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## The flow dependency of Tie2 expression in endotoxemia

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**Abstract** *Rationale:* Tie2 is predominantly expressed by endothelial cells and is involved in vascular integrity control during sepsis. Changes in Tie2 expression during sepsis development may contribute to microvascular dysfunction. Understanding the kinetics and molecular basis of these changes may assist in the development of therapeutic intervention to counteract microvascular dysfunction. *Objective:* To investigate the molecular mechanisms underlying the changes in Tie2 expression upon lipopolysaccharide (LPS) challenge. *Methods and results:* Studies were performed in LPS and pro-inflammatory cytokine

challenged mice as well as in mice subjected to hemorrhagic shock, primary endothelial cells were used for in vitro experiments in static and flow conditions. Eight hours after LPS challenge, Tie2 mRNA loss was observed in all major organs, while loss of Tie2 protein was predominantly observed in lungs and kidneys, in the capillaries. A similar loss could be induced by secondary cytokines TNF- $\alpha$  and IL-1 $\beta$ . Ang2 protein administration did not affect Tie2 protein expression nor was Tie2 protein rescued in LPS-challenged Ang2-deficient mice, excluding a major role for Ang2 in Tie2 down regulation. In vitro, endothelial loss of Tie2 was observed upon lowering of shear stress, not upon LPS and TNF- $\alpha$  stimulation, suggesting that inflammation related haemodynamic changes play a major role in loss of Tie2 in vivo, as also hemorrhagic shock induced Tie2 mRNA loss. In vitro, this loss was partially counteracted by pre-incubation with a pharmacologically NF- $\kappa$ B inhibitor (BAY11-7082), an effect further substantiated in vivo by pre-treatment of mice with the NF- $\kappa$ B inhibitor prior to the inflammatory challenge. *Conclusions:* Microvascular bed specific loss of Tie2 mRNA and protein in vivo upon LPS, TNF $\alpha$ , IL-1 $\beta$  challenge, as well as in response to

hemorrhagic shock, is likely an indirect effect caused by a change in endothelial shear stress. This loss of Tie2 mRNA, but not Tie2 protein, induced by TNF $\alpha$  exposure was shown to be controlled by NF- $\kappa$ B signaling. Drugs aiming at restoring vascular integrity in sepsis could focus on preventing the Tie2 loss.

**Keywords** Lipopolysaccharide · Endothelial Tie2 · Flow · In vitro · In vivo

#### Non-standard abbreviations and acronyms

LPS Lipopolysaccharide  
TNF- $\alpha$  Tumor Necrosis Factor- $\alpha$   
IL-1 $\beta$  Interleukin-1 $\beta$   
TLR4 Toll-like receptor-4

NF- $\kappa$ B Nuclear factor kappa B  
KLF2 Kruppel-like factor-2  
Ang1/2 Angiotensin-1/-2  
HUVEC Human umbilical vein endothelial cells  
ciGEnC Conditionally immortalized glomerular endothelial cells  
WPB Weibel-Palade Bodies

## Introduction

Septic shock is a life-threatening condition that is characterized by a severe inflammatory response to infection and hypotension [1]. While more clinically relevant models are available, lipopolysaccharide (LPS) administration to mice is a model that mimics in a highly reproducible manner many of the initial clinical manifestations of sepsis, including endothelial activation and vascular hyperpermeability [1]. The binding of LPS to toll-like receptor (TLR)-4 expressing endothelial cells activates intracellular NF- $\kappa$ B signaling pathways which induce the expression of endothelial adhesion molecules, cytokine release, and increased vascular permeability [2]. Moreover, hypotension is observed upon LPS challenge [3, 4], and studies by Pries et al. [5] suggested that this reduction in blood pressure is accompanied by a reduction in wall shear stress in microvascular beds. This can be sensed by endothelial cells via mechanosensory systems that transduce force through signaling complexes [6], leading among other events to decreased expression of flow response transcription factors, in particular Kruppel-like factor-2 (KLF2) [6].

One of the molecular regulatory systems which has been reported to contribute to endothelial activation and vascular permeability control in sepsis and other diseases is the Angiotensin/Tie2 system [7]. Tie2 is a receptor tyrosine kinase that is expressed primarily by the vascular endothelium [8]. Angiotensin (Ang)-1 and Ang2 are the ligands of Tie2. The binding of Ang1 to Tie2 is important for anti-inflammatory effects and maintenance of endothelial integrity, while the binding of the antagonist Ang2 is associated with endothelial activation and vascular leakage [7].

We recently demonstrated that LPS administration induced loss of Tie2 in the kidneys, which was paralleled by vascular integrity loss [9]. This relationship between loss of Tie2 and loss of vascular integrity was also observed in a mouse model of ventilator-induced lung injury [10]. Although therapy aimed at restoring Ang1 and diminishing Ang2 in sepsis are promising in pre-clinical models [11, 12], the molecular mechanisms behind the loss of Tie2 remain unclear. The purpose of this study was to examine

the extent of changes in Tie2 expression during the first phase of endotoxemia, and to identify the molecular mechanisms affecting Tie2 mRNA and protein expression upon LPS challenge. Understanding the molecular mechanisms that affect Tie2 expression can possibly assist in defining rational therapeutic approaches to inhibit microvascular dysfunction in sepsis in the future.

## Materials and methods

Extensive materials and methods are described in the supplementary materials and methods.

### Cells

Human umbilical vein endothelial cells (HUVEC), and conditionally immortalized glomerular endothelial cells (ciGEnC), [13]: were used [9, 14]. Moreover, primary mouse heart endothelial cells and kidney cortex peritubular endothelial cells were used [15].

