Investigations into the influence of pretubulysin and other microtubule-targeting agents on the interaction of endothelial cells with tumor cells and leukocytes

Dissertation

zur Erlangung des Doktorgrades

der Naturwissenschaften

vorgelegt beim Fachbereich 14

der Johann Wolfgang Goethe-Universität

in Frankfurt am Main

von

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aus Offenbach am Main

Frankfurt (2018)

(D 30)

Vom Fachbereich Biochemie, Chemie und Pharmazie (FB 14) der
Johann Wolfgang Goethe-Universität als Dissertation angenommen.

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Datum der Disputation: 16.10.2018

with deep gratitude and love dedicated to my family

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Abbreviations

Table 1: List of abbreviations

Abbreviation	Full name
AML	Acute monocytic leukemia
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid assay
Bcl-2	B-cell lymphoma/leukemia-2
BSA	Bovine serum albumin
BSA-T	Bovine serum albumin with Tween® 20
CAM	Cell adhesion molecule
CCL	C-C motif chemokine
CCR	C-C chemokine receptor
cDNA	Complementary DNA
COL	Colchicine
CXCL	C-X-C motif chemokine
CXCR	C-X-C chemokine receptor
DMEM	Dulbecco’s modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
dsDNA	Double-stranded DNA
DTT	1,4-Dithiothreitol
ECGM	Endothelial cell growth medium
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
E-selectin	Endothelial-leukocyte adhesion molecule 1
FCS	Fetal calf serum
FGF	Fibroblast growth factor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GPCR	G-protein-coupled receptor

Table 1: Continue list of abbreviations

Abbreviation	Full name
GTP	Guanosine triphosphate
HER	Human epidermal growth factor receptor
HFS	Hypotonic fluorochrome solution
HFS-PI	Hypotonic fluorochrome solution with PI
HMEC	Human dermal microvascular endothelial cell
HRP	Horseradish peroxidase
HUVEC	Human umbilical vein endothelial cell
i. a.	Intra-arterial
ICAM	Intercellular adhesion molecule
Ig	Immunoglobulin
IKK	IκB kinase
IL	Interleukin
INF	Interferon
INT	Iodonitrotetrazolium violet
IVM	Intravital microscopy
IκB	Inhibitor of NFκB
JAM	Junctional adhesion molecule
Lamp	Lysosomal-membrane-associated glycoprotein
LDH	Lactate dehydrogenase
LFA	Lymphocyte function-associated antigen
LPS	Lipopolysaccharide
MAP	Microtubule-associated protein
MICL	Myeloid inhibitory C-type lectin-like receptor
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MRP	Multidrug resistance-associated protein
MSU	Monosodium urate
MTA	Microtubule-targeting agent
MTOC	Microtubule organizing center
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced NAD
NALP	NOD-like receptor family pyrin
N-cadherin	Neural cadherin
NEMO	NFκB essential modulator

Table 1: Continue list of abbreviations

Abbreviation	Full name
NFκB	Nuclear factor-κB
NGS	Normal goat serum
NOD	Nucleotide-binding oligomerization domain
PAC	Paclitaxel
PBS	Phosphate-buffered saline
PBSA	Phosphate-buffered saline with BSA
PCR	Polymerase chain reaction
PECAM	Platelet/endothelial cell adhesion molecule
PI	Propidium iodide
PMSF	Phenylmethylsulfonyl fluoride
PSGL	P-selectin glycoprotein ligand
PT	Pretubulysin
PVDF	Polyvinylidene difluoride
qRT-PCR	Quantitative real-time polymerase chain reaction
RD	Reagent diluent
RNA	Ribonucleic acid
RT	Room temperature
SDF	Stromal cell-derived factor
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SEM	Standard error of the mean
sLe	Sialyl lewis
TA	Thomsen-Friedenreich antigen
TBS-T	Tris-buffered saline with Tween® 20
TEMED	Tetramethylethylenediamine
TF	Tissue factor
TGF	Transforming growth factor
TMB	Tetramethylbenzidine
TNF	Tumor necrosis factor
Tup	Tubuphenylalanine
Tuv	Tubuvaline
VCAM	Vascular cell adhesion molecule
VDA	Vascular disrupting agent
VE-cadherin	Vascular endothelial cadherin

Table 1: Continue list of abbreviations

Abbreviation	Full name
VEGF	Vascular endothelial growth factor
VIN	Vincristine
VLA	Very late activation antigen

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I INTRODUCTION

1 The vascular endothelium in health and disease

The vascular endothelium is composed of a monolayer of endothelial cells lining the entire circulatory system forming an almost 1 kg endocrine organ from the heart to the smallest capillaries.[1,2] Instead of only facilitating selective permeability to water and electrolytes, the vascular endothelium plays a pivotal multifunctional role in fluid filtration, hormone trafficking, modulation of the vascular tone and growth, regulation of blood vessel formation, modulation of blood flow, regulation of thrombosis and thrombolysis, hemostasis, platelet adherence, coordination of platelet and leukocyte interactions, regulation of immune and inflammatory responses by controlling neutrophil, leukocyte, monocyte and lymphocyte interactions with the blood vessel wall.[1-3] The vascular endothelium can be influenced through specific junctional proteins and receptors that govern cell-cell and cell-matrix interactions as well as through membrane-bound receptors for various molecules such as proteins, metabolites, hormones and many more.[4] Due to the broad spectrum of vascular endothelial functions it is no surprise that endothelial injury, dysfunction and activation is involved in several diseases, for example, chronic kidney failure, hypertension, atherosclerosis, vascular leakage, congestive heart failure, stroke, cancer, diabetes, sepsis and inflammatory syndromes.[1,2] Thus, numerous advances in the design of therapeutic agents targeting the vascular endothelium have been made to restore the normal endothelial function.[5-8]

