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The TNF-receptor subtype 2 mediates prothrombotic effects *in vivo*

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1 INTRODUCTION

1.1 TNFα – a proinflammatory cytokine

1.1.1 What is TNFα?

Tumor necrosis factor alpha (TNFα) is a proinflammatory cytokine and the prototypical member of the TNF protein superfamily. The polypeptide of approximately 17 kDa is produced by a variety of immune cells including T-lymphocytes, B-lymphocytes, natural killer cells and macrophages (1). TNFα plays a key regulatory role in several inflammatory processes, in host defense against bacterial infection as well in autoimmune disorders (2,3). TNFα was given its name as it was first discovered in 1975 with the cytotoxic ability to kill mouse fibrosarcoma cells (4).

1.1.2 TNF-receptors

The cellular response to TNFα is mediated through its interaction with two different membrane receptors (5-7). These receptor subtypes, namely TNF-receptor 1 (TNF-R1) and TNF-receptor 2 (TNF-R2), are of different size and are expressed to different levels in different cells (8). While TNF-R1, with its 55 kDa also referred to as p55-receptor, is widely expressed, expression of the 75-kDa TNF-R2 (p75-receptor) is mainly found on immune and endothelial cells (9). Whereas the extracellular domains of the two receptors are strikingly similar in structure, their intracellular domains appear to be unrelated (10).

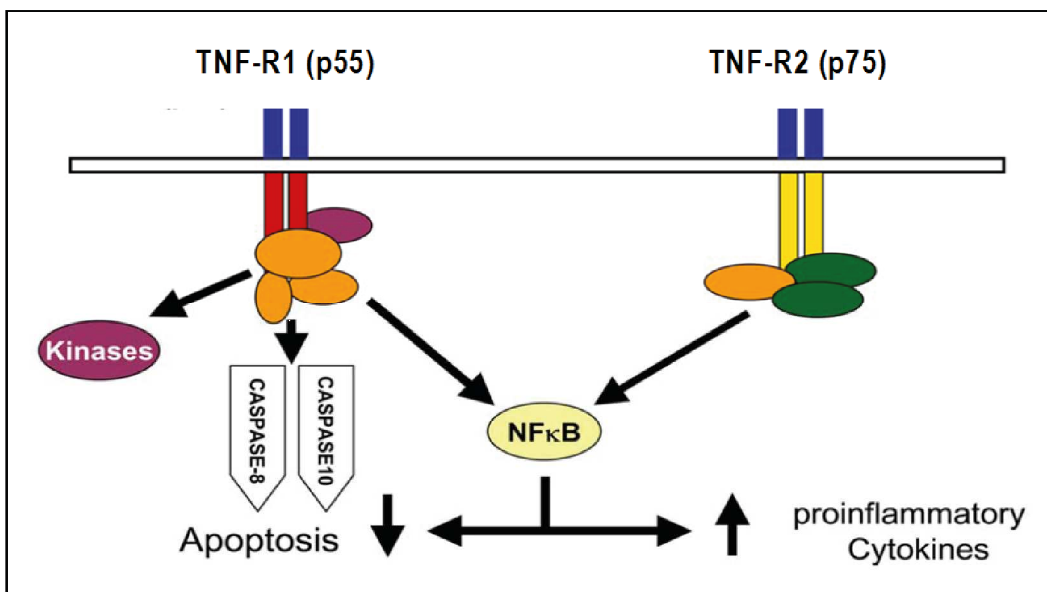
1.1.3 Signaling pathways

By interacting with its membrane receptors TNFα can activate distinctive signaling pathways in the cell (1,6). Since TNF-R1 and TNF-R2 are differentially expressed on cells and tissues TNFα can lead to a range of cellular responses and initiate both distinct and overlapping signal transduction pathways resulting in either cell death on one side or survival, differentiation, proliferation, and migration on the other side.

Among many intracellular factors involved in TNF-receptor signaling the families of the death domain homologues and of the TNF-receptor associated factors (TRAFs) should be mentioned (11-13). TRAF-signaling is described upon activation of both TNF-receptor subtypes and associated with the activation (TRAF2) or inactivation (TRAF1) of NF-κB and consecutive regulation of proinflammatory effects (14-16). TNF-R1 has an intracellular death domain, which can interact with the death domains of other cytosolic proteins such as TRADD (TNF-receptor associated death domain), FADD (Fas associated death domain) or RIP (receptor interacting protein). So, by activation of caspase-8 and other caspases TNFα similarly to the cell death signaling receptor Fas (CD95) induces apoptosis (17). Other cellular responses described upon activation of TNF-R1 include the activation of kinases such as PKC (proteinkinase C), JNK (c-Jun NH2-terminal kinase) and NIK (NF-κB induced kinase) and the activation of intracellular lipases (18). PKC is well described to activate

NADPH-oxidase in several tissues and therefore leading to the production of reactive oxygen species (ROS) causing oxidative stress (19,20).
Despite multiple variations and modulations in the downstream signaling upon activation of either of the two receptors the regulation of the transcription factor NF-κB plays a pivotal role in TNFα signaling. However, only recently different and even opposite receptor specific effects have been reported (21,22), concluding a much more complex downstream signaling than simply NF-κB activation and consecutive modulation of inflammatory processes. As most information regarding TNFα signaling is derived from TNF-R1, the role of TNF-R2 is likely underestimated.

Figure 1.1



Signaling pathways of TNFα through TNF-R1 and TNF-R2
TNFα can act through different TNF-receptor-subtypes. Activation of NF-κB and subsequent induction of proinflammatory cytokines play a central role and are associated with both TNF-receptor signaling pathways (Figure modified after *Holtmann and Neurath, Current Molecular Medicine 2004*).

1.1.4 TNFα's crucial role in inflammatory processes

Considering the downstream effects of the intracellular signaling pathways mentioned above it is easily conceivable why TNFα plays a central role in inflammatory diseases. While the original significance of TNFα in mediating necrosis in tumor cells could not lead to successful treatment options for cancer and remained limited to local therapy of limb soft tissue sarcoma (23), the role as a central player in inflammatory processes has become more and more evident. Finally as antibodies against TNFα have been used successfully in the treatment of several chronic inflammatory disorders including rheumatoid arthritis, ankylosing spondylitis, inflammatory bowel disease and psoriasis when other drugs failed,

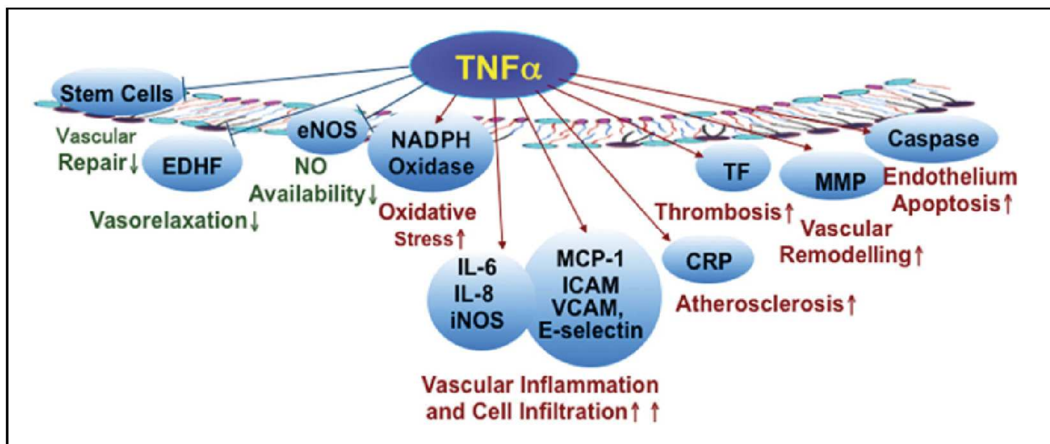
TNFα's role, not only in the development, but also in the maintenance of these diseases is undisputable (24,25). Different independent clinical studies show that inflammatory cytokines are not limited to the inflamed tissue but released to the systemic circulation leading to increased serum levels in these patients (26-30).

1.2 Inflammation and cardiovascular diseases

1.2.1 Links between inflammation and atherothrombotic diseases

It is well known that inflammation plays a critical role in the development of atherosclerosis and its fatal outcomes, such as myocardial infarction and ischemic stroke (31-33). Proinflammatory cytokines including IFNγ, TNFα, IL-1β or IL-4 facilitate monocyte and lymphocyte recruitment to the vascular wall via induction of endothelial cell adhesion molecules, such as VCAM-1, even before plaque formation and contribute to progression of atherosclerotic plaque size (34,35). Lastly, also the disruption or fracture of the fibrous cap can be promoted by CD40L, IL-1 and TNFα (36-38). The pivotal role of inflammatory cytokines in atherosclerosis is further sustained by studies showing fewer atherosclerotic lesions in mice lacking TNFα or IL-1 (39,40). Hence, it is not surprising that patients with atherosclerosis and atherothrombotic disorders have been found to have increased serum levels of proinflammatory cytokines such as TNFα and IL-6 (41).

Figure 1.2



Vascular effects of TNFα

TNFα signaling results in several cellular effects, which can promote the development of cardiovascular diseases (Figure modified after *Zhang et al., Clinical Science 2009*).

1.2.2 Vascular effects of TNFα

In endothelial cells, which form the inner layer of blood vessels, a variety of inflammatory responses upon TNFα stimulation are described leading to endothelial activation, which could facilitate atherothrombosis. These include upregulation of certain leukocyte recruiting molecules, such as E-selectin, intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) as well as release of chemokines including IL-8, MCP-1 and IL-10. Through activation of cyclooxygenase 2 (COX-2) and consecutive vasodilation by prostaglandin I_2 (prostacyclin, PGI_2) and increased vascular permeability even the classical clinical signs of inflammation “rubor”, “calor” and “tumor” can be observed as local effects of TNFα on endothelial cells (42). NF-κB translocation, increased production of ROS (reactive oxygen species) or influence on eNOS expression seem to be central steps in mediating the inflammatory effects (43-50). Figure 1.2 summarizes effects of TNFα on cells in the cardiovascular system.

1.3 Platelet activation and arterial thrombosis in inflammation

1.3.1 Activation of platelets and inflammation

The scenario would be simplified thinking endothelial function alone is involved in inflammatory processes. Platelets are not only the final actors in the event of arterial thrombosis but are also playing an important role in the pathophysiology of atherosclerotic lesion formation, which in most cases creates the breeding ground for the thrombotic events. It is well described that platelets represent an important link between inflammation, atherogenesis and thrombosis (51-57). Platelets store several mediators and growth factors that play a role in several inflammatory processes, including CD40 ligand (CD40L), cyclooxygenase (COX), epithelial neutrophil activating protein 78 (ENA-78), interleukin 1β, macrophage inflammatory protein 1α (MIP-1α), platelet derived growth factor (PDGF), platelet factor 4 (PF-4), p-selectin, chemokine ligand 5 (CCL5, earlier also known as RANTES) and transforming growth factor β (TGF-β) (58). Similar to leukocytes, platelets can roll on the endothelium, which takes place particularly when the endothelium is activated (59). Under shear stress p-selectin and its counter-receptors PSGL-1 or GPIb and vWF are mediating platelet interaction with the endothelium. In the absence of further stimulation these interactions remain transient and platelets return to the circulation after seconds or minutes (57). The intact endothelium has several mechanisms to prevent adhesion of platelets. These include next to the release of nitric oxide (NO), which earlier was referred to as endothelium derived relaxing factor (EDRF), prostacyclin (PGI_2) and adenosine (60-62) also the production of so called endothelium dependent hyperpolarizating factor (EDHF). The latter among other factors and substances include epoxieicosatrienoic acid (EET), which has recently characterized to affect platelets membrane potential through calcium dependent potassium channels (63,64). In the recent years our group could show that the inhibition of such endothelium released factors or potassium channels go along with increased platelet endothelium interaction and arterial thrombus formation *in vivo* (65-69).

In the case of activated endothelium or loss of endothelium derived platelet inhibiting factors respectively, it comes to firm platelet adhesion to the endothelium through integrins and other

adhesion molecules, which mediate platelet activation and induction of surface exposure of p-selectin and the release of proinflammatory cytokines stored in α-granules. Among the long and permanently expanding list of adhesion proteins, growth factors, chemokines and coagulation factors, which exert pleiotropic effects on blood and vascular cells, next to p-selectin (70) the chemokines or chemokine-like factors IL-1β and CD40L should be mentioned, as their ability to induce endothelial inflammation is well investigated (71,72). Subsequently, stimulated endothelial cells via several mechanisms promote atherosclerotic plaque formation and finally more platelet activation. Thus platelets activate and maintain the vicious circle of inflammation (57).

1.3.2 Arterial thrombus formation

While under normal conditions, platelets are at rest and flow through blood vessels without interacting with other cells, once a site of vessel damage is detected, passing platelets adhere to the endothelium within seconds of the injury occurring. A number of components of the exposed subendothelium, first of all collagen and von-Willebrand-factor (vWF), but also fibronectin, laminin, and thrombospondin directly or indirectly mediate platelet adhesion through a range of platelet surface membrane receptors (51,73,74). Upon binding of receptors with their ligands, platelets change shape to increase their surface and intracellular signals cause degranulation of storage vesicles. These contain more platelet activating substances such as ADP (adenosine diphosphate) and serotonin. In addition thromboxane A_2 is released, which also activates platelets. At the same time phospholipid membrane components on the platelets trigger the coagulation cascade resulting in the generation of insoluble fibrin, which provides stabilizing cross-links between platelets and exposed tissue (75,76). Platelet aggregation, which is following, together with the last stages of the coagulation cascade promote thrombus formation and growth eventually resulting in complete vessel occlusion.

1.3.3 Clinical observations

Clinical studies have pointed out several similar inflammatory and immunologic responses in atherosclerosis and rheumatoid arthritis, which is still representing the prototype of chronic inflammation (36,77). In both diseases a lot of common responses have been reported including increased markers of T- and B-cell-, macrophage- as well as mast cell activation and consequently also elevated serum levels of the proinflammatory cytokines TNFα and IL-6 (41). Considering the crucial role of these cytokines in the pathophysiology of cardiovascular diseases it is not surprising that patients with rheumatoid arthritis are at increased risk for cardiovascular morbidity and atherothrombotic events such as acute myocardial infarction (36). More than one study in these patients could show a not ignorable increase in cardiovascular events, which could not be explained by traditional cardiovascular risk factors (78-82). However, anticytokine therapy in these patients has been reported both to reduce as well as to induce thromboembolic complications (83,84).

1.3.4 TNFα and arterial thrombosis *in vivo*

Putting clinical data and *in vitro* effects of TNFα observed in endothelial cells together could lead to the assumption that TNFα in the vascular bed might shift the balance to a more

prothrombotic state and deteriorate thrombotic events. Surprisingly *Cambien et al.* could show in an elegant study that TNFα has antithrombotic activity *in vivo* after short term treatment, a setting found for instance in the acute phase of sepsis. This was explained by an increase in NO production in the vascular wall (85). However this effect was reversible after two hours and there is a lack of studies referring to long term effects of TNFα concerning arterial thrombosis *in vivo*.

2 OBJECTIVE OF THE STUDY

Considering an increased risk of atherothrombotic events in systemic inflammatory conditions with elevated serum levels of TNFα, but antithrombotic effects (albeit short term and reversible) in the only *in vivo* study for arterial thrombosis, the role of TNFα in this context remains unclear. Very limited data are available about the more long term effects and clinical data as well as *in vitro* effects on vascular cells rather support prothrombotic properties of the inflammatory cytokine. Moreover the underlying mechanisms specially referring to distinctive signaling through the different TNF-receptor subtypes are still to be elucidated.

Therefore the aim of this study was to investigate the following issues:

- Does TNFα increase arteriolar thrombosis and transient platelet-vessel wall-interaction *in vivo*?
- If so, what are the underlying mechanisms? Is it due to a direct effect on platelets or through endothelium-mediated mechanisms?
- What role do the TNF-receptor subtypes TNF-R1 and TNF-R2 play in this context?

To address these questions the dorsal skinfold chamber microcirculatory model was used for *in vivo* assessment of arteriolar thrombus formation and transient platelet-endothelium-interaction in wildtype and TNF-receptor deficient mice.

3 MATERIALS AND METHODS

3.1 Chemicals

Cell culture: Endothelial cell growth medium was purchased from Promocell (Heidelberg, Germany), fetal calf serum (FCS) and new born calf serum (NBCS) were from Biochrom (Berlin, Germany). Accutase was purchased from PAA (Cölbe, Germany) and Collagenase was from Roche (Basel, Switzerland). DMEM199 cell medium, EDTA, Penicillin-Streptomycin, Trypsin and Trypsin-EDTA solution were from Sigma-Aldrich (Taufkirchen, Germany).

Buffers and physiological solutions: $CaCl_2$, D-Glucose, Glycerine, Glycine, Hepes, KCl, KH_2PO_4, $MgCl_2$, Na_2HPO_4, NaCl and TRIS were purchased from Applichem (Darmstadt, Germany).

Chemicals and dyes: CFDA-SE was from Bachem AG (Bubendorf, Switzerland), FACS-lysing-solution was from BD (Franklin Lakes, NJ, USA), L-NAA was from Enzo Life Sciences (Lörrach, Germany), Prestained protein ladder was from Fermentas GmbH (St. Leon-Rot, Germany), Bromophenol blue was from Merck Chemicals Ltd. (Nottingham, UK), Nitrocellulose blocking membrane was from PEQLAB Biotechnologie GMBH (Erlangen, Germany), RLT buffer was from Qiagen (Hilden, Germany), Pefabloc was from Roche (Basel, Switzerland), Ilomedin was from Schering AG (Berlin, Germany). Murine leukemia virus reverse transcriptase was from Superscript; Invitrogen (Darmstadt, Germany), BCA reagens was from Thermo Scientific (Rockford, IL, USA). Bovine serum albumin (BSA), Acrylamid, Ammonium persulfate, Hydrogen peroxide, Luminol, Methanol, Paraformaldehyde, Sodium Dodecyl Sulfate (SDS), sodium-citrate, Temed and Tween were all purchased from Applichem (Darmstadt, Germany). Betamercaptoethanol, Cytochrom C, DAPI, Leupeptin, Na_3VO_4, $Na_4O_7P_2$, NaF, P-cumaric acid, Pepstatin, Poinceau-S solution, RIPA-Buffer, Superoxide dismutase (SOD), Triton, Ferric chloride and FITC-Dextran were all purchased from Sigma-Aldrich (Taufkirchen, Germany).

Cytokines and platelet agonists: Murine TNFα was from Chemicon International (Billerica, USA), human TNFα was from Reliatech GMBH (Wolfenbüttl, Germany), Thrombin Receptor Activator Peptide 6 (TRAP-6) was from Bachem AG (Bubendorf, Switzerland), collagen for platelet stimulation was from Nycomed GmbH (Konstanz, Germany) and ADP was purchased from Sigma-Aldrich (Taufkirchen, Germany).

