEST vector (Invitrogen) with Clonase II enzyme mix according to the manufacturer’s instructions (Invitrogen). Adenovirus expressing GFP-TrioD1, GFP-TrioD2 or GFP-TrioN was produced by transfecting Pac1 (Westburg, Leiden) digested pAd-GFP-TrioD1, pAd-GFP-TrioD2 or pAd-GFP-TrioN into HEK293T cells.

shRNA lentivirus production

shRNA constructs (Sigma Mission library) targeting Trio (Trio1, TRC_878; Trio2, TRC_872; Trio3, TRC_877; Trio4, TRC_874; Trio5, TRC_15061), Rac1 (TRC_4872), RhoA (TRC_4771), CDC42 (TRC_47630) and RhoG (TRC_48019) or non-targeting shCtrl (shc002) construct were packaged into lentivirus in HEK293T cells by means of third generation lentiviral packaging plamsids (Dull et al., 1998). Lentivirus-containing supernatant was harvested on day 2 and 3 after transfection. Lentivirus was concentrated by centrifugation at 20,000 g for 2 hours.

Western blotting

SDS-PAGE samples were analyzed on 7.5, 10, or 15% (w/v) polyacrylamide gels, depending on the size of the proteins of interest, and transferred onto nitrocellulose (Whatman) or PVDF membrane (Bio-Rad). Following blocking in 5% (w/v) low fat milk in TBST (Tris-buffered-saline, Tween-20) the blots were incubated with the primary antibody for 1 hour at room temperature, washed 3× for 20 minutes in TBST, and were subsequently incubated with horseradish peroxydase-conjugated secondary antibodies (dilution 1:7000) in TBST for 1 hour at room temperature, followed by washing 3× with TBST for 20 minutes each and development of the blot by ECL or SuperSignal west nano ECL (ThermoScientific, Amsterdam, Netherlands). Alternatively, blots were incubated with IR 680 or IR 800 dye-conjugated secondary antibodies (dilution 1:500) in TBST for 1 hour at room temperature followed by washing 3× for 20 minutes in TBST. Infrared signal was detected and analyzed with the Odyssey infrared detection system (LI-cor Westburg). For Trio protein expression, 3–8% (w/v) Tris-acetate pre-cast gels (Invitrogen) were used according to the manufacturer’s instructions and samples were transferred onto nitrocellulose membrane by blotting for 18 h at 20 mA.

Confocal laser scanning microscopy

For immunofluorescence, cells were grown on fibronectin-coated 14-mm coverslips. After treatment, the cells were washed in cold PBSA (PBS, 0.5 mM CaCl₂, 0.5 mM MgCl₂) for 10 min. After fixation, the samples were permeabilized in PBS-T (PBS + 1% [v/v] Triton X-100 and 0.1% [v/v] glyceral) for 4 min, followed by a blocking step in PBS supplemented with 2% (w/v) BSA. The cells were incubated with primary and secondary antibodies, and after each step washed 3 times in PBSA. Coverslips were mounted with Vectashield with DAPI (Vector Laboratories Inc., Peterborough, UK) on microscope slides. For live cell imaging, the cells were seeded on 30-mm coverslips and transfected or transduced as indicated. After 24–72 hours, the cells were placed in a heating chamber at 37°C and 5% CO₂ and imaged with a confocal microscope (LSM510 META; Carl Zeiss Microimaging, Inc.).

Immunohistochemistry

For immunohistochemical analysis, we analyzed synovial biopsies of inflamed joints of patients meeting established criteria for the diagnosis of RA (Arnett et al.,
Regulation of TNF-α signaling by Trio

1998) and patients displaying mild synovitis who did not meet classification criteria for RA or other forms of arthritis. Acquisition and use of the material was approved by the Medical Ethics Committee of the VU University Medical Center, and patients provided informed written consent prior to the study. For immunohistochemical analysis, 4 µm sections were dewaxed, rehydrated and incubated in methanol/H2O2 (0.3% v/v) for 30 minutes to block endogenous peroxidases. Next, antigen retrieval was performed in TRIS-EDTA (pH 9.0). This was followed by incubation with rabbit Trio Ab (1:100, Tebu-Bio, Heerhugowaard, Netherlands) for 1 h at RT. Sections were then incubated with Envision (undiluted, Dako Cytomation, Eindhoven, The Netherlands) for 30 minutes at room temperature. Staining was visualized using 3,3’-diaminobenzidine (0.1 mg/ml, 0.02% H2O2). Sections were then counterstained with hematoxylin, dehydrated and covered.

