Cardiovascular Research (2010) **86**, 283–292
doi:10.1093/cvr/cvq050

ILK mediates LPS-induced vascular adhesion receptor expression and subsequent leucocyte trans-endothelial migration†

Sonsoles Hortelano[1‡], Raquel López-Fontal[1‡], Paqui G. Través[2], Natividad Villa[2], Carsten Grashoff[3], Lisardo Boscá[2], and Alfonso Luque[1*]

[1]Department of Regenerative Cardiology, Fundación Centro Nacional de Investigaciones Cardiovasculares Carlos III, Melchor Fernandez Almagro 3, E-28029 Madrid, Spain; [2]Instituto de Investigaciones Biomédicas Alberto Sols (CSIC-UAM), Madrid, Spain; and [3]Robert M. Berne Cardiovascular Research Center, University of Virginia, Charlottesville, VA 22908, USA

Received 8 June 2009; revised 15 January 2010; accepted 8 February 2010; online publish-ahead-of-print 17 February 2010

Time for primary review: 31 days

Aims The inflammatory response to injurious agents is tightly regulated to avoid adverse consequences of inappropriate leucocyte accumulation or failed resolution. Lipopolysaccharide (LPS)-activated endothelium recruits leucocytes to the inflamed tissue through controlled expression of membrane-associated adhesion molecules. LPS responses in macrophages are known to be regulated by integrin-linked kinase (ILK); in this study, we investigated the role of ILK in the regulation of the LPS-elicited inflammatory response in endothelium.

Methods and results This study was performed on immortalized mouse endothelial cells (EC) isolated from lung and coronary vasculature. Cells were thoroughly characterized and the role of ILK in the regulation of the LPS response was investigated by suppressing ILK expression using siRNA and shRNA technologies. Phenotypic and functional analyses confirmed that the immortalized cells behaved as true EC. LPS induced the expression of the inflammatory genes E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). ILK knockdown impaired LPS-mediated endothelial activation by preventing the induction of ICAM-1 and VCAM-1. Blockade of the LPS-induced response inhibited the inflammatory-related processes of firm adhesion and trans-endothelial migration of leucocytes.

Conclusion ILK is involved in the expression of cell adhesion molecules by EC activated with the inflammatory stimulus LPS. This reduced expression modulates leucocyte adhesion to the endothelium and the extravasation process. This finding suggests ILK as a potential anti-inflammatory target for the development of vascular-specific treatments for inflammation-related diseases.

Keywords ILK • Endothelial cells • LPS • Adhesion molecules • Inflammation

1. Introduction

The development of new treatments for cardiovascular diseases requires the identification of molecules that play specific roles in these pathologies. The endothelium forms a barrier between the vessel lumen and the surrounding tissue and is critical for host immune responses during inflammation and infection. When exposed to inflammatory mediators, such as tumour necrosis factor-α (TNF-α) or lipopolysaccharide (LPS), the quiescent endothelium becomes activated and expresses additional pro-inflammatory factors and cell-specific adhesion molecules that enable the extravasation of circulating leucocytes to sites of inflammation. The initial step involves recruitment of leucocytes to the endothelial surface through the binding of leucocyte-expressed L-selectin and endothelium-expressed E- and P-selectins to carbohydrate moieties on the contacting cell. Firm adhesion is mediated by intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on the endothelium, which bind β2 and β1 integrins on leucocytes, respectively.[1–4] Excessive leucocyte accumulation contributes to the development of inflammatory disorders, whereas a deficiency in leucocyte trafficking results in host immunosuppression.

† Work was performed at: Department of Regenerative Cardiology, Fundación Centro Nacional de Investigaciones Cardiovasculares Carlos III, Melchor Fernandez Almagro 3, E-28029 Madrid, Spain.

‡ These authors have equally contributed to this work.

* Corresponding author. Tel: +34 91 453 12 00; fax: +34 91 453 12 65, Email: aluque@cnic.es

An intermediary component of LPS signalling is integrin-linked kinase (ILK), which mediates LPS-induced NOS-2 and COX-2 expression and NO production in macrophages.[5] ILK is a widely distributed pseudokinase located in specialized signalling complexes called focal contacts, and regulates signalling from integrins and growth factor receptors. The ILK carboxyl terminal region contains a kinase-like domain, the integrin-binding domain, and motifs for binding to the actin cytoskeleton via alpha and beta parvin adaptor proteins. The ILK amino terminus binds to PINCH, which interacts with the adapter protein Nck2. Nck2 recruits actin regulatory proteins and can bind tyrosine phosphorylated residues of activated growth factor receptors.[6] The formation of the ILK-PINCH-Parvin ternary complex is necessary for ILK function and for its recruitment to focal contacts.[7]

The ability of ILK to integrate signals from integrins and growth factor receptors suggests an involvement in many biological situations. Activation of ILK induces cell survival and cell proliferation,[8,9] and ILK closely regulates capillary formation and the survival of endothelial and progenitor cells.[10,11] The role of ILK in pathological processes has been confirmed in several *in vivo* models, including cardiac repair,[12] cardiomyopathy,[13,14] tumour angiogenesis,[15] and skeletal muscle regeneration.[16] ILK knockout models, both germline[17] and conditional,[16,18–20] have revealed essential roles in development and adult physiology. Endothelial ILK appears to play a specific role in the development of the coronary vasculature (A. Luque *et al.*, in preparation) confirming the variability of endothelial function between different organs.[21]

In this study, we investigated the role of ILK in the regulation of LPS-elicited inflammatory responses in endothelial cells (EC). Using immortalized EC lines of lung or coronary origin, we show that ILK is required for LPS-induced endothelial expression of ICAM-1 and VCAM-1. Moreover, LPS-stimulated EC lacking ILK show reduced adhesion of primary lymphocytes and monocytic lines and impaired trans-endothelial migration of lymphocytes. These results propose ILK as a potential target for the design of vascular-specific treatments for bacterial associated inflammation.

2. Methods

2.1 Animals and cell isolation

EC were isolated and maintained as described[22] from whole lungs and coronary vasculature of C57/BL6 wild-type animals and the genetically modified ILK lox/lox strain.[17,18] Spleen lymphocytes were isolated from C57/BL6 wild-type animals (see Supplementary material online, Methods). The study was approved by 'Comité de Bioética y Bienestar Animal. Instituto de Salud Carlos III' conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2 Positive endothelial selection with antibody-bound magnetic beads

EC were isolated by positive selection using PECAM-1 or ICAM-2 antibodies bound to magnetic beads (Dynabeads®, Invitrogen) as described previously.[22] Cells were further purified by FACS (MoFlo, Beckman Coulter) using standard procedures.

2.3 Immortalization of EC

EC lines were established using a modified version from previous reports.[22,23] Subconfluent primary cultures were incubated in the presence of polybrene (8 μg/mL, Sigma) with supernatant from packaging cells producing recombinant retroviruses that transduced polyoma middle T antigen (virus concentration about 10–200 pfu/cell). Cells were subjected to a second round of infection. Non-infected cells died within few passages. *Table 1* summarizes specific procedures performed.

2.4 Flow cytometry

Flow cytometry was performed following standard protocols (see Supplementary material online, Methods). As required, EC transfected with siRNAs or shRNA were incubated with TNF-α (PeproTech), Poly I:C (InvivoGen), or LPS (E. coli 0111:B4 LPS, InvivoGen). Concentrations used are specified for each figure.

2.5 Immunofluorescence

Cell immunostaining was performed as described[24] and analysed with a Leica TCS SP5 Multiphoton Confocal microscope.