### Animals

The C57BL/6 mice and were maintained on mouse chow and tap water ad libitum in a temperature-controlled chamber at 24 °C with a 12-hours light/dark cycle. All animal experiments were approved by the local Animal Care and Use committee of the University of Groningen (protocol number 4360A), The Netherlands and of the Regierungspräsidium Karlsruhe (protocol number 35-9185.81/G-148/09), Germany, and were performed according to governmental and international guidelines on animal experimentation.

### In vivo experiments

The LPS and hemorrhagic shock models were performed as described previously [9, 16].

The TNF- $\alpha$  and IL-1 $\beta$  were administered via the orbital plexus (*o.p.*) 200 ng/mouse TNF- $\alpha$  (Biosource Netherlands, Etten-Leur, The Netherlands) or 200 ng/mouse IL-1 $\beta$  (Biosource Netherlands), mice were sacrificed 2 h after TNF- $\alpha$  and IL-1 $\beta$  administration. The NF- $\kappa$ B inhibitor BAY11-7082 (400  $\mu$ g/mouse; Sigma) was administered *i.v.* 30 min prior to TNF- $\alpha$  administration.

#### In vitro experiments

The HUVEC and ciGEnC were grown to confluence of mRNA and stimulated with LPS (Sigma) at 300 EU/mL (0.1  $\mu$ g/ml) or human TNF- $\alpha$  (Boehringer, Ingelheim, Germany) at 10 ng/mL for 4 and 24 h.

The HUVEC were seeded into 1 % gelatin coated channels (ibidi, Martinsried, Germany) for flow experiments. Cells were exposed to 20 dyne/cm<sup>2</sup> shear stress for 48 h, where appropriate cells were next exposed to LPS at 300 EU/mL (0.1  $\mu$ g/ml) or TNF- $\alpha$  10 ng/mL for 8 h, and harvested for further analysis. The 10  $\mu$ M NF- $\kappa$ B inhibitor BAY11-7082 or DMSO 0.1 % as vehicle control were added 30 min prior to stopping the flow.

#### Gene expression analysis by quantitative RT-PCR

Total RNA from brain, heart, liver, kidneys and cultured cells were isolated with RNeasy Mini Plus Kit (Qiagen, Leusden, The Netherlands), and from lungs with RNeasy Mini Kit (Qiagen), according to the manufacturer's guidelines as previously described [9].

#### Microarray analysis

Mouse heart endothelial cells and kidney endothelial cells were subjected to RNA isolation using Rneasy mini kit (Qiagen, USA). The RNA samples were used for further analysis with Illumina Mouse Ref8 BeadChips (Illumina, San Diego, CA, USA) and the Tie2 value for each group was normalized to pan-endothelial marker VE-cadherin, expression immediately after isolation was set at 100 %.

#### Immunohistochemistry

Frozen organs were cryostat-cut at 5  $\mu$ m, mounted onto glass slides, and fixed with acetone. Immunohistochemical staining of Tie2 protein in tissue was performed as previously described [9].

#### Quantification of Tie2 protein expression

Quantification of Tie2 protein in mouse organs was determined by ELISA as previously described [9].

Quantification of Tie2 protein in HUVEC and ciGEnC was performed by western blot as previously described with some modifications [11].

#### Statistical analysis

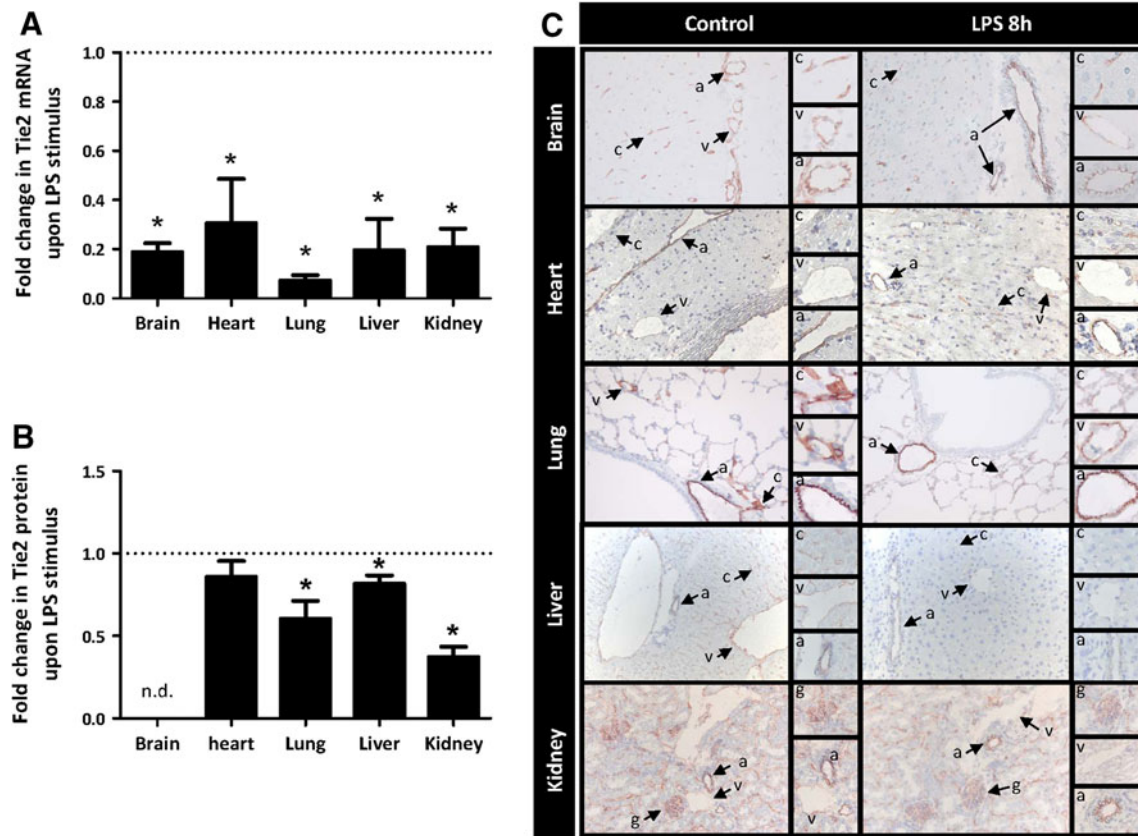
Statistical significance of differences was analysed by means of the Student's *t* test or ANOVA with post hoc comparison using Bonferroni correction. All statistical analyses were performed using GraphPad Prism software (GraphPad Prism Software Inc., San Diego, CA, USA). Differences were considered to be significant when  $P < 0.05$ .