RhoG, Rac1 and CDC42 activation assay
A confluent monolayer of HUVEC in a 100-mm Petri-dish was washed with cold PBS (+ 1 mM CaCl2 and 0.5 mM MgCl2) and lysed in 50 µl Tris, pH 7.4, 0.5 mM MgCl2, 500 mM NaCl, 1% (v/v) Triton X-100, 0.5% (w/v) deoxycholic acid (DOC), and 0.1% (w/v) SDS supplemented with protease inhibitors. Lysates were cleared at 14,000 g for 5 min. GTP-bound RhoG was isolated by rotating supernatants for 30 min with 60–90 µg of GST-ELMO (GST fusion protein containing the full-length RhoG effector ELMO) conjugated to glutathione-Sepharose beads (GE Healthcare, Zent, The Netherlands) (van Buul et al., 2007). Wittenberg and Burrage, 2008) GTP-bound Rac1 and CDC42 was isolated with biotynlated Pak1-Crib peptide coupled to streptavidin-agarose (ten Klooster et al., 2006). Beads were washed four times in 50 mM Tris, pH 7.4, 0.5 mM MgCl2, 150 mM NaCl, 1% (v/v) Triton X-100, and protease inhibitors. Pull-downs and lysates were immunoblotted with monoclonal RhoG, Rac1 and CDC42 antibodies.

Fusion proteins
GST-ELMO and GST-Rac1 G15A fusion proteins were purified from BL21 Escherichia coli cells (Agilent technologies, Amstelveen, The Netherlands) with glutathione-Sepharose 4B as previously described (Ellerbroek et al., 2004). GST fusion proteins were stored in 50% (v/v) glycerol at −80 °C.

ELISA for siCAM-1
Triol-silenced or control HUVECs were cultured on fibronectin-coated 12-well plates and were treated with TNF-α for 20 h. Supernatant was harvested and the ELISA for soluble ICAM-1 was performed according to the manufacturer’s protocol (Diaclone, Sanquin Reagents, Amsterdam, The Netherlands).

RNA isolation, cDNA synthesis and quantitative RT-PCR
Total cellular RNA was extracted with a QiAamp RNA blood mini kit (Qiagen, Venlo, The Netherlands) according to the protocol provided by the manufacturer. cDNA was synthesized with oligo(dT) from the superscript III First-strand synthesis system for RT-PCR kit (Invitrogen, Breda, Netherlands) according to the protocol provided by the manufacturer. PCR reactions were performed with Lightcycler FastStart DNA Master PLUS SYBR Green I (Roche Diagnostics, Indianapolis, IN, USA). PCR amplification was performed on a LightCycler instrument (Roche Applied Science), and analyzed with LightCycler Software version 3.5 (Roche Molecular Biochemicals, Mannheim, Germany). The reaction mixture consisted of 4 µl of cDNA, 1 µl of relevant primer combination at a concentration of 10 µM, and 4 µl of SYBR Green I mix in a total volume of 20 µl. cDNA input was determined by measuring a household gene in parallel with the assay of the genes of interest. For amplification, the following LightCycler protocol was used. The chemical clef of the Taq polymerase was removed by preincubation for 10 minutes at 95°C; the template was amplified for 45 cycles, at the end of 45 cycles, a melting curve was generated to determine the unique features of the DNA amplified. The primers used for detection in the PCR were Em-F (GAAAAATATGGTTGAGAGACCTATT), Em-R (CCCGAGTGAAGATCCCCTTTTTA); ICAM-1-F (CTTCCTCACCTGTTTGGTCT), ICAM-1-R (TGGCCTCATGGATCCTCCTCA); VCAM-1-F (TGAACTTAGAAATGGAAAAAGGA); VCAM-1-R (CAGAGGGCCACCTAATAGCATT); E-selectin-F (TACCCGTAAGACTCCCTGAGTACTGTC); E-selectin-R (GTGTTAATCTTGTATCCCTCCTAG).

Statistical analysis
Statistical comparisons between experimental groups were performed by the student t-test. A two-sided α-value of ≥0.05 was considered significant. Unless otherwise stated, a representative experiment out of at least three independent experiments is shown.

Results
Rac1 mediates TNF-α-induced VCAM-1 expression
TNF-α induces rapid changes in the cytoskeleton of endothelial cells, indicative of GTPase signaling (Wójciak-Stothard et al., 1998). Upon TNF-α treatment of HUVEC, we observed enhanced Rac1 activation after 15 minutes, and Rac1-GTP levels remained increased for up to 20 h (Fig. 1A). Analysis of the Rac1-related GTPases RhoG and CDC42 showed no increase in RhoG-GTP levels (Fig. 1B), whereas CDC42-GTP levels were transiently elevated after TNF-α (Fig. 1C).

To specifically examine Rac1 function in TNF-α-mediated up-regulation of adhesion molecules, shRNA produced by lentivirus was used to reduce Rac1 expression in HUVEC. Silencing of Rac1 significantly impaired TNF-α-induced up-regulation of VCAM-1 (Fig. 1D). ICAM-1 levels were only mildly affected upon Rac1 silencing. Manipulation of other Rho-family GTPases RhoG or RhoA showed no effect on TNF-α-induced expression levels of VCAM-1 or ICAM-1 (Fig. 1E; supplementary material Fig. S1A). Silencing CDC42 expression did not change TNF-α-induced VCAM-1 and ICAM-1 expression. These results demonstrate that Rac1 is activated upon TNF-α stimulation and mediates TNF-α-induced VCAM-1 expression.