2.6 LDL uptake

Alexa Fluor 488 acetylated low-density lipoprotein (Ac-LDL, L-23380, Molecular Probes) was incubated with EC following manufacturer's recommendation and incorporation was evaluated by flow cytometry.

2.7 Western blotting

Endothelial activation and protein expression were monitored by standard immunoblot analysis of downstream phosphorylated targets (see Supplementary material online, *Table S1*).[24] Bands were quantified with Quantity One software (BioRad).

2.8 Wound healing assay

Denuded areas ('wounds') were made in endothelial monolayers by scraping with a plastic pipette tip. Plates were then washed and cells stimulated with VEGF (500 ng/mL, Peprotech). Cell migration into the denudated area was monitored by time-lapse photomicroscopy and quantified with AxioVisionLE 4.1 software.

Table 1 Timetable of procedures for the generation of immortalized endothelial lines

MCoEC-25	MLEC-04	MCoEC-31	MLEC-12
Mouse strain: C57/Bl6	C57/Bl6	C57/Bl6	ILKlox/lox-C57/Bl6
Process date: 25/04/2006	04/05/2006	31/05/2006	12/07/2006
03/05/06, ICAM-2 selection	09/05/06, PECAM-1 selection	01/06/06, first virus-infection	13/07/06, first virus-infection
08/05/06, first virus-infection	12/05/06, first virus infection	02/06/06, PECAM-1 select	17/07/06, PECAM-1 select
12/05/06, second virus-infection	16/05/06, second virus-infection	05/06/06, second virus-infection	24/07/06, second virus-infection
19/05/06, PECAM-1 selection	30/05/06, PECAM-1 FACS-sorting	14/07/06, PECAM-1 FACS-sorting	22/08/06, first PECAM-1 FACS-sorting
30/06/06, PECAM-1 FACS-sorting			03/10/06, second PECAM-1FACS-sorting

2.9 ILK silencing

ILK expression was suppressed by two rounds of transient transfection with ILK siRNA (Ambion) using Lipofectamine 2000 (Invitrogen). Alternatively, ILK-silenced EC were generated by the transfection with shRNA plasmids followed by neomycin selection of positively transfected cells (KM05291N, SABiosciences).

2.10 Real-time pcr

Transcript expression of adhesion receptors was measured by the SyBr Green method with an ABI Prism 7900 HT Sequence Detection system (see Supplementary material online, Methods).[25]

2.11 Leucocyte-endothelium co-adhesion assay

Wild-type or ILK-deficient endothelial monolayers in 96 well plates were stimulated with TNF-α, Poly I:C, or LPS for 6 h. EC were then washed several times and CFSE-(10 μM/20 min C1157, Molecular probes)-labelled mouse monocyte/macrophage J774 cells, human T cell line Jurkat or spleen lymphocytes were added (125.000–250.000 cells/well). After 1 h, wells were washed and fluorescently-labelled cells attached were measured after detergent treatment with a spectrofluorometer plate reader (485 nm/535 nm, Fluoroskan Ascent, Thermo Labsystems). Alternatively, wells were fixed in 3% paraformaldehyde and evaluated by fluorescence microscopy. Each condition was performed in triplicate and adhesion was calculated as a percentage of the adhesion to fibronectin (10 μg/mL, Sigma). Identification of adhesion molecules involved was assessed by including blocking antibodies (see Supplementary material online, *Table S1*).

2.12 Trans-endothelial migration

shRNA-ILK or shRNA-control transfected EC were cultured to confluency on gelatin-coated polycarbonate filter inserts (5 μm pores, Corning) and either left unstimulated or stimulated with LPS or TNF-α for 6 h. After washing the wells, $3-5 \times 10^5$ CFSE-labelled splenocytes in RPMI media/0.5% BSA were placed in the upper compartment and allowed to migrate for 2 h towards SDF-1 (50 ng/mL, PeproTech) placed in the lower chamber. Transmigrated lymphocytes were quantified by microscopy. Migrated splenocytes were represented as the percentage of the total cellular input. Assays were run in triplicate.

2.13 Statistical analysis

All data are means ± standard deviations of three independent experiments. Statistical significance was estimated by Student's *t*-test for unpaired observations, with $P < 0.05$ considered significant. Analysis was performed with the Prism 3 program (GraphPad Software).

3. Results

3.1 Generation and phenotypic description of novel immortalized mouse EC

To examine the role of ILK in inflammatory response of different vascular beds (A. Luque *et al.*, in preparation), we isolated EC from the mouse lung and coronary vasculatures. Since the number of EC obtained, especially from coronary vessels, was insufficient for the experimentation proposed, we generated immortalized cultures to provide infinite biological material. Procedures for generating cell lines included several rounds of endothelial positive selection and two rounds of infection with the polyoma virus middle T (summarized in *Table 1*).

Four cell lines were established: two mouse lung endothelial cell (MLEC) cultures, MLEC-04 and MLEC-12; and two mouse coronary endothelial cell (MCoEC) cultures, MCoEC-25 and MCoEC-31. The identification numbers refer to the isolation date. MLEC-12 cells were isolated from ILK lox/lox mice,[17,18] although the other lines were obtained from wild-type C57/BL6 mice.

Light field microscopy revealed the cobblestone monolayer morphology typical of endothelial cultures (*Figure 1A*). In all immortalized cell lines, flow cytometry detected expression patterns of the endothelial markers PECAM-1, ICAM-1, and ICAM-2 similar to those