## Results

Tie2 mRNA and protein are down regulated in all major organs of mice challenged with LPS

First, we determined the overall changes in Tie2 mRNA and protein to ascertain whether they are restricted to certain organs or occur in multiple microvascular beds. Brain, heart, lung, liver and kidney were chosen as the main organs to study. While significant differences in levels of expression of Tie2 between organs was observed (Suppl. Fig. 1a), these primarily reflected endothelial prevalence in the organs (Suppl. Fig. 1c), 8 h after LPS challenge, Tie2 mRNA was lost to 70–90 % of its original level in all organs. In contrast, loss of Tie2 protein was only observed in the lungs and kidneys, while in the heart Tie2 protein levels did not change (Fig. 1a, b). In the brain, Tie2 protein levels were the lowest among organs in basal conditions (Suppl. Fig. 1b), and 8 h after LPS administration its levels became too low to be detected by ELISA (Fig. 1b).

To investigate whether the Tie2 protein downregulation was restricted to specific microvascular beds, we immunohistochemically determined Tie2 protein localization. In basal conditions, Tie2 protein was expressed in arterioles, capillaries and venules of brain, heart, lungs, liver, and kidneys, though expression in the capillaries of the lungs was less abundant compared to that in the capillaries of the other organs. Eight hours after LPS challenge, loss of Tie2 protein was visible in all microvascular segments in all organs, yet least prominent in the heart. The loss of Tie2 in capillaries showed the most extensive reduction (Fig. 1c). Thus, LPS-induced loss of Tie2 mRNA was extensive and occurred in all organs studied, while the loss of Tie2 protein predominantly took place in lungs and kidneys, particularly in the capillaries.



**Fig. 1** Down regulation of Tie2 mRNA and protein induced by LPS occurs in major organs. Organ Tie2 mRNA (a) and protein (b) levels 8 h after LPS challenge (1,500 EU/g, 0.5 mg/kg mouse body weight) in wild type mice are expressed as fold change compared to control (vehicle treated) group. Bars show mean  $\pm$  SD of 4–5 mice. \* $P < 0.05$ , control group vs. LPS-

challenged group. n.d., protein not detectable. c Localization of Tie2 protein in the major mouse organs in control conditions, respectively, at 8 h after LPS administration. Pictures show representative immunohistochemical staining as described in “Materials and Methods”. Original magnification: 200 $\times$ . a arteriole, v venule, c capillary, g glomerulus

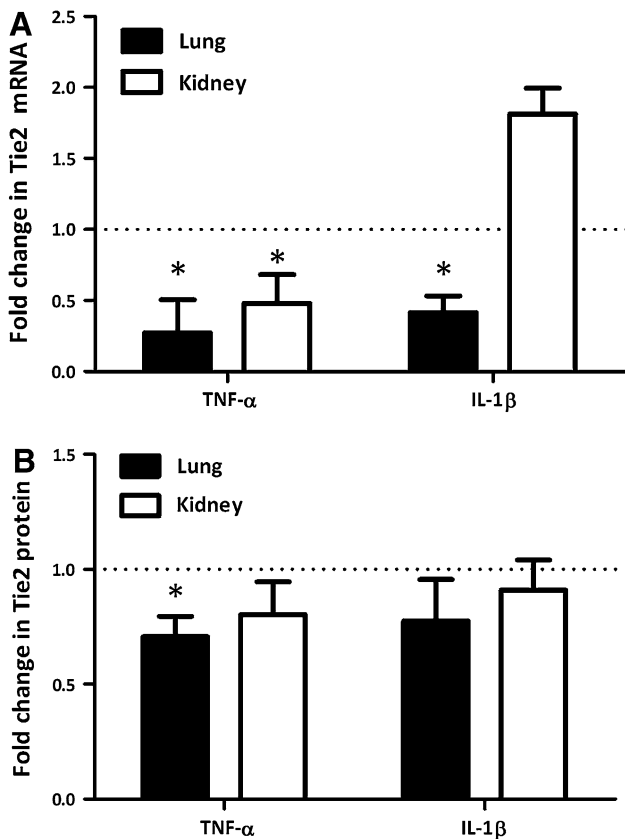
Loss of Tie2 mRNA and protein can be induced by cytokines