The Rac1 GEF Trio is upregulated under inflammatory conditions
The GEF Trio is a 350 kD multi-domain enzyme that encompasses two GEF units. The N-terminal GEF unit (TrioD1) mediates nucleotide exchange on RhoG and Rac1, whereas the C-terminal GEF unit (TrioD2) is specific for RhoA. We observed that Trio protein levels were elevated in TNF-α-stimulated HUVEC, whereas other GEFs showed no changes in expression levels upon TNF-α treatment (Fig. 2A). These data suggest that TNF-α-induced GEF upregulation is specific for Trio. Quantification of Trio mRNA upregulation after 20 h TNF-α treatment showed that its expression was increased 3.5-fold (Fig. 2B), whereas mRNA levels of the GEF Vav2 remained unaltered.

In addition to TNF-α, other inflammatory stimuli such as IL-1β and LPS, but not IFN-γ, also significantly induced the expression of Trio (Fig. 2C). In contrast to endothelium, no enhanced Trio protein expression by TNF-α in epithelial A549 cells was measured (Fig. 2D), indicating that up-regulation of Trio is an endothelial-specific event. To investigate whether Trio is also expressed in endothelium under inflammatory conditions in vivo, we analyzed synovial biopsies of inflamed joints of patients meeting established criteria for the diagnosis of RA and patients displaying mild synovitis, who did not meet classification criteria for RA or other forms of arthritis. Interestingly, Trio-specific staining was particularly intense in the endothelium lining the blood vessels (Fig. 2E). In contrast, this intense staining was reduced in patients with mild synovitis (Fig. 2E). Quantification analysis showed that Trio expression was significantly increased in vessels of RA patients (Fig. 2E). Additionally, immunofluorescence imaging of the histopathology samples showed that Trio co-localized with ICAM-1 and VCAM-1 in vessels from RA patients (Fig. 2F). These results show that the expression of Trio is increased by various inflammatory mediators and that increased Trio expression in the endothelium correlates with progressed inflammatory conditions, such as RA.

Silencing of Trio expression reduces adhesion molecule expression
To investigate whether Trio has a role in TNF-α-induced activation of endothelial cells, we tested five different lentivirally-produced shRNA constructs for their potential to
reduce Trio expression in HUVEC. Three out of five constructs tested, shTrio1, 3 and 5, reproducibly reduced Trio expression (Fig. 3A). As a control, Vav2 expression was unaltered upon Trio silencing (supplementary material Fig. S1B). Since shTrio5 was most efficient in silencing Trio expression, this construct was used in subsequent experiments. Given that Rac1 has been implicated in TNF-α-mediated F-actin rearrangements, leading to endothelial cell elongation and alignment (Cain et al., 2010; Wojcik-Stothard et al., 1998), we examined whether Trio also regulates these processes. Under control conditions, TNF-α induced F-actin stress fibers that resulted in elongated and aligned morphology of the endothelial cells (Fig. 3B; supplementary material Movie 1). However, in shTrio-treated endothelial cells, TNF-α-induced elongation and alignment were severely impaired (Fig. 3B; supplementary material Movie 2), whereas VE-cadherin expression was unaltered (supplementary material Fig. S1C). These data suggest that Trio is involved in TNF-α-induced cytoskeletal changes.

To examine whether reduction in Trio expression also perturbs upregulation of VCAM-1, ICAM-1 and E-selectin by TNF-α, we analyzed protein expression of these adhesion molecules by Western blotting. The expression of VCAM-1, ICAM-1 and E-selectin was reduced after TNF-α stimulation in HUVEC upon Trio silencing (supplementary material Fig. S1D). However, in shTrio-treated endothelial cells, TNF-α-induced elongation and alignment were severely impaired (Fig. 3B; supplementary material Movie 2), whereas VE-cadherin expression was unaltered (supplementary material Fig. S1C). These data suggest that Trio is involved in TNF-α-induced cytoskeletal changes.

Next, we focused on the mechanism how Trio regulates TNF-α-induced adhesion molecule expression. Reducing Trio expression in endothelium showed loss of cell–cell junctions and appearance of intercellular gaps (Fig. 3B). To study if loss of cell–cell junctions resulted in altered expression of VCAM-1 and ICAM-1, junctional protein VE-cadherin was silenced using shRNA constructs. No reduction in VCAM-1 and ICAM-1 expression was measured upon loss of VE-cadherin-mediated cell–cell junctions (supplementary material Fig. S2B). In addition, to study whether Trio silencing affects VCAM-1 and ICAM-1 expression under inflammatory conditions in general. To study the relevance of this, we tested monocyte TEM under physiological flow conditions, since monocyte TEM depends on ICAM-1 and VCAM-1 (Ley et al., 2007). Reducing Trio expression severely impaired both monocyte adhesion (Fig. 3F) and TEM (Fig. 3G), thus showing that Trio-mediated adhesion molecule expression is required for efficient monocyte diapedesis.