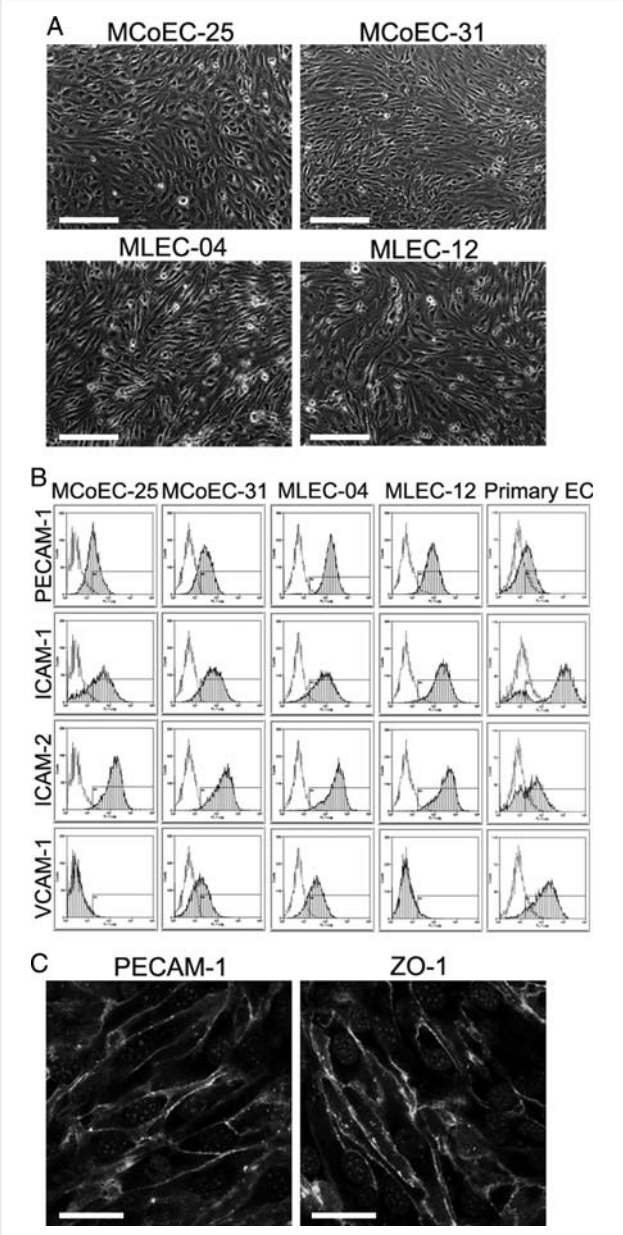


Figure 1 Generation and phenotypic description of novel immortalized mouse EC. (*A*) Light field microscopy showing typical cobblestone-like morphology of the four immortalized endothelial lines: MCoEC-25, MCoEC-31, MLEC-04, and MLEC-12. Scale bar equals 50 μm. (*B*) Flow cytometry analysis of the endothelial surface markers PECAM-1, ICAM-1, ICAM-2, and VCAM-1 on the immortalized EC lines and primary MLEC. Dark profiles corresponded to expression of the selected antigen, isotype matched negative controls are shown in white. (*C*) PECAM-1 and ZO-1 immunofluorescence staining of confluent MLEC-04. Scale bar equals 10 μm.

seen in primary MLEC. In contrast, only MCoEC-31 and MLEC-04 resembled primary EC regarding VCAM-1 levels (*Figure 1B*). Unless otherwise stated, data shown in subsequent experiments for an individual cell line are representative of similar results obtained with the other immortalized EC lines.

Endothelial polarity and selective permeability depend on specific intercellular connections.[26] Immunocytochemical analysis of confluent MLEC-04 confirmed localization of the endothelial homophilic adhesion protein PECAM-1 at intercellular contacts and of the tight junction molecule ZO-1 at the cell–cell boundaries along lateral cell borders (*Figure 1C*).

3.2 Functional characterization of the mouse endothelial lines

The usefulness of the immortalized cell lines as *in vitro* models of endothelial processes was evaluated through a series of functional assays. EC lines labelled with Alexa Fluor 488 Ac-LDL were brilliantly fluorescent comparable to primary MLEC, indicating characteristic high endothelial Ac-LDL uptake and metabolism;[27] in contrast, fluorescence intensity of a murine keratinocyte cell line (PB) was only slightly above background (*Figure 2A*). The four endothelial lines also express the receptor tyrosine kinase VEGFR2 (*Figures 2B* and *3A*), which mediates most of the angiogenic actions of VEGF.[28] Stimulation of EC with VEGF induced VEGFR2 autophosphorylation, detected by immunoblotting with the anti-phosphotyrosine antibody PY20 followed by anti-VEGFR2 antibody. Sequential membrane reprobing with phospho-specific antibodies detected VEGF-induced phosphorylation of Akt, GSK-3β, and ERK (*Figure 2B*). VEGF-stimulated endothelial migration was evaluated in wound healing assays.[29] Increased migration of EC in the presence of VEGF is evident from the pronounced closure of the denuded region at 24 h compared with control (*Figure 2C*).

The induced expression of adhesion molecules—essential for leucocyte recruitment in response to inflammatory stimulation—was tested by activating quiescent EC with LPS or TNF-α. Upon activation, immortalized cultures overexpressed E-selectin, VCAM-1, and ICAM-1 similarly as occurred in primary MLEC. As expected, ICAM-2 and PECAM-1 expression was invariable under these conditions (*Figure 2D*). Together these results confirm the immortalized cultures as true EC, thus characterizing a useful resource for vascular research.

3.3 Expression of the ILK signalosome by EC lines and ILK down-regulation

The four endothelial lines expressed similar levels of ILK protein and its adaptor proteins PINCH-1 and α- and β-parvin (*Figure 3A*), suggesting normal signalling through ILK in these cell types.

The function of ILK in endothelial biology was evaluated by knockdown approaches. ILK expression in all four EC lines was suppressed with siRNA reagents. Approximately 65–75% of ILK protein was eliminated after two rounds of transfection (*Figure 3B*). In addition, we used shRNA reagents to generate stable ILK-deficient lines. After shRNA-EC transfection and neomycin selection, ILK expression was reduced to around 25% compared with the shRNA control (*Figure 3C*). In another approach, MLEC-12 derived from ILK lox/lox animals were positively tested for ILK elimination after cre enzyme expression (Supplementary material online, *Figure S1*). However,

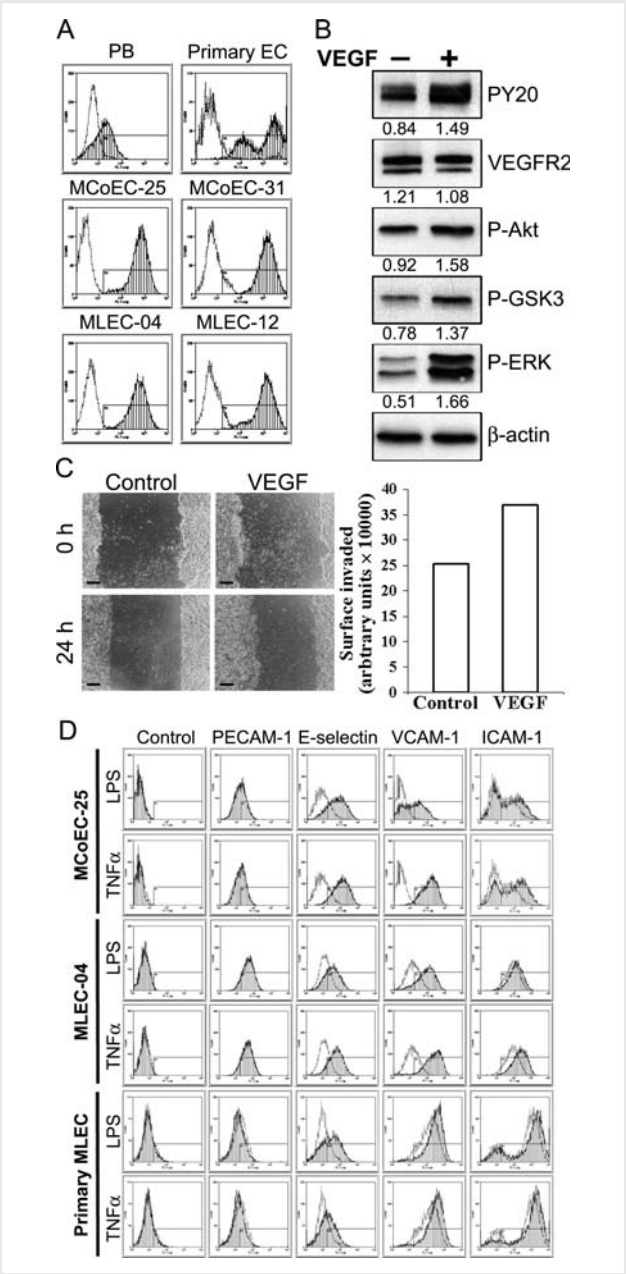


Figure 2 Functional characterization of mouse endothelial lines. (*A*) AcLDL uptake. EC were incubated with Alexa Fluor 488-AcLDL (dark histograms) and examined by flow cytometry. PB cells were used as control. (*B*) VEGF signalling. Resting (−) or VEGF-stimulated (+) MLEC-12 were analysed by western blot with sequential antibody stripping and reprobing of the same membrane. Protein levels are quantified below each blot as a fold-increase relative to β-actin densities. (*C*) Wound healing assays. Left-hand panels show light field images of scraped VEGF-stimulated or control MCoEC-31 monolayers at 0 and 24 h. Scale bar equals 100 μm. Quantification (right panel) shows surface invaded = a.u.(0 h) − a.u.(24 h). (*D*) Inflammatory response of EC. MCoEC-25, MLEC-04, and primary MLEC were left untreated (white histograms) or stimulated (dark histograms) with LPS (500 ng/mL) or TNF-α (15 ng/mL) for 6 h and expression of surface adhesion molecules was assessed by flow cytometry. 'Control' shows isotype matched negative controls.