Anaesthetics: Fentanyl was from CuraMED Pharma GmbH (Karlsruhe, Germany), Medetomidinhydrochloride was from Orion Pharma (Espoo, Finland), Midazolam was from Ratiopharm GmbH (Ulm, Germany) and Sodium-Pentobarbital was purchased from Merial GmbH (Halbergmoos, Germany).

Antibodies and primers: Anti-human p65 was from Santa Cruz Biotechnology (Santa Cruz, USA), Anti-mouse IgG alexa fluor 546 was from Invitrogen (Darmstadt, Germany), Anti-rabbit IgG was from Calbiochem/Merck Chemicals Ltd. (Nottingham, UK), Anti-mouse GR-1-FITC and Rat IgG Negative control-FITC were purchased from EuroBioSciences GmbH (Friesoythe, Germany), Anti-human TNF-R1 and Anti-human TNF-R2 were from Cell

Signaling Technology (Danvers, MA, USA), Anti-human CD142-FITC, Anti-human CD62P-RPE, Anti-mouse-TNF-R1-RPE, Anti-mouse-TNF-R2-RPE, Mouse IgG Negative control-FITC, Hamster IgG Negative control-RPE and Mouse IgG Negative control-RPE were all purchased from Abd Serotec (Oxford, UK). Predesigned GAPDH and PAI-1 TaqMan primers were purchased from Applied Biosystems (Carlsbad, California, USA).

3.2 Cell culture

To investigate signaling and mechanisms on endothelial cells Human Microvascular Endothelial Cells (HMEC), which is an immortalized cell line (86), as well as primary Human Umbilical Vein Endothlial Cells (HUVEC) were used.

Table 3.1 Cell media for HMEC

HMEC growth medium (10%)	HMEC starvation medium (1%)
DMEM-199	DMEM-199
+ Fetal calf serum (FCS) 10%	+ Fetal calf serum (FCS) 1%
+ Endothelial growth media 10%	+ Penicilline/Streptamycine 1%
+ Penicilline/Streptamycine 1%	pH 7.4
pH 7.4	

Table 3.2 Cell media for HUVEC

HUVEC growth medium (20%)	HUVEC starvation medium (1%)
DMEM-199	DMEM-199
+ New born calf serum (NBCS) 20%	+ New born calf serum (NBCS) 1%
+ Endothelial growth media 10%	+ Penicilline/Streptamycine 1%
+ Penicilline/Streptamycine 1%	pH 7.4
pH 7.4	

Table 3.3 Collagenase A solution for detaching umbilical vein endothelial cells

Collagenase A solution	
Collagenase type I (1700 U/mg)	1 g
PBS+	ad 1000 ml
The solution was passed through the filter Membrex 25 CA with a pore size of 0.2 µm for sterilisation.	

Table 3.4 Phosphate buffered saline (PBS)

	PBS+	**PBS–**
NaCl	8 g	8 g
KCl	0.20 g	0.20 g
Na_2HPO_4	1.15 g	1.42 g
KH_2PO4	0.20 g	0.20 g
$MgCl_2$	0.04 g	
$CaCl_2$	0.50 g	
Distilled H_2O	ad 1000 ml	ad 1000 ml
pH 7.4		

3.2.1 Isolation and cultivation of human umbilical vein endothelial cells

The human umbilical cords were provided by the obstetrical department of the Ludwig Maximilians University hospital of Munich. Written and informed consent was obtained from all patients. To isolate human umbilical vein endothelial cells (HUVEC) the vein in the umbilical cord was first rinsed with warm phosphate buffered saline supplemented with Ca^{2+} (PBS+, see table 3.4). To detach the endothelial cells from the vessel wall, the umbilical cord vein was incubated with 1 mg/ml collagenase A solution (table 3.3) in a humidified incubator for 10 minutes. The primary endothelial cells were then washed out of the vessel with HUVEC growth medium (table 3.2). Following collection of the cells, they were pelleted by centrifugation at 1200 rpm (Heraeus Megafuge 1.0R from Kendro, Langenseldbold, Germany) for 5 minutes at room temperature and subsequently washed with M199 medium. Finally, the purified primary endothelial cells were obtained in standard culture flasks in endothelial growth medium and left to grow at 37°C in a humidified incubator with 5% CO_2. When reaching confluency, the cells were passaged and maintained in HUVEC growth medium at 37 °C with 5% CO_2 in a humidified incubator. The primary HUVEC were used until the third passage only. Prior to the experiments cells were starved for 3 hours in HUVEC starvation medium (table 3.2). The investigation conforms with the principles outlined in the *Declaration of Helsinki.*

3.2.2 Cultivation of human microvascular endothelial cells

Human microvascular endothelial cells (HMEC) were provided by *Ades et al.* (86) and cultured in HMEC growth medium (table 3.1). The cells were maintained at 37 °C with 5% CO_2 in a humidified incubator. Prior to the experiments cells were starved for 24 hours in HMEC starvation medium (table 3.1).

3.2.3 Passaging of cells

Confluent cells were washed once with warm PBS- followed by incubation with trypsin-EDTA for approximately 2 minutes to allow the majority of the cells to detach from the cell culture dish. The enzymatic activity of trypsin was inhibited by application of the respective media containing at least 10% serum. Non attached cells were gently scraped off the dish with

a rubber scraper and the cell suspension diluted in the respective media followed by seeding onto new culture dishes.

3.3 Animals

All animal experiments were performed in Wildtype (WT)- or TNF-receptor deficient C57-BL/6 mice. WT mice were purchased from Charles River, Sulzfeld, Germany. TNF-R1-/- and TNF-R2-/- mice were originally obtained from the Jackson Laboratory (Bar Harbor, ME, USA), and TNF-R1-/-R2-/- mice were subsequently generated by cross-breeding of single TNF-receptor deficient mice. Animals were bred and kept at the animal department of the Institute of Physiology Munich, where they had a 12 hour light-dark cycle, were allowed to move freely in their cages and had free access to food and water. Ambient air temperature was 24 °C and air humidity 50%. All experiments were conducted in full accordance with the German animal protection law (TierSchG) paragraphs §1, §2 and §2a and approved by the government of Oberbayern. The investigation conforms with 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*).

3.4 *In vitro* and *ex vivo* experiments

3.4.1 Flow cytometry

Flow cytometry (FACS) is a technique to analyze several cell parameters simultaneously. By letting cells pass one at a time through a focused laser beam, the scattering of the light gives information about the cell size, so called forward scatter (FSC) and the internal complexity of a cell, such as the extent of granulation, called sideward scatter (SSC). In addition to scatter, a cytometer is also able to detect fluorescence. By incubating cells with fluorescent labeled antibodies the measured fluorescence can give relative information about the density of the respective antigen on the cell. Flow cytometry was used to assess the expression of p-selectin and tissue factor on the surface of cultured endothelial cells, as well as to detect the presence of TNF-R1 and TNF-R2 on murine neutrophils.

3.4.1.1 Assessment of surface molecules on endothelial cells

HMEC or HUVEC were grown to sub-confluence, serum-starved for 24 hours or 3 hours respectively and incubated with sham or TNFα (5 ng/ml) for 30 minutes or 4 hours. After incubation cells were washed with PBS- and detached using Accutase. They were then collected, spun down (3000 rcf, 2 min) and resuspended in PBS+. After 10 minutes of fixation with 5% formaldehyde in PBS+, cells were washed again and then incubated using fluorescent-labeled antibodies (see table 3.5) or corresponding negative controls for

30 minutes at room temperature and darkness. They were then washed again and resuspended in PBS+ before fluorescence intensity was measured using a FACSCanto II flow cytometer (Becton Dickinson, USA). Data were analyzed using FACSDiva software (Becton Dickinson) and mean or median fluorescence, depending whether values were normally distributed or not, was calculated.

Table 3.5 Antibodies used for FACS analysis of endothelial surface molecules

Antibody	Host	Dilution	Conjugate	Company
Anti-human-CD62P (p-selectin)	Mouse (MC)	1:20	FITC	Abd Serotec, Oxford, UK
Anti-human-CD142 (tissue factor)	Mouse (MC)	1:20	RPE	Abd Serotec, Oxford, UK

3.4.1.2 Assessment of TNF-R1 and TNF-R2 on murine neutrophils

Mice were anesthetized and whole blood was drawn with a 27G syringe by cardiac puncture. To avoid coagulation 3.13% sodium citrate was added 1:10. Whole blood was then incubated with fluorescent-labeled antibodies against TNF-R1 and TNF-R2 (table 3.6) or respective isotype control for 20 minutes at room temperature in the dark. Erythrocytes were lysed and white blood cells were fixed by adding 2 ml of FACS lysing solution for 10 minutes. Cells were spun down (700 rcf, 5 min) and washed with PBS+ before being analyzed on a FACSCanto II flow cytometer. Neutrophil identification was done by forward and sideward scatter and verified by staining with a FITC-labeled GR-1 (granulocyte antigen 1) antibody.

Table 3.6 Antibodies used for FACS analysis of TNF-receptors on neutrophils

Antibody	Host	Dilution	Conjugate	Company
Anti-mouse-TNFR1	Hamster (MC)	1:10	RPE	Abd Serotec, Oxford, UK
Anti-mouse-TNF-R2	Hamster (MC)	1:10	RPE	Abd Serotec, Oxford, UK
Anti-mouse-GR1	Rat (MC)	1:10	FITC	EuroBioSciences Friesoythe, Germany

3.4.2 Immunofluorescent staining

The principle is to detect an antigen with a primary antibody and to visualize it by staining with a so called secondary fluorescent-labeled antibody against the Fc-fragment of the primary antibody. This technique was used to localize the p65-subunit of NF-κB within endothelial cells.

3.4.2.1 Assessment of NF-κB translocation

HUVEC were grown to sub-confluence in 8-well μ-slides for fluorescence microscopy (Ibidi GmbH, Martinsried, Germany), serum-starved for 3 hours and incubated with sham or TNFα (5 ng/ml, 4 hours). After incubation the cells were washed with PBS+ and fixed in 2% formaldehyde for 12 minutes. They were permeabilised by a 2 minute incubation with 0.2% Triton and unspecific binding of the antibody was blocked with 5% BSA for 1 hour. Cells were then incubated with an anti-p65-antibody (first antibody) for 45 minutes and DAPI (4′,6-Diamidin-2-phenylindol) to stain the nucleus of the cells, followed by incubation with a secondary fluorescent antibody for 30 minutes. After washing several times with 5% BSA the cells were analyzed by fluorescent imaging using a LSM II confocal microscope (Zeiss, Germany).

Table 3.7 Antibodies used to detect the p65-subunit of NF-κB in endothelial cells

	Antibody	Host	Dilution	Company
Primary antibody	Anti-human-p65 (monoclonal)	Mouse	1:200	Santa Cruz, Heidelberg, Germany
Secondary antibody	Anti-mouse IgG alexa fluor 546	Goat	1:200	Invitrogen, Eugene, Oregon, USA

For quantification of the distribution of p65 between cellular cytosol and nucleus, cells were first identified in the microscopic transmission mode where five pairs of regions of interest (ROI) were placed in every image, of which one ROI was placed in the cytosolic compartment, the corresponding one in the nucleus of a cell. The size of a ROI was defined at 5 μm^2. After placement of ROIs the microscopic mode was switched to fluorescence within the very same image. The mean relative fluorescence in every ROI was then measured and the value of the ROI within the nucleus divided by the value of the ROI in the corresponding cytosolic compartment. For every condition of an experiment 10 different images were analyzed and the mean value of the ratios counted as one experiment.

3.4.3 Western blotting

Western blotting or immunoblotting is an analytical technique used to detect specific proteins in a given sample of cell or tissue lysate. Gel electrophoresis is used to separate denaturated proteins by the length of the polypeptide. The proteins are then transferred to a nitrocellulose membrane, where they can be detected using antibodies to the specific target protein. Western blotting in this study was used to see whether or which TNF-receptor subtypes are expressed in cultured endothelial cells.

3.4.3.1 Cell lysates

To obtain protein lysates the cells were washed once with PBS+ and immediately put on ice and lysed for 10 minutes using ice-cold lysis buffer (table 3.8). The cells were then scraped off the dish with a rubber scraper and passed repeatedly through a 29G needle. The debris was removed from the lysate by centrifugation at 10,000 g for 10 minutes and at a temperature of

4 °C. The protein concentration for the remaining solution was determined using BCA (bicinchoninic acid) protein assay reagent kit according to the manufacturer's protocol. The protein lysates were either directly analyzed or kept frozen at -20 °C until further handling.

Table 3.8 Buffer to lyse tissue culture cells grown in monolayer

Lysis buffer	
KH_2PO_4	20 mM
EDTA (Ethylenediaminetetraacetic acid)	1 mM
Pefablock	1 mM
Leupeptin	1 µM
Pepstatin	1 µM
NaF	50 mM
$Na_4O_7P_2$	40 mM
Na_3VO_4	2 mM
pH 7.3	
Before usage of the buffer, Triton X-100 was added to a final concentration of 1%.	

3.4.3.2 Assessment of protein concentration

Protein concentration was measured using the BCA (bichinconinic acid) protein assay reagent kit and is based on the biuret reaction, where Cu^{2+} is reduced to Cu^{1+} by proteins in an alkaline medium. Addition of bichinconinic acid leads to a purple-coloured end product which can be detected colometrically at 562 nm. This end product is formed by the chelation of two molecules of BCA with one Cu^{1+} and is almost linear with increasing protein concentrations. 10 µl of protein lysate was added to a well in a 96 well plate formate prior to application of 200 µl BCA solution (mixed according to the supplier´s protocol). Every sample was measured in triplicates with SpectraFluor (Tecan, Crailsheim, Germany). A dilution series with BSA (2 mg/ml in distilled water) was run alongside with the protein lysates for the establishment of a standard curve. The mixture was incubated for 30 minutes at 37 °C prior to measurement at 550 nm.
The protein concentration was calculated according to the following formula:

$$f(x) = m\,x + b$$

where x has to be dissolved to reveal the protein amount in µg. m is the gradient of the standard curve, b is the axis intercept of the standard curve and $f(x)$ is the absorbance data obtained.

3.4.3.3 SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis)

Proteins were thawed on ice and 25-40 µg were mixed with 1x loading dye, denaturated at 100 °C for 5 minutes before separated on a 10% SDS-PAGE consisting of a 4% stacking gel and a 10% separation gel covered in 1x running buffer (composition of gel and buffers are listed in table 3.9). As a reference for protein size a prestained protein ladder solution was separated together with the proteins. The current was set to 35 mA and the proteins were left to run until the loading dye front was approximately 1 cm from the edge of the gel.

Table 3.9 Gel and buffers for electrophoresis

Separation gel (10%):	
Distilled H20	20 ml
Tris 1.5 M pH 8.8	12.5 ml
Sodium dodecyl sulfate (SDS) 10%	0.5 ml
Acrylamide/Bisacrylamide 30% / 0.8% (w/v)	16.6 ml
Ammoniumpersulfate 10% (w/v)	0.25 ml
Temed	0.025 ml

Stacking gel (4%):	
Distilled H_2O	6 ml
Tris 0.5 M pH 8.8	2.5 ml
SDS 10% (w/v)	0.1 ml
Acrylamide/Bisacrylamide 30% / 0.8% (w/v)	1.3 ml
Ammoniumpersulfate 10% (w/v)	0.05 ml
Temed	0.01 ml

For both gel solutions, H_2O, Tris, Acrylamide/Bisacrylamide were mixed and degassed before addition of SDS, Ammoniumpersulfate and TEMED. Upon pouring the separation gel, a layer with butanol was added on top of the gel to prevent it from drying while polymerising. The butanol layer was meticulously washed off with distilled water before pouring the stacking gel on top of the separation gel.

Loading dye (4x)	
Tris HCl pH 6.8	0.25 M
SDS	8%
Glycerine	40%
Bromphenolblue	0.02%
Mercaptoethanol	400 mM

Running buffer (5x)	
Tris Base	123.8 mM
Glycine	959.1 mM
SDS	17.3 mM

3.4.3.4 Blotting

The separated proteins were then transferred onto a nitrocellulose membrane by electrophoresis using semi-dry blotting. For this, the nitrocellulose membranes together with filter papers were soaked in transfer buffer (table 3.10). The membrane and filters were stacked as a "sandwich" in the following order: filter paper, membrane, gel and filter paper. To check for equal amounts of protein and successful blotting, the membrane was incubated with Poinceau S solution (Sigma-Aldrich, Taufkirchen, Germany) which was removed by washing before further handling. To prevent unspecific binding of the antibodies the

membrane was blocked with blocking buffer for 30 minutes prior to a 1 hour incubation with the primary antibody diluted in the respective blocking buffer at 37 °C or at 4 °C over night. Unbound antibody was removed by washing 3 times for 5 minutes with TBS/T (table 3.10). The membrane was then incubated for 1 hour at room temperature or at 4 °C over night with a secondary antibody conjugated with horseradish peroxidase. After washing 3 times as described above, the enzymatic activity was detected upon addition of chemiluminescence detection kit for horseradish peroxidase according to the supplier’s protocol with a bioluminescence detection system attached to a digital camera (Sequoia).

Table 3.10 Buffers for blotting

Transfer buffer	
Glycine	39 mM
Tris Base	48 mM
SDS	0.037%
Methanol	10%

Tris buffered saline (TBS) (10x)	
Tris	24.2 g
NaCl	58.44 g
Distilled H_2O	ad 1000 ml

Tris buffered saline with Tween (TBS/T)	
TBS	1 x
Tween 20	0.1%
pH 7.6	

Blocking buffer	
Bovine serum albumine (BSA)	5% in TBS/T

3.4.4 Quantitative real-time PCR

Real-time polymerase chain reaction (RT-PCR), also called *quantitative real time polymerase chain reaction* is a method based on PCR, which is used to amplify and simultaneously quantify a targeted DNA molecule. If combined with reverse transcription real-time PCR can be used to quantify messenger RNA (mRNA) in cells or tissues. RT-PCR was used to assess mRNA-amount of plasminogen-activator-inhibitor 1 (PAI-1) in cultured endothelial cells.

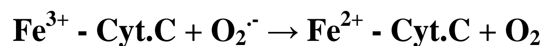
3.4.4.1 PAI-1 transcription in endothelial cells

HMEC were grown to sub-confluence, serum-starved for 24 hours and incubated with sham or TNFα (5 ng/ml) for 30 minutes or 4 hours. After incubation cells were washed with PBS- and lysed quickly in 300 µl/3-cm-dish RLT buffer (Qiagen, Hilden, Germany) containing 1% beta-mercaptoethanol to eliminate ribonuclease released during cell lysis. RNA isolation was

performed immediately. Total RNA was isolated by RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol including digestion of DNA during the procedure by RNase-Free DNase Set (Qiagen). Purity was checked by RNA-gel electrophoresis and absorbance at 230 to 320 nm. For RT-PCR, 2 µg of isolated total RNA underwent random primed reverse transcription using a modified murine leukemia virus reverse transcriptase (Superscript; Life Technologies, Germany). In parallel, 2 µg aliquots were processed without reverse transcription to control for contaminating genomic DNA.
Real-time PCR was performed on a TaqMan ABI 7700 sequence detection system (Applied Biosystems, Darmstadt, Germany) using heat-activated *Taq*DNA polymerase. GAPDH was used as reference housekeeping gene. All controls consisting of distilled H_2O were negative for target and housekeeper. Quantification was done by the Pfaffl method (87). Commercially available pre-developed TaqMan reagents were used for the human target gene PAI-1 (Applied Biosystems) and the endogenous control gene GAPDH (Applied Biosystems). The data shown in the figures are normalized to GAPDH. All measurements were performed in duplicates.