Since Trio expression was upregulated by multiple inflammatory stimuli, we assessed whether Trio also played a role in adhesion molecule expression induced by stimuli other than TNF-α. Based on Haraldsen et al., two time points for cytokine stimulation were used. ICAM-1 expression is known to appear as early as 4 h and peaks at 20 h. VCAM-1 expression is observed at 4 h and is maximal at 18 h. However, we wish to note that in particular for VCAM-1, the expression levels at early time points, i.e. 4 h, can vary due to the use of primary endothelium and donor variation. Interestingly, silencing of Trio expression also significantly impaired VCAM-1 and ICAM-1 upregulation by IL-1β, IFN-γ and LPS (Fig. 3E; supplementary material Fig. S2A), suggesting that Trio regulated endothelial adhesion molecule gene expression under inflammatory conditions in general. To study the relevance of this, we tested monocyte TEM under physiological flow conditions, since monocyte TEM depends on ICAM-1 and VCAM-1 (Ley et al., 2007). Reducing Trio expression severely impaired both monocyte adhesion (Fig. 3F) and TEM (Fig. 3G), thus showing that Trio-mediated adhesion molecule expression is required for efficient monocyte diapedesis.
ICAM-1 protein stability, we tested whether proteosomal or lysosomal inhibition rescued VCAM-1 and ICAM-1 expression upon Trio silencing. We used chloroquine to block lysosomal degradation and MG-132 to prevent proteosomal degradation. However, VCAM-1 and ICAM-1 expression was still reduced in Trio-silenced endothelial cells (supplementary material Fig. S2C). These data demonstrated that Trio does not regulate ICAM-1 and VCAM-1 protein degradation.

In addition, we examined whether Trio increased shedding of the extracellular domain of ICAM-1. Using ELISA to detect the soluble extracellular domain of ICAM-1, we did not observe increased soluble ICAM-1 in the culture medium (supplementary material Fig. S2D). A decrease in soluble ICAM-1 was measured that correlated with decreased ICAM-1 protein expression in Trio-silenced endothelial cells (supplementary material Fig. S2E). This showed that Trio silencing did not promote shedding of the extracellular domain of ICAM-1. Together with the real-time PCR analysis, showing that reduced Trio expression decreased VCAM-1, ICAM-1 and E-selectin mRNA levels (Fig. 3D), these data indicate that Trio regulates TNF-α-induced adhesion molecule expression at the level of gene expression.

Trio regulates VCAM-1 expression through Rac1

To examine whether TNF-α activated Trio to exchange GTP on Rac1, we used a nucleotide-free mutant of Rac1 (G15A) fused to GST for pull-down assays. This nucleotide-free Rac1 mutant possesses a high affinity for activated GEFs (Garcia-Mata et al., 2006). Upon treatment with TNF-α, increased binding of Trio to GST-Rac1-G15A was detected (Fig. 4A), demonstrating Trio is activated to bind Rac1. To assess whether TNF-α-induced Rac1 activation is Trio-mediated, TNF-α-induced Rac1 activation was significantly impaired in Trio-silenced cells (Fig. 4B). To study whether Trio activity is required for TNF-α-induced up-regulation of VCAM-1 and ICAM-1, we used the inhibitor ITX3, recently found to be specific for the TrioD1-GEF domain, responsible for Rac1/RhoG activation (Bouquier et al., 2009; van Rijssel et al., 2012). Pretreatment of endothelium with ITX3 reduced TNF-α-induced Rac1 activation (Fig. 4C). Interestingly, ITX3 treatment impaired TNF-α-induced VCAM-1 expression, but not ICAM-1 expression (Fig. 4D). In addition, pretreatment of endothelium with NSC23766, an inhibitor preventing Rac1 binding to TrioD1 (Gao et al., 2004), likewise reduced VCAM-1 expression (Fig. 4E), showing that TNF-α-induced VCAM-1 upregulation is
controlled by the activity of the Rac1-activating, N-terminal GEF domain of Trio.

Expression of TrioN partially rescues VCAM-1, but not ICAM-1 expression in Trio-silenced endothelial cells

We investigated whether TrioD1 activity is sufficient to restore the impaired VCAM-1 expression in Trio-silenced endothelium. With an adenoviral approach GFP, GFP-TrioD1 or GFP-TrioD2 was expressed in control and Trio-silenced HUVEC. However, neither expression of GFP-TrioD1 (supplementary material Fig. S3A) nor GFP-TrioD2 (supplementary material Fig. S3B) rescued VCAM-1 or ICAM-1 expression in TNF-α-stimulated endothelial cells, showing that TrioD1 activity is necessary, but not sufficient for TNF-α-induced VCAM-1 and ICAM-1 expression. F-actin staining showed that TrioD1 and TrioN were still able to induce membrane ruffles in Trio-deficient cells (supplementary material Fig. S3C), indicative for endogenous Rac1 activation.

The TrioD1 domain is preceded by ~1300 amino acids containing a putative lipid transfer SEC14 domain and 9 spectrin-like repeats, which may have scaffolding functions. To assess whether these domains are required for TrioD1 to mediate VCAM-1 expression, we expressed a Trio N-terminal construct, GFP-TrioN, including the SEC14 domain, spectrin repeats and TrioD1 GEF domain, in control and Trio-silenced HUVEC. TrioN partially rescued VCAM-1 but not ICAM-1 expression in Trio-silenced endothelial cells (Fig. 5A). Quantification analysis showed that TrioN significantly rescued VCAM-1 expression (Fig. 5B). Additionally, under control conditions GFP-TrioN expression did already enhance TNF-α-stimulated VCAM-1 expression, although not significantly. These data show that the N-terminal region of TrioD1-GEF domain partially regulates VCAM-1 expression. These data indicate that Trio is more dominantly involved in regulating VCAM-1 expression than ICAM-1 expression. Therefore, we focused our research on Trio-mediated VCAM-1 expression.