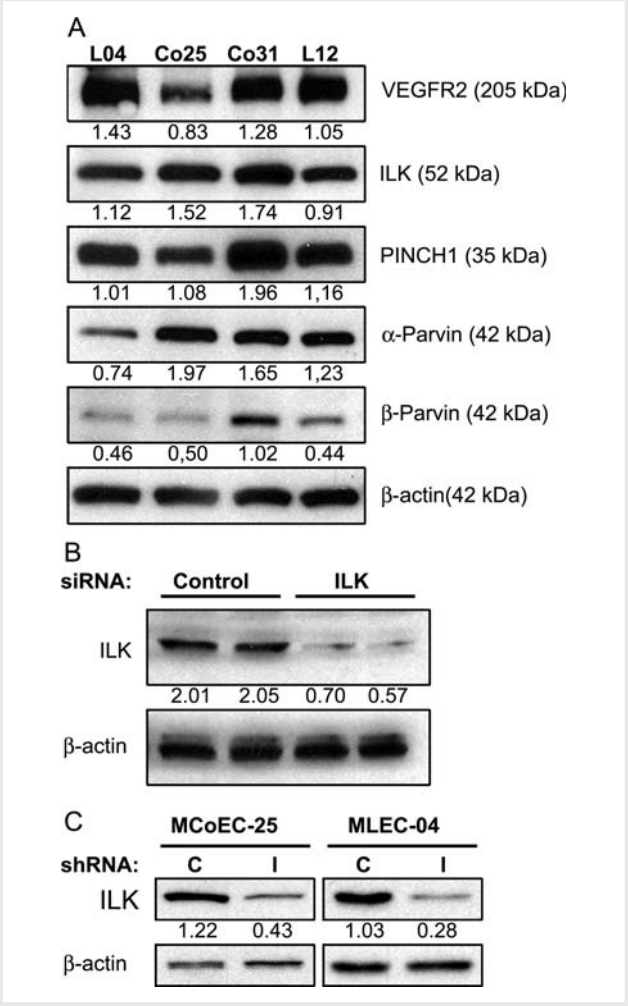


Figure 3 Expression of the ILK signalosome by EC lines and ILK down-regulation approaches. (*A*) MLEC-04 (L04), MCoEC-25 (Co25), MCoEC-31 (Co31), and MLEC-12 (L12) express ILK and the adaptor proteins PINCH1 and α- and β-parvins as shown western blot of whole cell lysates. (*B*) Down-regulation of ILK by siRNA. MCoEC-25 were subjected to two rounds of transfection with ILK or control siRNA, and ILK levels were evaluated by western blot. (*C*) MCoEC-25 and MLEC-04 transfected with ILK-shRNA (I) reduced ILK protein expression compared with control-shRNA (*C*). In all cases β-actin was assessed as loading control and the protein levels are quantified below each blot as a fold-increase relative to the correspondent β-actin densities.

since siRNA technology is applicable to all four EC lines and shRNA EC show similar reductions in ILK expression, siRNA/shRNA approaches were used in all subsequent experiments.

3.4 ILK regulates LPS-induced transcription of ICAM-1 and VCAM-1 in EC

To test the role of endothelial ILK in LPS-induced responses, we first analysed the effect of ILK knockdown on the expression of inflammation-related genes. LPS stimulation of MLEC-04 increased the transcription rate of E-selectin by three-fold, and ICAM-1 and VCAM-1 by about 10- and 17-fold. Transfection with ILK-siRNA weakened LPS-induced ICAM-1 and VCAM-1 expression to 60 and 20% of the control values but did not affect LPS-induced expression of E-selectin. ICAM2 was examined as a non-varying endothelial marker (*Figure 4A*). We next evaluated ILK function in response to different pro-inflammatory conditions. Increasing amounts of LPS (10–500 ng/mL) induced ICAM-1 expression in a concentration-dependent manner, and suppression of this expression in ILK-siRNA-transfected MLEC-04 was significant at higher LPS concentrations (100 and 500 ng/mL). In contrast, ILK knockdown did not significantly interfere with EC expression of ICAM-1 mRNA induced by TNF-α or Poly I:C (*Figure 4B* and see Supplementary material online, *Figure S2*).

3.5 LPS-induced VCAM-1 and ICAM-1 protein expression is partially inhibited in ILK-deficient EC

Resting MCoEC-25 cells do not express VCAM-1 protein, shown by flow cytometry (*Figure 1B*) and western blot ('resting' in *Figure 5A*). LPS induced VCAM-1 protein expression on the cell surface (*Figure 2D*), coinciding with increased amounts of VCAM-1 protein in total cell lysates ('LPS-C' in *Figure 5A*). ILK knockdown significantly inhibited the LPS-induced increase in VCAM-1 protein expression ('LPS-I', *Figure 5A*, top panel) to about 50% of that in LPS-stimulated controls ('LPS-C'), as shown by densitometry analysis ('L-ILK' compared with 'L-4611', *Figure 5A*, lower panel).

Non-stimulated MCoEC-25 cells express ICAM-1 (*Figures 1B* and *5B* 'resting'). This expression was increased by LPS (*Figures 2D* and *5B* 'LPS-C'), a response partially inhibited in ILK knockdown cells (*Figure 5B* 'LPS-I'); densitometry analysis revealed ICAM-1 protein expression in LPS-treated knocked-down cells to be reduced ~35% of the value in LPS-stimulated controls (*Figure 5B*, lower panel).

Further flow cytometry analysis of MCoEC-25 (*Figure 5C* and *D*) and MLEC-04 (data not shown) confirmed that the reduced VCAM-1 and ICAM-1 expression in LPS-treated ILK-deficient EC correlates with lower cell surface localization. In contrast, VCAM-1 and ICAM-1 over-expression induced by TNF-α and Poly I:C was not affected by the absence of endothelial ILK. ICAM-2 expression was invariable under all conditions (data not shown).

3.6 Down-regulation of endothelial ILK reduces the adhesion of leucocytes to LPS-activated endothelium

ILK-mediated regulation of LPS-induced cell adhesion molecules suggests a regulatory action for ILK on leucocyte adhesion to endothelium, event that was investigated with functional co-adhesion experiments. CFSE labelled J774 cells were added to resting or stimulated siRNA transfected EC and cell attachment was evaluated by fluorescence microscopy (*Figure 6A*, left panel). LPS and TNF-α treatments increased monocyte adhesion to MLEC-04 monolayers. Although endothelial ILK knockdown did not affect co-adhesion under TNF-α stimulation or resting conditions, it severely inhibited LPS (500 ng/mL)-stimulated adhesion. As it was expected, no ILK regulation occurred at 25 ng/mL because very low EC activation was achieved in this condition (*Figure 4B* and data not shown). Parallel experiments were quantified by spectrofluorometry of lysed cells (*Figure 6A*, right panel).