1.1 The role of the vascular endothelium in inflammatory diseases

In general, the tightly regulated process of acute inflammation is a beneficial event since it is strongly required for providing protection against infections or injury restoring homeostasis in the healthy organism. Acute inflammation occurs predominantly at postcapillary venules and its main function is, at a basic level, to recruit plasma proteins and leukocytes (mainly neutrophils) to the site of infection or injury, a process that is based on the selective extravasation through the activated endothelium.[9] However, a successful acute inflammatory response crucially includes a timely resolution and repair phase to prevent a pathological outcome that is associated with a broad variety of diseases characterized by constant infiltration of leukocytes leading to tissue damage or even cancer.[10] Resolution of inflammation is primarily mediated by recruited macrophages and is accompanied with an active biosynthesis of anti-inflammatory and pro-resolution lipid mediators such as lipoxins, resolvins and protectins as well as growth factors such as the transforming growth factor-β (TGFβ) to inhibit neutrophil recruitment and to promote the recruitment of monocytes, which initiate tissue remodeling.[10-13] Besides macrophages, epithelial and mesenchymal cells as well as

extracellular matrix (ECM) and the vascular endothelium are crucially involved in a successful tissue repair. Incomplete remodeling of the vasculature is associated with altered tissue oxygenation promoting the manifestation of atrophy and fibrosis.[14,15] Moreover, much less is known about the mechanisms causing chronic inflammatory diseases such as atherosclerosis, diabetes, chronic obstructive pulmonary disease, asthma, inflammatory bowel disease, neurodegenerative disease, multiple sclerosis, rheumatoid arthritis or cancer. Nonetheless, although non-resolving inflammation is not a primary cause of these diseases it significantly contributes to their pathogenesis.[15] Due its pivotal role in the inflammatory response, it is not surprising that the vascular endothelium is in the focus for the treatment of chronic inflammatory diseases. In fact, diverse therapies or therapeutic approaches are intent on the decrease of leukocyte-endothelial cell interactions mediated *via* reduced expression or blocking of cell adhesion molecules for example by the treatment with NFκB inhibitors or neutralizing antibodies.[16-19]

1.2 The role of the vascular endothelium in cancer

According to healthy cells and tissues, tumors have to be supplied with nutrients and oxygen and require the ability to get rid of metabolic wastes and carbon dioxide. Hence, a functional vascular network is essential for tumor growth and survival and metastatic dissemination. Angiogenesis, the sprouting of new blood vessels which is in an healthy adult only activated transiently as part of physiologic processes such as wound healing, is, therefore, continually activated during tumor progression leading to the permanent sprouting of new blood vessel surrounding the tumor.[20] On the one hand, constant angiogenesis is enabled by the upregulated expression of angiogenic factors such as the vascular endothelial growth factor (VEGF) or the fibroblast growth factor (FGF) due to both hypoxia and oncogene signaling.[21-24] On the other hand, angiogenesis can be induced by signals derived from cells of the innate immune system such as macrophages, neutrophils, mast cells and myeloid progenitors that are recruited to the tumor microenvironment and infiltrate the progressed tumor.[25-29] Thus, the tumor vasculature represents a promising therapeutic target since it is easily accessible to blood-borne drugs and its destruction prevents the nutrient and oxygen supply of tumors leading to tumoral cell death.[30] In this context, microtubule-targeting agents (MTAs) are the most frequently used chemotherapeutic drugs: besides the inhibition of endothelial cell proliferation due to their mitosis-blocking actions MTAs show mitosis-independent anti-angiogenic effects as well as vascular-disruptive actions on already existing tumor vessels.[31-39]

Besides the induction of angiogenesis the activation of invasion and metastasis was described as another hallmark of cancer – distinctive and complementary capability that enable tumor

growth and metastatic dissemination.[40] Three crucial steps in metastatic dissemination are (i) the intravasation of tumor cells into the circulatory system, (ii) the attachment of tumor cells to the healthy endothelium and finally (iii) the extravasation of tumor cells through the endothelium into the underlying tissue where the formation of a secondary tumor is initiated.[41,42] Although endothelial cells of the non-tumor vasculature are directly involved in all of these steps playing an essential role in their regulation, the impact of chemotherapeutic drugs such as MTAs on the healthy endothelium has been largely neglected.[42-44]

2 Cell interactions of the vascular endothelium

2.1 The interaction of endothelial cells with leukocytes or tumor cells

During the process of acute inflammation, leukocyte recruitment is initiated by the presentation of chemokines to leukocytes travelling in the circulatory system leading to the secretion of pro-inflammatory cytokines such as tumor necrosis factor-α (TNFα) to activate the vascular endothelium. Endothelial activation in turn results in the enhanced expression of selectins and cell adhesion molecules leading to the attachment of leukocytes to the endothelium. Since leukocyte attachment is initiated *via* weak interactions to selectins, the leukocytes are still pulled along with the blood flow, which leads to a rolling motion of the cells on the vascular surface (Figure 1).[45-47] In the next step of the cascade, leukocytes are activated by endothelial derived chemoattractants and chemokines that regulate leukocyte integrin affinity and avidity *via* inside-out signaling.[48-50] After activation, leukocytes tightly adhere to the endothelium enabled by cell adhesion molecules such as the vascular cell adhesion molecule-1 (VCAM-1) or ICAM-1. Finally, leukocytes extravasate through the endothelial barrier into the underlying tissue, a process referred to as diapedesis, where monocytes differentiate into classically or alternatively activated macrophages.[45-47]

