

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 NF κ B or MAP-kinase pathways,

but rather acts on the transcriptional level by increasing phosphorylation and nuclear translocation 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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Key words: Endothelium, GEF, GTPase, Inflammation, Signal transduction

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 IL1 β , 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) (Nourshargh 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- κ B 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; Tsoyi et al., 2010; Umetani et al., 2001). Although NF- κ B 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 N-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- κ B 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 μ g/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-1 β , 100 ng/ml IFN- γ or 0.5 μ g/ml lipopolysaccharide (LPS) for periods as indicated.

Luciferase reporter assays

Adenoviral 4 \times NF- κ B-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 \times 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 (Korchynskiy and ten Dijke, 2002).

Monocyte adhesion and TEM under flow

HUVECs were cultured in a fibronectin-coated Ibidi μ -slide V1^{0.4} (ibidi, München, Germany) for 2–3 days until confluency and stimulated overnight with TNF- α (10 ng/ml). Freshly isolated monocytes (0.4 \times 10⁶ 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 \times /0.45 M27 Zeiss-objective) equipped with a motorized stage. Transmigrated monocytes were distinguished from those 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 D-20 and clone H-120), DOCK180 (clone H-4), Vav2 (clone H-200), Sos1 (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 (cloneA) and p50 (clone E-10) were purchased from Tebu-Bio (Heerhugowaard, 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 from Millipore (Leiden, Netherlands). E-selectin (BBA-18) antibody and IFN- γ were purchased from R&D systems (Abingdon, UK). mAbs against Rac1 (clone 102

and CDC42 (clone 44) were purchased from BD biosciences (Breda, Netherlands). Antibodies against I κ B α , p-I κ B α , p(S536)-p65 (93H1), P38, p-P38, Jnk, p-Jnk, p-ATF2 were from Cell Signaling (Danvers, MA, USA). mAb RhoG 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 (JL-8) antibody, secondary goat anti-rabbit IR 680, goat anti-mouse IR 800 and donkey anti-goat IR 800 antibodies were purchased from Licor Westburg (Leusden, Netherlands). Texas Red- and alexa-633-conjugated phalloidin were from Invitrogen (Breda, Netherlands). TNF- α and IL-1 β were purchased from Peprotech EC (London, UK). NSC23766 was purchased from Merck (Nottingham, UK). ITX3 was purchased from Chembridge (San Diego, CA, USA).

Adenovirus production

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 (GAGATCGTCGACCTGGTTTGTGAACCGTCAGA) and JR4R (GAGATCGAATTCCTAGGCGTCATTGCTGGAGAC), JR22F and JR20R (GAGATCGAATTC CTATAAAAAATTGCGCTGGTTTTCT), JR23F (GAGATCCAATTGCTGGTTTGTGAACCGTCAGA) and JR24R (GAGATCGTCGACCTAGGCGTCATTGCTGGAGAC) respectively. The PCR product was cloned as a *SalI/EcoRI* (TrioD1/D2) or *MfeI/SalI* (TrioN) fragment into pENTR1A (Invitrogen) with *SalI/EcoRI* (TrioD1/D2) or *EcoRI/XhoI* (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 *PacI* (Westburg, Leusden)-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_871; Trio2, TRC_872; Trio3, TRC_873; Trio4, TRC_874; Trio5, TRC_10561), Rac1 (TRC_4872), RhoA (TRC_47711), 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 plasmids (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 \times for 20 minutes in TBST, and were subsequently incubated with horseradish peroxidase-coupled secondary antibodies (dilution 1:7000) in TBST for 1 hour at room temperature, followed by washing 3 \times 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 1:5000) in TBST for 1 hour at room temperature followed by washing 3 \times 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-casted 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% (w/v) BSA, 1 mM CaCl₂, 0.5 mM MgCl₂) and fixed in 4% (v/v) formaldehyde in PBS (supplemented with 1 mM CaCl₂, 0.5 mM MgCl₂) for 10 min. After fixation, the cells were permeabilized in PBS-T (PBS + 1% [v/v] Triton X-100 and 10% [v/v] glycerol) 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 $^{\circ}$ 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.,

1988) 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 μ m 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 mM 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 μ g of GST-ELMO (GST fusion protein containing the full-length RhoG effector ELMO) conjugated to glutathione-Sepharose beads (GE Healthcare, Zeist, The Netherlands) (van Buul et al., 2007; Wittchen and Burridge, 2008). GTP-bound Rac1 and CDC42 was isolated with biotinylated 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 30% (v/v) glycerol at -80°C .