To identify the molecule pairs involved in J774-endothelium binding, first we tested adhesion molecules expressed by the monocytic line. J774 cells express CD11a, CD11b, and CD11c associated with integrin β2, which are receptors for ICAM-1, whereas CD49d

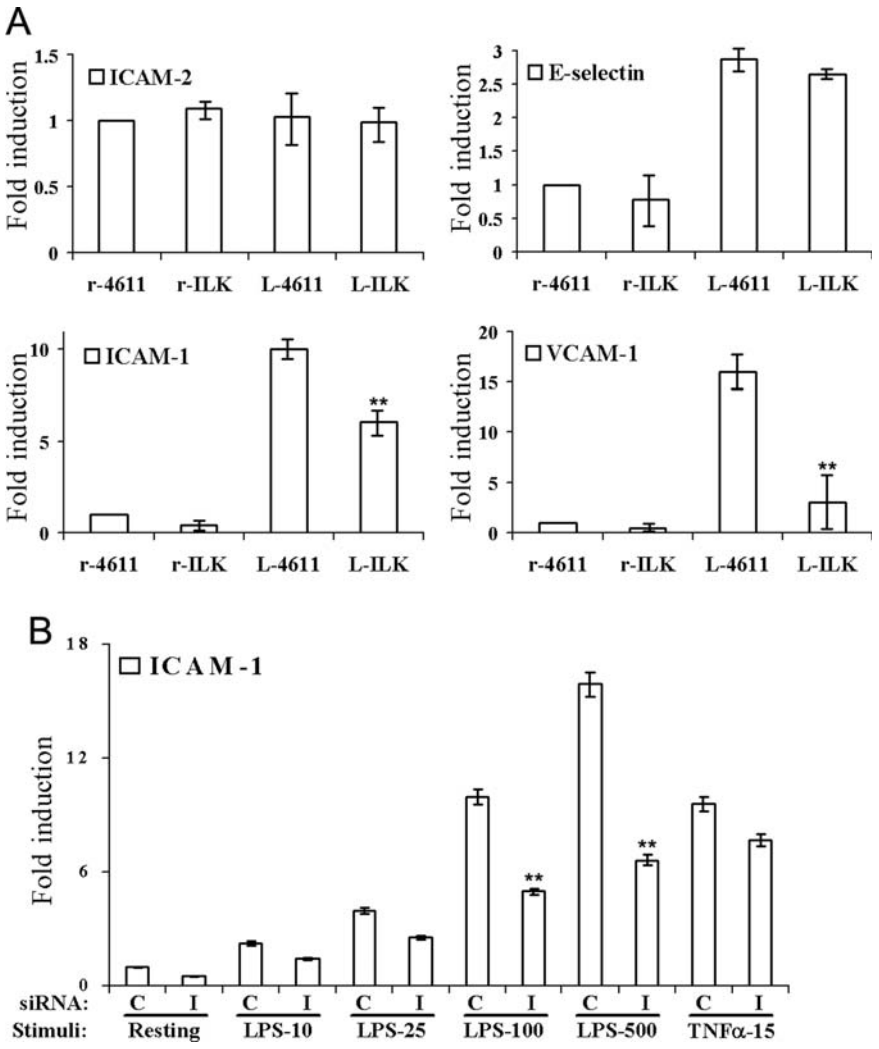


Figure 4 ILK regulates LPS-induced transcription of ICAM-1 and VCAM-1 in EC. (*A*) MLEC-04 transfected with ILK siRNA or control siRNA (4611) were stimulated with LPS (500 ng/mL) for 6 h and expression of selected genes was evaluated by Real-Time PCR. r, resting cells; L, LPS-stimulated cells. (*B*) MLEC-04 transfected with ILK (I) or control (C) siRNAs were incubated with indicated stimuli for 6 h and expression of ICAM-1 gene was evaluated as before. Data are means ± SEM of three independent experiments conducted in triplicate. $^{**}P < 0.01$ with respect to the control condition.

associated with β1 integrin, the receptor for VCAM-1. CD45 was examined as leucocytic positive control (*Figure 6B*). Next we conducted co-adhesion experiments in the presence of blocking antibodies to adhesion components (see manufacturer's datasheet, Supplementary material online, *Table S1*). Anti-β2 integrin antibody partially reduced adhesion of J774 cells to resting MLEC-04, and severely reduced the adhesion to LPS-activated endothelium (*Figure 6C*). Thus the main adhesive complex in this experimental setting appears to be endothelial ICAM-1, upregulated in response to LPS, and β2 integrins on J774 cells.

To mimic a more physiological situation, next we used mouse spleen lymphocytes. Adhesion of CFSE-splenocytes to MCoEC-25 (shRNA-Con) was increased after LPS, Poly I:C, or TNF-α endothelial activation. As before, endothelial ILK knockdown (shRNA-ILK) did not affect co-adhesion involving resting EC or stimulated with Poly I:C or TNF-α, but significantly inhibited LPS-stimulated adhesion (*Figure 6D*). Similar results were obtained with Jurkat cells (see Supplementary material online, *Figure S3*).

3.7 Endothelial ILK regulates extravasation of lymphocytes through LPS-activated endothelium

An effective inflammatory response involves leucocytes attachment to activated endothelium and trans-endothelial migration to the inflamed focus. We therefore next evaluated the role of endothelial ILK in extravasation in transwell migration assays.

shRNA transfected MCoEC-25 (control and ILK) were cultured to confluence on polycarbonate filter and either left unstimulated or stimulated with LPS or TNF-α. After washing the wells, CFSE-splenocytes were placed in the upper compartment and allowed to migrate towards SDF-1 gradient. LPS and TNF-α treatments increased lymphocyte trans-endothelial migration, and