3.4.5 Cytochrome C assay

Cytochrome C is a heme protein and part of the mitochondrial electron transport chain. The ferricytochrome C can be directly reduced by superoxide ($O_2^{\cdot-}$) to ferrocytochrome C.



$$\mathbf{Fe^{3+} - Cyt.C + O_2^{\cdot-} \rightarrow Fe^{2+} - Cyt.C + O_2}$$

This reduction leads to an increase in the absorbance spectrum of cytochrome C at 550 nm, which can be photometrically monitored. To detect the superoxide dependent part of cytochrome C reduction the difference in absorbance between samples incubated with or without superoxide dismutase has to be calculated. From Lamber-Beers-Law $E = \varepsilon\ c\ d$ the concentration of produced superoxides can be determined (E = extinction; ε = extinction coefficient = 21 mmol/l cm^{-1} for cytochrome C at 550 nm; c = concentration; d = layer thickness). The cytochrome C reduction method was used to measure superoxide release of cultured endothelial cells.

3.4.5.1 Assessment of endothelial superoxide release

HUVEC were grown to sub-confluence and serum-starved for 3 hours before the experiments. To prevent modulating effects of NO all measurements were performed in the presence of the NO synthase inhibitor L-NAA (N^G-Amino-L-argninine; 30 µmol/l), which was added to the cell medium 30 minutes before starting TNFα (5 ng/ml) stimulation. After 4 hours of treatment with TNFα or sham endothelial cells were incubated in colorless DMEM medium containing 40 µmol/l cytochrome C with or without SOD (200 units/ml). After 30 minutes the supernatant was removed, and the reduction of cytochrome C was spectometrally measured at 550 nm (Ultrospec 2000, Amersham Pharmacia Biotech). Superoxide release was calculated as described above.

3.4.6 Platelet aggregation studies

Platelet aggregation was measured in human or mouse PRP (platelet rich plasma) using the turbidimetric method described by Born (88). The optical aggregometer is a fixed wavelength spectrophotometer, where a beam of infrared light shines through the test sample (PRP) and another shines through the reference sample, which contains PPP (platelet poor plasma). When the PRP is stirred and stimulated by platelet agonists platelets start to form increasingly larger aggregates and the PRP begins to clear, allowing more light to pass through. The increase in light transmittance is directly proportional to the amount of aggregation and is amplified and recorded digitally.

3.4.6.1 Platelet aggregation in human PRP

Blood was drawn from the cubital vein of healthy human volunteers. Written informed consent was obtained from all blood donors. To prevent blood from clotting syringes were containing 10% of 0.11 mol/l sodium citrate. Human PRP was obtained by centrifugation of the whole citrated blood at 340 rcf for 15 minutes. PRP was incubated with TNFα (5 ng/ml) or sham for different times. PPP was obtained by centrifugation of PRP for 5 minutes at 3000 rcf. Aggregation was started by adding ADP, collagen or TRAP to PRP under continuous stirring at 1000 rpm at 37 °C and measured by using a 2-channel-aggregometer (ChronoLog 490-2D, Havertown, PA, US). Percentage of maximal platelet aggregation was analyzed 6 minutes after addition of the stimulator using Aggrolink software (ChronoLog, USA).

3.4.6.2 Platelet aggregation in murine PRP

For platelet aggregation in mouse PRP the protocol was analogue to the procedure for platelet aggregation in human PRP with minor modifications. Whole blood was drawn by cardiac puncture from anesthetized mice with 27G syringes containing 1:10 sodium citrate to prevent coagulation. Murine whole blood was diluted with platelet resuspension buffer (table 3.12) to increase the volume and centrifugation was done at 130 rcf for 10 minutes. Platelet aggregation was then performed as described above.

3.5 *In vivo* experiments

3.5.1 Anaesthesia

Animal surgical procedures were performed under short term anesthesia. As anaesthetics the drugs listed in table 3.11 were used. Anaesthetics were diluted in saline and administered intraperitoneally. After the experiments the animals were killed by injection of an overdose (2 g/kg) of sodium pentobarbital (Merial GmbH, Halbergmoos, Germany).

Table 3.11 Anaesthetics used for surgical procedures in mice

Anaesthetic	Dosis	Company
Midazolam	3 mg/kg	Ratiopharm GmbH, Ulm, Germany
Fentanyl	0.03 mg/kg	CuraMED Pharma GmbH, Karlsruhe, Germany,
Medetomidinhydrochloride	0.3 mg/kg	Pfizer, Berlin, Germany; produced by Orion Pharma, Espoo, Finland

3.5.2 Dorsal skinfold chamber implantation

The dorsal skinfold chamber, which was developed at the Institute of Surgical Research, LMU Munich, Germany, is a well established model for microcirculation and already used in several studies in mice and other rodents (89-91).
After the animals were anaesthetized as described above the hair on the back was removed by a hair clipper (Aesculap) and chemical depilation (Pilcamed, Schwarzkopf). Eyes were protected by application of Dexpanthenol ointment (Roche Germany, Grenzach-Whylen, Germany). After disinfection with alcohol 70% subcutaneous vessels were visualized diaphanoscopically and an extra manufactured chamber made up of two inversed plates of titanium was implanted. The chamber and the surgical instruments were sterilized prior to the surgical procedure. In the chamber a circular window is building the frame for an extra cut circular coverslip (ø 11.7 mm, Menzel, Braunschweig), which covers a circular area where the skin was removed on one side in a way that the skin of the opposite side is attached via adhesion and its blood vessels can be observed macroscopically and microscopically (see figure 3.1). All experiments were performed at a minimum of 24 hours after chamber implantation. Animals showing abnormal blood flow within the vessels of the dorsal skinfold chamber arterioles were excluded from the experiments.

3.5.3 Carotid catheterization

Animals with an intact microcirculation underwent carotid artery catheterization, which was needed for application of drugs or injection of isolated platelets to investigate platelet-vessel wall-interaction.
The surgical procedure of catheterization was performed under the same short time anaesthesia protocol as described above. The right carotid artery was dissected from connective tissue and a 20 cm polyethylene tube (0.28 mm ID, 0.61mm OD; SIMS Portex, U.K.), which was prior thinned by mechanic elongation, was introduced in the common carotid. The tube was fixed to the artery and the subcutaneous tissue by a nonabsorbable surgical silk suture (5-0, Fine Science Tools, Heidelberg, Germany), placed subcutaneously and led out of the body on the back. The tube was flushed by saline, closed at the distal end and fixed to the dorsal skinfold chamber to avoid manipulation by the animals.

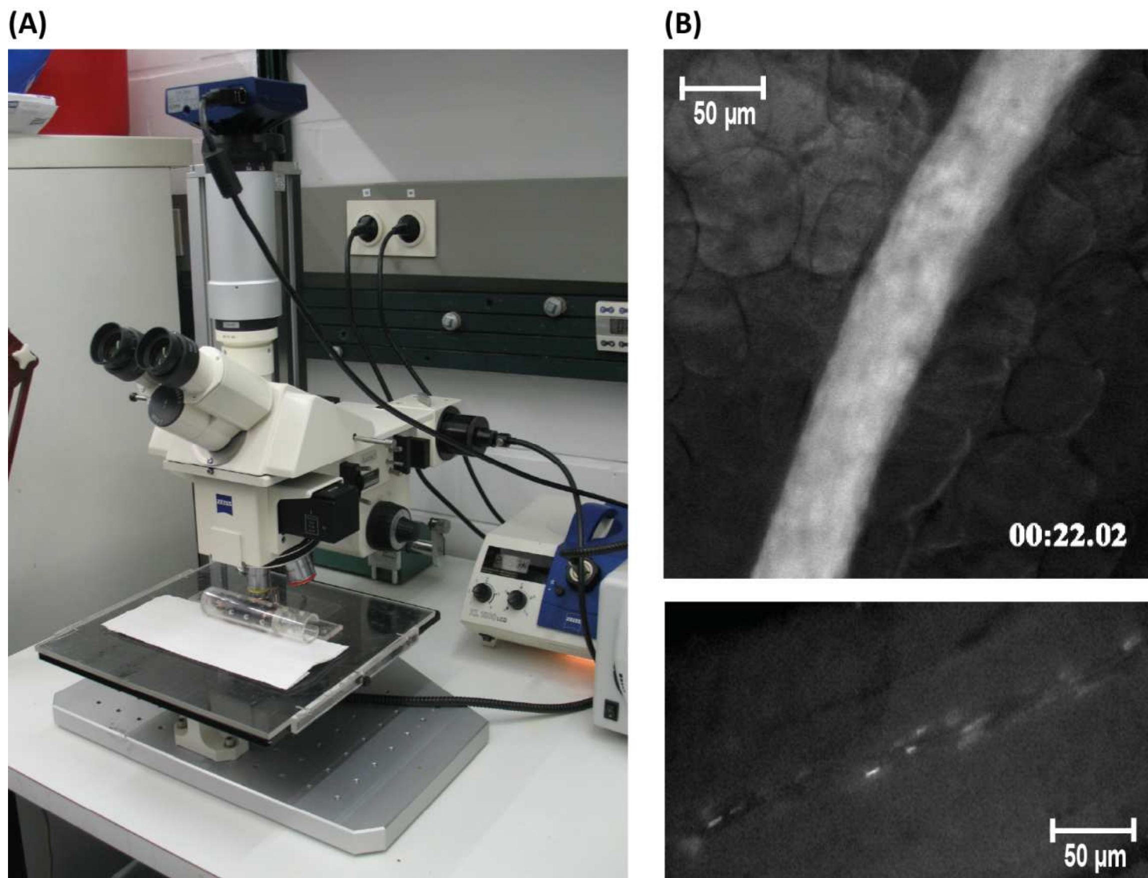
Figure 3.1

Dorsal skinfold chamber mouse model
The dorsal skinfold chamber is made up of two inversed plates of titanium, where a circular window is building the frame for an extra cut circular coverslip (the paper clip is shown as a size comparison) through which the microcirculation can be observed. We used this model to investigate arterial thrombosis and platelet-endothelium-interaction *in vivo*. The right upper panel shows a mouse carrying the implantation.

3.5.4 Intravital microscopy

Intravital fluorescence microscopy was performed using a modified microscope (Zeiss Axiotech Vario, Germany, figure 3.2). For the experiments mice were positioned in special plexiglas tubes, where the dorsal skinfold chamber can be fixed on the table not allowing mice to maneuver, which is required for the experiments. Fluorescence illumination was provided by an X-Cite® 120Q microscope light source system (Exfo life sciences division, Ontario, Canada) and images were recorded with a digital camera (AxioCam HSm, Carl Zeiss Germany). The exposure time for intravital recordings of blood flow or platelet-endothelium-interaction was 60 ms.

Figure 3.2



Intravital microscopy of the microcirculation in the dorsal skin
(A) A specially modified microscope linked to a digital camera was used for intravital imaging. Mice were positioned in a Plexiglas tube on the table in a way they were not allowed to maneuver the dorsal skinfold chamber during the experiments. **(B)** Fluorescent dyes allow visualization of blood flow (upper panel) or blood cells (e.g. platelets as shown in the lower panel) in the vessels.

3.5.5 *In vivo* TNFα-stimulation

For all intravital experiments TNFα was administered via the carotid catheter in a dose which was calculated to match serum concentrations of 5 ng/ml, a dose that caused effects *in vitro* and was previously used in studies of similar design (85).

3.5.6 Intravital assessment of arterial thrombosis

Intravital thrombotic vessel occlusion time was assessed in arterioles of WT- or TNF-receptor deficient mice in the dorsal skinfold chamber model. For induction of intraarterial thrombosis, the ferric chloride superfusion method was used (65,69). Before the experiments blood vessel flow was digitally recorded and regular blood flow was confirmed for all analyzed arterioles.

To visualize vessel lumina before vessel injury 50 µl of a 5% fluoresceine-isothiocyanate-labeled dextran solution (FITC-Dextran, MW 150,000; excitation maximum λ = 490 nm, emission maximum λ = 520 nm) was infused via the carotid catheter. This way formation of thrombi was apparent as sparing in the fluorescent-labeled plasma (see figure 3.2, panel B and also figure 4.7, panel B). Injury to the vascular wall was then performed by application of 30 µl of a ferric chloride solution (25 mmol/l) onto arterioles, using a standardized protocol. Movies were recorded until blood flow ceased or for at least 6 minutes if no vessel occlusion occurred.

3.5.7 Mouse platelet isolation and staining

For observation of *in vivo* platelet-endothelium-interaction by intravital microscopy platelets from donor mice were isolated and fluorescently labeled. Whole blood was drawn from anesthetized mice by cardiac puncture. To prevent blood from clotting syringes were containing 10% of sodium citrate. The citrated whole blood was spun at 130 rcf and the obtained PRP was incubated with the fluorescent dye CFDA-SE (5-Carboxyfluorescein diacetate; 17 µmol/l; excitation maximum λ = 492 nm, emission maximum λ = 517 nm) at dark for 30 minutes. Labeled platelets were then spun at 340 rcf and resuspended in a buffered calcium free physiologic solution (table 3.12). For centrifugation Iloprost (10 ng/ml) was added to prevent platelet activation. The ability of the isolated and stained platelets to aggregate was tested by platelet aggregometry (see 3.4.6).

Table 3.12 Buffer for platelet resuspension

Calcium free physiologic solution for platelet resuspension	
NaCl	4,03 g (138 mM)
KCl	0,1 g (2,7 mM)
$NaHCO_3$	0,5 g (12 mM)
NaH_2PO_4	0,03 g (0,4 mM)
$MgCl_2$ x $6H_2O$	0,1 g (0.49 mM)
D-Glucose	0,45 g (5 mM)
Hepes	0,6 g (5 mM)
Ad 500 ml distilled water	
pH 7.35	

3.5.8 Intravital analysis of platelet-vessel wall-interaction

For intravital studies of transient platelet interaction with the intact vessel wall isolated and fluorescent-stained murine platelets were injected via the carotid artery catheter and observed in the dorsal skinfold chamber model. Movie sequences of 30 seconds in 4-6 vessel segments in each animal were recorded and analyzed using AxioVision Software (Carl Zeiss, Germany). Vessels with abnormal flow were excluded from analysis. Velocities of single platelets were calculated with the formula $v = \Delta x / \Delta t$ being Δx the assessed length of the platelet trace in single images and Δt the exposure time of each single picture. Platelet-vessel wall-interaction (PVWI) was expressed in frequency histograms consisting of all platelet

velocities analyzed. Histograms were normalized to the maximum platelet speed within a vessel to exclude biasing influences of altered blood flow velocities between different arterioles. As a consequence a rightward shift in platelet velocity distribution within a histogram expresses less PVWI, whereas a leftward shift signalises increased PVWI at the arteriolar wall. Platelets with less than 5% of the velocity of the fastest platelets were defined as *rolling platelets* (see figure 3.2, panel B and also figure 4.8). A platelet was considered as firmly adherent when not moving for at least 10 seconds.

3.6 Statistical analysis

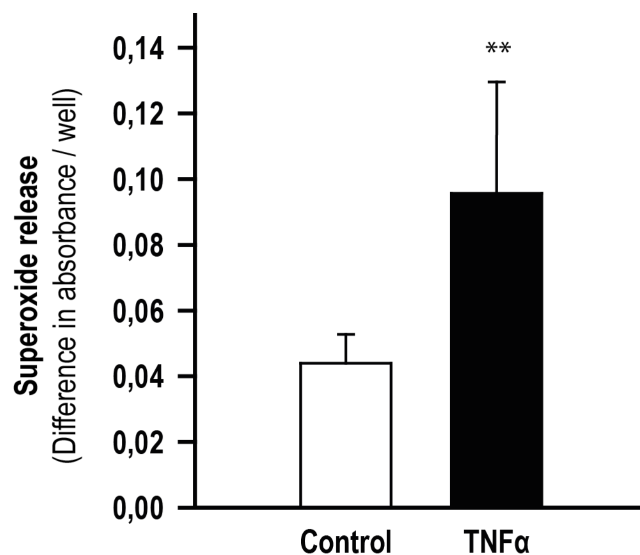
All statistical analyses were performed using Sigma Stat version 3.5. With normally distributed data, the Student's t-test was used to compare two groups while the one-way ANOVA with Dunn's correction was used for multiple comparisons. The Mann-Whitney Rank sum test was performed when comparing two groups which were not normally distributed, whereas comparisons between several groups not exhibiting normal distribution was achieved by analyzing the data with the analysis of variance on ranks. All data are presented as means +/- SEM (standard error of the mean). Differences were considered significant when the error probability level was $P<0.05$.

4 RESULTS

4.1 Superoxide formation in endothelial cells

To confirm previously described effects of TNFα on the cellular redox system the release of superoxide ($O_2^{\cdot-}$) was measured using the cytochrom C assay in HUVEC. The photometrically recorded SOD (superoxide dismutase) sensitive fraction of cytochrome C (expressed as difference in absorbance) depicts the production of $O_2^{\cdot-}$ and was 0.044 +/-0.01 /well in untreated cells, whereas it was significantly elevated to 0.099 +/-0.03 /well in cells stimulated with TNFα for 1 hour in a dose of 5 ng/ml (n=15, P< 0.01 vs. control; figure 4.1).