As septic shock is accompanied by the release of pro-inflammatory cytokines, which is mimicked by LPS injection, we asked the question whether the rapidly released secondary pro-inflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$  per se might play a role in Tie2 mRNA and protein down regulation. We focused on the loss of Tie2 mRNA and protein in the lungs and kidneys as these organs showed the largest protein down regulation, and can fail dramatically during sepsis. Upon TNF- $\alpha$  administration, Tie2 mRNA was significantly downregulated in both organs, while loss of Tie2 protein was only observed in the lungs (Fig. 2a, b). Upon IL-1 $\beta$  administration, on the other hand, loss of Tie2 mRNA was only observed in the lungs, while Tie2 mRNA in the kidneys was upregulated approximately twofold (Fig. 2a). Furthermore, the levels of Tie2 protein upon IL-1 $\beta$  administration did not change in both organs (Fig. 2b). Taken together, these

data show that the loss of Tie2 mRNA and protein can also be partially induced by pro-inflammatory cytokines released in reaction to systemic LPS exposure in an organ dependent manner.

In vitro Tie2 expression is modulated by shear stress

We proceeded to in vitro studies to explore the potential molecular mechanisms controlling Tie2 mRNA and protein down regulation by using two different endothelial cell subtypes, i.e., human umbilical cord derived HUVEC and human glomeruli derived ciGenC. For this purpose, cells were stimulated with LPS or TNF- $\alpha$  for 4 and 24 h. Neither Tie2 mRNA (Suppl. Fig. 3a, b) nor Tie2 protein (Suppl. Fig. 4) were down regulated in both cell models, irrespective of the time period of exposure or the stimulus used. Instead of downregulating Tie2, LPS and TNF- $\alpha$  induced Tie2 mRNA significantly in HUVEC at 24 h after stimulation. Since the loss of Tie2 could not be



**Fig. 2** TNF- $\alpha$  induces loss of Tie2 mRNA and protein in the kidney and Tie2 mRNA in the lung in vivo. Tie2 mRNA (a) and protein (b) levels at 2 h after orbital puncture TNF- $\alpha$  or IL-1 $\beta$  challenge of wild type mice are expressed as fold change compared to control (vehicle treated) group. Bars show mean  $\pm$  SD of three mice. \* $P < 0.05$ , control group vs. cytokine challenged group

mimicked in vitro, we hypothesized that changes in the microenvironment upon taking cells into culture cause changes in Tie2 expression. To test this, we isolated HUVEC from human umbilical cords and grew them under standard conditions. The cells were lysed directly after isolation, and at day 1, 3, and 5 after isolation and we analyzed the changes in Tie2 expression upon culture. We found that Tie2 mRNA expression was down regulated by more than 60 % in the first 3 days of culture (Fig. 3a). Similar observations were made using freshly isolated cultured mouse heart capillary and mouse renal cortex endothelial cells (Fig. 3a). These findings indicate that a commonly experienced environmental change such as variations in flow per se could be one cause of Tie2 mRNA down regulation.

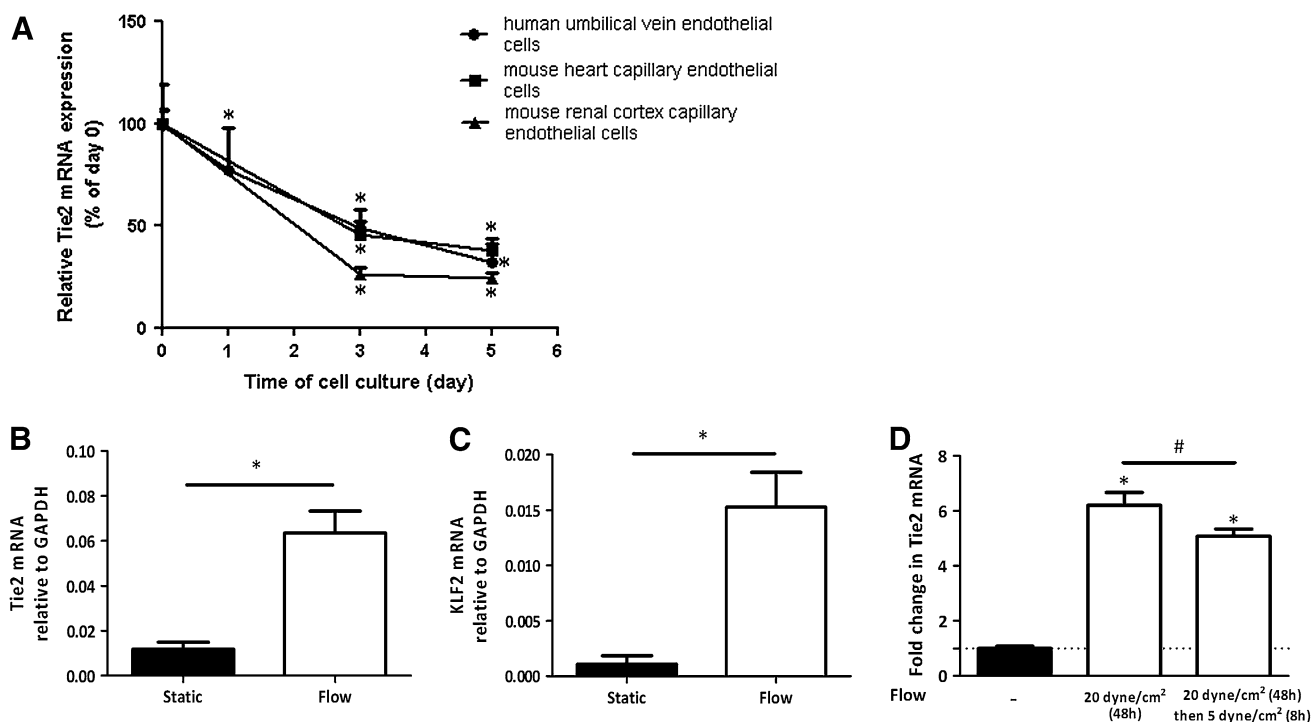
The LPS challenge has been previously shown to lower the blood pressure [3]. Moreover, it represents a factor that is absent in the static in vitro conditions used in most studies on the effect of sepsis mediators on endothelial cells so far. To test the hypothesis that Tie2 expression is regulated by diminished blood flow, we