Trio silencing does not affect MAPK signaling

We have demonstrated that Trio-mediated VCAM-1 expression involves Rac1 activation. Rac1 has been shown to induce gene transcription through regulating the activation of the MAPKs JNK and P38 (Jaffe and Hall, 2005). To confirm that JNK and P38 are involved in the upregulation of VCAM-1 and ICAM-1 in...
HUVEC, we used pharmacological inhibitors to target MAPK activity. Inhibition of JNK and P38, but not ERK or MEK, reduced TNF-α-induced VCAM-1 upregulation (supplementary material Fig. S4A,B). Also TNF-α-induced ICAM-1 upregulation was perturbed. To investigate whether JNK or P38 signaling is involved in downstream Trio signaling, we analyzed JNK and P38 phosphorylation in time induced by TNF-α in the presence or absence of Trio. However, phosphorylation of both JNK1/2 and P38 (supplementary material Fig. S4C,D) was not altered in the absence or presence of Trio in HUVEC. In addition, phosphorylation of the transcription factor AP-1 subunit ATF-2, known to be under control of JNK and P38, was also still induced by TNF-α in Trio-silenced endothelium (supplementary material Fig. S4E). Thus, although JNK and P38 are activated upon TNF-α-stimulation and are involved in adhesion molecule expression, this pathway is Trio-independent.

Fig. 4. Trio and Rac1 activity are required for TNF-α-induced ICAM-1 and VCAM-1 expression. (A) Trio and Vav2 activation following TNF-α treatment was examined by means of pull-down assays with GST-Rac1-G15A mutants as described in Methods in SM. Total Trio and Vav2 expression in the cell lysates is shown as loading control. Rac1 activation (Rac1.GTP) after TNF-α stimulation is shown in the lower panels. This experiment was carried out four times. (B) Reduced Trio expression in endothelial cells (third panel) prevented Rac1 activation (first panel; Rac1.GTP) upon TNF-α treatment as well as VCAM-1 and ICAM-1 expression (lower panels). Graph at the right shows the quantification of Rac1.GTP levels in fold increase. The experiment was carried out at least three times. (C) Inhibition of TrioD1 activity using ITX3 showed impaired Rac1 activity (Rac1.GTP). Second panel shows equal Rac1 protein expression. Graph at the right shows the quantification of Rac1.GTP levels in fold increase. Experiment is performed three times. Data are mean±SEM. ITX3 (D) and NSC-23766 (E) prevented upregulation VCAM-1 and ICAM-1 after TNF-α, respectively. Experiments were carried out four times. Data presented in all graphs are means±SEM. *P<0.05.
Regulation of TNF-α signaling by Trio

**Trio silencing does not affect NF-κB activation**
Rac1 influences gene transcription also through mediating the activation of the transcription factor NF-κB (Boyer et al., 2004; Sulciner et al., 1996). To examine whether NF-κB activation is regulated by Trio, we first analyzed TNF-α-induced phosphorylation and degradation of the NF-κB inhibitory protein IκBα. In both control and Trio-silenced cells, IκBα became rapidly phosphorylated within 5 minutes of TNF-α stimulation and was subsequently degraded (supplementary material Fig. S5A). In addition, nuclear translocation of both the p65/RelA and p50 (supplementary material Fig. S5B, Fig. S6A) subunits of NF-κB in response to TNF-α stimulation was not affected in Trio-silenced endothelial cells. Phosphorylation of serine-536 on the p65 subunit of NF-κB has been shown to promote its transcriptional activity (Hu et al., 2004; Jiang et al., 2003) and some reports have suggested that Rac1 signaling regulates p65 phosphorylation on serine-536 (Chen et al., 2009; Orr et al., 2008). However, under both control and Trio-silenced conditions, TNF-α-induced serine-536 phosphorylation on p65 equally well (supplementary material Fig. S5C). To study NF-κB transactivation, a specific NF-κB reporter construct was used, which showed that silencing of Trio did not reduce TNF-α-induced NF-κB transactivation in endothelial cells (supplementary material Fig. S5D). These results therefore demonstrate that Trio does not regulate TNF-α-induced NF-κB activation in endothelium. To exclude the role of Trio in TNF-α-induced apoptosis, we measured phosphatidylserine (PS) exposure using Annexin V. No effect on apoptosis by TNF-α and/or Trio silencing was measured in endothelial cells (supplementary material Fig. S6B,C). Additionally, no change was found in the cell cycle of all treated cells (supplementary material Fig. S6D).

**Trio mediates Ets-2 translocation and phosphorylation**
Regulation of TNF-α-mediated VCAM-1 gene expression by Trio occurs either on transcriptional level or on post-transcriptional level by affecting mRNA turnover. To explore the latter possibility, we analyzed the stability of VCAM-1 mRNA levels in time upon inhibition of mRNA synthesis with actinomycin-D after TNF-α stimulation or under resting conditions. Under both control and Trio-silenced conditions, VCAM-1 mRNA levels rapidly decayed upon blocking of new mRNA synthesis, i.e. actinomycin D treatment (Fig. 6A).