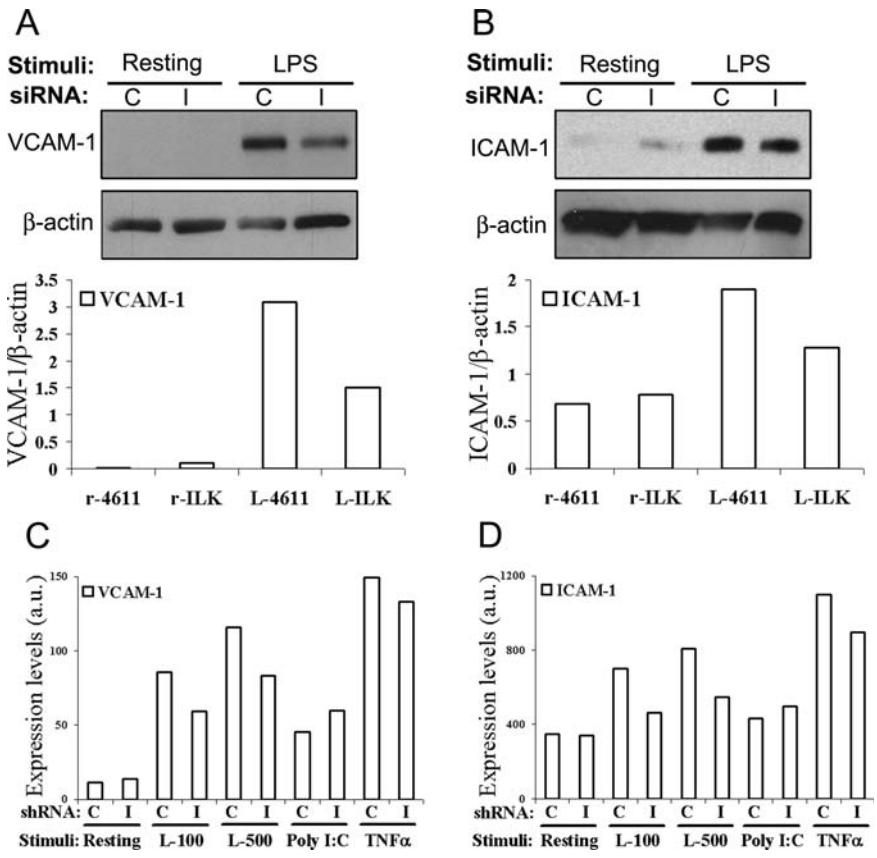


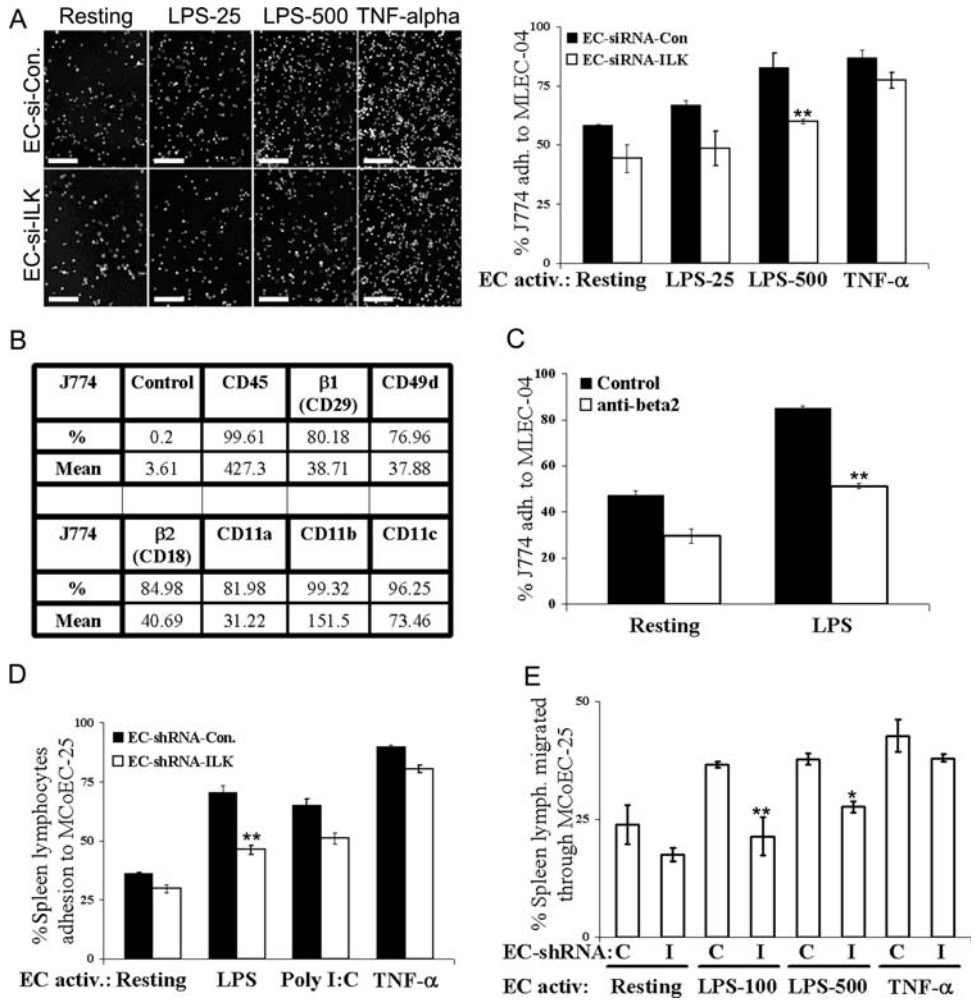
Figure 5 LPS-induced VCAM-1 and ICAM-1 protein expression is partially inhibited in ILK-deficient EC. (*A*, *B*) MCoEC-25 transfected with ILK siRNA (I, ILK) or control siRNA (C, 4611) were left resting (r) or treated with LPS (L, 500 ng/mL) for 6 h. The representative western blots show expression of VCAM-1 (*A*) and ICAM-1 (*B*), with β-actin detected as a loading control. The histograms show semi-quantification of relative band densities from four experiments. (*C*, *D*) Flow cytometry profiles of VCAM-1 and ICAM-1 on ILK-silenced (I) or control (C) shRNA transfected MCoEC-25 on resting conditions or stimulated for 6 h with LPS (100 and 500 ng/mL), Poly I:C (30 μg/mL), or TNF-α (15 ng/mL). Data are the mean fluorescence intensities of a representative experiment of three performed.

whereas endothelial ILK knockdown did not affect extravasation across TNF-α stimulated or resting EC, it significantly inhibited LPS-stimulated trans-endothelial migration (*Figure 6E*).

4. Discussion

EC play a critical role in regulating inflammation through their capacity to direct leucocyte traffic via the controlled expression of homeostatic and inflammatory mediators.[1] A consequence of the stepwise procedure of leucocyte recruitment is that blockade of any step will interrupt the extravasation process and prevent leucocytes entering the surrounding tissue. Therefore, anti-inflammatory strategies to inhibit the function of cell adhesion molecules involved in leucocyte-endothelium interaction are a promising means of regulating the cellular response in inflammation. Our results show that ILK regulates the inflammatory reaction triggered by LPS in four new mouse immortalized EC lines. These EC express ILK and the co-adaptor molecules PINCH and parvin, suggesting normal ILK signalling. LPS induced transcription of the inflammatory genes E-selectin, ICAM-1, and VCAM-1. LPS signals through ILK, as shown by down-regulation of ICAM-1 and VCAM-1 expression in LPS-activated ILK-deficient cells. Moreover, suppression of adhesion molecule expression impaired both the adhesion of monocytic cells and lymphocytes to LPS-activated endothelium and subsequent trans-endothelial migration. In our system, ILK seems to specifically regulate LPS-triggered EC activation, since the endothelial responses induced by TNF-α or Poly I:C were unaffected. This finding is particularly relevant because endothelial TLR4, the LPS receptor, has been identified as the primary intravascular bacterial sensor, being entirely sufficient to recruit neutrophils to peripheral tissues.[30] These findings thus identify a novel regulatory function of endothelial ILK in LPS-induced leucocyte recruitment, suggesting ILK as a potential target for therapeutic modulation of inflammatory disorders triggered by infection with Gram-negative bacteria.

We compared EC cultures derived from lung and coronary circulation because our unpublished results with conditional knockouts indicate that ILK makes a distinct contribution to the endothelium of coronary vessels during development (A. Luque *et al.*, in preparation). However, the experiments presented here revealed no differences between adult vascular beds in the ILK-mediated regulation of LPS triggered responses. Nonetheless, all four endothelial lines were comparable to freshly isolated EC for a range of endothelial phenotypes, as listed in the Supplementary material online, *Table S2*, positively identifying them as true EC.[31] In addition to data presented in this



J774	Control	CD45	β1 (CD29)	CD49d
%	0.2	99.61	80.18	76.96
Mean	3.61	427.3	38.71	37.88
J774	β2 (CD18)	CD11a	CD11b	CD11c
%	84.98	81.98	99.32	96.25
Mean	40.69	31.22	151.5	73.46