Figure 4.1



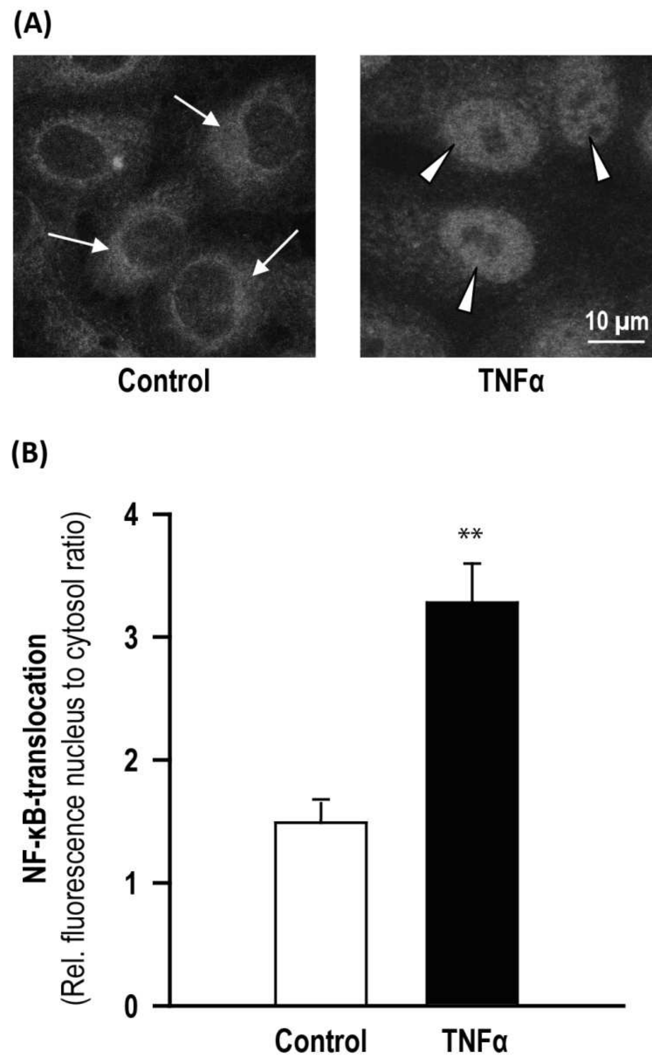
Superoxide ($O_2^{\cdot-}$) formation in cultured endothelial cells
$O_2^{\cdot-}$ formation was assessed by measuring the SOD (superoxide dismutase) sensitive fraction of cytochrome C reduction in HUVEC and was significantly increased when cells were treated with TNFα (5ng/ml, 1h).
** P<0.01 vs. control (n=15)

4.2 Translocation of the p65-subunit of NF-κB

ROS such as $O_2^{\cdot-}$ can be involved in several pathways of intracellular signaling and are well known to activate the transcription factor NF-κB. Therefore the translocation of the p65-subunit of NF-κB to the nucleus was assessed upon TNFα stimulation in cultured endothelial cells using immunofluorescence imaging. TNFα treatment (5 ng/ml; 1 h) of HUVEC resulted

in threefold increase in the ratio of relative fluorescence between nucleus and cytosol (3.28 +/-0.28) in comparison to controls, where it was 1.49 +/-0.16 (n=15, P<0.01 vs. control; figure 4.2).

Figure 4.2

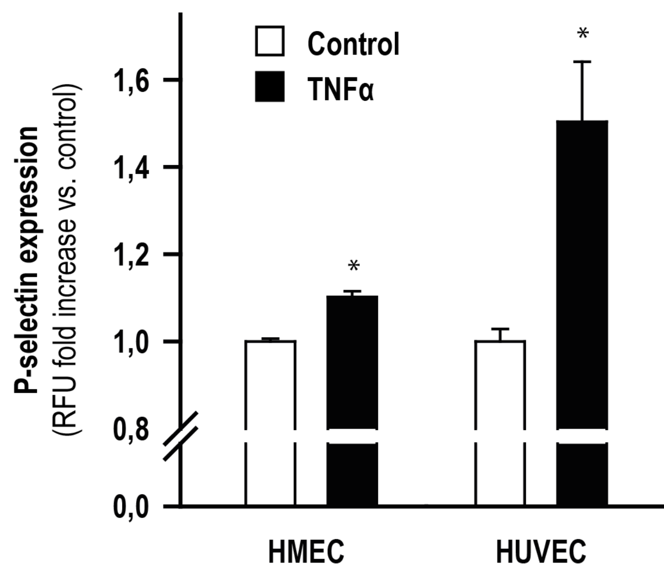


Translocation of the p65-subunit of NF-κB in cultured endothelial cells
The localization of the p65-subunit of the transcription factor NF-κB was analyzed in HUVEC by immunofluorescence imaging. **(A)** The immunofluorescent staining shows p65 localized mostly in the cytosol when cells were sham treated (left image, arrows), while a translocation to the nucleus could be observed in HUVEC stimulated with TNFα (right image, arrowheads). **(B)** Quantitative analysis shows an increased nuclear to cytosol ratio of p65-fluorescence in cells treated with TNFα (5ng/ml, 1h).
** P<0.01 vs. control (n=15)

4.3 Endothelial adhesion molecule expression

Since the transcription factor NF-κB can upregulate several cell surface membrane molecules it was investigated whether and at what time TNFα leads to an up-regulation of the adhesion molecule p-selectin (CD62P), which is described to play a critical role in mediating platelet-endothelium-interaction. The expression of p-selectin was measured on the cell membrane of HMEC and HUVEC by flow cytometric analysis. While p-selectin was barely detectable on the surface membrane of either non stimulated HMEC or only 30 minutes after stimulation with TNFα (5 ng/ml), the adhesion molecule was significantly upregulated in HMEC stimulated for 4 hours, where the mean of the relative fluorescence was elevated by 10.2 +/-1.3 % compared to non stimulated cells ($P<0.05$, n=9; figure 4.3). To see whether TNFα leads to an increase in adhesion molecules also in primary endothelial cells, p-selectin surface expression was assessed in HUVEC. The effect of TNFα in these cells was much more striking, as the mean in the relative fluorescence was 50.4 +/-13.7 % higher than in untreated cells ($P<0.05$, n=5; figure 4.3).

Figure 4.3

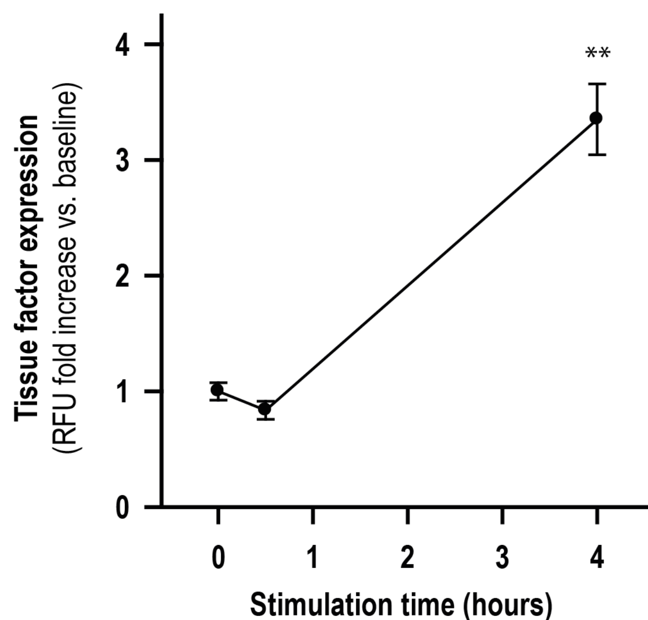


P-selectin (CD62P) expression on the surface of cultured endothelial cells
The appearance of the adhesion molecule p-selectin was measured on the surface of HMEC and HUVEC by FACS analysis. In HMEC as well as in primary endothelial cells (HUVEC) p-selectin was significantly upregulated in cells being treated with TNFα (5ng/ml, 4h) compared to sham treated cells.
RFU: Relative fluorescence units.
* $P<0.05$ vs. control (n=5-9)

4.4 Tissue factor expression on endothelial cells

To investigate whether mechanisms independent from the primary haemostasis could contribute to prothrombotic properties of TNFα the amount of tissue factor (CD142) expressed on cultured endothelial cells was measured. Tissue factor is a protein playing a crucial role in the activation of the extrinsic pathway of the coagulation cascade and is described to be upregulated under inflammatory conditions. Thus tissue factor was detected on the surface membrane of HMEC by FACS analysis after treatment with TNFα 5 ng/ml. The expression of tissue factor expression was slightly but not significantly decreased when stimulating cells for 30 minutes but raised up to the threefold after stimulating cells with TNFα for 4 hours (fold increase vs. baseline 3.35 +/-0.31 compared to non stimulated cells; P<0.01, n=10; figure 4.4).

Figure 4.4



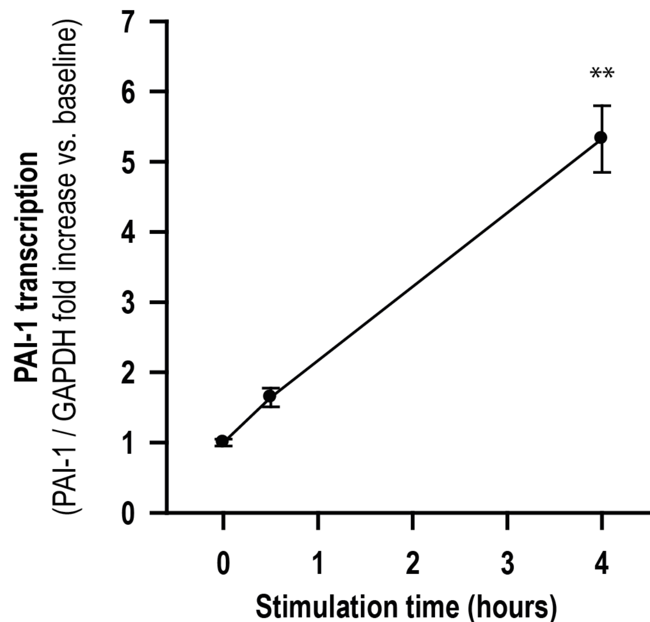
Tissue factor on the cell membrane of cultured endothelial cells
Tissue factor was detected on HMEC by FACS analysis. While no increase was found half an hour after stimulation with TNFα (5ng/ml), the expression on the cell membrane was significantly enhanced when cells were treated for 4 hours.
RFU: Relative fluorescence units
** P<0.01 vs. baseline (n=10)

4.5 PAI-1 mRNA in endothelial cells

As another primary haemostasis independent mechanism possibly contributing to prothrombotic effects of TNFα the transcription of PAI-1 (Plasminogen activator inhibitor 1)

was analyzed, which is one of the most potent inhibitors of fibrinolysis. Real time polymerase chain reaction (RT-PCR) was used to measure mRNA levels in cultured endothelial cells (HMEC) upon stimulation with TNFα 5 ng/ml for 30 minutes or 4 hours. The mRNA-ratio PAI-1 to GAPDH was slightly increased after half an hour but more than fivefold higher compared to baseline when stimulating endothelial cells for 4 hours (fold increase 5.32 +/-0.47 vs. baseline; $P<0.01$, n=16; figure 4.5).

Figure 4.5

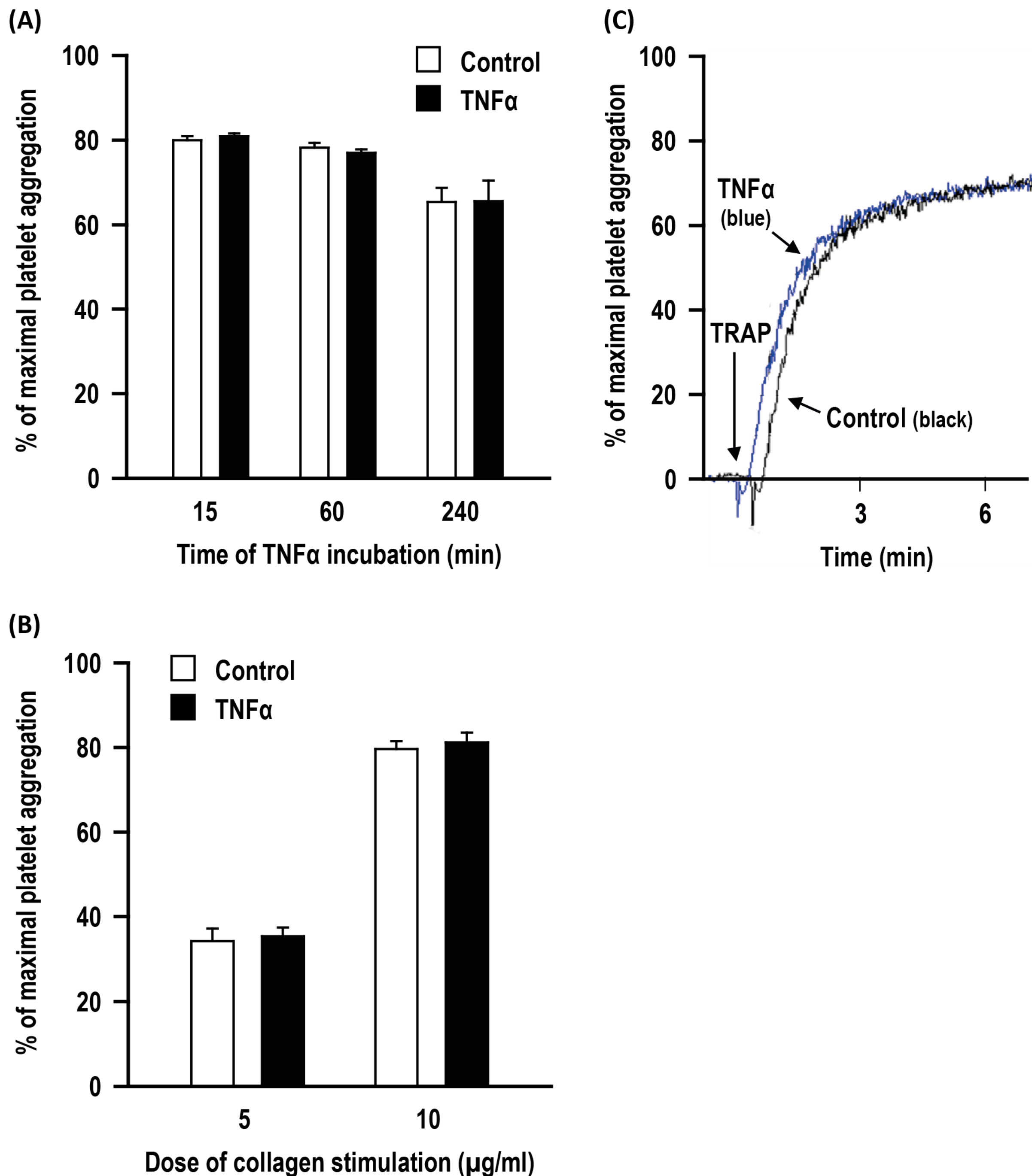


PAI-1 (plasminogen activator inhibitor 1) transcription in endothelial cells
mRNA levels of PAI-1 were analyzed by RT-PCR after endothelial cells were stimulated with TNFα. In HMEC TNFα (5ng/ml) raised PAI-1 mRNA ratio slightly after 30min but a striking increase was observed after 4 hours.
** $P<0.01$ vs. baseline (n=12)

4.6 Platelet aggregation in PRP

To consider direct effects of TNFα on platelets, aggregation was assessed *in vitro* in human PRP using light transmission aggregometry (Born's method). Incubation of PRP with TNFα 5 ng/ml for different times did not affect the ability of platelets to form aggregates upon stimulation with several commonly used platelet agonists. The ADP (5 µmol/l) dependent aggregation was not influenced by TNFα, neither after a short time of incubation nor after 4 hours (figure 4.6, panel A). No changes could be seen with other stimuli like collagen 5-10 µg/ml (figure 4.6, panel B) or TRAP (thrombin receptor activating protein) 5 µmol/l (figure 4.6, panel C) and TNFα itself did not cause aggregation either (data not shown).

Figure 4.6



Platelet aggregation in human PRP (platelet rich plasma)
Aggregation studies were performed in human PRP using light transmission aggregometry. **(A)** ADP (5µmol/l) dependent platelet aggregation at different time points was not changed when PRP was incubated with TNFα (5ng/ml) compared to the respective control PRP. **(B)** TNFα (5ng/ml, 30min) did not change aggregation when platelets were stimulated with different doses of collagen. **(C)** The representative image is showing characteristic platelet aggregation traces upon stimulation of PRP with TRAP (5µmol/l). No significant difference in either slope or maximal amplitude of the curves was apparent between TNFα and sham treated platelets. TRAP: Thrombin-receptor-activating-protein. (n=9-10, each)

4.7 Arterial thrombus formation *in vivo*

To analyze the effect of TNFα on thrombus formation *in vivo*, next the time to thrombotic arterial vessel occlusion following injury to the vascular wall was assessed. Therefore the ferric chloride superfusion model in the dorsal skinfold chamber in the mouse was used. The time to complete microvascular thrombotic vessel occlusion upon injury was significantly accelerated from 259.5 +/-30.6 s in control animals to 157.8 +/-31.3 s in animals treated with TNFα 5 ng/ml (which was the calculated plasma level when given TNFα at a dose of 0.4 µg/kg) 4 hours prior to the experiment (n=8, P<0.05 vs. control animals). Time to thrombotic vessel occlusion was not significantly accelerated compared to control animals when TNFα was given only half an hour prior to the experiment (241 +/-54.3 s; n=5; figure 4.7 and table 4.1).

Table 4.1 Thrombotic arterial occlusion time upon vessel injury *in vivo* after TNFα treatment (5ng/ml) in WT mice. Values are expressed as mean +/- SEM.

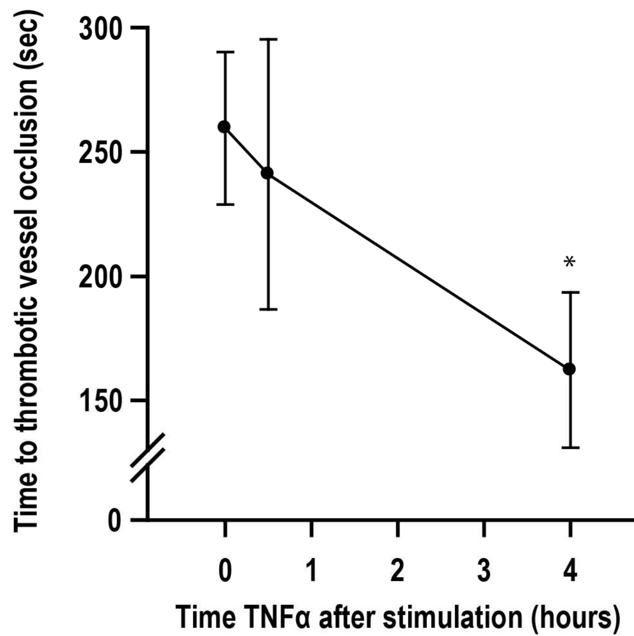
Time of treatment (min)	n	Occlusion time (s)	P-value vs. non treated animals
0	13	259.5 +/-30.6	-
30	5	241.0 +/-54.3	P=0.837
240	8	157.8 +/-31.3	P<0.05

4.8 Platelet-endothelium-interaction *in vivo*

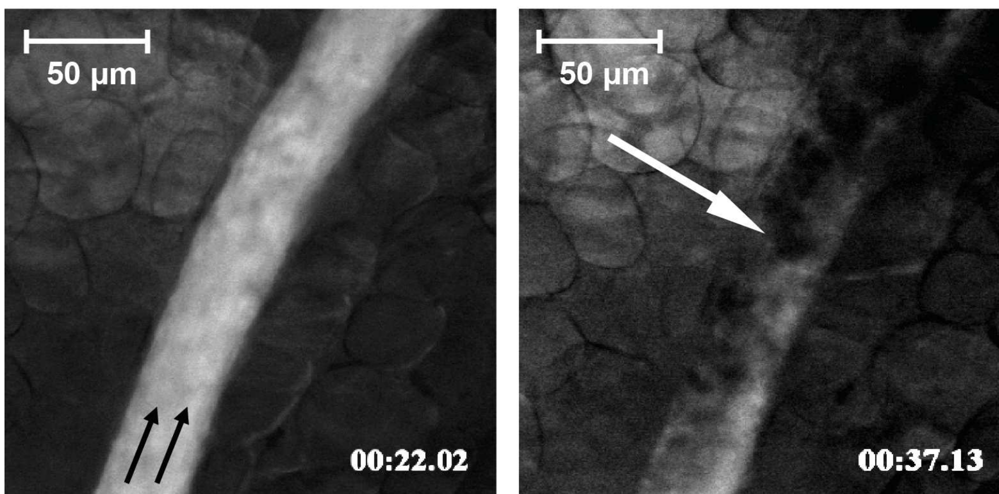
To have a more distinctive look at the endothelium transient platelet-vessel wall-interaction (PVWI) and firm adhesion of platelets to the vessel wall was investigated *in vivo* by intravital microscopy in the dorsal skinfold chamber. Following TNFα treatment (5 ng/ml calculated plasma level, 4 h) PVWI was enhanced as indicated by a leftward shift in platelet flow velocity distribution pattern and a significant decrease in the median platelet velocity from 4.0 mm/s in control animals (n=3294 platelets, 5 different animals, range: 0.3-11.5 mm/s) to 3.7 mm/s in TNFα treated animals (n=3239 platelets, 5 different animals, range: 0.6-15.3 mm/s, P<0.05 vs. control animals; figure 4.8). When observing *rolling* platelets that were defined as platelets with a velocity of less than 5% of the maximal platelet velocity measured within a vessel, i.e. platelets directly interacting with the endothelium, their fraction relating to all analyzed platelets was only 0.1 +/-0.1 % in control animals and not significantly higher in TNFα treated animals, where it was 0.4 +/-0.6 % (n=5).