studied its expression in a hemorrhagic shock (HS) model. Also in this model, Tie2 mRNA downregulation occurred in all major organs (Fig. 5b). These in vivo data were corroborated by the observation that when HUVEC were exposed to shear stress at 20 dyne/cm<sup>2</sup> for 48 h, increased expression was observed of Tie2 mRNA up to eightfold (Fig. 3b) and of Tie2 protein up to sevenfold (Fig. 4c) compared to expression in static conditions. At the same time, the shear stress responsive gene KLF2 [17] was up regulated in vitro (Fig. 3c). Moreover, when we reduced the shear stress to 5 dyne/cm<sup>2</sup>, Tie2 mRNA levels dropped significantly (Fig. 3d). These combined data sets suggest that changes in Tie2 expression during endotoxemia are likely to be regulated by flow differences.

To determine the potential contribution of pro-inflammatory cytokines to the observed loss of Tie2, HUVEC were exposed to shear stress at 20 dyne/cm<sup>2</sup> for 48 h and next stimulated with LPS or TNF- $\alpha$  for 8 h before lysing the cells. While Tie2 was increased under flow, subsequent exposure to LPS and TNF- $\alpha$  did not lead to a decrease (Fig. 4a–c). The increased levels of E-selectin observed upon LPS and TNF- $\alpha$  challenge validated HUVEC responsiveness toward LPS with respect to TNF- $\alpha$  exposure (Suppl. Fig. 5a, b). This suggests further substantiation of the model that loss of Tie2 mRNA and protein in sepsis is likely caused by diminished blood flow or altered flow patterns, and not directly by pro-inflammatory endothelial cell activation.

#### Loss of Tie2 mRNA, but not Tie2 protein, can be counteracted by inhibition of NF- $\kappa$ B signaling

Previously, involvement of NF- $\kappa$ B signaling in shear stress-exposed endothelial cells has been reported [18], and it was shown that a decrease in shear stress enhances NF- $\kappa$ B activation [19]. We next studied in vitro whether NF- $\kappa$ B signaling played a role in the lower shear stress induced loss of Tie2 by treating the cells with the NF- $\kappa$ B inhibitor BAY11-7082 prior to cessation of the flow. The effects of the NF $\kappa$ B inhibitory drug on TNF $\alpha$  challenged microvascular endothelial cells in vitro and in vivo in mouse organs are available via our website <http://irs.ub.rug.nl/ppn/304222879>. In the absence of the inhibitor, the levels of Tie2 and KLF2 mRNA were reduced significantly to 75 and 95 % of initial levels, respectively, after ceasing the flow. When flow was stopped in the presence of NF- $\kappa$ B inhibition, a small but likely biological insignificant, raise in Tie2 mRNA was observed (Fig. 5a). Again, proper action of the drug to inhibit NF- $\kappa$ B [14], was substantiated by the fact that the increased E-selectin expression could be reduced by pre-treatment with the NF- $\kappa$ B inhibitor (Fig. 5a). To substantiate a role for NF- $\kappa$ B signaling in loss of endothelial Tie2 in vivo, mice were treated with the NF- $\kappa$ B inhibitor prior to TNF- $\alpha$  administration. The results showed that in



**Fig. 3** Shear stress controls Tie2 gene expression. **a** Endothelial cells which were isolated from human umbilical cord veins, mouse heart and mouse kidney cortex capillaries, were either analysed immediately after isolation, or after being cultured for indicated times. Tie2 mRNA was determined by quantitative RT-PCR (HUVEC) and by Illumina expression arrays (mouse heart and kidney cortex capillary endothelial cells). Tie2 mRNA was calculated relative to pan-endothelial marker VE-cadherin. Mean of day 0 (directly after isolation) was set as 100%. Values represent mean  $\pm$  SD of three independent experiments.  $*P < 0.05$

vs. day 0. **b**, **c**, **d** HUVEC were cultured in gelatin-coated microchannels and exposed to shear stress of 20 dyne/cm<sup>2</sup> for 48 h or kept under static conditions, respectively. After 48 h of 20 dyne/cm<sup>2</sup>, the shear stress was reduced to 5 dyne/cm<sup>2</sup> for 8 h. Tie2 mRNA and KLF2 mRNA were determined by quantitative RT-PCR. **b**, **c** Values represent mean  $\pm$  SD of three independent experiments.  $*P < 0.05$ . **d** Values represent mean  $\pm$  SD in triplicate and are representative of two independent experiments.  $*P < 0.05$ , vs. static group;  $\#P < 0.05$