ELISA for sICAM-1

Trio-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 (Diacclone, Sanquin Reagentia, 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 μ l of cDNA, 1 μ l of relevant primer combination at a concentration of 10 μ M, and 4 μ l of SYBR Green I mix in a total volume of 20 μ l. 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 cleft of the Taq polymerase was removed by preincubation for 10 minutes at 95 $^{\circ}\text{C}$; the template was amplified for 45 cycles, with annealing of the primers at 60 $^{\circ}\text{C}$. The fluorescence was measured at the end of each cycle at 72 $^{\circ}\text{C}$. 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 En-F (GAAAATATGTGGTTGGAGAGCTCATT); En-R (CCGAGTGAAGATCCCCTTTTA); ICAM-1-F (CTTCTCACCGT-GACTGGACTC); ICAM-1-R (TGGCTCCATGGAGATCTCTCTCCTCA); VCAM-1-F (TGAATCTAGGAAATTGAAAAAGGA); VCAM-1-R (CAGAGGGCCACTCAAATGAATC); E-selectin-F (TACCTGTGAAGCTCCCACTGAGTC); E-selectin-R (GTAACTTCAGTGTATCCCTCTAG).

Statistical analysis

Statistical comparisons between experimental groups were performed by the student *t*-test. A two-sided *P*-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 (Wójcicki-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-1 β 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