Figure 4.7

(A)

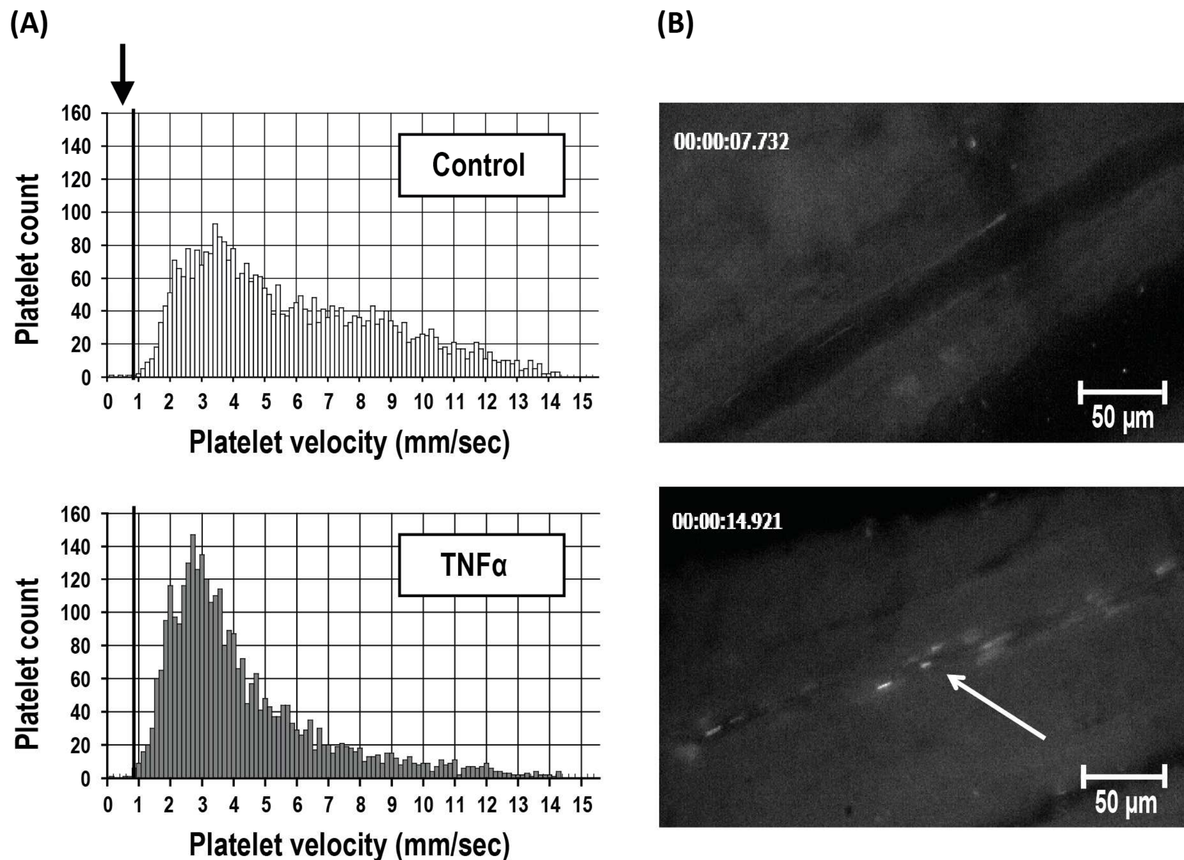


(B)



Arterial thrombus formation *in vivo*
Time to thrombotic arterial vessel occlusion was measured *in vivo* in the ferric chloride superfusion model in the dorsal skinfold chamber in mice. **(A)** TNFα (5ng/ml) treatment for 30 minutes did not enhance thrombotic vessel occlusion, but 4 hours after stimulation with the same dose time to complete thrombotic vessel occlusion was markedly accelerated (see also table 4.1). **(B)** The image shows thrombus formation in arterioles of the dorsal skinfold chamber after injury with ferric chloride. The white arrow is pointing at a thrombus, which is spared by the fluorescin-labeled plasma and almost occluding the vessel. Black arrows are indicating blood flow.
* $P<0.05$ vs. non treated animals (n=8).

Figure 4.8



Platelet-vessel wall-interaction (PVWI) *in vivo*
Transient platelet interaction and firm adhesion to the endothelium was analyzed by intravital microscopy in the dorsal skinfold chamber in mice after injection of fluorescently labeled platelets from a donor animal. Quantitative analysis is performed by assessment of platelet velocities, which can be calculated from the length of the trace of a platelet in one single picture when knowing the exposure time. **(A)** Platelet velocities were displayed in frequency histograms. The median platelet velocity was decreased in mice treated with TNFα (5ng/ml, 4h; lower histogram) compared to control animals (upper histogram) as indicated by a leftward shift in platelet velocity distribution pattern concluding more PVWI in TNFα treated mice. Platelets with a velocity of less than 5% of the maximal platelet velocity (platelets left of the vertical bold line in the histograms, indicated by arrow) were defined as *rolling* platelets. The fraction of rolling platelets relating to all analyzed platelets was very low and not significantly different between animals stimulated with TNFα and control animals. **(B)** The images show digital records of carboxy-fluorescin labeled platelets in arterioles of the dorsal skinfold chamber. The lower image is showing increased platelet-endothelium-interaction with *rolling* platelets (arrow).

4.9 TNF-receptor subtypes on endothelial cells

Since TNFα is known to exert its effects through different receptor subtypes, namely TNF-R1 and TNF-R2, the presence of these receptors on endothelial cells was checked for. Therefore western blotting of whole cell lysates of cultured endothelial cells was performed and the TNF-receptor subtypes were immunologically detected. Both TNF-R1 and TNF-R2 were present in HMEC and could be found also in primary endothelial cells (HUVEC) showing their characteristic protein band at 55 kDa for TNF-R1 and 75 kDa for TNF-R2 (figure 4.9, panel A).

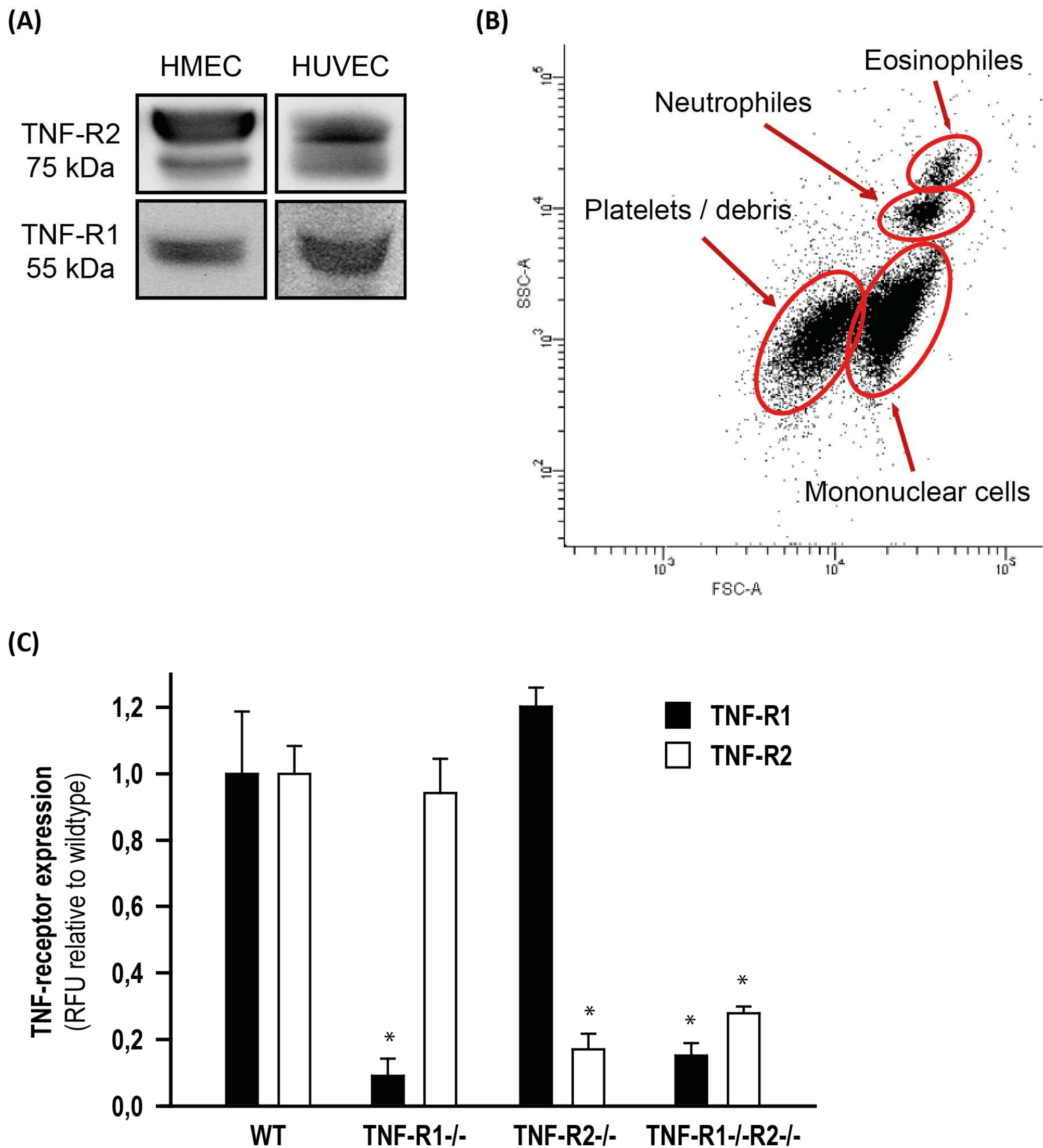
4.10 TNF-receptor subtype expression in WT and TNF-receptor KO mice

Next it was investigated whether and to what amount the two TNF-receptor subtypes TNF-R1 and TNF-R2 are expressed in WT, TNF-R1-/-, TNF-R2-/- and TNF-R1-/-R2-/- mice. Since TNF-receptors are known to play a role in white blood cells and these cells are relatively easy available, the density of TNF-R1 and TNF-R2 was measured on the surface of neutrophil granulocytes by FACS analysis of whole blood drawn by cardiac puncture. While on neutrophils of WT mice both receptor subtypes were present in a considerable amount, in TNF-receptor KO mice the respective receptors were successfully knocked out. In TNF-R1 or TNF-R2 deficient animals the expression of the respective non knocked-out receptor subtype was not significantly different from the expression observed in WT mice (fig. 4.9, panel C).

4.11 Arterial thrombus formation *in vivo* in TNF-receptor KO mice

After detection of the presence of both TNF-R1 and TNF-R2 on endothelial cells, distinctive effects by the two receptor subtypes regarding arterial thrombosis were analyzed after stimulation with their ligand TNFα. Time to complete thrombotic occlusion of the arteries upon exogenic injury of the vessel by ferric chloride superfusion was measured in the dorsal skinfold chamber model as described in the method section. The ability of TNFα (5 ng/ml, 4 h) to accelerate arterial thrombus formation *in vivo* observed in WT mice (figure 4.7) was much more striking in TNF-R1-/- mice, where the mean vessel occlusion time after treatment with TNFα was reduced to 62.4 +/-13.1 s (n=5, $P<0.05$ vs. sham treated TNF-R1-/-; figure 4.10, panel A). In TNF-R1-/- animals also the relative acceleration of thrombotic arterial vessel occlusion by TNFα was enhanced compared to WT animals. While the mean arterial vessel occlusion time was reduced by TNFα in WT mice by 37.5 +/-12.1 %, it was decreased by 71.1 +/-6.1 % in TNF-R1-/- mice after treatment with TNFα (n=5-8; $P<0.05$ vs. WT treated with TNFα; figure 4.10, panel B and table 4.2). In mice lacking TNF-R2 or both, TNF-R1 and TNF-R2, TNFα did not significantly accelerate arterial thrombus formation *in vivo*. Without treating animals with TNFα time to thrombotic vessel occlusion was not significantly affected in either of the TNF-receptor deficient animals compared to WT animals (fig. 4.10, panel A and table 4.2).

Figure 4.9



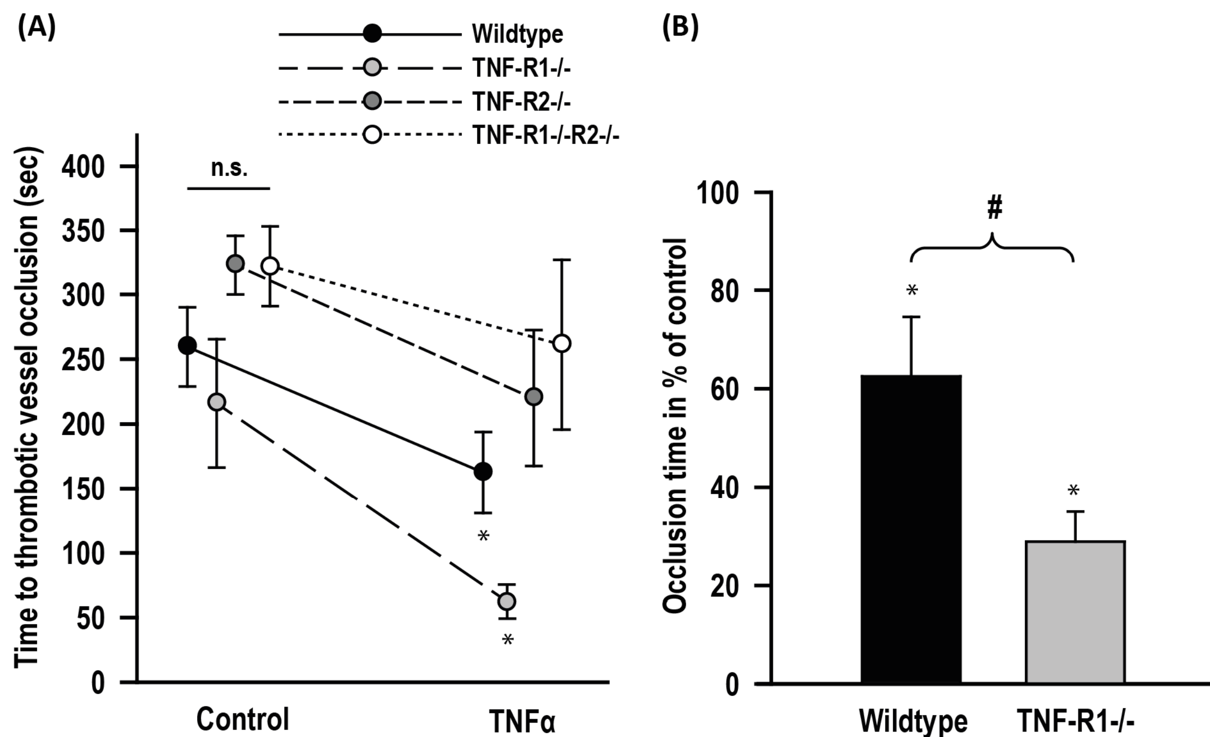
TNF-receptor expression

Expression of TNF-R1 and TNF-R2 was assessed in cultured human endothelial cells by Western blotting and in white blood cells of WT and TNFR-KO mice by FACS analysis. **(A)** The representative blots show the characteristic protein bands for TNF-R1 and TNF-R2 detected at 55 kDa and 75 kDa respectively in whole cell lysates of both HMEC and HUVEC. **(B)** Murine neutrophils were identified in the Dot blot by FACS analysis of murine whole blood by forward (FSC) and sideward scatter (SSC). **(C)** Quantitative analysis reveals TNF-R1 and TNF-R2 were present on the surface of neutrophils in WT mice. In TNFR-KO mice the respective receptor was successfully knocked out, whereas the respective non-knocked-out receptor was expressed to a similar level as in WT animals. * P<0.05 vs. WT (n=4).

Table 4.2: Relative reduction of thrombotic arterial vessel occlusion time *in vivo* after TNFα treatment (5ng/ml, 4h) in TNFR-KO mice. Values are expressed as mean +/- SEM.

Genotype	n	Relative reduction of time to vessel occlusion by TNFα (%)	P-value vs. untreated animals
WT	8	37.5 +/-12.1	P<0.05
TNF-R1-/-	5	71.1 +/-6.1	P<0.05
TNF-R2-/-	5	31.9 +/-16.3	P=0.11
TNF-R1-/-2-/-	5	18.9 +/-20.4	P=0.43

Figure 4.10



Arterial thrombus formation *in vivo* in TNF-receptor KO mice

(A) As already shown in figure 4.7 for WT mice the time to thrombotic arterial occlusion assessed upon vessel injury with ferric chloride was significantly accelerated with TNFα (5ng/ml, 4h; black circles, solid line). This effect was even stronger when TNF-R1 deficient mice were treated with TNFα (light gray circles, long-dashed line). In mice lacking TNF-R2 only (dark gray circles, short-dash line) or both TNF-receptor subtypes (white circles, spotted line) TNFα did not significantly affect arterial thrombosis. **(B)** In TNF-R1-/- mice (grey bar) also the relative acceleration of arterial thrombus formation by TNFα was significantly stronger compared to the relative acceleration by TNFα in WT mice (black bar).

* P<0.05 vs. respective sham treated animals (n=5-10); # P<0.05 (n=5)

4.12 Platelet-vessel wall-interaction *in vivo* in TNF-R1-/- mice

Considering TNF-R1 deficient mice showing enhanced arterial thrombus formation upon TNFα stimulation in the ferric chloride superfusion model platelet-vessel wall-interaction *in vivo* in this genotype was investigated in our model in the dorsal skinflold chamber.
The leftward shift in platelet velocity pattern indicating increased platelet-endothelium-interaction in WT mice after treatment with TNFα (5 ng/ml calculated plasma level, 4 h) in the group of TNF-R1-/- mice treated likewise was much more prominent. Median platelet velocity in these animals was significantly lower than in WT animals treated in the same manner (figure 4.11, panel A and table 4.3). In these animals TNFα raised the fraction of *rolling* platelets (defined as those platelets with a velocity below 5% of the maximum platelet velocity), which was only 0.4 +/-0.3 % in WT-mice, but 4.1 +/-1.0 % in TNF-R1-/- mice treated with TNFα (n=5, P<0.01 vs. WT; figure 4.11, panel B and table 4.3). When comparing maximum platelet velocities in the analyzed vessels between animals as an approximate measure of flow velocity, no significant difference was found between the genotypes.