Figure 6 Down-regulation of endothelial ILK reduces adhesion and extravasation of lymphocytes and monocytes to LPS-activated endothelium. (*A*) Left panels: Fluorescence microscopy showing CFSE-J774 cells attached to resting or activated MLEC-04 cells transfected with control or ILK siRNA; EC were activated before co-attachment for 6 h with LPS (25, 500 ng/mL) or TNF-α (15 ng/mL). Scale bar, 50 μm. Right panel: Spectrophotometric quantification of CFSE-J774 fluorescence in parallel experiments. Results are the means ± SEM of four experiments. (*B*) Summary of cell adhesion molecule expression on J774 cells analysed by flow cytometry. '%', percentage of positive cells; 'Mean', mean fluorescence intensity. Control indicates to isotype matched negative controls. (*C*) CFSE-J774 cells were incubated with resting or LPS-treated MLEC-04 in the absence (control, solid bars) or presence of integrin β2 subunit blocking antibody (anti-beta2, clear bars). Results are the means ± SEM of three experiments. (*D*) Quantification of the fluorescence signal from CFSE-spleen lymphocytes adhered to resting, LPS (500 ng/mL), Poly I:C (30 μg/mL), or TNF-α (15 ng/mL)-activated MCoEC-25 expressing control-shRNA (solid bars) or ILK-shRNA (clear bars). Results are the means ± SEM of three experiments. (*E*) Splenocyte trans-migration towards SDF-1 across control (C) or ILK (I) shRNA-transfected MCoEC-25 monolayers resting or previously activated with LPS (100, 500 ng/mL) or TNF-α (15 ng/mL). Results are the means ± SEM of three experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ with respect to the control.

study, all four cultures express endothelial NOS constitutively and NOS-2 upon LPS activation (Supplementary material online, *Figure S4*). These well-defined EC lines provide homogeneous cell systems for the reproducible study of physiological and pathological processes including inflammation, vascular tone, blood coagulation, angiogenesis, haemostasis, and transport across blood vessels.

Crosstalk between ILK and FAK signalling has recently been described in the regulation of vascular smooth muscle cells and cardiac maintenance.[13,32] FAK regulates ICAM-1 expression upon β1 integrin adhesion, influencing cell viability and maturation.[33] Another focal contact protein, Pyk2, mediates up-regulation of LPS-induced VCAM-1 in smooth muscle cells.[34] The fact that ILK knockdown did not fully block ICAM-1 and VCAM-1 up-regulation in LPS-treated EC might indicate the activity of compensatory mechanisms arising from crosstalk among different pathways located at the focal contact area, a phenomenon that remains to be investigated.

Another possible explanation for the incomplete suppression of LPS-induced adhesion molecule expression includes the deficient elimination of ILK protein with the knockdown strategies used. The remnant 25–35% ILK protein expression might reflect limited efficiency of the reagents used or the long half life of the protein.[35] The use of ILK knockout cells was not considered because they have low survival and proliferation rates.[17–19] Cell counts indicate that cell proliferation and survival in our immortalized cell cultures

were not affected by ILK-siRNA transfection or ILK-shRNA expression (data not shown). Another possibility is that only part of the signalling triggered by LPS in EC is dependent on ILK: the moderate effect of ILK suppression on adhesion molecule expression might reflect complexity of LPS-induced signalling, involving several pathways (ERK, JNK, p38, PI3K/Akt, and NF-κB).[2]

Several EC responses to LPS *in vitro* are similar to events occurring during sepsis, including the production of pro-inflammatory cytokines and the increased surface expression of E-selectin, ICAM-1, and VCAM-1.[3,4] Co-adhesion experiments are a widely used *in vitro* model for studying the regulation of interactions between lymphoid cells and the endothelial layer.[34,36,37] The invariable attachment in the presence of conditioned media derived from LPS-activated EC (data not shown) did not motivate us to analyse the regulation of endothelial cytokine production by ILK in this system.

The regulation of LPS-induced ICAM-1 and VCAM-1 overexpression by ILK, but not of E-selectin, suggests a role for ILK in firm adhesion of lymphoid cells to the inflamed endothelium, whereas rolling of circulating cells would be unaffected. Co-adhesion assays in the presence of blocking antibodies revealed that of the molecules tested only β2 integrins mediate J774 attachment to endothelium. Nonetheless, the incomplete suppression of adhesion suggests that other adhesion couples are involved in this process.

To our knowledge, this study provides the first description of the involvement of ILK in the endothelial expression of cell adhesion molecules in response to LPS. The impact of ILK suppression on lymphoid adhesion and subsequent transmigration suggests that vascular ILK could be targeted to moderate the inflammatory response, possibly improving the prognosis of LPS-mediated inflammatory diseases. A previous report showed the involvement of ILK in the expression of SDF-1 and ICAM-1 in EC in hypoxia.[11] ILK also regulates the expression of various endothelial markers in an extracellular matrix dependent manner.[37] Targeting of ILK has been proposed as a potential anti-cancer approach because of its role in proliferation, migration, and survival of tumour cells.[38] The identification of an additional anti-adhesive potential for agents that target ILK suggests further applications in the treatment of inflammatory disorders. Several clinical trials targeting cell adhesion molecules are in progress, based on the ability of agents such as pharmacological drugs, antibodies, antagonist peptides, and antisense nucleotides to reduce injury in animal models of inflammatory and immune disease.[39] However, in some cases systemic treatment with these agents has shown no therapeutic effect or has adverse outcome. There is therefore a need to redirect strategies to target the cells specifically involved in the disease. Much work has been done to map different endothelium niches,[40] but further research is needed to identify the specific markers expressed during pathological events.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Acknowledgements

We thank Simon Bartlett for editing the manuscript, Gemma Benito, Lidia Jiménez, and Alberto Tierrez for technical assistance and Reinhard Fässler for providing the ILK lox/lox mice.

Conflict of interest: none declared.

Funding

This work was supported by Ministerio de Ciencia e Innovación from Spain, RYC-2004-000367 to A.L.; BFU2009-02161 and RECAVA to L.B.; PI080070 and the Fundación Mutua Madrileña to S.H.