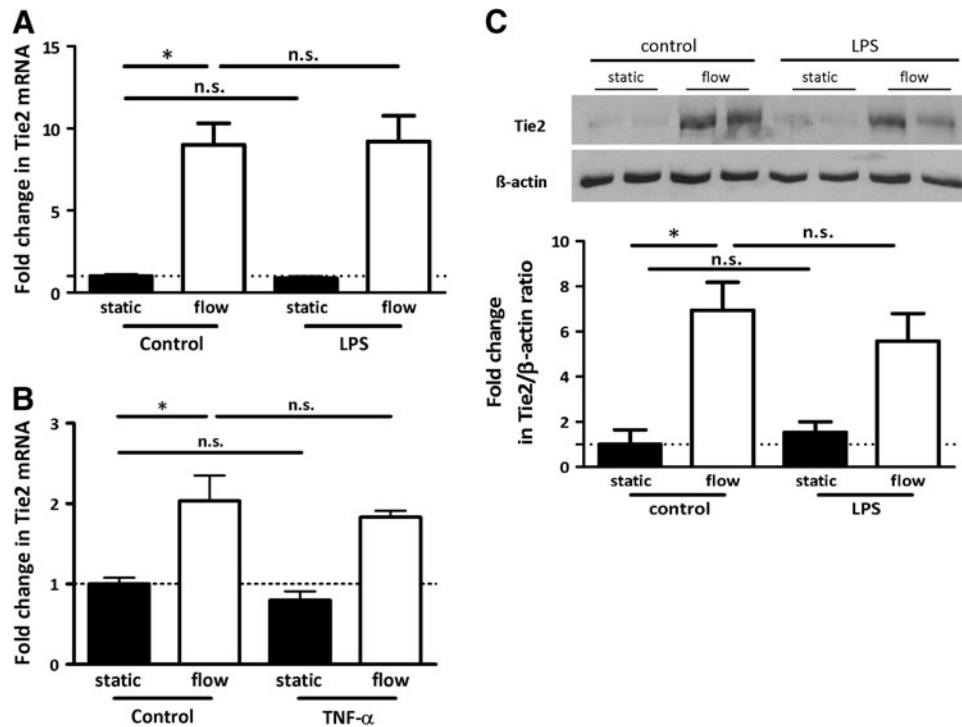
the lungs Tie2 mRNA was completely rescued in the presence of the inhibitor (Fig. 5c). These data indicate that NF- $\kappa$ B is a major controlling molecular factor in inflammation related loss of Tie2 mRNA, which most likely is induced by low blood pressure. In contrast, loss of Tie2 protein could not be rescued by blocking NF- $\kappa$ B activity, suggesting a different mode of regulation of Tie2 protein under conditions of diminished blood flow or altered flow patterns (Fig. 5b).

## Discussion

In this study we show that the endothelial expression of Tie2 in vivo is dependent on flow. A decrease of flow leads to a decrease in Tie2 expression. This has major implications for basal research on sepsis mediators and its effects on endothelial cells. Research performed in cell systems investigating the inflammatory response can only be interpreted when the local flow status is taken into

account [20]. The translation from this preclinical findings to human sepsis is more complicated [7]. In sepsis, cardiac output, and, therefore, flow in larger vessels and arterioles, is increased, while in capillaries flow almost ceases [21]. Flow characteristics are different in different microvascular beds. The difference of flow characteristics and the peritubular vasculature are, for instance, largely unknown [22]. The decrease in blood pressure is one of the defining characteristics of sepsis, using the paradigm that oxygen delivery to the cells has to be maintained. The restoration of blood pressure and blood flow has always been one of the hallmarks of the treatment of sepsis. Our study suggests that manipulating flow itself influences the inflammatory response of the microvasculature.

The Angiopoietin/Tie2 system has been reported to contribute to endothelial activation and vascular permeability control in sepsis. Human sepsis and the endotoxaemia mouse model are characterized by microvascular endothelial activation and loss of vascular integrity, which contribute to hypotension, vascular



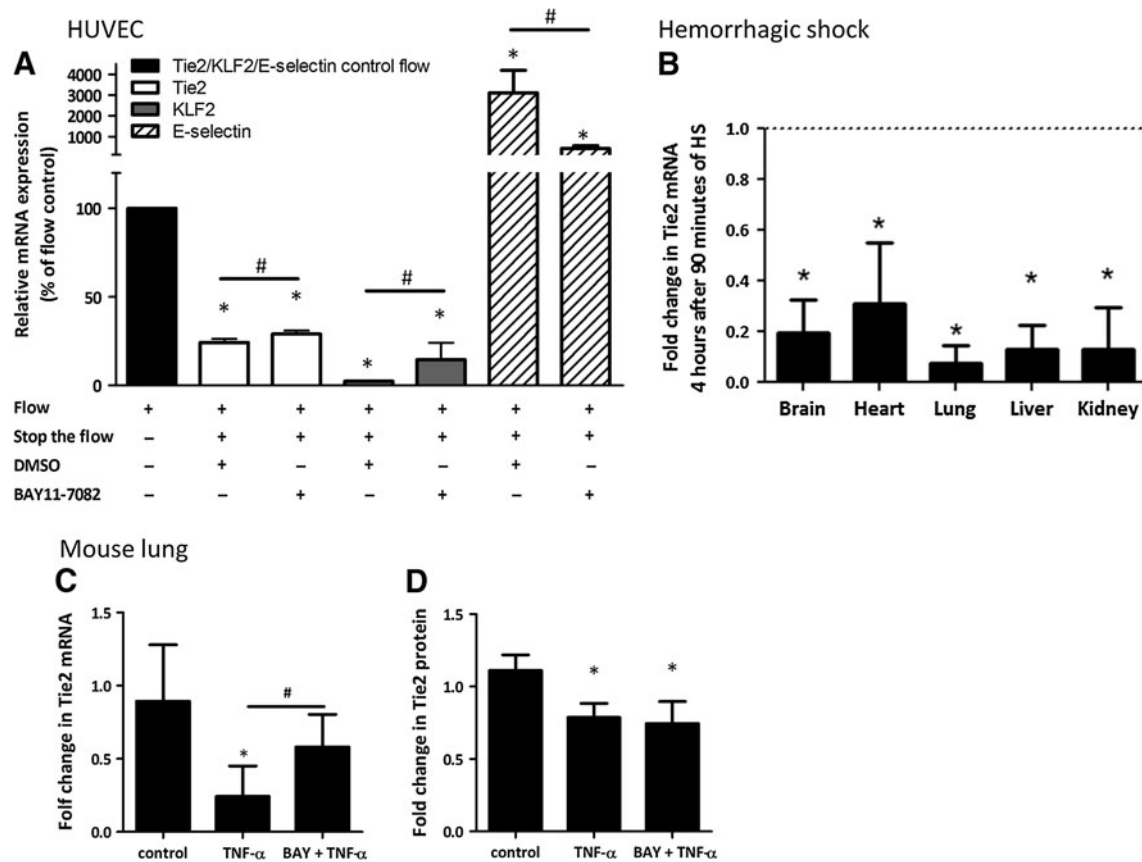
**Fig. 4** LPS nor TNF $\alpha$  challenge of flow-exposed cells does not downregulate Tie2 mRNA and protein expression. HUVEC were exposed to shear stress at 20 dyne/cm<sup>2</sup> for 48 h, then LPS (300 EU/mL, 0.1  $\mu$ g/ml) or TNF- $\alpha$  (10 ng/mL) was added for 8 h before lysing the cells for mRNA (a, b) and for protein (c). The HUVEC cultured in static conditions were used as a control for flow exposure and LPS stimulation. Tie2 mRNA and protein levels were determined by quantitative RT-PCR and Western Blot,