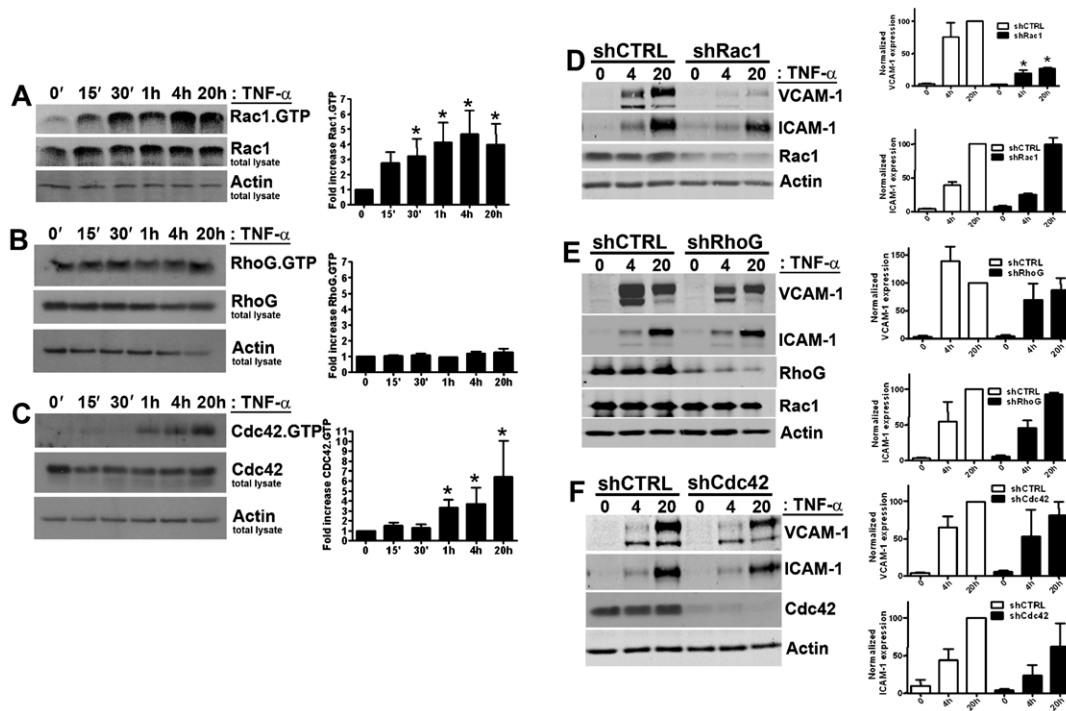


Fig. 1. The small GTPase Rac1 is activated by TNF- α and regulates the expression of VCAM-1 and ICAM-1. HUVEC were treated with TNF- α as indicated and a Rac1.GTP (A), RhoG.GTP (B) and CDC42.GTP (C) pull-down assay was performed as described in Online Methods. Second and third panels show protein loading. Graphs on the right show quantification. All experiments described above were repeated at least four times. Data are mean \pm SD. * P < 0.05. Rac1 (D), RhoG (E) and CDC42 (F) protein levels were reduced in HUVEC with lentivirally-delivered shRNA and TNF- α -induced VCAM-1 and ICAM-1 expression was examined. Actin is included for protein loading (lower panels). All experiments described above were repeated at least four times. Data are mean \pm SD. * P < 0.01.

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; Wójciak-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 by shTrio5 (Fig. 3C). These data were confirmed when shTrio3 was used (supplementary material Fig. S1D). In addition, also mRNA levels of ICAM-1, VCAM-1 and E-selectin were reduced upon TNF- α treatment when Trio was silenced (Fig. 3D).

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-1 β , 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.