References

1. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol* 2007;**7**:678–689.
2. Dauphinee SM, Karsan A. Lypopolysacharide signalling in endothelial cells. *Lab Invest* 2006;**86**:9–22.
3. Chen CC, Rosenbloom CL, Anderson DC, Manning AM. Selective inhibition of E-selectin, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1 expression by inhibitors of IκB-α phosphorylation. *J Immunol* 1995;**155**:3538–3545.
4. Jersmann HP, Hii CS, Ferrante JV, Ferrante A. Bacterial lipopolysaccharide and tumor necrosis factor-α synergistically increase expression of human endothelial adhesion molecules through activation of NF-κB and p38 mitogen-activated protein kinase signaling pathways. *Infect Immun* 2001;**69**:1273–1279.
5. Tan C, Mui A, Dedhar S. Integrin-linked kinase regulates inducible nitric oxide synthase and cyclooxygenase-2 expression in an NF-kappa B-dependent manner. *J Biol Chem* 2002;**277**:3109–3116.
6. Brakebusch C, Fässler R. The integrin-actin connection, an eternal love affair. *EMBO J* 2003;**22**:2324–2333.
7. Legate KR, Montañez E, Kudlacek O, Fässler R. ILK, PINCH and parvin: the tIPP of integrin signalling. *Nat Rev Mol Cell Biol* 2006;**7**:20–31.
8. Attwell S, Roskelley C, Dedhar S. The integrin-linked kinase (ILK) suppresses anoikis. *Oncogene* 2000;**19**:3811–3815.
9. Troussard AA, Mawji NM, Ong C, Mui A, St-Arnaud R, Dedhar S. Conditional knock-out of integrin-linked kinase demonstrates an essential role in protein kinase B/Akt activation. *J Biol Chem* 2003;**278**:22374–22378.
10. Kaneko Y, Kitazato K, Basaki Y. Integrin-linked kinase regulates vascular morphogenesis induced by vascular endothelial growth factor. *J Cell Sci* 2004;**117**:407–415.
11. Lee SP, Youn SW, Cho HJ, Li L, Kim TY, Yook HS *et al.* Integrin-linked kinase, a hypoxia-responsive molecule, controls postnatal vasculogenesis by recruitment of endothelial progenitor cells to ischemic tissue. *Circulation* 2006;**114**:150–159.
12. Bock-Marquette I, Saxena A, White MD, Dimaio JM, Srivastava D. Thymosin beta4 activates integrin-linked kinase and promotes cardiac cell migration, survival and cardiac repair. *Nature* 2004;**432**:466–472.
13. White DE, Coutu P, Shi YF, Tardif JC, Nattel S, St Arnaud R *et al.* Targeted ablation of ILK from the murine heart results in dilated cardiomyopathy and spontaneous heart failure. *Genes Dev* 2006;**20**:2355–2360.
14. Knöll R, Postel R, Wang J, Krätzner R, Hennecke G, Vacaru AM *et al.* Laminin-alpha4 and integrin-linked kinase mutations cause human cardiomyopathy via simultaneous defects in cardiomyocytes and endothelial cells. *Circulation* 2007;**116**:515–525.
15. Tan C, Cruet-Hennequart S, Troussard A, Fazli L, Costello P, Sutton K *et al.* Regulation of tumor angiogenesis by integrin-linked kinase (ILK). *Cancer Cell* 2004;**5**:79–90.
16. Wang HV, Chang LW, Brixius K, Wickström SA, Montanez E, Thievessen I *et al.* Integrin-linked kinase stabilizes myotendinous junctions and protects muscle from stress-induced damage. *J Cell Biol* 2008;**180**:1037–1049.
17. Sakai T, Li S, Docheva D, Grashoff C, Sakai K, Kostka G *et al.* Integrin-linked kinase (ILK) is required for polarizing the epiblast, cell adhesion, and controlling actin accumulation. *Genes Dev* 2003;**17**:926–940.
18. Grashoff C, Aszódi A, Sakai T, Hunziker EB, Fässler R. Integrin-linked kinase regulates chondrocyte shape and proliferation. *EMBO Rep* 2003;**4**:432–438.
19. Liu E, Sinha S, Williams C, Cyrille M, Heller E, Snapper SB *et al.* Targeted deletion of integrin-linked kinase reveals a role in T-cell chemotaxis and survival. *Mol Cell Biol* 2005;**25**:11145–11155.
20. Akhtar N, Marlow R, Lambert E, Schatzmann F, Lowe ET, Cheung J *et al.* Molecular dissection of integrin signalling proteins in the control of mammary epithelial development and differentiation. *Development* 2009;**136**:1019–1027.
21. Chi JT, Chang HY, Haraldsen G, Jahnsen FL, Troyanskaya OG, Chang DS *et al.* Endothelial cell diversity revealed by global expression profiling. *Proc Natl Acad Sci USA* 2003;**100**:10623–10628.
22. Balconi G, Spagnuolo R, Dejana E. Development of endothelial cell lines from embryonic stem cells: A tool for studying genetically manipulated endothelial cells in vitro. *Arterioscler Thromb Vasc Biol* 2000;**20**:1443–1451.
23. Stanchi F, Bordoy R, Kudlacek O, Braun A, Pfeifer A, Moser M *et al.* Consequences of loss of PINCH2 expression in mice. *J Cell Sci* 2005;**118**:5899–58910.
24. Luque A, Carpizo DR, Iruela-Arispe ML. ADAMTS1/METH1 inhibits endothelial cell proliferation by direct binding and sequestration of VEGF165. *J Biol Chem* 2003;**278**:23656–23665.
25. Zeini M, Través PG, López-Fontal R, Pantoja C, Matheu A, Serrano M *et al.* Specific contribution of p19(ARF) to nitric oxide-dependent apoptosis. *J Immunol* 2006;**177**:3327–3336.
26. Dejana E. Endothelial cell–cell junctions: happy together. *Nat Rev Mol Cell Biol* 2004;**5**:261–270.

27. Voyta JC, Via DP, Butterfield CE, Zetter BR. Identification and isolation of endothelial cells based on their increased uptake of acetylated-low density lipoprotein. *J Cell Biol* 1984;**99**:2034–2040.
28. Olsson AK, Dimberg A, Kreuger J, Claesson-Welsh L. VEGF receptor signalling - in control of vascular function. *Nat Rev Mol Cell Biol* 2006;**7**:359–371.
29. Mahadev K, Wu X, Donnelly S, Ouedraogo R, Eckhart AD, Goldstein BJ. Adiponectin inhibits vascular endothelial growth factor-induced migration of human coronary artery endothelial cells. *Cardiovasc Res* 2008;**78**:376–384.
30. Andonegui G, Zhou H, Bullard D, Kelly MM, Mullaly SC, McDonald B *et al*. Mice that exclusively express TLR4 on endothelial cells can efficiently clear a lethal systemic Gram-negative bacterial infection. *J Clin Invest* 2009;**119**:1921–1930.
31. Bachetti T, Morbidelli L. Endothelial cells in culture: a model for studying vascular functions. *Pharmacol Res* 2000;**42**:9–19.
32. Li JJ, Han M, Wen JK, Li AY. Osteopontin stimulates vascular smooth muscle cell migration by inducing FAK phosphorylation and ILK dephosphorylation. *Biochem Biophys Res Commun* 2007;**356**:13–19.
33. Nakayamada S, Saito K, Fujii K, Yasuda M, Tamura M, Tanaka Y. beta1 integrin-mediated signaling induces intercellular adhesion molecule 1 and Fas on rheumatoid synovial cells and Fas-mediated apoptosis. *Arthritis Rheum* 2003;**48**:1239–1248.
34. Lin WN, Luo SF, Wu CB, Lin CC, Yang CM. Lipopolysaccharide induces VCAM-1 expression and neutrophil adhesion to human tracheal smooth muscle cells: involvement of Src/EGFR/PI3-K/Akt pathway. *Toxicol Appl Pharmacol* 2008;**228**:256–268.
35. Chen H, Huang XN, Yan W, Chen K, Guo L, Tummalapali L *et al*. Role of the integrin-linked kinase/PINCH1/alpha-parvin complex in cardiac myocyte hypertrophy. *Lab Invest* 2005;**85**:1342–1356.
36. Pfaff D, Héroult M, Riedel M, Reiss Y, Kirmse R, Ludwig T *et al*. Involvement of endothelial ephrin-B2 in adhesion and transmigration of EphB-receptor-expressing monocytes. *J Cell Sci* 2008;**121**:3842–3850.
37. Ruiz-Torres MP, Pérez-Rivero G, Rodríguez-Puyol M, Rodríguez-Puyol D, Díez-Marqués ML. The leukocyte-endothelial cell interactions are modulated by extracellular matrix proteins. *Cell Physiol Biochem* 2006;**17**:221–232.
38. Hannigan G, Troussard AA, Dedhar S. Integrin-linked kinase: a cancer therapeutic target unique among its ILK. *Nat Rev Cancer* 2005;**5**:51–63.
39. Cantor JM, Ginsberg MH, Rose DM. Integrin-associated proteins as potential therapeutic targets. *Immunol Rev* 2008;**223**:236–251.
40. Corti A, Curnis F, Arap W, Pasqualini R. The neovasculature homing motif NGR: more than meets the eye. *Blood* 2008;**112**:2628–2635.