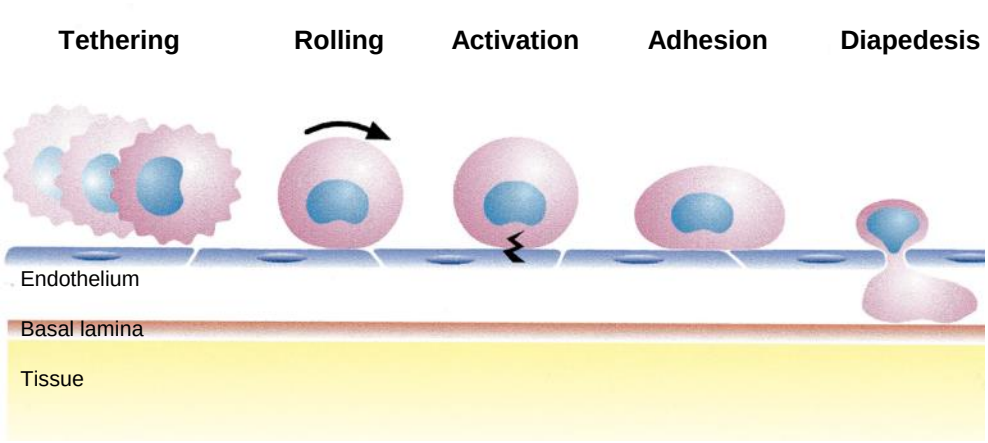

Figure 1: Important steps in the process of inflammation. The initial attachment (tethering) of leukocytes recruited to the vascular endothelium represents the first step in the inflammatory process. Due to the weak leukocyte-endothelial cell interactions leukocytes are still pulled along with the blood flow (rolling). Then, firm adhesion of leukocytes is mediated by endothelial cell-induced leukocyte activation. Finally, leukocytes transmigrate through the endothelium to extravasate into the underlying tissue (diapedesis). The figure is partially adapted and modified by permission from Springer Customer Service Centre GmbH, Springer Nature, Lab. Incest., Leukocyte-endothelial cell interactions in the inflammatory response, Muller WA, © 2002.[51]

Although only a small subset of cancer cells in a primary tumor are able to develop into metastases, cancer cell dissemination is responsible for 90 % of cancer-related mortality.[40,52,53] In general, the multistep process of tumor metastasis (Figure 2) is initiated by the detachment of malignant cells from a primary vascularized tumor that migrate and invade the surrounding tissue followed by direct transmigration through nearby blood vessels (or lymphatics) to enter the circulatory system (intravasation). If these spreading cancer cells are not eliminated by the immune system and survive the shear stress in the bloodstream, they can subsequently attach onto endothelial cells lining the blood vessels. This process commonly involves platelets, lymphocytes and other blood components. Then the disseminating cells either directly extravasate through the vascular endothelium into the underlying tissue and start to proliferate or they already proliferate in the blood vessels before they extravasate through the vascular endothelium into the underlying tissue collectively as groups.[41,54-57] These secondary tumors, referred to as micrometastases, can develop into clinically detectable macrometastases, provided that they are able to survive and proliferate in the new environment.[41,58,59]