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

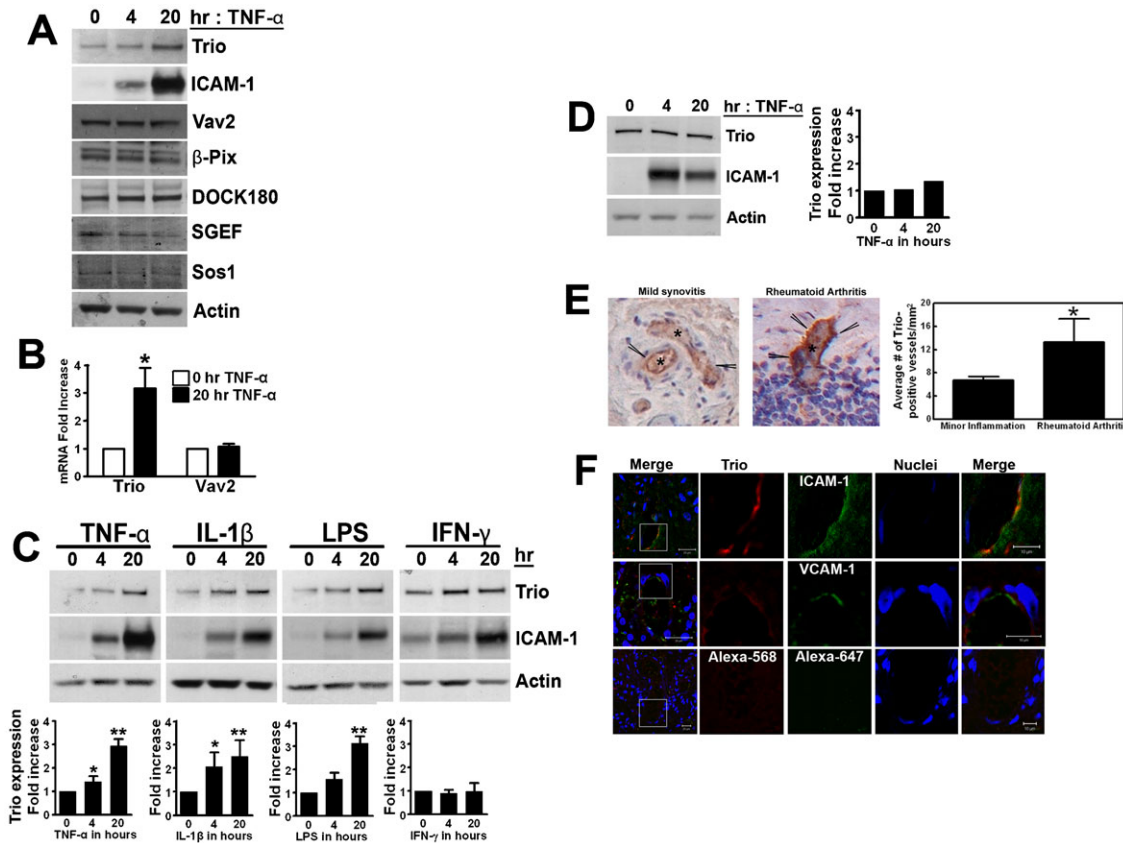


Fig. 2. Trio expression in endothelial cells and in RA patients. (A) Protein expression of GEFs Trio, Vav2, β -Pix, DOCK180, SGEF and Sos1 in TNF- α -treated HUVEC. (B) Trio and Vav2 mRNA levels examined by qRT-PCR in HUVEC after TNF- α . Data are means of four independent experiments \pm SEM. * P < 0.05. (C) Trio protein expression in HUVEC was examined following TNF- α , IL-1 β , LPS and IFN γ . Data are means of four independent experiments \pm SEM. * P < 0.05; ** P < 0.05 (D) Trio protein expression was examined in A549 lung epithelial cells after TNF- α treatment. Graph shows quantification of Trio expression. (E) Immuno-histochemical staining of Trio in synovial biopsies of inflamed joints of patients with mild synovitis and rheumatoid arthritis (RA) patients. Arrowheads indicate Trio-positive endothelial cells. Asterisk indicates blood vessel lumen. Graph shows quantification of Trio-positive vessels in mild synovitis and RA patients. In total, 863 vessels from mild synovitis and 2695 vessels from RA patients were analyzed. Data are mean \pm SD. * P < 0.01. (F) Immunofluorescent staining was carried out on samples from mild synovitis and RA patients. Overview shows merge of nuclei (blue), Trio (red) and ICAM-1 or VCAM-1 (green). Magnification shows localization of Trio and ICAM-1 or VCAM-1 at sites of vessels. Scale bars: 20 μ m and (zoom) 10 μ m.

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 regulated 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 (García-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