respectively. Tie2 mRNA expression is expressed as fold change compared to static control group. For Tie2 protein,  $\beta$ -actin was used as a loading control. The level of Tie2 and  $\beta$ -actin were quantified as described in “Materials and Methods”. The Tie2/ $\beta$ -actin ratio is expressed as relative change compared to the control static group arbitrarily set at 1. Bars show mean  $\pm$  SD; of three independent experiments; \* $P$  < 0.05

leakage and leukocyte extravasation that are all hallmarks of the severe septic shock syndrome. Although therapies aimed at restoring Ang1 and diminishing Ang2 in sepsis are promising in pre-clinical models [11, 12], until now in the regulation pattern of the receptor, Tie2 was insufficiently known. In a previous study we showed that LPS induced loss of Tie2 mRNA and protein expression in the renal microvasculature [9]. In the present study, we showed that this loss was prominent in all major mouse organs, while loss of Tie2 protein was predominantly observed in lungs and kidneys, in particular in capillaries. The pro-inflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$  could partially recapitulate these effects. There was no role for systemically released Ang2 in regulating loss of Tie2 protein (Supplementary Fig. 2, Supplementary results and discussion). In vitro, loss of Tie2 was observed upon lowering shear stress but not upon LPS and TNF- $\alpha$  stimulation of cells cultured under flow, suggesting that sepsis related haemodynamic changes may be the cause of loss of Tie2 expression in vivo. This flow related expression control was further substantiated in a mouse model of hemorrhagic shock. A definite role for NF- $\kappa$ B in in vivo controlled loss of Tie2 mRNA was established by

pre-treatment of mice with an NF- $\kappa$ B inhibitor prior to inflammatory challenge with TNF- $\alpha$ . Although LPS and pro-inflammatory cytokine injections represent models with limited resemblance to human sepsis, we chose to use these models based on the fact that they are highly standardized and frequently used to study important inflammatory components of sepsis/septic shock. This standardization makes the results reproducible and allows comparison with published research. Furthermore, while LPS effects are highly complex due to the spatiotemporal systemic release of cytokines in time, single cytokine injections allowed to (partly) dissect their contribution to observations made with LPS.

One of the most striking observations in this study was the extent of Tie2 mRNA loss that took place throughout all microvascular beds in the body. Moreover, the disparate behavior of Tie2 mRNA and protein was unexpected. To our knowledge, in vivo Tie2 mRNA half-life has not been reported, while the half-life of Tie2 protein was reported in HUVEC to be approximately 9 h [23]. Would Tie2 protein half-life in vivo also be 9 h, the rapid loss of Tie2 mRNA could not be directly responsible for the observed Tie2 protein loss. A differential



**Fig. 5** NF- $\kappa$ B activation contributes to the loss of Tie2 mRNA, not Tie2 protein. **a** In vitro study. NF- $\kappa$ B inhibitor BAY11-7082 (final concentration of 10  $\mu$ M) was added 30 min prior to stopping the flow after 48 h of flow-exposure of the HUVEC. Twenty-four hours after stopping the flow, the cells were lysed for mRNA analysis. The mRNA expression of Tie2, KLF2 and E-selectin was determined by quantitative RT-PCR and normalized to GAPDH expression. mRNA level of flow control group was set as 100%. Bars show mean  $\pm$  SD of three independent experiments. **b** In vivo study. Down regulation of Tie2 mRNA induced by hemorrhagic shock (HS) followed by resuscitation occurs in major organs. In the hemorrhagic shock model, mice were subjected to blood withdrawal until a mean arterial pressure of 30 mmHg was achieved for