Since silencing of Trio expression did not alter VCAM-1 mRNA turnover, Trio may regulate VCAM-1 expression by transactivation of its promoter. Measuring total mRNA levels of TNF-α-induced VCAM-1 showed a significant reduction of VCAM-1 mRNA levels in Trio-silenced endothelial cells (supplementary material Fig. S7A). Next, we expressed a construct of the VCAM-1 promoter coupled to a luciferase reporter gene in endothelial cells using an adenoviral approach and analyzed luciferase reporter activity upon TNF-α stimulation. Reduction of Trio expression in endothelial cells resulted in a significant reduction of approximately 60% of the TNF-α-induced VCAM-1 promoter activity compared to control conditions (Fig. 6B). These data show that Trio mediates VCAM-1 upregulation by controlling its mRNA transcription.

Recently, Cheng and colleagues showed that the transcription factor Ets-2 translocates to the nucleus upon TNF-α treatment and regulates VCAM-1 mRNA expression (Cheng et al., 2011). Our data showed that in Trio-deficient cells, TNF-α-induced Ets-2 translocation to the nucleus is prevented (Fig. 6C; supplementary material Fig. S7B). By overexpressing TrioN, but not TrioD1, nuclear translocation of Ets-2 was partially restored (Fig. 6D). Ets-2 translocation was quantified by reduced Ets-2 levels in the cytosol (supplementary material Fig. S7C). Wei and co-workers reported that Ets-2 phosphorylation on threonine-72 was increased upon persistent TNF-α treatment (Wei et al., 2004). In endothelium, TNF-α-induced threonine phosphorylation of Ets-2 (Fig. 6E). Interestingly, this induction was prevented in Trio-deficient cells (Fig. 6E), indicating that Trio regulates threonine phosphorylation and nuclear translocation of Ets-2. Recently, Harris and colleagues showed that Ets-2 may also mediate VCAM-1 expression through miR-126 (Harris et al., 2008; Harris et al., 2010). We additionally tested if the expression level of miR-126 was changed upon Trio silencing. As expected, miR-126 expression declined after TNF-α treatment, allowing VCAM-1 expression, but no difference was measured between shCTRL and shTrio-treated endothelium (supplementary material Fig. S7D), indicating that Trio did not regulate miR-126.

Together, our data indicate that Trio regulates TNF-α-induced expression of VCAM-1 on promoter activity level through the transcription factor Ets-2 in a Rac1-dependent fashion. These results provide a novel target, i.e. Trio activity, to develop new strategies to improve treatment of inflammatory diseases such as RA.

**Discussion**
Expression of vascular adhesion molecules plays a key role in coordinating leukocyte recruitment and the initiation of inflammation (Ley et al., 2007). In this study, we demonstrate that the GEF Trio regulates the expression of endothelial adhesion molecules VCAM-1, ICAM-1 and E-selectin, induced by inflammatory stimuli, and that this is required for efficient monocyte adhesion and diapedesis. We focused our studies on VCAM-1 regulation and showed that VCAM-1 expression is mediated by a pathway involving Rac1 activation through the
N-terminal GEF domain of Trio. Moreover, this involves the activation and translocation of the transcription factor Ets-2. The rescue experiments show that simply expressing the Trio-GEF unit only does not induce VCAM-1 expression. The N-terminal part of Trio was required to partially rescue VCAM-1 expression. Moreover, we demonstrate that Trio expression itself is elevated in endothelium treated with various inflammatory stimuli. Interestingly, Trio expression is also particularly intense in

![Graph A](image1.png) VCAM-1 mRNA levels were measured as described in Materials and Methods. Left graph: untreated HUVEC; Right graph: TNF-α treated. Data are means of three independent experiments ± SEM. *P<0.001. (B) VCAM-1 promoter activity in HUVEC (TNF-α 20 h) was measured as described in Materials and Methods. Data are means of three independent experiments ± SEM. *P<0.001. HUVEC were treated as indicated and immunostained for Ets-2. Left graph: untreated HUVEC; Right graph: TNF-α treated. Data are means of three independent experiments ± SEM. *P<0.05. (C) HUVEC were treated as indicated and immunostained for Ets-2. Profile on the right shows increase in fluorescence according to drawn line in image, and graph on the right shows quantification. Data are means of three independent experiments ± SEM. *P<0.01. (D) Trio was silenced in HUVEC, treated as described under C and GFP-TrioN (open bars) or GFP-TrioD1 (closed bars) was expressed. Data are means of three independent experiments ± SEM. *P<0.001. (E) Immunoprecipitation of Ets-2 showed increased threonine/serine (Thr/Ser) phosphorylation of Ets-2 upon TNF-α treatment, which was abrogated in Trio-deficient cells. Graph on the right shows quantification. Data are means of three independent experiments ± SEM. *P<0.01.
endothelium in inflamed joints of RA patients, suggesting that increased Trio expression may contribute to the severity of inflammatory diseases such as RA.