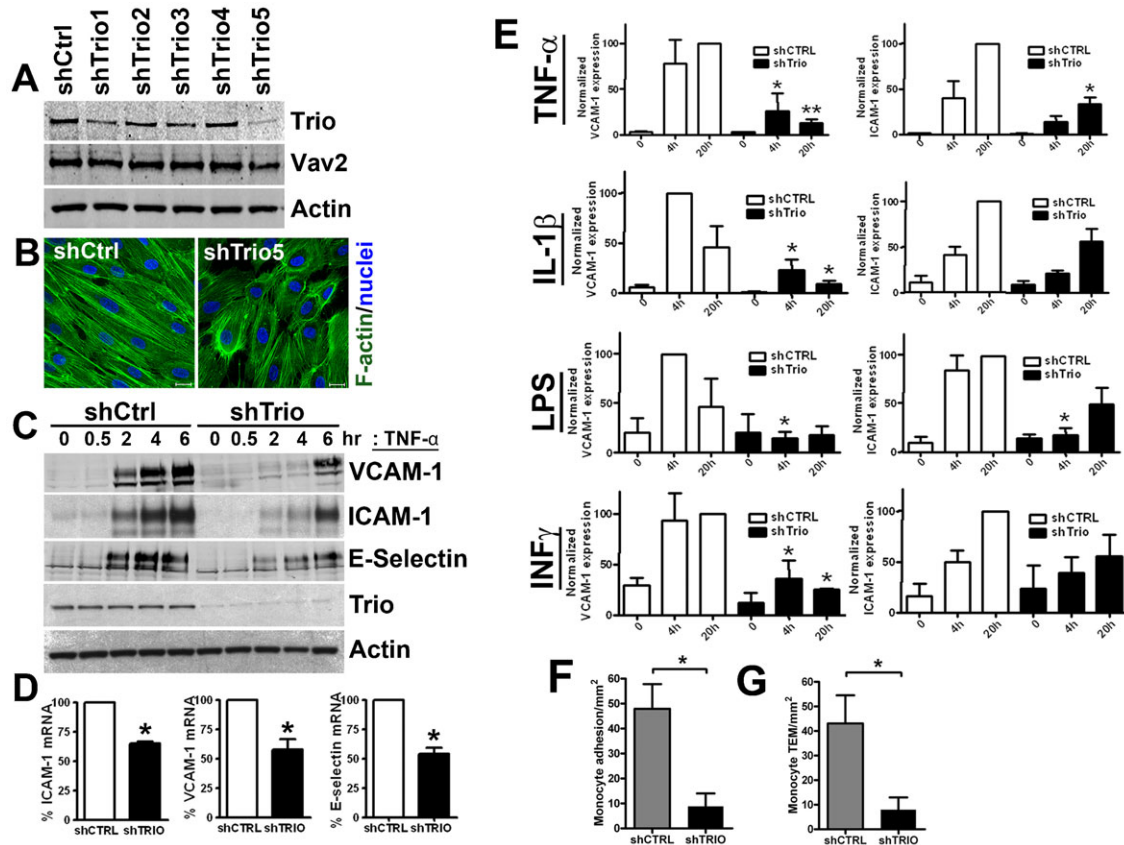


Fig. 3. Trio silencing reduces inflammatory cytokine-induced expression of ICAM-1 and VCAM-1 and reduces leukocyte adhesion and transendothelial migration. (A) Five lentivirally-delivered shRNA constructs targeting Trio expression were examined for their potential to downregulate Trio protein expression. The GEF Vav2 and actin are shown as loading controls. (B) The effect of Trio silencing on the F-actin cytoskeleton in HUVEC that were stimulated with TNF- α . Immunofluorescent images show actin in green and nuclei in blue. Scale bars: 20 μ m. (C) ICAM-1, VCAM-1 and E-selectin protein expression upon TNF- α time-course in shCTRL and shTrio-treated HUVEC. (D) ICAM-1, VCAM-1 and E-selectin mRNA levels by qRT-PCR in shCTRL and shTrio-HUVEC after TNF- α . Adhesion molecule mRNA levels in shTrio-treated HUVEC as a percentage of the adhesion molecule mRNA levels in controls. Data are means of three independent experiments \pm SEM. (E) Upregulation of VCAM-1 (left graphs) and ICAM-1 (right graphs) by TNF- α , IL-1 β , IFN- γ and LPS in shCTRL (open bars) and shTrio (closed bars)-treated HUVEC. Primary monocytes were perfused over HUVEC and adhesion (F) and TEM (G) was quantified as described in Materials and Methods. This experiment was carried out four times in duplicates. Data presented in all graphs are means \pm SEM. * P <0.01.

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 \sim 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