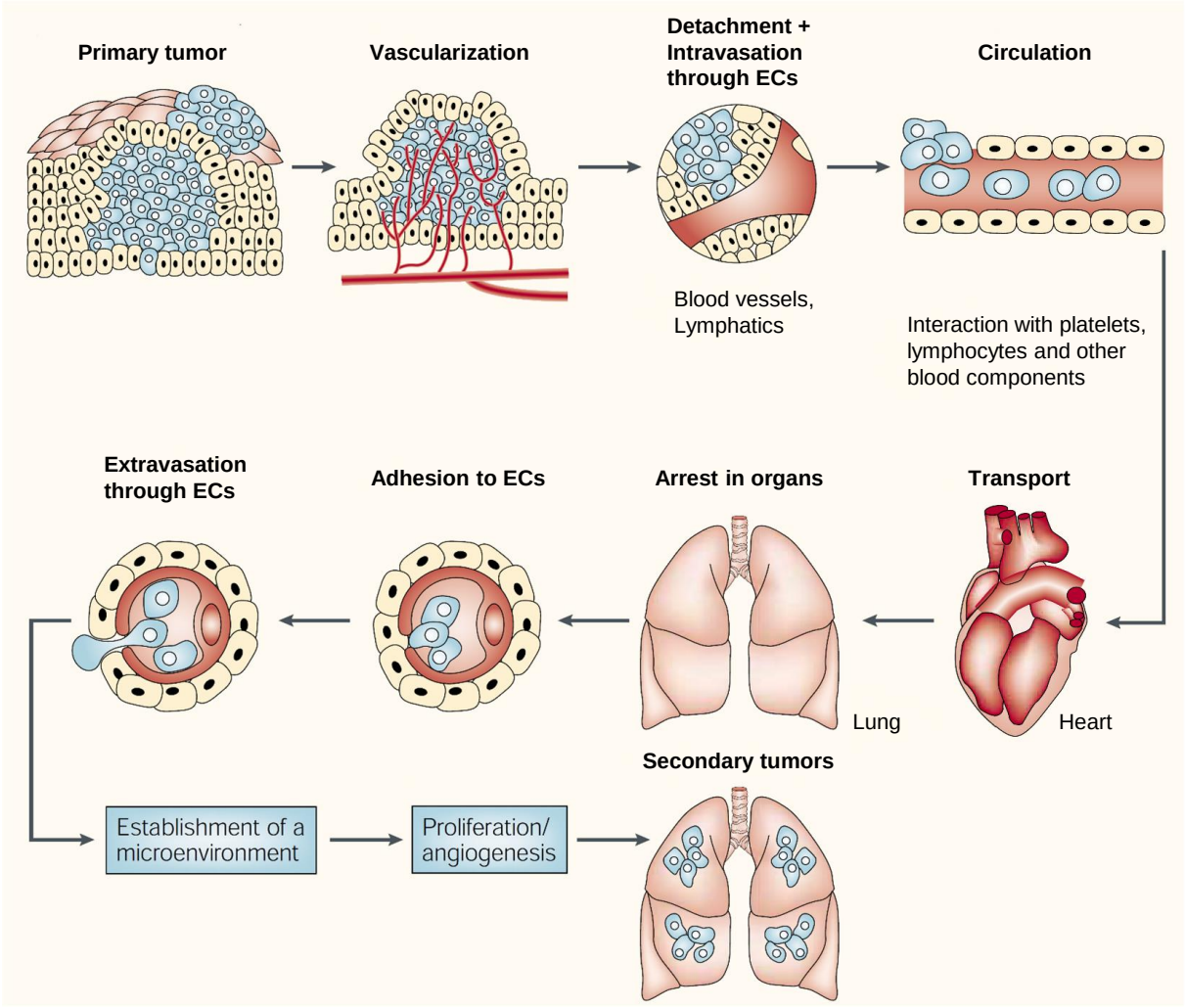

Figure 2: Important steps in the process of tumor metastasis. Single malignant cells are detached from a primary vascularized tumor, migrate and invade the surrounding tissue and intravasate through the endothelium into the circulatory system of vascular or lymphatic vessels. The disseminating tumor cells are transported into distant organs where they adhere onto and extravasate through the endothelium into the underlying tissue followed by the initiation of proliferation and angiogenesis leading to the formation of secondary tumors. The figure is partially adapted and modified by permission from Springer Customer Service Centre GmbH, Springer Nature, Nat Rev Cancer, The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited, Fidler IJ, © 2002.[60] ECs: endothelial cells.

2.2 Cell adhesion molecules involved in endothelial cell interactions with leukocytes or tumor cells

The interaction between vascular endothelial cells and leukocytes in the process of acute inflammation or tumor cells in the metastatic process is crucially regulated by cell adhesion molecules (CAMs) (Figure 3). Since the structure, function and hemodynamic pattern of vascular endothelial cells differs between the diverse organs and tissues of the human body, there are differences in how endothelial cells interact with tumor cells or leukocytes.[61,62] For instance, the rolling of leukocytes on the vascular surface seems to play a minor role in the

brain microvasculature while this process is even non-existent in pulmonary capillaries and hepatic sinusoids.[61-64]

2.2.1 Cell adhesion molecules involved in the rolling of leukocytes or tumor cells on the endothelium

Tethering and rolling of leukocytes on the endothelium (Figure 3) is mainly mediated by the selectin family of adhesion molecules and their ligands bearing the terminal core tetra-saccharide structure sialyl Lewis$^{x/a}$ (sLe^x/sLe^a).[65-67] The family of selectins comprises three related molecules: L-selectin (CD62L) is constitutively expressed in most leukocytes, E-selectin (CD62E) is expressed in the inflammatory activated endothelium and P-selectin (CD62P) is expressed in both the activated endothelium and activated platelets.[67] Accordingly, the diverse ligands for L-selectin on endothelial cells must be properly decorated with the correct carbohydrate residues upon endothelial activation, whereas P-selectin glycoprotein ligand-1 (PSGL-1), the major ligand for E-selectin and P-selectin, is constitutively expressed on all blood lymphocytes, monocytes and neutrophils.[47,51] Besides selectins, interactions between the integrin $\alpha_4\beta_1$ (also known as very late activation antigen-4, VLA-4) on leukocytes and the vascular-cell adhesion molecule-1 (VCAM-1) on the activated endothelium as well as homophilic interactions of N-cadherin on neutrophils and endothelial cells have been identified to be involved in the rolling of leukocytes on the vascular surface.[68,69]

Although the mechanisms by which tumor cells adhere to endothelial cells are not yet fully understood, it is very likely that they mimic mechanisms described for leukocyte-endothelial cell interactions.[70] Thus, as with leukocytes, the rolling of tumor cells on the endothelium is crucially mediated *via* selectins (Figure 3).[71] However, in contrast to leukocytes, tumor cells do not express selectins, but their respective ligands, since malignant transformation is associated with alterations of cell surface glycans leading to enhanced expression of sLe^x/sLe^a and other structures.[72,73] For instance, expression of the selectin ligands PSGL-1 and CD24 was determined for several tumors such as breast and colon cancer.[74-78] Hence, it is no surprise that several studies indicate that the enhanced expression of selectin ligands is associated with tumor progression, enhanced metastasis formation, organ-specific metastatic spreading and poor prognosis in various cancers.[79-81] The observation that the rolling of tumor cells on the vascular surface is enabled *via* interactions between endothelial E- and P-selectin and their ligands expressed on tumor cells implies the need of inflammatory processes in metastasis formation since these endothelial selectins are only expressed upon inflammatory activation.[71,82] Actually, several studies demonstrate the role of pro-inflammatory cytokines for the attachment of tumor cells to the endothelium.[83-86] As with neutrophils, besides selectins

hemophilic interactions of N-cadherin were reported to be involved in the rolling of tumor cells on the endothelium.[68]

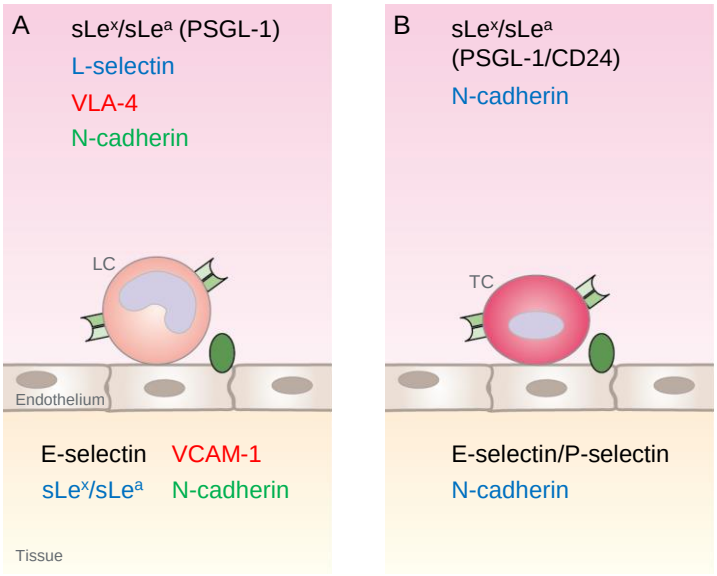

Figure 3: CAMs involved in the rolling of leukocytes or tumor cells on the vascular endothelium. (A) Prominent molecules involved in the rolling of leukocytes (LC) on the vascular surface are sLe^x/sLe^a (PSGL-1), L-selectin and VLA-4 expressed on leukocytes that interact with endothelial E-selectin, sLe^x/sLe^a or VCAM-1, respectively. **(B)** Prominent molecules involved in the rolling of tumor cells (TC) on the vascular surface are sLe^x/sLe^a (PSGL-1/CD24) expressed on tumor cells that interact with endothelial E-selectin or P-selectin, respectively. (A/B) Homophilic interactions of N-cadherin play a role in the rolling of both leukocytes and tumor cells on the vascular surface.