90 min, after which they were resuscitated with Voluven as described in “Materials and Methods”. Wild type HS mice are expressed as fold change compared to control (untreated) group. Bars show mean  $\pm$  SD of 3. \* $P$  < 0.05, control group vs. hemorrhagic shock group. **c, d** In vivo study. Wild type mice were *i.v.* injected with NF- $\kappa$ B inhibitor BAY11-7082 (400  $\mu$ g/mouse) prior to TNF- $\alpha$  (200 ng/mouse) challenge, and sacrificed 2 h after orbital puncture TNF- $\alpha$  injection. Quantitation of expression of Tie2 mRNA and protein in the lungs of these mice was performed by quantitative RT-PCR (**c**) and ELISA (**d**), respectively. Bars represent mean  $\pm$  SD of three mice. \* $P$  < 0.05, vs. control group; # $P$  < 0.05; ns not significant

regulation is furthermore implied by the observation that, e.g., in the heart, mRNA loss was not followed by protein loss, and that the loss of Tie2 mRNA, but not Tie2 protein, could be rescued by blocking NF- $\kappa$ B activation prior to pro-inflammatory challenge.

TNF- $\alpha$  and IL-1 $\beta$  are two pro-inflammatory cytokines that are rapidly released upon LPS challenge [24]. While administration of LPS and either one of these cytokines induced loss of Tie2 mRNA and protein in vivo, this loss could not be mimicked in vitro. This outcome corroborates a previous study that used TNF- $\alpha$ -challenged microvascular endothelial cells [25]. Culturing freshly isolated cells in static conditions for several days clearly demonstrated an extensive loss of Tie2 mRNA, in HUVEC as well as in

primary mouse capillary endothelial cell isolates. Since this loss of Tie2 in vitro was associated with a condition of absence of flow, and since LPS-induced endotoxemia is associated with a decrease in blood pressure [3, 4], we hypothesized that a change in endothelial shear stress could be one of the major mechanisms in regulating loss of Tie2 mRNA upon LPS challenge in vivo. Indeed, by applying discontinuing flow, we could diminish Tie2 expression in vitro. Furthermore, in vivo administering of an NF- $\kappa$ B inhibitor prior to pro-inflammatory challenge, previously shown to prevent systemic hypotension [4], could rescue loss of Tie2 mRNA, thereby further substantiating a likely the role of haemodynamic changes in the loss of Tie2 mRNA.



A role for haemodynamic changes in regulating Tie2 expression was previously reported in ischemia-reperfusion studies [26], in which, analogous to our studies, cytokines are being released during the pathophysiological processes that is taking place [27]. Although TNF $\alpha$  and IL-1 $\beta$  have been associated with lowering of the blood pressure [28], it remains speculative what their contribution to the loss of Tie2 is relative to direct LPS effects. Our studies reported here do not formally show that changes in local blood flow are responsible for microvascular Tie2 downregulation after LPS administration. Measuring systemic blood pressure in shock models also does not formally prove this, as systemic blood pressure does not reflect the flow status in the microvasculature. In studies from the group of Bellomo, sheep were instrumented with transit time flow probes which were placed around the feeding arteries of the heart, gut, kidney and the intestine. The authors show that after i.v. bolus injection of *Escherichia coli* blood flow to the heart, gut, and kidney increased [29]. Contrary to these data, the Parikh group showed the opposite in male C57BL/6J mice injected with 10 mg/kg LPS. In this model renal perfusion decreased 18 h after LPS administration [30]. From this, one has to conclude that no consensus exists on what the effect of LPS administration on microvascular blood flow is. Our observation that also in hemorrhagic shock (Fig. 5b) and in renal ischemia/reperfusion (data not shown) Tie2 downregulation is prominent, indicates a general flow-related response. In future studies, we plan to dissect the effects of local hypoperfusion, inflammation and tissue hypoxia and the behavior of the smallest blood vessels in critically ill mice by renal microvascular flow measurements using micro bubble echocardiography and assess different flow responsive genes. Furthermore, in our studies we used a setup with continuous laminar flow, while flow characteristics along the vascular tree vary from pulsatile to steady and sometimes even interrupted flow [31].

Unpublished studies from our own laboratory on LPS challenge showed that Tie2 was not rescued in TNFR1

knock-out mice, implying that a direct LPS effect is a major contributor to Tie2 loss. The in vivo data, showing that blockade of NF- $\kappa$ B activity prior to TNF- $\alpha$  exposure could rescue loss of Tie2 mRNA, is an important starting point for future studies to determine the role of endothelial specific NF- $\kappa$ B activation in regulating flow dependent loss of Tie2 mRNA in vivo. We will address this by using a vascular drug targeting strategy to pharmacologically knock out endothelial specific NF- $\kappa$ B [32, 33]. The observation furthermore provides an important starting point for therapeutic studies aimed at inhibiting NF- $\kappa$ B prior to LPS administration. In summary (Supplementary Fig. 6), we demonstrated that LPS-induced loss of Tie2 mRNA is extensive and occurs in all organs studied, while the loss of Tie2 protein predominantly takes place in the lungs and kidneys, in particular in the capillaries. The likely origin of loss of Tie2 mRNA lies in a change in endothelial shear stress, with NF- $\kappa$ B signaling induced by diminished shear stress contributing significantly to this process. It is conceivable that therapy aimed at restoring Ang1 and diminishing Ang2 levels in sepsis are effectively combined with efforts to restore/rescue the expression of the Tie2 receptor. Our study suggests that interventions in sepsis patients aimed at normalizing diminished blood flow may be able to prevent down regulation of Tie2 and potentially counteract microvascular dysfunction and permeability in this devastating condition.

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**Conflicts of interest** None.

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