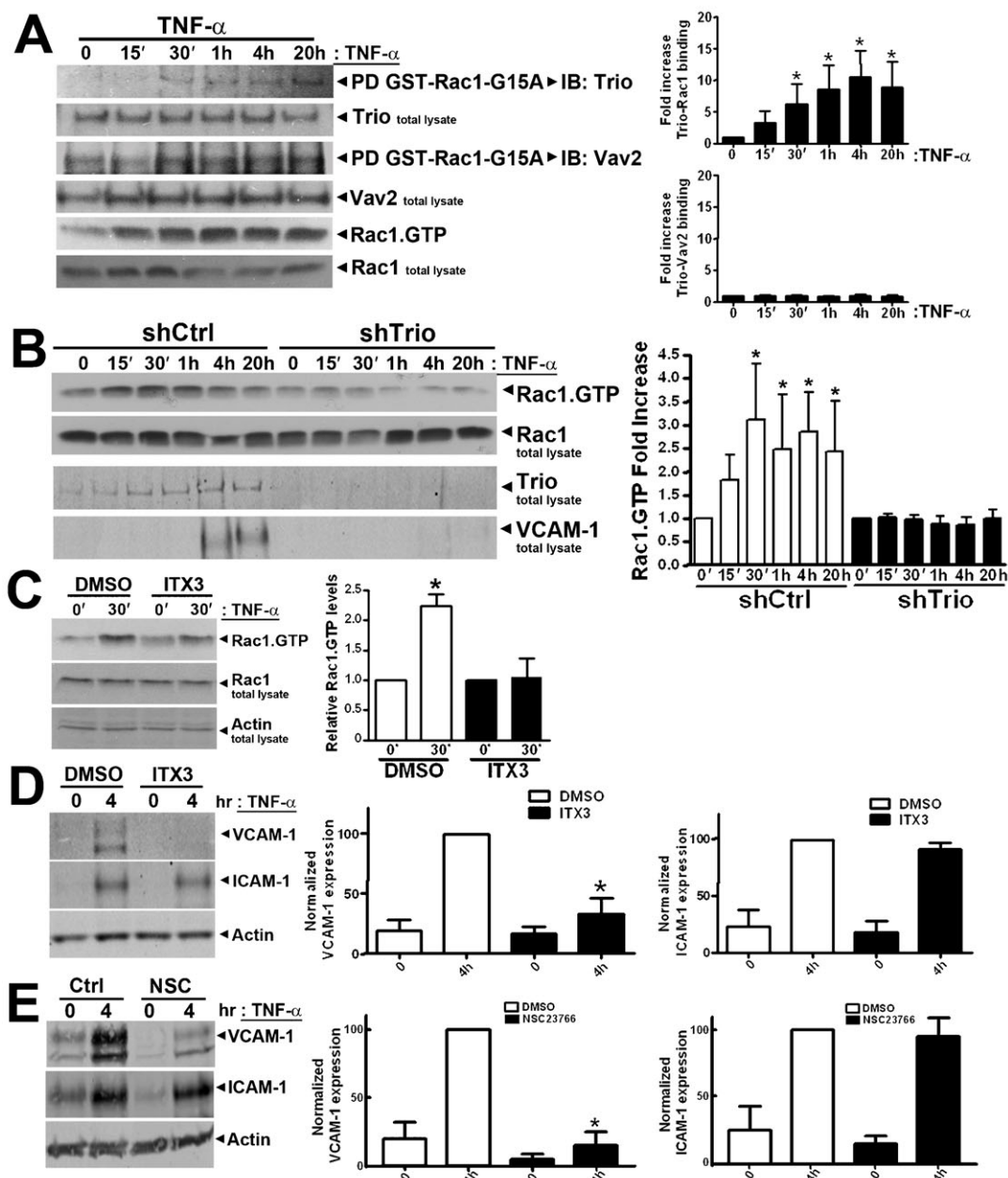


Fig. 4. Trio and Rac1 activity are required for TNF- α -induced ICAM-1 and VCAM-1 expression. (A) Trio and Vav2 activation following TNF- α treatment was examined by means of pull-down assays with GST-Rac1-G15A mutants as described in Methods in SM. Total Trio and Vav2 expression in the cell lysates 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 \pm 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 \pm SEM. * P < 0.05.

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.