2.2.2 Cell adhesion molecules involved in the firm adhesion of leukocytes or tumor cells onto the endothelium

The rapid transition from rolling to firm adhesion of leukocytes on the endothelium (Figure 4) is mediated by the integrin family of cell adhesion molecules expressed by leukocytes and their interaction partners, immunoglobulin superfamily members, on endothelial cells.[87,88] Integrins are heterodimeric receptors consisting of two non-covalently associated α- and β-chains.[47,51] Within the eight groups of the integrin family, β1-integrins, which mediate mainly interactions between cells and extracellular matrix, and β2-integrins, which mediate mainly cell-cell interactions, are the most widely studied groups. The most important integrins involved in the firm adhesion of leukocytes on the endothelium are the αLβ2-integrin (also known as lymphocyte function-associated antigen-L (LFA-1)), the αMβ2-integrin (also known as Mac-1) and VLA-4.[70,89] The integrin interaction partners on the endothelium are the intercellular adhesion molecule-1 (ICAM-1) and -2 (ICAM-2) that interact with LFA-1 and Mac-1 and VCAM-1 that interacts with VLA-4.[47] Classical chemoattractants and chemokines secreted from inflammatorily activated endothelial cells play a crucial role in the integrin-mediated adhesion

since they rapidly regulate integrin affinity and avidity in a cell-specific manner by inside-out signaling leading to conformational changes of integrins from a favored low-affinity state to an high-affinity state with an opened ligand-binding pocket.[48-50] The endothelial integrin counterparts ICAM-1/-2 and VCAM-1 are expressed on the surface of the resting endothelium only at very low levels, but their surface expression is crucially increased in response to inflammatory cytokines such as TNFα or interleukin 1β (IL-1β).[90,91] Moreover, besides their role in the regulation of apoptosis, cell migration, adhesion and angiogenesis, there is a growing evidence that endothelial galectins such as galectin-1, -3 and -9 are also involved in the firm adhesion of leukocytes to the endothelium.[92-94]

Although the expression of several integrins, especially of the β2-subgroup, is mainly restricted to leukocytes, alterations in the integrin expression is associated with malignant transformation.[47,82] The β1-integrin subfamily constitutes a major class of integrins that mediate cell interactions with ECM proteins such as collagen, fibronectin and laminin and is critically involved in the adhesion of tumor cells to the basal lamina underlying the endothelium.[95,96] For instance, it was demonstrated that α5β1 and α2β1-integrins expressed by the breast cancer cell line MDA-MB-231 mediate tumor cell adhesion to fibronectin and type I collagen (Figure 4).[97] Moreover, it was reported that VLA-4 plays a crucial role in the firm adhesion of cancer cells to either fibronectin or endothelial VCAM-1.[98-102] In addition, as with leukocyte-endothelial cell interactions, there is growing evidence that tumor cells are able to tightly adhere to endothelial galectins what might be facilitated by an altered glycosylation and exposure of glycoproteins by the disseminating cells.[103,104] For instance, the Thomsen-Friedenreich antigen (TA) represented by MUC1 and CD44v6 is expressed on T-cell lymphomas and most human carcinomas and bears β-galactose as a terminal sugar which serves as a ligand for galectin-3.[105-111] Moreover, the lysosomal-membrane-associated glycoprotein-1 and -2 (Lamp1/2), whose surface expression is upregulated in several human tumor cells, were also described as ligands for galectin-3.[112] Besides the direct adhesion of tumor cells onto the endothelium, leukocytes are critically involved in tumor cell adhesion since they act as bridge or linker cells. For instance, MDA-MB-468 breast carcinoma cells expressing ICAM-1 were found to be connected *via* β2-integrins expressed on neutrophil granulocytes to endothelial cells expressing ICAM-1 that was also linked to β2-integrins of the bridging leukocytes.[47] A similar mechanism has been described for tumor cells expressing the α5β3-integrin that is linked *via* fibrinogen to αIIbβ3-integrins of platelets whose attachment to the endothelium is in turn mediated by selectins.[113-117]

It is worth of note that, in the case of fenestrated or discontinuous endothelia such as the hepatic endothelium, leukocytes and tumor cells can also directly attach to the underlying basal

lamina, which is mediated *via* the interaction between integrins and ECM components such as collagen and fibronectin.[47]