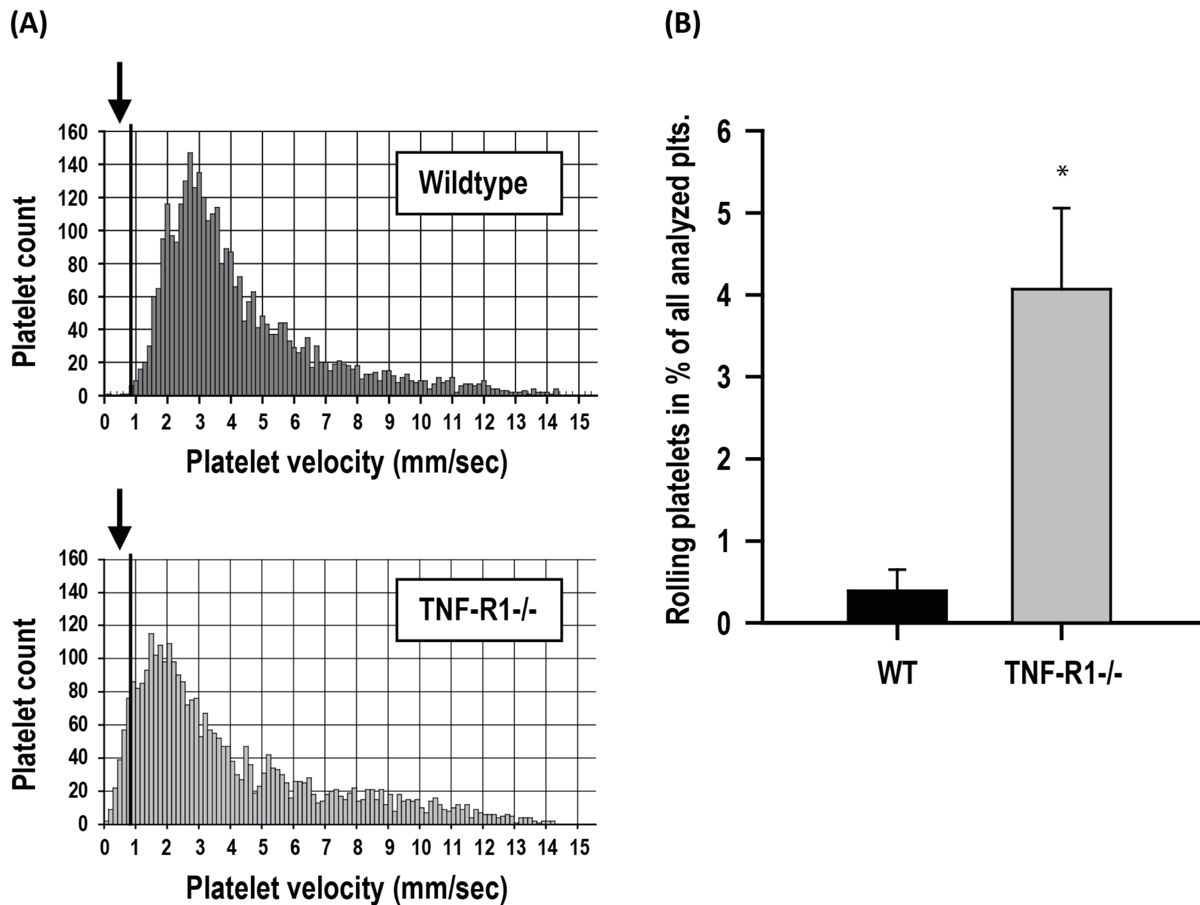
Table 4.3 Platelet-endothelium-interaction after TNFα treatment (5ng/ml, 4h) *in vivo* in WT and TNF-R1-/- mice. Values are expressed as median and range for platelet velocity and as mean +/- SEM for fraction of rolling platelets.

Genotype	n	Analyzed platelets	Platelet velocity (mm/s)	Rolling platelets (fraction in %)
WT	5	3239	3.7 (0.6-15.3)	0.4 +/-0.6
TNF-R1-/-	5	3091	2.7 (0.1-13.0)	4.1 +/-1.0

4.13 Platelet aggregation in TNF-R1-/- mice

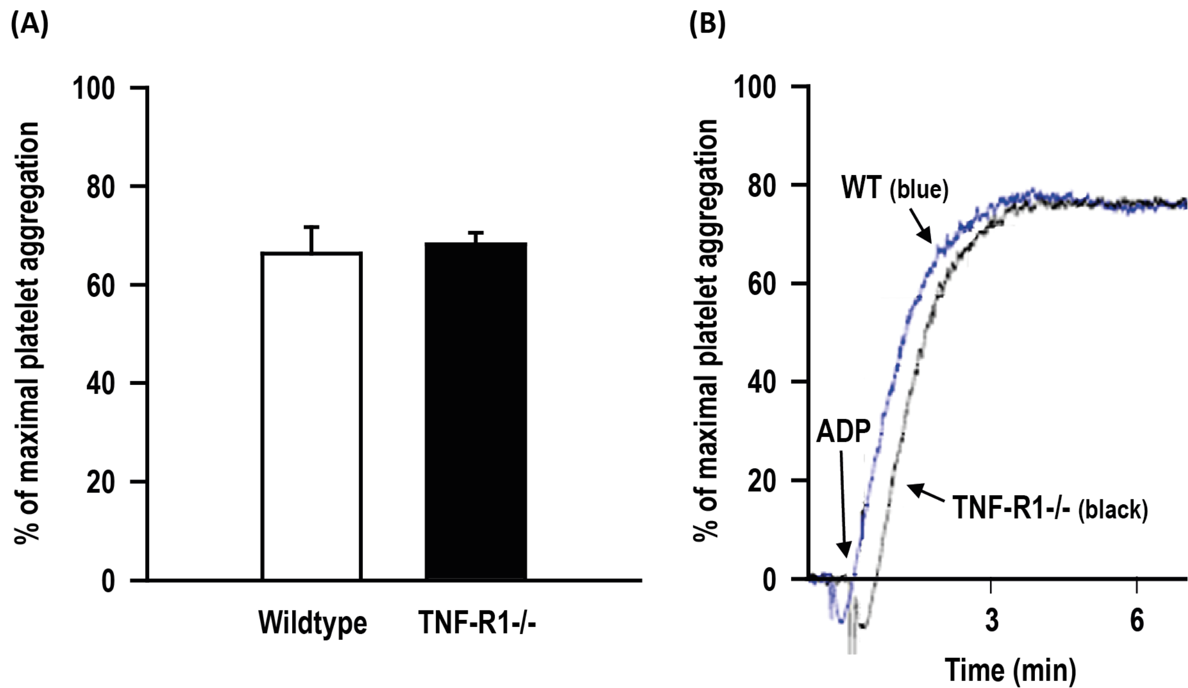
To check whether the prothrombotic effects observed in TNF-R1-/- mice could be due to a direct activation of platelets platelet aggregation studies were performed *ex vivo* in PRP from TNF-R1-/- mice. Platelets from these animals showed no differences in the ability to aggregate with or without prior incubation with TNFα 5 ng/ml to different stimuli including ADP (n=5; figure 4.12), collagen and TRAP and in different time ranges. TNFα alone did not induce aggregation in the murine PRP either.

Figure 4.11



Platelet-vessel wall-interaction (PVWI) *in vivo* in TNF-R1 deficient mice
Platelet-endothelium-interaction was analyzed by intravital microscopy in the dorsal skinfold chamber after injection of fluorescently labeled platelets from a donor animal. **(A)** Platelet velocities are displayed in frequency histograms. The left shift in the distribution pattern of platelet velocities when TNF-R1-/- mice (lower histogram) were treated with TNFα (5ng/ml, 4h) indicates more PVWI in these animals compared to WT animals (upper histogram) treated likewise. Those platelets with a velocity of less than 5% of the maximal platelet velocity, which are settled on the left of the vertical bold line in the histograms (indicated by arrow), were defined as rolling platelets and were significantly more frequent in the TNF-R1 deficient group compared to the WT group if both were treated with TNFα. **(B)** When quantifying rolling platelets after TNFα treatment the fraction related to all analyzed platelets was significantly higher in mice lacking TNF-R1 (gray bar) compared to WT animals (black bar; see also table 4.3).
* $P<0.05$ vs. WT mice (n=5)

Figure 4.12



Platelet aggregation in PRP (platelet rich plasma) of TNF-R1 deficient mice
PRP from WT and TNF-R1-/- mice was obtained from anticoagulated whole blood drawn by cardiac puncture and aggregation studies were performed using light transmission aggregometry. **(A)** TNFα did not influence ADP (20µmol/l) dependent platelet aggregation *ex vivo* in PRP from either WT or TNF-R1 deficient mice. The platelet aggregation maximum after incubation with TNFα (5ng/ml, 30min) was not significantly different in PRP from TNF-R1-/- mice compared to PRP from WT mice either. **(B)** The representative traces upon stimulation with ADP for WT (blue trace) and TNF-R1-/- mice (black trace) show a similar pattern of aggregation (n=5).

5 DISCUSSION

5.1 TNFα and atherothrombotic disease

Cardiovascular diseases are still the number one of death causes in developed countries and not only of scientific but also of economical interest. The understanding of the pathophysiology of these diseases is fundamental to approach this immense challenge for our health systems.

It is now more than a decade ago, when *Russ et al.* claimed atherosclerosis to be an inflammatory disease (31) and hereby got a ball rolling to put cardiovascular diseases more and more in a wider spread context. The role of inflammation has not only shown to be essential in the development of cardiovascular diseases but also seems to influence heavily the fatal endpoints as myocardial infarction and stroke (32,33). As these events are lastly the consequence of arterial occlusion by thrombus formation, treatment and prevention so far has focused primary on direct inhibition of platelets, but also on improving endothelial dysfunction e.g. by blocking deleterious effects of Angiotensin-2 (ACE-inhibitors) or catecholamines (betablockers) or on lowering lipid levels (statines). However, modifying the inflammatory component of cardiovascular diseases so far has not been established in routine medical treatment.

In the classical chronic inflammatory disorders as rheumatoid arthritis or inflammatory bowel diseases the modulation of inflammatory cytokines such as TNFα or IL-1 has been very successful, especially when the traditional anti-inflammatory drugs are failing. The reasons why anticytokine therapy so far could not succeed in the treatment and prevention of cardiovascular diseases (92) can be numerous and based on scientific as well as economical aspects. Nevertheless this approach remains of pharmacological interest since the role of inflammation in these illnesses has more and more turned out to be of great importance – even if more complex as initially expected.

The aim of this study was to address the role of TNFα as one of the best described inflammatory cytokines and to characterize its effects concerning arterial thrombus formation and platelet activation, the latter being also important in the early pathophysiological steps of atherosclerotic plaque formation. The role of platelets and endothelial cells in these processes was analyzed and the specific signaling by the different TNF-receptor subtypes TNF-R1 and TNF-R2 was elucidated.

5.2 The dorsal skinfold chamber as a mouse model to investigate arterial thrombosis and platelet-vessel wall-interaction *in vivo*

As described in detail in the methods section intravital microscopy was used as a tool to study arterial thrombus formation and platelet-vessel wall-interaction *in vivo*. The dorsal skinfold chamber mouse model allows a direct view into the microcirculation and effects can be observed for an extended time period (91). It has been used for a number of applications ranging from investigation of tumor growth to the effects of vasoactive agents (89-91).

Compared to other *in vivo* models, anesthesia is not required during the experiments so that bias by anesthetic drugs must not be considered. A disadvantage of this model in the setting used here is that it assesses effects on the microcirculation, whereas atherothrombosis is predominantly a problem of large vessels. Indeed, findings from the microcirculation need to be applied very carefully to other vascular beds. However, the model used in this study has been successfully used for similar studies before (65,66,69) and the findings did very well correlate with the pathophysiology and clinical observations in larger vessels (93,94). Finally the microcirculation has the advantage that processes can be observed via intravital microscopy and digitally recorded in a very high quality so that it is possible to visualize and analyze also very moderate effects in a precise and selective manner.

5.2.1 Assessment of arterial thrombosis *in vivo*

A range of murine thrombosis models in different vascular beds are described in the literature including chemical injury, photochemical induced oxidation injury (light-dye technique) (95,96), direct laser injury, temporary ligation of the vessel, intraluminal mechanical as well as electrical injury (97). In principle, almost every technique can be applied in the microcirculation, however, some methods such as ligation of these small arterioles or venules are very difficult to apply without damaging the vessel and so causing relevant bias. Chemically induced vascular injury by ferric chloride superfusion is a well approved model for arterial thrombosis, which is reliably applicable also for very small vessels and established in our group (65,69). It must be said, however, that superfusion in the microcirculation does not allow very selective injury, i.e. all cells of the vessel wall are hit by the chemical. The technique was applied in the *in vivo* model, where FITC-dextran was used as a fluorescent dye to stain plasma so that the progressive growth of thrombi can be visualized as increasing negative contrast in the labeled plasma.

5.2.2 Analysis of platelet-endothelium-interaction *in vivo*

Based on the dorsal skinfold chamber model in our group a method to analyze platelet-endothelium-interaction was developed (65,69). Platelet activation is not only relevant when it comes to vascular injury, but in the last years activation of platelets and transient interaction with the endothelium have also been described to influence the development of atherosclerotic lesions (51-58). Digital records of high quality using a high speed camera permit to selectively investigate these transient interactions of labeled platelets from a donor animal with the intact endothelium.

To quantify platelet-interaction with the vascular wall platelet velocities were calculated as described in detail in the method section and platelet velocity profiles within a vessel were analyzed. There is no fixed definition of a *rolling* platelet in a movie sequence. In this study those platelets were defined as “rolling platelets” that showed a velocity of less than 5 % of the velocity of the fastest analyzed platelet within a vessel representing the blood flow. Hereby a more strict definition of *rolling* was applied compared to that used in other studies of platelet-vessel wall-interaction (98). The definition reflects the intention to only detect those platelets that really interact considerably with the endothelium through cell surface adhesion molecules such as p-selectin or GPIb/vWF and hereby play a role in the pathophysiology of atherosclerosis.

A limitation of this method is certainly that the analyzed platelets are from another individual. Even if *in vivo* selective targeting of specific platelet surface antigens by antibodies is possible, it would not be sufficient for adequate visualization and analysis of platelet rolling on the vessel wall. However, the platelet isolation from allogenous donors is well established and activation by the isolation or staining processes as well as the ability of the platelets to aggregate was tested by *ex vivo* platelet aggregation studies. Moreover by using platelets from donor animals, there is the possibility to investigate platelet-endothelium-interaction between individuals with different genetic backgrounds and thereby to find out something about whether platelet or endothelium dependent factors are contributing to the findings.
Another point to mention is the role of leukocytes, which in the model used in this study are not stained and therefore not visible for analysis. Since white blood cells are well known to interact with platelets and their interaction with platelets and the endothelium is playing an important role in the development of atherosclerotic lesions (74,99-101) this would be an interesting aspect and should be subject for further experiments.

5.3 TNFα – pro- or antithrombotic?

There is compelling evidence that TNFα has prothrombotic properties from several clinical studies proclaiming an increased risk for atherothrombotic events in diseases associated with high plasma levels of TNFα (36,41,77-82) independently from the classic cardiovascular risk factors. Additionally a lot of *in vitro* studies in endothelial cells have been published, which show effects of TNFα potentially favoring prothrombotic effects (43-50). However, there is a lack of *in vivo* studies concerning direct links between inflammation and atherothrombotic diseases. Surprisingly, in the only *in vivo* study investigating the influence of TNFα on arterial thrombosis *Cambien et al.* describe an antithrombotic effect of TNFα after exogenous administration (85). This effect was observed after short term administration of TNFα peaking 30 minutes after application and is explained by a rapid generation of NO in the vessel wall. The antithrombotic effect was fully reversed after 2 hours and the observation was not extended for a longer period. Even if these observations are not necessarily inconsistent with findings of clinical studies there is a need for further studies to clear this problem.

In the *in vivo* model of arterial thrombosis used in this study a clear prothrombotic effect of TNFα could be observed. Time to thrombotic vessel occlusion upon injury was significantly accelerated after animals had been stimulated systemically with TNFα for 4 hours. These prothrombotic properties were not seen, when stimulating mice for only half an hour. When analyzing platelet interaction with the intact endothelium after stimulation with TNFα, i.e. without endothelial injury, a slight leftward shift in the platelet velocity histogram was noticed, indicating a tendency towards increased communication of platelets with the endothelium. However the amount of *rolling* platelets, i.e. platelets mechanically interacting with endothelial cells through surface adhesion molecules such as p-selectin or GPIb and vWF, was not changed by TNFα.

Different paramenters in endothelial cells and platelets potentially resulting in increased arterial thrombosis or favoring interaction of platelets with the endothelium were investigated. In cultured human endothelial cells activation of NF-κB, release of $O_2^{\cdot -}$ and regulation of

adhesion molecules as well as parameters independent from the primary haemostasis were assessed upon stimulation with TNFα. Indeed, increased production of $O_2^{\cdot -}$ and translocation of the p65-subunit of NF-κB already could be measured after an hour of incubation with TNFα. After 4 hours – but not after 30 min – of stimulation with TNFα there was a significant increase in endothelial surface membrane p-selectin, which is well known to play an important role in platelet-endothelium-interaction (59,98,102). Also effects not related to the primary haemostasis, which are going along with prothrombotic effects, such as the expression of tissue factor (CD 142) on endothelial cells and endothelial mRNA of PAI-1 were manifold increased upon TNFα stimulation. Similar to p-selectin expression these parameters were not elevated after only half an hour of stimulation but markedly after 4 hours.

Taken together, these results show prothrombotic properties of TNFα *in vivo*. These effects could not be observed after a short stimulation with TNFα of only half an hour but arterial thrombus formation was clearly enhanced after 4 hours. Also the observations in endothelial cells are highly supporting evidence for prothrombotic properties of TNFα.
The *in vivo* data do well support clinical studies reflecting correlations between chronic inflammatory disorders and atherothrombotic events and the endothelial effects agree with those already described in the literature. TNFα exerted prothrombotic effects *in vivo* only 4 hours after stimulation and also the *in vitro* effects on endothelial cells as p-selectin or tissue factor upregulation and increased mRNA levels of PAI-1 were not seen after half an hour of incubation with TNFα but after 4 hours.
The results did not confirm antithrombotic effects of TNFα observed in another *in vivo* study of arterial thrombosis by *Cambien et al.* (85). In the mentioned study antithrombotic effects were due to a rapid release of endothelial NO and fully reversed after two hours. Acute elevation of TNFα can be found e.g. in the acute phase of sepsis, but chronic inflammation is associated with ongoing increased levels of inflammatory cytokines. The results obtained in this study do not put potential antithrombotic effects of TNFα after short term administration into question, but rather refer to the more long lasting effects of the cytokine as found in chronic inflammatory disorders. Indeed, several effects of TNFα were not observed after half an hour but already after four hours. After a short time of stimulation TNFα in our model did not reveal any significant effects – neither pro- nor antithrombotic. Local differences between distinctive vascular beds could be responsible for this, since antithrombotic effects were described in mesenteric arterioles while the experiments in this work were performed in the microvascular bed of the dorsal skin. It should also be said, that our *in vivo* model of arterial thrombosis is rather eligible to detect prothrombotic than antithrombotic effects.