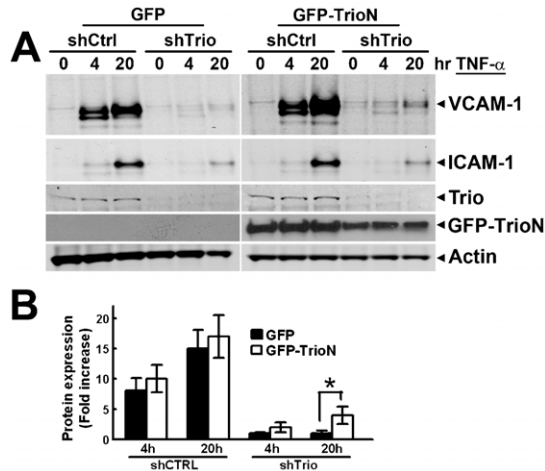


Fig. 5. TrioN partially rescues TNF- α -induced VCAM-1 expression in Trio-deficient cells. (A) Trio expression was reduced in endothelial cells with shTrio5, resulting in impaired VCAM-1 and to a minor extent ICAM-1 expression after TNF- α treatment. GFP-TrioN was expressed in HUVEC by adenoviral delivery, as described in Online Methods. Transduction with adenovirally-delivered GFP was used as a control. Actin expression is shown for protein loading. (B) Graphs show quantification of VCAM-1 expression rescue by TrioN in Trio-deficient cells. The experiment was carried out four times. Data are mean \pm SEM. * $P < 0.05$.

Trio silencing does not affect NF- κ B activation

Rac1 influences gene transcription also through mediating the activation of the transcription factor NF- κ B (Boyer et al., 2004; Sulciner et al., 1996). To examine whether NF- κ B activation is regulated by Trio, we first analyzed TNF- α -induced phosphorylation and degradation of the NF- κ B inhibitory protein I κ B α . In both control and Trio-silenced cells, I κ B α 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- κ B in response to TNF- α stimulation was not affected in Trio-silenced endothelial cells. Phosphorylation of serine-536 on the p65 subunit of NF- κ B 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- κ B transactivation, a specific NF- κ B reporter construct was used, which showed that silencing of Trio did not reduce TNF- α -induced NF- κ B transactivation in endothelial cells (supplementary material Fig. S5D). These results therefore demonstrate that Trio does not regulate TNF- α -induced NF- κ B activation in endothelium. To exclude the role of Trio in TNF- α -induced apoptosis, we measured phosphatidylserine (PS) exposure using AnnexinV. 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