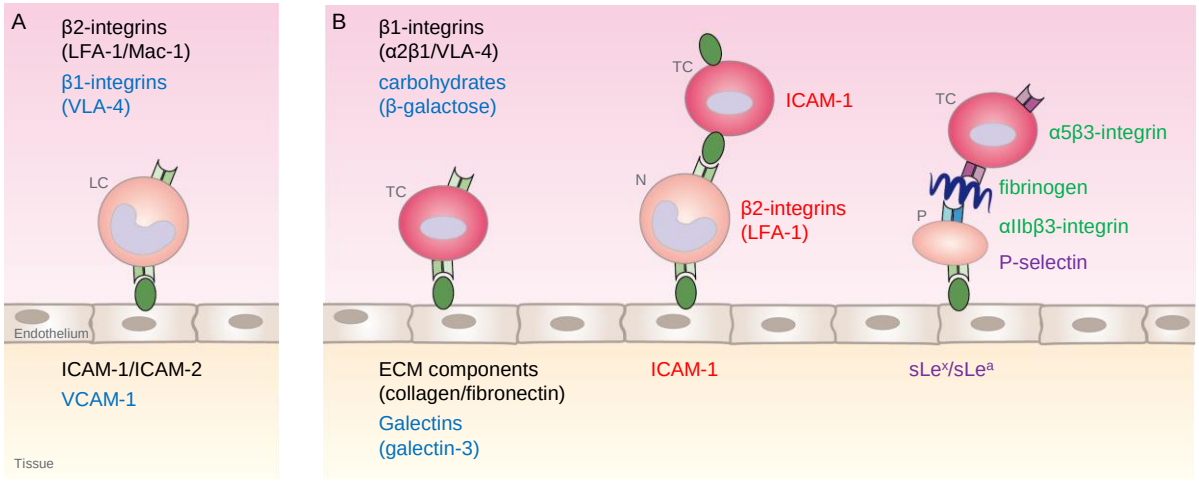

Figure 4: CAMs involved in the firm adhesion of leukocytes or tumor cells on the vascular endothelium. (A) Prominent molecules involved in the firm adhesion of leukocytes (LC) onto the vascular surface are β2-integrins (LFA-1/Mac-1) and β1-integrins (VLA-4) expressed on leukocytes that interact with endothelial ICAM-1, ICAM-2 and VCAM-1, respectively. **(B)** Prominent molecules involved in the direct firm adhesion of tumor cells (TC) onto the vascular surface are β1-integrins (α2β1/VLA-4) and carbohydrates (β-galactose) expressed on tumor cells that interact with extracellular matrix (ECM) components (collagen/fibronectin) and endothelial galectins (galectin-3), respectively. Tumor cells often use neutrophils (N) or platelets (P) as a linker to firmly adhere onto endothelial cells. Prominent molecules involved in this indirect firm adhesion of tumor cells onto the vascular surface are ICAM-1 or α5β3-integrin expressed on tumor cells, β2-integrins (LFA-1) expressed on neutrophils, αIIbβ3-integrin and P-selectin expressed on platelets and endothelial ICAM-1 and sLe^x/sLe^a, respectively.

2.2.3 Cell adhesion molecules involved in the transmigration of leukocytes or tumor cells through the endothelium

The transendothelial migration of leukocytes (Figure 5) is the final step in acute inflammation with respect to leukocyte-endothelial cell interactions. On this occasion, leukocytes can either transmigrate between two endothelial cells referred to as paracellular diapedesis or they can transmigrate through a single endothelial cell referred to as transcellular diapedesis.[118] Paracellular transmigration, where leukocytes have to pass endothelial tight junctions and adherens junctions, is mainly mediated by homophilic interactions of the platelet/endothelial cell adhesion molecule-1 (PECAM-1) and of CD99, both of which are expressed on most leukocytes as well as vascular endothelial cells.[119-121] PECAM-1, a member of the immunoglobulin (Ig) superfamily, is supposed to play a role in the early phase of leukocyte diapedesis, since extravasating cells were found to be tightly adherent to the apical surface of the endothelial monolayer and cannot pass pseudopods into the intercellular junctions upon PECAM-1 blockade.[119,120,122,123] In contrast, upon the blockade of CD99 transmigrating leukocytes were found to arrest part-way across the intercellular junctions whereby the leading edge of the extravasating cell was below the endothelial monolayer whereas the trailing edge

remained on the apical surface of the endothelium. Hence, CD99, a type 1 transmembrane protein which does not belong to any known protein family, is supposed to play a role in the late phase of leukocyte diapedesis.[121] While both PECAM-1 and CD99 are involved in the diapedesis of monocytes and neutrophilic granulocytes to the site of inflammation, only CD99 seems to be relevant for lymphocyte diapedesis.[121,122,124-127] Besides PECAM-1 and CD99, junctional adhesion molecules (JAMs), further members of the Ig superfamily, are associated with paracellular diapedesis, since it was reported that JAMs expressed on endothelial cells can interact with integrins on leukocytes (JAM-1 binds to LFA-1, JAM-2 binds to VLA-4 and JAM-3 binds to Mac-1).[118,128-130] Much less is known about transcellular diapedesis, however, it is assumed that it is mediated *via* the same molecules as described for the paracellular transmigration of leukocytes.[130]

In contrast to the diapedesis of leukocytes, where the endothelial integrity is only transiently and reversibly impaired, the transendothelial migration of tumor cells is associated with an irreversible damage caused by active endothelial retraction that is required for tumor cells to overcome the endothelial barrier function.[131-134] Endothelial integrity is primarily dependent on the organization and function of transmembrane adhesion proteins of both tight and adherens junctions. Endothelial adherens junctions formed by homophilic interactions of vascular endothelial cadherin (VE-cadherin), which builds a complex with cytoskeletal and signaling proteins including α-, β- and γ-catenin, represents one of the major adhesion systems in this context.[135] Disseminating tumor cells are thought to increase the vascular permeability by inducing changes in endothelial cells such as tyrosine phosphorylation of VE-cadherin which leads to the dissociation of cytoskeletal proteins and is accompanied by the reorganization of the actin cytoskeleton, the formation of actin stress fibers and interendothelial gaps and the induction of apoptosis (Figure 5).[131,136-140] It is suggested that these changes in endothelial function can be caused solely by the attachment of tumor cells on the endothelium and/or the secretion of endothelial cell-influencing factors.[132,133] For instance, it could be demonstrated that the adhesion of invasive breast, prostatic and ovarian cancer cells onto the endothelium led to endothelial retraction caused by tyrosine phosphorylation of VE-cadherin, which was mediated by activation of the H-Ras/Raf/MEK/ERK signaling cascade triggered by endothelial cell interactions with tumoral α2β1-integrins.[132] Furthermore, the transmembrane protein tissue factor (TF), which is highly expressed on metastatic melanoma cell lines, is crucially involved in the generation of the serine protease thrombin.[141,142] Tumor-derived thrombin in turn facilitates the transendothelial migration of melanoma cells since it is able to disrupt the endothelial integrity by both tyrosine phosphorylation and ubiquitination-coupled internalization of VE-cadherin.[134] Thus, instead of a direct interplay between cell adhesion molecules as described for the diapedesis of leukocytes, transendothelial migration of tumor cells

substantially depends on the loss of endothelial barrier function caused by an active breakdown of endothelial junction integrity.