5.4 What are the mechanisms underlying the prothrombotic effects of TNFα?

The striking prothrombotic effects of TNFα in the *in vivo* model raise the question about what mechanisms are responsible for the observations. The main issue is whether accelerated thrombus formation is due to a direct effect of TNFα on platelets or based on indirect, endothelium-mediated mechanisms. The endothelium is building the inner layer of a blood vessel so that endothelial cells can directly interact or communicate with the blood cells. An

intact endothelium is necessary to keep thrombosis and thrombolysis, coagulation and fibrinolysis in balance. Several pathological conditions can affect endothelial cells and thereby influence the physiological equilibrium. Numerous studies have investigated effects of TNFα on endothelial cells *in vitro*. When looking at the cytokine's properties being able to influence thrombus formation or to activate platelets, effects as downregulation of eNOS, increased production of ROS and activation of NF-κB are well described (43-50). In this study a cultured endothelial cell line (HMEC) as well as primary endothelial cells (HUVEC) were used to investigate effects potentially resulting in increased susceptibility for arterial thrombosis. Additionally direct effects of TNFα on platelets were considered.

5.4.1 ROS formation and activation of NF-κB in endothelial cells

Reactive oxygen species (ROS) are molecules containing oxygen that are highly reactive due to the presence of unpaired electrons. ROS are not only a product of the normal oxygen metabolism, but have also important roles in cell signaling by functioning as second messengers (103,104). An increase in ROS levels, also known as oxidative stress, which happens for instance upon environmental stress such as UV or heat exposure can lead to significant cell damage. Endothelial or platelet derived ROS can exert prothrombotic effects by directly activating platelets or decreasing the threshold for platelet activation (68,105,106). As a scavenger of endothelial and platelet derived NO, earlier also referred as EDRF (endothelium derived relaxing factor), $O_2^{\cdot -}$ has prothrombotic properties by decreasing the bioavailability of this very important inhibitor for platelets under physiological conditions (48,107,108). Also in their function as intracellular messengers e.g. by activation of the transcription factor NF-κB (109,110) $O_2^{\cdot -}$ can contribute to endothelial dysfunction and therefore lead to a more prothrombotic state. In this study a markedly elevated formation of $O_2^{\cdot -}$ in HUVEC and a translocation of the p65-subunit of NF-κB could be found after incubating the cells with TNFα. The transcription factor NF-κB is well described to play a role in many inflammatory processes and its activation is also known to induce up-regulation of cell adhesion molecule expression (50) mediating platelet-endothelium-interaction.

5.4.2 Upregulation of endothelial adhesion molecules

Considering activation of NF-κB, an interesting question was whether TNFα would influence endothelial surface membrane adhesion molecules. Indeed, a considerable increase in p-selectin expression on HMEC as well as on HUVEC could be measured after 4 hours of incubation with TNFα. Endothelial p-selectin is known to play an essential role in platelet-endothelium-interaction and is best described in the context of platelet rolling (59,98,102). Elevated amounts of p-selectin on the surface of endothelial cells can promote rolling and adhesion of platelets and hereby not only facilitate thrombus formation but also contribute to the development and progression of atherosclerotic lesions.

5.4.3 Mechanisms other than primary hemostasis

As mentioned in the introduction, arterial thrombus formation and following occlusion of the vessel is a very complex pathophysiological process, where next to platelets and endothelial cells also other influencing factors play a role. The plasmatic coagulation, also referred to as

secondary hemostasis, interacts with the primary hemostasis and the cleavage of fibrinogen by thrombin is necessary to stabilize a thrombus.
Tissue factor is a protein playing a crucial role in the activation of the extrinsic pathway of the coagulation cascade (111). In a physiological environment endothelial cells express, if at all, very little tissue factor. However, the protein is described to be upregulated under inflammatory conditions (112-114). The results in this study agree with data in the literature and are showing a severalfold upregulation of tissue factor on endothelial cells *in vitro* after 4 hours of TNFα stimulation. By binding activated coagulation factor VII, tissue factor activates the extrinsic pathway of the coagulation cascade and contribute to the growth and stabilization of a thrombus. Additionally tissue factor can induce the generation of microparticles, which are recruited to the site of an injury and can contribute to increased thrombin generation (111).
Another protein which independently from primary hemostasis could affect arterial thrombosis is plasminogen activator inhibitor 1 (PAI-1). It is the major physiologic inhibitor of tissue-type plasminogen activator in plasma, which serves as an endogenous defense mechanism to prevent intravasal thrombosis (115). PAI-1 is elevated in a variety of clinical situations that are associated with increased risk of ischemic cardiovascular events (116-120). Consistently with similar experiments previously described (121,122), the experiments performed in this study showed an increase of PAI-1 mRNA in human endothelial cells upon stimulation with TNFα after 4 hours. Increased PAI-1 levels can shift the hemostasiologic balance to the prothrombotic side by inhibiting plasminogen activation and consequently fibrinolysis and therefore display another explanation for TNFα's prothrombotic properties.

5.4.4 Direct effects of TNFα on platelets

Prothrombotic properties of TNFα could also be due to a direct effect on platelets. Therefore platelet aggregation was performed in PRP (platelet rich plasma). Different platelet agonists such as ADP, TRAP and collagen were tested and no changes in platelet aggregation could be seen when the PRP was preincubated with TNFα. Stimulation for a time of 4 hours, where the endothelial effects of TNFα were most pronounced did not show any difference in platelet aggregation either and TNFα itself did not induce aggregation. There are descriptions of weak direct activation of platelets by TNFα (123-125). Platelets are indeed described to express TNF-R1 and TNF-R2 even if (at least under physiological conditions) in a very low amount (126). However, in this study no influence of TNFα on platelets could be observed, neither in human nor in murine PRP.

These data clearly speak for the endothelium being the main mediator of the observed prothrombotic effects exerted by TNFα in our experiments.
All of the effects of TNFα observed in endothelial cells *in vitro* can increase platelet responses, activation or aggregation. While increased endothelial superoxide production and NF-κB activation indirectly contribute to a greater susceptibility for arterial thrombosis (68), elevated p-selectin directly facilitate the interaction of platelets with the endothelium (98,111). Tissue factor and PAI-1 as important regulators of the plasmatic coagulation finally can promote the growth and stabilization of thrombi (112,115). The fact that in additionally performed aggregation studies in PRP TNFα did not have an impact on platelet aggregation, endorses the hypothesis of an endothelium mediated pathomechanism. These observations do fit well to the findings in our *in vivo* model of arterial thrombosis after vessel injury, where a

clear prothrombotic effect was mediated by TNFα. However, one must be very careful when transferring data obtained under the bench to the living organism, and indeed it must be said that from the *in vivo* findings a direct effect of TNFα on platelets cannot be excluded. The environment, where platelets roll and adhere on the endothelium and finally aggregate and form thrombi *in vivo*, is much more complex than any platelet aggregation measurements *ex vivo*. Nevertheless our *in vivo* model of platelet-vessel wall-interaction, where a slight increase in transient interaction of freshly isolated (i.e. non TNFα treated) platelets with a pretreated endothelium was apparent, demonstrates that TNFα can lead to a prothrombotic or platelet activating state by stimulating endothelium alone. *In vitro* observed findings cannot be seen isolated *in vivo*, either. Effects as increased superoxide production or NF-κB activation are involved in numerous processes and signaling pathways in several cellular systems and tissues (50,103,108), but some of their abilities can help to explain prothrombotic properties of TNFα *in vivo*.
Taken together the observed prothrombotic effects in this study are mediated mainly through the endothelium rather than through direct effects on platelets.

5.5 What TNF-receptor subtype is responsible for the effects?

It is well known that TNFα is acting through different receptor subtypes and distinctive signaling upon activation of either receptor has been described (1,5,6). The signaling pathways mediated by the two receptors are complex and partly crosslinked. However, both TNF-receptor-subtypes are described to be expressed on endothelial cells and associated with the activation of NF-κB (1). Although the discovery of the different TNF-receptor subtypes dates back to year 1984, for a long time most of TNFα's effects were attributed to signaling trough TNF-R1 as this receptor was better characterized and known to be expressed in most tissues and cell types. As it is not ubiquitously expressed, the role of TNF-R2 is less described, if not to say underestimated. The receptor specific signaling in the recent years has moved more and more into the focus of interest, not only because of intensive investigations in the context of the meanwhile widely used TNFα-inhibitors. The treatment of inflammatory disorders led to a more extensive focus on the TNF-receptor subtypes, even suggesting specific signaling through the different subtypes being responsible for the failing of the therapy in certain occasions. TNFα-blockade in more than one clinical study in heart failure could not show the expected improvements (92,127) and indeed a recent study by *Hamid et al.* highlighted opposite effects upon activation of TNF-R1 and TNF-R2 in the setting of induced heart failure in mice (22). In recent years TNF-R2 has achieved more attention as probably playing the crucial role in TNFα induced progression of atherosclerosis (128-130). Considering these findings it is of great interest to find out which one of the two main TNF-receptor subtypes would be responsible for the prothrombotic effects observed in this study. To address this very interesting issue arterial thrombus formation and platelet-endothelium-interaction was compared in TNF-R1 KO mice, in TNF-R2 KO mice and in animals lacking both of the two TNF-receptor subtypes. In these animals also platelet aggregation was analyzed.

5.5.1 TNF-receptor subtype expression on endothelial cells

To investigate receptor specific effects of TNFα it is fundamental to know, whether they are expressed in the observed cells. As many parameters were influenced by TNFα in endothelial cells, within this work it was analyzed whether and if so what TNF-receptors the used cells are carrying. Qualitative analysis of TNF-receptor subtypes in whole cell lysates clearly proves both the 55 kDa TNF-R1 and the 75kDa TNF-R2 are present on cultured endothelial cells in a considerable amount. Characteristic bands could be observed in our endothelial cell line (HMEC) as well as the primary cells (HUVEC). As we know TNFα can activate both receptor-subtypes we assume that the outcomes observed are the result from signaling through both receptor subtypes, TNF-R1 as well as TNF-R2.
However, one must also be very careful transmitting the information from the immunoblotting to the *in vivo* experiments. To assure that the TNF-receptor subtypes are really expressed in endothelial cells of the murine microcirculation immunohistochemnistry is required and to obtain information about the localization of the receptors within the cell immunofluorescence imaging studies are needed. As specific gene knockdown of a single receptor would be the necessary next step in the investigation of receptor specific effects of TNFα the information from the blotting will be essential to prove effectiveness of knockdown techniques. Some of the mentioned experiments to further clarify the localization of TNF-receptors are in progress, but unfortunately not yet included in this work. Nevertheless, it can be said that in general both TNF-receptor subtypes are expressed in endothelial cells.

5.5.2 TNF-receptor expression in TNF-receptor deficient mice

To analyze receptor specific effects *in vivo* C57-BL/6 mice deficient for either one of the two TNF-receptor subtypes or both receptors were used. To exclude significant overexpression of the respective non knocked-out receptor the expression on the neutrophils of these animals were quantitatively analyzed. Neutrophils were used as they are cells of the immune system and best described to express both receptor subtypes (8,9). No difference was seen in the amount of the non knocked-out receptor in TNF-receptor deficient mice compared to wildtype controls, thus concluding there is no compensation in terms of the expression of the other receptor. Additionally, this confirms the functional knock-down of these receptors in the TNF-receptor deficient animals.

5.5.3 Arterial thrombosis and platelet-endothelium-interaction in TNF-receptor deficient mice

Upon the observations that TNFα enhances arterial thrombosis *in vivo*, the very same experiments were performed in mice lacking one or both of the TNF-receptor subtypes. Arterial thrombus formation, which was already significantly accelerated in wildtype mice receiving TNFα 4 hours prior to the experiment, was even more increased in mice deficient for TNF-R1 and could not be seen in mice lacking TNF-R2 only or both TNF-receptor subtypes. These observations indicate that TNF-R2 is required to mediate the prothrombotic effects of TNFα *in vivo*, as TNFα was not able to enhance thrombus formation, when TNF-R2 is missing as it is the case in TNF-R2- or TNF-R1-R2- double-knockout mice. The fact, that the prothrombotic effect of TNFα is less pronounced in wildtype mice, where signaling through both receptor subtypes is possible, leads to the suggestion that TNF-R1 may exert antithrombotic effects or at least may have a compensatory function. However, mice lacking

TNF-R2, meaning only TNF-R1 activation is possible, do not show a vessel occlusion time which is significantly longer than in mice either not treated with TNFα or lacking both receptor subtypes. Therefore, activation of TNF-R1 does not seem to have antithrombotic activity itself, but can attenuate the apparent prothrombotic effects exerted upon activation of TNF-R2.
Not only arterial thrombus formation after injury but also the amount of *rolling* platelets, which was not significantly changed by TNFα stimulation in wildtype mice, was increased in TNF-R1 KO mice. P-selectin is known to be an important mediator of leukocyte and platelet *rolling* and indeed TNFα induced p-selectin surface expression on endothelial cells. Interestingly *in vivo* increased *rolling* of platelets could not be observed in wildtype mice, when both of the TNF-receptor subtypes are expressed on the endothelium. We therefore conclude signaling through TNF-R2 is responsible for transient platelet-endothelium-interaction while TNF-R1 is partly compensating for the effect.

5.5.4 Direct effects of TNFα on TNF-R1 deficient platelets

As the most striking prothrombotic effects of TNFα *in vivo* were seen in TNF-R1 deficient mice and these animals also showed an increased amount of *rolling* platelets, platelet aggregation was performed in PRP (platelet rich plasma) of TNF-R1 deficient mice. Similar as in human platelets and in PRP from wildtype mice no direct influence of TNFα on platelets obtained from TNF-R1 deficient mice could be observed. These data further confirmed the assumption that the prothrombotic effects of TNFα were due to endothelial activation and not due to a direct effect on platelets. Regarding the observation of TNF-R2 mediating prothrombotic effects while TNF-R1 being the counterregulating player, these effects are due to receptor specific signaling on endothelial cells but not on platelets.

The results of this study show that signaling through the different TNF-receptor subtypes differentially contributes to the prothrombotic effects of TNFα. In fact it seems like even opposite effects are mediated through the receptors. While TNF-R2 is the prothrombotic player in this game, TNF-R1 rather represents the antithrombotic counterpart. The results go along with observations in other studies where TNF-receptor subtypes upon activation with TNFα do not activate the same downstream signaling pathways and do not result in the same effects (22). TNF-R2 has been described to be crucial for TNFα induced atherosclerotic lesions in mouse models for atherosclerosis (129). Since it has been shown that platelets play an important role in the pathophysiology of atherosclerosis (51-56,58), the findings in this work that TNFα mediates platelet activation and enhances platelet-endothelium-interaction via signaling through endothelial TNF-R2 could be an explanation. Another study could show an important role for TNF-R2 in TNFα induced leukocyte rolling and proposed upregulation of e-selectin, vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) as possible mechanisms (21). There are several similarities in terms of involvement of cell adhesion molecules between leukocyte and platelet rolling on the endothelium (74). So the present data strongly support a role for TNF-R2 in mediating *rolling* of either platelets or leukocytes on the endothelium. For how TNF-R1 is counterregulating TNFα's prothrombotic effects *in vivo* so far it can only be speculated. However, it can be excluded that results are due to over- or underexpression of the receptor subtypes in the knockout animals. The downstream signaling pathways of the two TNF-receptor subtypes are very complex and partly crosslinked (6,7,9). Further studies are needed to find out how the

pathways interact with one another and are able to modulate the effects in the context of the prothrombotic actions of TNFα.
In summary, it can be said that TNF-R2 is required for thrombotic or platelet activating effects of TNFα *in vivo*, while signaling through TNF-R1 may exert compensatory i.e. antithrombotic effects.

5.6 Pathophysiological and clinical consequences

Inflammation plays a crucial role in the pathophysiology of cardiovascular diseases (32). TNFα is one of the best characterized inflammatory cytokines and together with its receptor system it is essential in many inflammatory processes (26). In this study the influence of TNFα and its receptors TNF-R1 and TNF-R2 on arterial thrombus formation and platelet activation *in vivo* was investigated. Arterial thrombosis is the final step in myocardial infarction or stroke, mostly on the basis of atherosclerotic plaques. In the last years activated platelets and their transient interaction with the endothelium have turned out to play a considerable role in the development of atherosclerotic lesions (51-58).
In our study TNFα promotes arterial thrombus formation and transient platelet endothelium interaction *in vivo*. These effects are mediated by the endothelial TNF-receptor subtype 2 while TNF-receptor subtype 1 can at least partly compensate for the effects. Hereby these data strongly support clinical observations describing chronic inflammatory disorders with elevated levels of TNFα going along with increased risk for atherothrombotic events (78-82). Even if several effects of TNFα shown in the past were suggestive for prothrombotic effects *in vivo* in this work accelerated thrombus formation upon TNFα stimulation is shown for the first time in an *in vivo* model of arterial thrombosis. Regarding the mechanism there is high evidence that our observations are endothelium-mediated and not due to direct activation of platelets. Finally by these findings this study contributes to a better understanding of the very complex distinctive signaling through the two TNF-receptor subtypes. While acceleration of thrombotic vessel occlusion by TNFα was apparent also in wildtype mice but much more striking in TNF-R1 deficient animals, increased transient platelet-endothelium-interaction also referred to as *rolling* was only seen in these animals. These observations may illustrate why a successful therapeutic approach must be very sophisticated and might explain why anticytokine therapy failed to treat cardiovascular diseases in the past. Indeed in the literature several clinical studies of “anti-TNFα therapy” for cardiovascular diseases are described (92,127). While monoclonal antibodies against inflammatory cytokines as TNFα or IL-1 are very successful in the treatment of certain disorders as rheumatoid arthritis or chronic inflammatory bowel diseases (24,25,71), these drugs clearly failed to help people with chronic heart failure and even led to premature termination of such clinical studies. Considering the fact that TNFα acts very specific and can exert even opposite effects by activating the two receptor subtypes, we can speculate that the use of TNFα-antibodies could also dismantle beneficial effects or at least anticipate compensatory mechanisms mediated by one of the receptors. Indeed, in terms of prevention of thrombotic events, also clinical data from studies in patients with rheumatoid arthritis receiving anticytokine therapy including the TNFα-antibodies Infliximab and Adalimumab as well as the soluble TNF-receptor Etanercept remain confusing, since both increased as well as decreased risks for thrombotic complications and thromboembolic events have been reported (83,84). However, in the

development and progression of atherosclerosis, TNF-R2 seems to mediate the unwanted effects while TNF-R1 being rather protective. We should not be discouraged by initial failing of anti-inflammatory therapy in cardiovascular disease, but try to further elucidate and clarify pathological mechanisms. Since aside from facilitating thrombotic events the TNFα-TNF-receptor-signaling plays a role in the development of atherosclerotic lesions, specific modulation of TNF-receptors could in general be a therapeutic target.