We have delineated a novel pro-inflammatory pathway leading to VCAM-1 expression through activation of Rac1 by the Rho-GEF Trio, involving the transcription factor Ets-2, but excluding NF-κB and MAP-kinase pathways. In addition to MAPK/AP-1 and NF-κB, several other transcription factors have been implicated in TNF-α-induced VCAM-1 upregulation. This includes IRF-1, GATA family transcription factors and the Krüppel-like transcription factor Sp1 (Neish et al., 1995a; Tsoyi et al., 2010; Umetani et al., 2001; Warfel and D’Agnillo, 2008). Upon TNF-α-induced synthesis, IRF-1 has been reported to synergize with NF-κB to induce VCAM-1 expression (Dagia et al., 2004; Neish et al., 1995b). However, our data show that in Trio-silenced endothelial cells in which IRF-1 synthesis was blocked with cyclohexamide, VCAM-1 expression was still reduced after TNF-α administration (J.v.R., unpublished data).

The GATA family transcription factor GATA-6 is also indicated to be involved in specifically regulating TNF-α-induced VCAM-1 expression (Tsoyi et al., 2010; Umetani et al., 2001). Interestingly, TNF-α-stimulated binding of GATA-6 to the VCAM-1 promoter has been demonstrated to be induced by a PI3K-Akt-GSK3β-pathway (Tsoyi et al., 2010). Since Rac1 stimulates PI3-Kinase activity (Murga et al., 2002), Trio and Rac1 may function upstream of this pathway. However, analysis of TNF-α-induced phosphorylation of Akt and GSK3β showed that both were unaffected in Trio-silenced endothelial cells, indicating that Trio and Rac1 do not likely mediate GATA-6 activation through this pathway.

Recently, Cheng and colleagues reported that the transcription factor Ets-2 determines the inflammatory state of endothelial cells in advanced atherosclerotic lesions (Cheng et al., 2011). They showed that Ets-2 is required for TNF-α-induced VCAM-1 expression, as Ets-2 silencing reduced VCAM-1 expression upon TNF-α treatment. Interestingly, we show that Ets-2 translocates to the nucleus upon TNF-α in a Trio-dependent manner. Surprisingly, our data show that expressing the Rac-1 GEF by guest on December 21, 2017http://bio.biologists.org/Downloaded from

Unexpectedly, Trio was similarly required for adhesion molecule expression, induced by IL-1β, LPS and IFN-γ. Therefore, we speculate that Trio is part of a common pathway that is employed by different inflammatory stimuli to regulate adhesion molecule expression. Indeed, Rac1 has also been reported to become activated by IL-1β and LPS stimulation in endothelium (Equils et al., 2004; Jefferys and O’Neill, 2000).

Overall, we identified a novel pathway downstream of inflammatory mediator TNF-α including the Rho-GEF Trio/Rac1/Ets-2 and excluding NFκB and MAP-kinase activity. The relevance of Trio in endothelial inflammatory signaling is underscored by the finding that Trio expression is detected in the synovial endothelium of RA patients. Therefore, compounds that can inhibit the enzymatic activities of Trio, such as ITX3 and NSC-23766 (Bouquier et al., 2009; Gao et al., 2004), may have therapeutic potential for treating inflammatory diseases such as RA.

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Author Contributions
J.v.R. and J.D.v.B. designed the experiments, performed the research, wrote the manuscript and analyzed the data. I.T., F.P.J.v.A., M.H., J.G. and H.W.M.N. performed the research. O.K., D.G. and K.A.R. contributed vital new reagents.

Competing Interests
The authors have no competing interests to declare.

References
Regulation of TNF-α signaling by Trio


Supplementary Material
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Fig. S1. (A) shRhoA does not affect VCAM-1 and ICAM-1 expression. RhoA protein levels were reduced after shRhoA treatment (third panel). shRhoA-treated endothelial cells showed no VCAM-1 (upper panel) or ICAM-1 (second panel) expression difference compared to shCTRL-treated cells after TNF-α treatment. Actin is shown for protein loading (lower panel). (B) Quantification of Trio silencing of several tested shTrio constructs. No effect on Vav2 expression was measured. (C) shTrio3 reduces TNF-α-induced ICAM-1 and VCAM-1 expression. Reduced Trio expression induced reduced ICAM-1 and VCAM-1 expression in endothelial cells after TNF-α treatment; time course as indicated. Actin is shown as loading control (lower panel) (D).