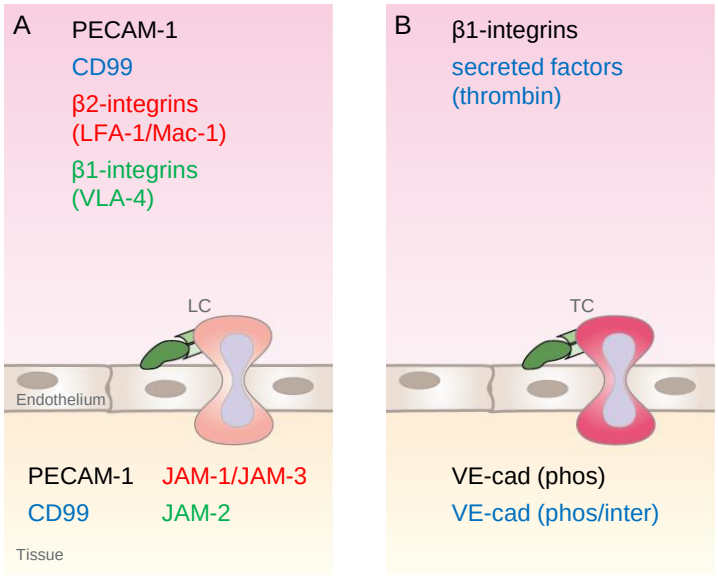

Figure 5: Transmigration of leukocytes or tumor cells through the vascular endothelium. **(A)** Homophilic interactions of PECMA-1 and CD99 are mainly involved in the transendothelial migration of leukocytes (LC). Further prominent molecules involved are β2-integrins (LFA-1/Mac-1) and β1-integrins (VLA-4) expressed on leukocytes that interact with endothelial JAM-1, JAM-2 and JAM-3, respectively. **(B)** The transendothelial migration of tumor cells (TC) through the endothelium is not mediated by typical molecule interactions. However, the β1-integrin-mediated adhesion of tumor cells onto the vascular surface or factors secreted by tumor cells (e.g. thrombin) induce endothelial VE-cadherin phosphorylation and, in the case of thrombin, also VE-cadherin internalization causing endothelial cell retraction.

2.3 Chemokines involved in endothelial cell interactions with leukocytes or tumor cells

Besides CAMs, various chemokines play a pivotal role in the regulation of leukocyte-endothelial cell interactions, since they provide the directional information for the movement of leukocytes during development, homeostasis and inflammation.[143] Moreover, chemokines are thought to be the most powerful physiological activators of the firm adhesion of leukocytes as they are able to convert the low-affinity, selectin-mediated interaction between leukocytes and the endothelium into the higher-affinity, integrin-mediated interaction within less than a second.[48,49] Chemokines are a superfamily of small molecules with highly conserved structural elements and are secreted by many different cell types of the body under inflammatory and non-inflammatory conditions.[143] Chemokine receptors belong to the family of G-protein-coupled receptors (GPCRs) and are mainly expressed on different types of leukocytes, where their expression is specific in certain subpopulations.[47,144,145] During inflammation, leukocytes are therefore selectively recruited into the inflamed tissue by increased secretion of the

respective chemokines at the site of infection or injury.[143] Early pro-inflammatory cytokines such as interleukin-1 and -4 (IL-1 and IL-4), tumor necrosis factor-α (TNFα) and interferon-γ (INFγ) as well as bacterial products such as lipopolysaccharide (LPS) and viral products represent the primary stimuli of chemokine secretion in the process of acute inflammation.[146-148] For instance, the prominent neutrophil activator interleukin-8 (IL-8), also known as C-X-C motif chemokine 8 (CXCL8), can be produced by activated endothelial cells or macrophages and binding to one of its receptors (C-X-C chemokine receptor type 1 or 2, CXCR1/2) triggers a signaling cascade leading to the activation of leukocyte integrins and thus firm adherence.[146,149,150] Due to the specific expression of chemokine receptors in leukocyte subpopulations the type of inflammatory infiltrate in acute and chronic diseases such as bacterial pneumonia, the acute respiratory distress syndrome, ulcerative colitis and Crohn's disease is, at least in part, controlled by the subgroup of chemokines expressed in the diseased tissue.[148,151-153]

Chemokines have also been suggested to play a crucial role in tumor growth and tumor cell migration as well as in the determination of the metastatic pattern of various malignant neoplasias such as breast cancer, prostate cancer, colon and colorectal cancer, melanoma and many more.[154-168] For instance, the chemokine receptors C-X-C chemokine receptor type 4 (CXCR4) and C-C chemokine receptor type 7 (CCR7) are highly expressed in human breast cancer cells, malignant tumors and metastases, where the expression of CXCR4 is mediated by overexpression of the receptor tyrosine-protein kinase c-erbB-2 (also known as HER2/neu) in about 30 % of all breast cancers.[169-171] Accordingly, the respective ligands for these receptors, C-X-C motif chemokine 12 (CXCL12, also known as stromal cell-derived factor-1α, SDF-1α) for CXCR4 and C-C motif chemokine 21 (CCL21) for CCR7, are prominently expressed in organs that are preferential destinations for breast cancer metastasis including bone marrow, brain, liver, lung and lymph nodes.[169] Similar correlations between the chemokine system CXCL12/CXCR4 and organ-specific metastasis formation could be observed for prostate cancers, colon and colorectal cancers and melanoma.[163,167,172-176] Furthermore, it was demonstrated that the administration of CXCL12 to PC-3 prostate cancer cells resulted in an increased expression of CXCR4 leading to an enhanced adhesion onto and transmigration through endothelial cells most likely due to the CXCL12/CXCR4-regulated expression and activation of α5β3-integrins.[177-179] In addition, the chemokine receptor C-X-C chemokine receptor type 3 (CXCR3), which is the receptor for the C-X-C motif chemokine 9, 10 and 11 (CXCL9, CXCL10, CXCL11), has been associated with colon cancer and melanoma cell dissemination into the lymph nodes.[180-182] Moreover, it was reported that the chemokine system CXCL9/CXCR3 triggers melanoma cell migration as well as the α5β1-integrin (also known as VLA-5) and VLA-4 dependent melanoma cell adhesion to fibronectin, which highlights the role of this chemokine system in melanoma cell metastasis formation.[182]