Anticytokine therapy to prevent atherothrombotic events will be challenging, not only because of distinctive TNF-receptor subtype specific signaling but also because of economical reasons, i.e. the extremely high expenses in a health system, which is forced to save costs. In any case, for selective receptor targeted therapy the complex signaling of TNFα certainly needs to be better understood. However, the current findings suggest that a specific blockade of TNF-R2 rather than TNF-R1 may be of potential therapeutic benefit in atherothrombotic diseases.

6 SUMMARY

Cardiovascular diseases are still the number one cause of death in the western civilization. Arterial thrombosis as it happens in myocardial infarction or ischemic stroke is intimately linked with inflammation. Elevated serum levels of proinflammatory cytokines correlate with an increased risk for atherothrombotic diseases. Several effects of TNFα, the best described inflammatory cytokine, on endothelial cells could result in prothrombotic *in vivo* properties. However, it was unclear so far, how TNFα influences arterial thrombosis *in vivo*, since short term but reversible antithrombotic effects have been described. Moreover it was not known, what role the two TNF-receptor subtypes TNF-R1 and TNF-R2 play in this context.

In this study the dorsal skinfold chamber was used as an *in vivo* mouse model to investigate arterial thrombosis and platelet-endothelium-interaction. Rolling and firm adhesion of fluorescently labeled platelets on the intact endothelium were analyzed by intravital microscopy and arterial thrombosis was assessed by measuring the time to thrombotic vessel occlusion upon exogenous vessel injury by ferric chloride superfusion.

In wildtype mice systemic stimulation with TNFα led to a significant acceleration of complete thrombotic vessel occlusion after injury. Transient platelet-interaction with the endothelium was slightly increased after treating mice with TNFα, but there was no significant increase in the amount of *rolling* platelets, that were defined as the platelets with a velocity of less than 5% of the maximal platelet velocity.

To find out, whether the underlying mechanisms for the observed prothrombotic effects of TNFα were mediated through a direct effect on platelets or rather through indirect endothelium-mediated mechanisms, several *in vitro* effects of TNFα on platelets and cultured endothelial cells were investigated.

In primary human endothelial cells (HUVEC) a significant increase of $O_2^{\cdot-}$ release was measured by cytochrome C reduction after stimulation of the cells with TNFα. In the same cells TNFα also led to a translocation of the p65-subunit of the transcription factor NF-κB to the nucleus, as assessed by immunofluorescence imaging. By FACS analysis the surface membrane expression of the adhesion molecule p-selectin was measured, which plays a role in platelet-endothelium-interaction and a significant upregulation could be observed after stimulating endothelial cells (HMEC) for 4 hours with TNFα. Moreover parameters non related to the primary hemostasis were investigated. Tissue factor on the surface of HMEC was assessed and quantified by flow cytometric analysis and was markedly increased, when cells were treated with TNFα for 4 hours. A similar effect could be observed for PAI-1, where significantly elevated mRNA levels assessed by RT-PCR were shown upon stimulation of HMEC with TNFα.

To exclude potential direct effects of TNFα on platelets *ex vivo* platelet aggregation studies using several agonists were performed in human and murine platelet rich plasma (PRP). TNFα stimulation of platelets in PRP did neither cause spontaneous aggregation, nor led to changes in ADP-, collagen- or TRAP-induced aggregation.

To find out, which TNF-receptor subtype was responsible for the observed prothrombotic effects, arterial thrombus formation and platelet-vessel wall-interaction were studied *in vivo* in TNF-R1-/-, TNF-R2-/- and TNF-R1-/-R2-/- mice. Time to thrombotic vessel occlusion, which was already decreased in wildtype mice after TNFα stimulation, was again accelerated in TNF-R1-/- animals. Compared to wildtype animals in TNF-R1-/- mice, TNFα also led to increased transient platelet interaction with the endothelium, resulting in a greater amount of *rolling* platelets. These effects could not be observed in TNF-R2-/- and in TNF-R1-/-2-/-

animals. As TNFα did not enhance platelet aggregation in PRP of TNF-R1-/- mice, these results suggest that the endothelial TNF-R2 mediates prothrombotic effects of TNFα.

Taken together, in this study, for the first time prothrombotic effects of the inflammatory cytokine TNFα are described using an *in vivo* model of arterial thrombosis. The data suggest that endothelium mediated mechanisms underlie these observations. The mechanisms involve an enhanced production of $O_2^{\cdot -}$, activation of NF-κB and expression of the adhesion molecule p-selectin on endothelial cells, as well as mechanisms not directly related to the primary hemostasis, such as increased tissue factor expression on endothelial cells and mRNA levels of PAI-1. The endothelial TNF-receptor subtype 2 was required for prothrombotic effects *in vivo*, while the TNF-receptor subtype 1 partly compensated for these effects.

These data lead to a better understanding of the role of TNFα and its receptors in the pathophysiology of atherothrombotic diseases. For the treatment and prevention of such diseases by selective receptor targeted anticytokine therapy further studies are needed, but nevertheless the findings could contribute to the development of such approaches in the future.

7 ZUSAMMENFASSUNG

Herz-Kreislauf-Erkrankungen bilden die häufigste Todesursache in den westlichen Industrieländern. Entzündliche Prozesse spielen in der Pathophysiologie kardiovaskulärer Erkrankungen eine entscheidende Rolle und zahlreiche Studien belegen, dass erhöhte Plasmaspiegel von proinflammatorischen Zytokinen wie TNFα das Risiko für atherothrombotische Erreignisse wie Myokardinfarkt und Schlaganfall erhöhen. Verschiedene in der Literatur beschriebene Effekte von TNFα auf Endothelzellen könnten eine prothrombotische Wirkung begünstigen. Es war bisher jedoch noch nicht geklärt, wie TNFα arterielle Thrombose *in vivo* beeinflusst, da nach kurzzeitiger Stimulation auch reversible antithrombotische Effekte beschrieben wurden. Weiterhin ist nicht bekannt, welche Rolle die beiden TNF-Rezeptor Subtypen TNF-R1 und TNF-R2 in diesem Zusammenhang spielen.

In dieser Studie wurde das Rückenhautkammermodell in der Maus als *in vivo* Modell zur Untersuchung von arterieller Thrombose und Thrombozyten-Endothel-Interaktion verwendet. Dabei wurden intravitalmikroskopisch Rollen und feste Adhäsion von Fluoreszenz-markierten Thrombozyten mit dem Endothel analysiert und arterielle Thrombose durch Messung der Zeit bis zum thrombotischen Gefäßverschluss nach exogener Gefäßverletzung durch Eisenchlorid-Superfusion untersucht.
In Wildtyp Mäusen führte die Stimulation mit TNFα zu einer signifikanten Beschleunigung des vollständigen thrombotischen Verschlusses nach Gefäßverletzung. Die transiente Interaktion von Thrombozyten mit dem Endothel war in Mäusen nach vierstündiger Stimulation mit TNFα zwar leicht vermehrt, der Anteil der am Endothel rollenden Thrombozyten unterschied sich jedoch nicht von dem in unbehandelten Tieren. Als rollende Thrombozyten wurden diejenigen mit einer Geschwindigkeit von weniger als 5% der maximal gemessenen Thrombozytengeschwindigkeit definiert.
Um herauszufinden, ob die beobachteten prothrombotischen Effekte Endothel-vermittelt oder durch direkte Wirkung auf Thrombozyten bedingt sind, wurden verschiedene Parameter in Thrombozyten und kultivierten Endothelzellen nach Stimulation mit TNFα untersucht. In primären humanen Endothelzellen (HUVEC) führte TNFα zu einer signifikanten Erhöhung der Superoxidproduktion, welche mittels Cytochrom-C Reduktion bestimmt wurde, sowie zur Translokation der p65-Untereinheit von NF-κB in den Zellkern, welche mittels Immunofluorescenz-Mikroskopie gemessen wurde. Durchflusszytometrisch wurde die Expression des für Thrombozyten-Endothel-Interaktion bedeutenden Adhäsionsmoleküls p-Selektin an der Oberflächenmembran von Endothelzellen bestimmt, wobei eine signifikante Hochregulation vier Stunden nach Behandlung mit TNFα gemessen werden konnte. Zudem wurden nicht direkt auf die primäre Hämostase bezogene Parameter untersucht und die Expression von Tissue Faktor an der Oberfläche von Endothelzellen bestimmt, welche durch TNFα Stimulation signifikant erhöht wurde. Ähnlich waren die mittels RT-PCR gemessenen mRNA Spiegel für PAI-1 (Plasminogen activator inhibitor 1) in HMEC vier Stunden nach Stimulation mit TNFα signifikant erhöht.
Um mögliche direkte Effekte von TNFα auf Thrombozyten auszuschließen wurden *in vitro* Thrombozytenaggregationsmessungen in humanem PRP (Plättchen reiches Plasma) nach Stimulation mit verschiedenen Agonisten durchgeführt. TNFα führte weder zu spontaner Aggregation, noch wurde die ADP-, Collagen- oder TRAP-abhängige Aggregation beeinflusst.

Um herauszufinden, welcher TNF-Rezeptor Subtyp für die beobachteten prothrombotischen Effekte verantwortlich ist, wurden arterielle Thrombose und Thrombozyten-Endothel-Interaktion *in vivo* in TNF-R1-/-, TNF-R2-/- und TNF-R1-/-R2-/- Mäusen analysiert. Die arterielle Gefäßverschlusszeit, welche bereits in Wildtyp Mäusen durch TNFα Stiumulation beschleunigt wurde, war in TNF-R1-/- Mäusen noch kürzer. Im Vergleich zu Wildtyp Tieren führte TNFα in TNF-R1-/- Mäusen auch zu einem erhöhten Anteil von rollenden Thrombozyten. Diese Effekte konnten in TNF-R2-/- und TNF-R1-/-R2-/- Mäusen nicht beobachtet werden. Da TNFα auch die Thrombozytenaggregation im PRP von TNF-R1 defizienten Mäusen nicht verstärkte, lassen diese Ergebnisse darauf schließen, dass die prothrombotischen Effekte von TNFα *in vivo* über den TNF-R2 Subtyp vermittelt werden.

Zusammenfassend konnten in dieser Studie zum ersten Mal prothrombotische Effekte des inflammatorischen Zytokins TNFα in einem *in vivo* Modell für arterielle Thrombose gezeigt werden. Die Ergebnisse weisen darauf hin, dass Endothel vermittelte Mechanismen diesen Beobachtungen zugrunde liegen. Zu diesen Mechanismen zählen vermehrte Superoxid Produktion, NF-κB Aktivierung und Hochregulierung von Adhäsionsmolekülen an der Zellmembran, sowie erhöhte Expression von Tissue Faktor und mRNA von PAI-1. Es konnte gezeigt werden, dass der endotheliale TNF-Rezeptor Subtyp 2 für die prothrombotischen Effekte *in vivo* verantwortlich ist und diese durch Signaling über den TNF-Rezeptor Subtyp 1 teilweise kompensiert werden können.

Diese Erkenntnisse führen zu einem besseren Verständnis der Bedeutung von TNFα und seinen Rezeptoren für die Pathophysiologie von artherothrombotischen Erkrankungen. Auch wenn für Behandlung oder Prävention dieser Erkrankungen durch gezielte Rezeptor spezifische Therapie sicherlich weitere Studien erforderlich sind, könnten die Ergebnisse zur Entwicklung solcher Ansätze in Zukunft beitragen.

8 APPENDIX

8.1 Non-standard abbreviations and acronyms

ADP	adenosine diphosphate
BSA	bovine serum albumin
CD40L	CD40 ligand
CD62P	p-selectin
Da	Dalton
eNOS	endothelial nitric oxide synthase
FACS	fluorescence activated cell sorting
FITC	fluorescein isothiocyanate
GPIb	glycoprotein Ib
HMEC	human microvascular endothelial cells
HUVEC	human umbilical vein endothelial cells
ICAM-1	intracellular adhesion molecule 1
IFNγ	interferon gamma
IL	interleukin
KO	knock out
NF-κB	nuclear factor kappa B
NO	nitric oxide
PAI-1	plasminogen activator inhibitor 1
PBS	phosphate buffered saline
PRP	platelet rich plasma
PSGL-1	p-selectin glycoprotein ligand 1
PVWI	platelet-vessel wall-interaction
RANTES	Regulated upon Activation, Normal T-cell Expressed and Secreted
RFU	relative fluorescence units
ROS	reactive oxygen species
RPE	R-phycoerythrin
RT-PCR	real time reverse transcriptase polymerase chain reaction
SOD	superoxide dismutase
TNF-R1	tumor necrosis factor-receptor subtype 1
TNF-R1-/-	tumor necrosis factor-receptor subtype 1 – knock-out
TNF-R1-/-2-/-	tumor necrosis factor-receptor subtype 1 and 2 – double knock-out
TNF-R2	tumor necrosis factor-receptor subtype 2
TNF-R2-/-	tumor necrosis factor-receptor subtype 2 – knock-out
TRAP-6	thrombin receptor activating protein
VCAM-1	vascular cell adhesion molecule 1
vWF	von Willebrand factor
WT	wildtype

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8.3 Publications

The results of this study have in part contributed or been submitted to publication of the following journal articles:

Struthmann L, Hellwig N, **Pircher J**, Bürkle M, Sohn H-Y, Klauss V, Mannell H, Pohl U, Krötz F. Effects of diclophenac on intravital platelet activation and atherothrombosis. *J Thromb Haemost. 2009 Oct;7(10):1727-35.*

Pircher J, Koch E, Vielhauer V, Chaudhry D, Sohn H-Y, Mannell H, Pohl U, Krötz F. The TNF-receptor-subtype 2 mediates prothrombotic effects in vivo. *Manuscript in preparation.*

The results of this study have in part been published as oral communications or poster presentations on the following congresses:

Pircher J, Vielhauer V, Mannell H, Sohn HY, Klauss V, Pohl U, Krötz F. The TNFα-receptor-subtyp 1 (TNFR1A) mediates antithrombotic effects in vivo. Jahrestagung Deutsche Gesellschaft für Kardiologie 2010, Mannheim, Germany.

Pircher J, Koch E, Vielhauer V, Mannell H, Sohn HY, Klauss V, Pohl U, Krötz F. The TNFα-receptor-subtyp 1 (TNFR1) mediates antithrombotic effects in vivo. Jahrestagung Deutsche Gesellschaft für Kardiologie 2009, Mannheim, Germany.

Pircher J, Vielhauer V, Mannell H, Sohn HY, Klauss V, Pohl U, Krötz F. The TNF-receptor-subtype 1 mediates antithrombotic effects in vivo. Nottingham Platelet Conference 2010, Nottingham, UK. (Abstract published in *Platelets, August 2010; 21(5): 393–419*).

Pircher J, Vielhauer V, Mannell H, Sohn HY, Klauss V, Pohl U, Krötz F. The TNF-receptor-subtype 1 mediates antithrombotic effects in vivo. World congress of Microcirculation 2010, Paris, France.

Further publications as journal articles:

Mannell H, **Pircher J**, Chaudhry D, Alig S, Koch E, Mettler R, Pohl U, Krötz F. ARNO regulates VEGF dependent tissue responses by stabilizing endothelial VEGFR-2 surface expression. *Accepted for publication in Cardiovasc Res 2011*

H. Mannell, F. Fochler, **J. Pircher**, T. Räthel, B. Gleich, C. Plank, O. Mykhaylyk, C. Dahmani, U. Pohl, F. Krötz. Site directed vascular gene delivery by ultrasonic destruction of magnetic nanoparticle coated microbubbles. *Submitted to Nanomedicine*

H. Mannell, **J. Pircher**, T. Räthel, K. Schilberg, K. Zimmermann, A. Pfeifer, O. Mykhaylyk, B. Gleich, U. Pohl, F. Krötz. Targeted endothelial gene delivery by ultrasonic destruction of lentiviral. *Submitted Pharm Res*

J. Pircher, F. Fochler, H. Mannell, C. Schuhmann, P. Del Soldato, A. Sparatore, U. Pohl, F. Krötz. The new hydrogen sulfide releasing aspirin derivative ACS14 is a strong antiplatelet drug effective beyond arachidonic acid dependent platelet activation. *Manuscript in preparation.*

Further publications as oral communications or poster presentations on congresses:

Pircher J, Fochler F, Mannell H, Del Soldato P, Pohl U, Krötz F. The new hydrogen sulfide releasing aspirin derivative ACS14 exerts strong antiplatelet effects in vitro and in vivo. Joint Meeting of the European Society of Microcirculation (ESM) and the German Society of Microcirculation and Vascular Biology (GfMVB) 2011, Munich, Germany.
Poster awarded by the ESM/GfMVB.

Pircher J, Fochler F, Mannell H, Del Soldato P, Pohl U, Krötz F. The new hydrogen sulfide releasing aspirin derivative ACS14 is a strong antiplatelet drug in vitro and in vivo. UK platelet meeting 2011, Cardiff, UK. (Abstract published in *Platelets, Online Supplement 10/2011*).

Pircher J, Fochler F, Schuhmann C, Mannell H, Del Soldato P, Pohl U, Krötz F. The new hydrogen sulfide-releasing Aspirin-derivative ACS14 is a highly effective platelet inhibitor in vitro and in vivo. Jahrestagung Deutsche Gesellschaft für Kardiologie 2011, Mannheim, Germany.

Pircher J, Koch E, Mannell H, Sohn HY, Pohl U, Krötz F. Die thrombozytäre Tyrosin-Phosphatase SHP-1 hemmt überschießende Collagen-induzierte Thrombozytenaggregation und arterielle Thrombose nach Gefäßverletzung in vivo. Jahrestagung Deutsche Gesellschaft für Kardiologie 2011, Mannheim, Germany.

Pircher J, Chaudhry D, Mannell H, Pohl U, Krötz F. The TNF-receptor-subtype 1 is responsible for ischemia-reperfusion induced endothelial dysfunction in vivo. Jahrestagung Deutsche Gesellschaft für Kardiologie 2011, Mannheim, Germany.

Pircher J, Chaudhry D, Mannell H, Pohl U, Krötz F. The TNF-receptor-subtype 1 is responsible for ischemia-reperfusion induced endothelial dysfunction in vivo. Annual meeting of the German physiological society 2011, Regensburg, Germany.

Pircher J, Hellwig N, Köhler R, Mannell H, Sohn HY, Klauss V, Pohl U, Krötz F. The intermediate conductance K^+-channel KCa3.1 prevents platelet activation in vivo. Jahrestagung Deutsche Gesellschaft für Kardiologie 2010, Mannheim, Germany.

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