Fig. S2. (A) Trio silencing impaired expression of VCAM-1 and partly ICAM-1 upon treatment for 4 or 20 h with different inflammatory stimuli. (B) shVE-cadherin does not affect ICAM-1 and VCAM-1 expression levels. Endothelial cells were silenced for VE-cadherin (second panel) and treated with TNF-α as indicated. ICAM-1 (third panel) and VCAM-1 (fourth panel) are upregulated after TNF-α treatment in shCTRL-treated cells. (C) Protein degradation is not involved in Trio-mediated ICAM-1 and VCAM-1 expression regulation. Trio expression was reduced in endothelial cells with shRNA (upper panel). Endothelial cells were treated with TNF-α or untreated and with proteosomal inhibitor MG-132 for 1 h, to avoid inactivation of the NF-κB pathway, or lysosomal inhibitor chloroquine. (D) Soluble ICAM-1 was measured in an ELISA, as described in SM Methods. HUVEC were treated for 20 h with TNF-α and the soluble extracellular domain of ICAM-1 was measured in the supernatant. The experiment was carried out twice. (E) Western blot shows protein controls for supplementary material Fig. S2D.
Fig. S4. MAP kinases are not involved in Trio-mediated TNF-α signaling. (A) HUVEC were incubated with inhibitors as indicated and treated for 30 minutes with TNF-α. All inhibitors showed specific inhibition of kinase activity after TNF-α. VE-cadherin shows equal protein loading (lower panel). (B) Jnk- and P38-inhibitor show reduced ICAM-1 and VCAM-1 expression upon 4 hours of TNF-α treatment in endothelial cells. No effect on the expression of ICAM-1 and VCAM-1 after TNF-α was measured for the Erk and MEK inhibitor. Endothelial cells were treated with 25 µM Jnk inhibitor (SB600125), 10 µM P38 inhibitor (SB203580), 2 µM ERK inhibitor (PD184352) or 50 µM MEK1 inhibitor (PD98059). (C) Jnk and P38 phosphorylation are not impaired upon TNF-α treatment of Trio-deficient endothelial cells. Trio expression was reduced in endothelial cells with shRNA and subsequently treated with TNF-α. Cell lysates were analyzed for phosphorylation levels of Jnk (p-Jnk; first panel). Graph shows quantification of Jnk phosphorylation. (D) This experiment was performed as described under B. No significant difference was measured between control vs. Trio-silenced cells for p-P38. Graph shows quantification. (E) Experiment performed as described under B. No difference was measured between control vs. Trio-silenced cells for ATF-2 phosphorylation.
Fig. S5. Trio does not control TNF-α-induced NF-κB pathway. (A) Trio expression was reduced in HUVEC with shTrio5. Phosphorylation (p-IκBα; first panel) and total levels of IκBα (second panel) were examined in HUVEC that were treated with TNF-α as indicated. Actin expression is shown for protein loading (lower panel). (B) Nuclear translocation of NF-κB-subunit p65 in HUVEC after stimulation with TNF-α for 30 minutes. Immunofluorescent images show p65 in red and F-actin in green. Merge/Dapi panel shows p65, F-actin and nuclei staining (in blue) together. Scale bars: 20 μm. (C) Phosphorylation of serine-536 of p65 (pS536-p65; upper panel) was examined in HUVEC that were treated with TNF-α as indicated. Lower panel shows p65 protein loading control. (D) NF-κB transactivation in HUVEC treated with TNF-α for 4 hours was measured as described in Materials and Methods. Data are means of three independent experiments ± SEM. All experiments shown are repeated at least three times.

Fig. S6. Translocation of NF-κB-subunit p50 to the nucleus. (A) Immunofluorescent images show NF-κB-subunit p50 in green and nuclei in violet (DAPI). Merge panel shows p50 and nuclei together. After 30 minutes of TNF-α treatment, p50 translocation to the nucleus (blue) was observed in shCTRL-treated cells. In shTrio-treated cells, p50 also translocated to the nucleus after TNF-α treatment. Scale bars: 20 μm. (B) HUVEC were treated with TNF-α overnight and assayed for apoptosis (annexin V-positive) by flow cytometry. To show TNF-α treatment, ICAM-1 (ICAM-1-405) and VCAM-1 (VCAM-1-PE) surface expression was measured using flow cytometry. (C) Quantification of apoptotic HUVEC after TNF-α treatment. No difference was measured under all tested conditions. (D) Quantification of cell cycle arrest showed no difference in cell cycle under all tested conditions.
Fig. S7. Trio depletion reduced VCAM-1 mRNA levels and reduced nucleus translocation of Ets-2. (A) VCAM-1 mRNA levels were measured as described in Materials and Methods. VCAM-1 mRNA levels are presented as a percentage of mRNA levels measured in cells at the starting time point (0 hour). Data are means of three independent experiments ± SEM. *P<0.05. (B) HUVEC were treated with shTrio or shCTRL, stimulated with TNF-α overnight or not and immunostained for Ets-2 (green), F-actin (red) and nuclei (blue). Scale bars: 50 μm. Zoom shows additionally VCAM-1 in white. Scale bars: 20 μm. (C) HUVEC were silenced for Trio (shTrio) and treated as described under C. GFP-TrioN or GFP-TrioD1 were expressed and shown in green. Ets-2 in red and nuclei in blue. White line indicates GFP-positive cell. Zoom shows magnification of red square. Images on the right show control conditions, i.e. translocation of Ets-2 to the nucleus upon 4 h TNF-α treatment. Scale bars: 20 μm. (D) miR-126 and miR-222 were measured after TNF-α stimulation in HUVEC and showed no difference in expression pattern in control versus Trio-depleted HUVEC. Experiments described are carried out at least three times.

Movie 1. TNF-α-induced alignment of shCTRL-treated HUVEC. HUVEC were treated with TNF-α for 17 hours during which every 15 minutes DIC images were recorded.

Movie 2. TNF-α-induced alignment of shTrio-treated HUVEC. HUVEC were treated with TNF-α for 17 hours during which every 15 minutes DIC images were recorded.