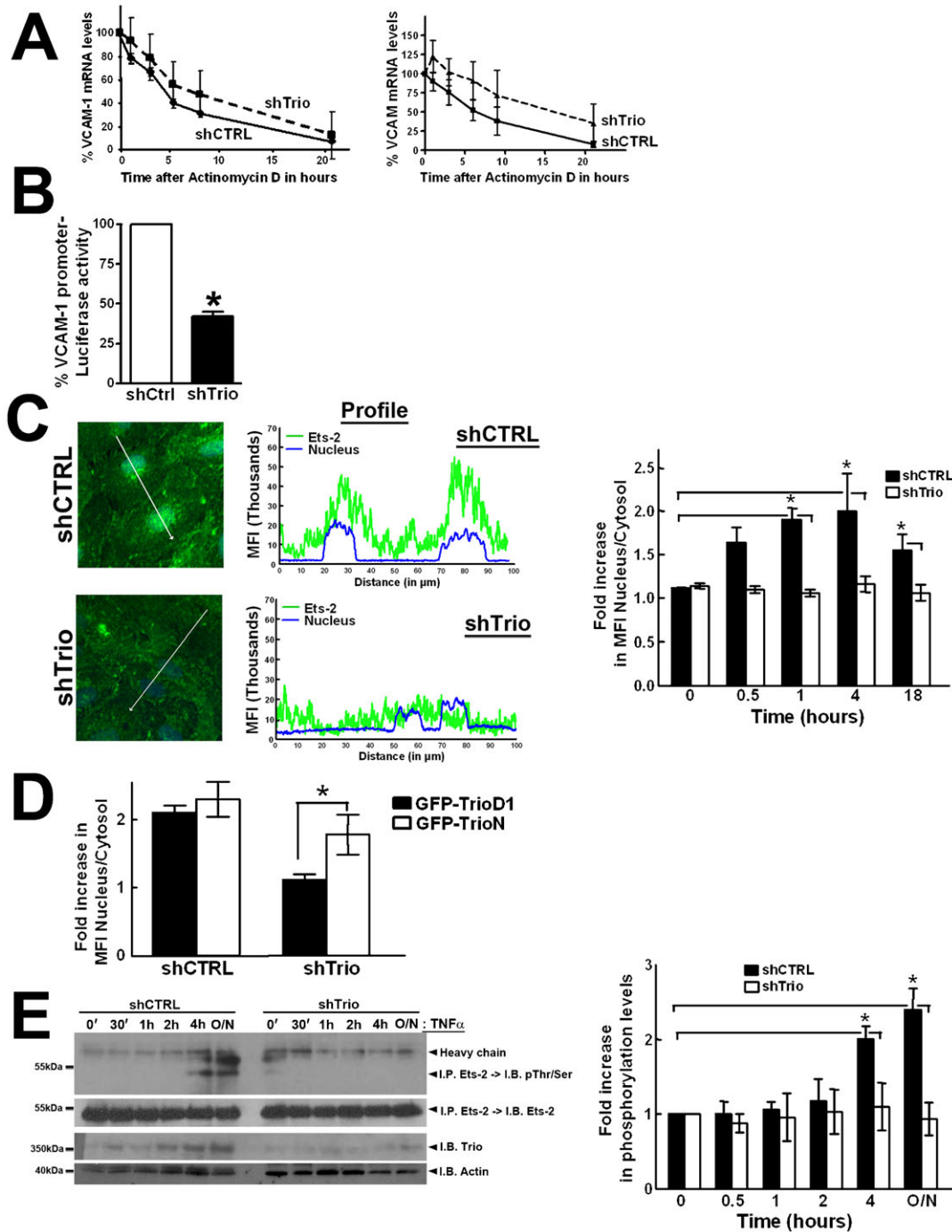


Fig. 6. Trio depletion inhibits VCAM-1 promoter activity and prevents Ets-2 activity and translocation. (A) VCAM-1 mRNA levels were measured as described in Materials and Methods. Left graph: untreated HUVEC; Right graph: TNF- α treated. Data are means of three independent experiments \pm SEM. (B) VCAM-1 promoter activity in HUVEC (TNF- α 20 h) was measured as described in Materials and Methods. Data are means of three independent experiments \pm SEM. * P <0.001. (C) HUVEC were treated as indicated and immunostained for Ets-2 in green and nuclei in blue. 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 \pm 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. Ets-2 nuclear translocation was determined by fold increase in nuclear vs. cytosolic fluorescent signal. Data are means of three independent experiments \pm SEM. * P <0.05. (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 \pm SEM. * P <0.01.

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

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- κ B and MAP-kinase pathways. In addition to MAPK/AP-1 and NF- κ B, 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- κ B 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-GSK3 β -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 GSK3 β 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 domain of Trio only is insufficient to restore Ets-2 nuclear translocation. For proper Ets-2 translocation, the N-terminal part of Trio is required. The N-terminus of Trio includes a SEC14 domain and several spectrin-repeats. Neubrand and colleagues reported that these domains bind specific proteins such as Kidins220/ARMS (Neubrand et al., 2010). They suggested that Kidins220/ARMS may target Trio to specific membrane sites, where it mediates its activity and induces local Rac1 activation.

In addition to that, Ets-2 activity is determined by its phosphorylation status, in particular through threonine-72 (Yang et al., 1996). Our data indicate that Trio is needed for TNF- α -induced Ets-2 phosphorylation. Harris and co-workers showed that miR-126 negatively regulates VCAM-1 expression (Harris et al., 2008). Interestingly, the same authors showed that Ets-1 and -2 are also responsible for the expression of miR-126 (Harris et al., 2010). Our data show that TNF- α reduced the levels of miR-126, in accordance to Harris et al. (Harris et al., 2008). However, Trio silencing did not affect these levels, indicating that Trio does not regulate miR-126 expression, but directly promotes Ets-2 phosphorylation.

In support of its role in mediating VCAM-1 upregulation, Trio expression is increased in endothelium stimulated for a prolonged period with inflammatory cytokines such as TNF- α and IL-1 β .

Unexpectedly, Trio was similarly required for adhesion molecule expression, induced by IL-1 β , 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-1 β and LPS stimulation in endothelium (Equils et al., 2004; Jefferies 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 NF κ B 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.

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