2.4 The NFκB signaling pathway

The nuclear factor-κB (NFκB) is an important, ubiquitously expressed transcription factor that is typically activated by pro-inflammatory cytokines such as TNFα and IL-1 and other specific stimuli and plays a critical role in the regulation of various biological processes including apoptosis, inflammation, innate and adaptive immunity and many more. This is achieved by regulating the transcription of several target genes such as cell adhesion molecules (e.g. ICAM-1, VCAM-1, E-selectin), pro-inflammatory cytokines and chemokines.[183,184] Thus, inflammatory activation of NFκB is crucially involved in leukocyte recruitment to the inflamed or injured tissue as well as in leukocyte activation.[183,185,186] Due to the broad spectrum of NFκB responsibilities it is no surprise that aberrant NFκB activity has been implicated in the pathogenesis of many diseases including acute respiratory distress syndrome and *H. pylori* infection as well as chronic diseases such as atherosclerosis and rheumatoid arthritis.[187-190]

In the simplest model of NFκB activation (Figure 6), an inflammatory stimulus, for example TNFα, activates the signal transduction cascade that induces the activation of the inhibitor of NFκB (IκB) kinase (IKK) complex by phosphorylation.[183,184] The IKK complex is composed of the two catalytic subunits IKKα (also known as IKK1) and IKKβ (also known as IKK2) as well as the regulatory subunit IKKγ (also known as NEMO).[191,192] Activation of IKKα and IKKβ in turn leads to the phosphorylation of members of the IκB protein family, which comprises the typical IκB proteins IκBα, IκBβ and IκBε, the precursor proteins p100 and p105 and atypical IκB proteins such as BCL-3 and IκBNS.[191,193-198] Phosphorylated IκBα then undergoes rapid ubiquitin-mediated degradation by the proteasome whereby NFκB homo- or heterodimers, which are in an non-activated cell sequestered in a complex with IκB proteins in the cytoplasm, are released from the IκB/NFκB complex.[183,184] Subsequently, released cytosolic NFκB dimers, for example the heterodimer p50/p65 as the most abundant form, translocates into the nucleus, where it is further activated by diverse posttranslational modifications followed by binding to promoters or enhancers of NFκB target genes.[183,184,193,194] Gene transcription is finally terminated by one of the well described negative feedback loops or by post-translational ubiquitination of the signaling intermediates.[199]

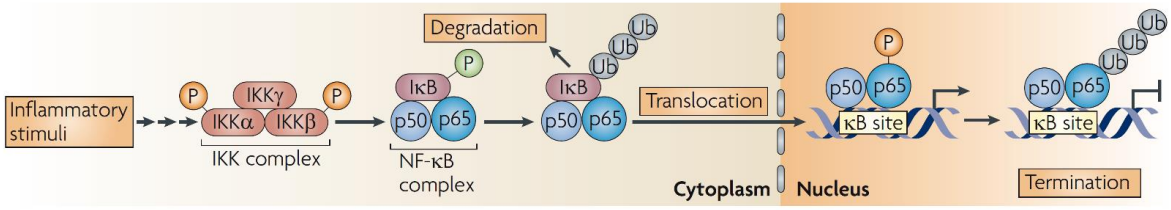

Figure 6: Crucial steps in the canonical NFκB signaling pathway. Inflammatory stimuli phosphorylate and thereby activate the catalytic subunits IKKα and IKKβ of the IKK complex, which in turn results in the phosphorylation of the NFκB inhibitor IκBα. Phosphorylated IκBα is subsequently ubiquitinated and degraded by the proteasome whereby the NFκB heterodimer p50/p65 is released. The released cytosolic p50/p65 heterodimer translocates into the nucleus where it binds to the NFκB binding site of promotors or enhancer of NFκB target genes. Gene transcription can be terminated by post-translational ubiquitination of the translocated subunits. The figure is adapted by permission from Springer Customer Service Centre GmbH, Springer Nature, Nat Rev Immunol., New regulators of NFκB in inflammation, Ghosh S and Hayden MS, © 2008.[183] IKK: inhibitor of IκB kinase, NFκB: nuclear factor-κB, P: phosphate, Ub: ubiquitin.

3 The microtubule network

Microtubules – key components of the eukaryotic cytoskeleton – are highly dynamic structures, which play an essential role in a wide range of cellular processes such as development and maintenance of cell shape, intracellular transport, cell signaling, cell division and mitosis, cell polarization and migration just to name a few.[200-203] Especially the importance of microtubules in cell division and the orchestration of mitotic events makes them, at least so far, the major target for anticancer drugs.[201,204]

3.1 The structure of microtubules

Microtubules are polarized hollow tube-like structures composed of 13 parallel protofilaments, which are formed by polymerization of α/β-tubulin heterodimers so that α- and β-tubulin subunits alternate along the longitudinal axis of the filament (Figure 7).[202,205] Due to the tandem arrangement of α/β-tubulin heterodimers, microtubules consists of a (+)-end, which is terminated by β-tubulin subunits and radiates in the whole cytoplasm, and a (-)-end, which is terminated by α-tubulin subunits and is anchored at the microtubule organizing center (MTOC), also known as centrosome by which microtubule assembly is controlled.[202,206,207] The dynamic behavior of microtubules is defined by their ability to undergo cycles of rapid growth (polymerization) and shrinkage (depolymerization) – referred to as dynamic instability, a process mediated by the exchange of guanosine triphosphate (GTP) by guanosine diphosphate (GDP).[200,208,209]

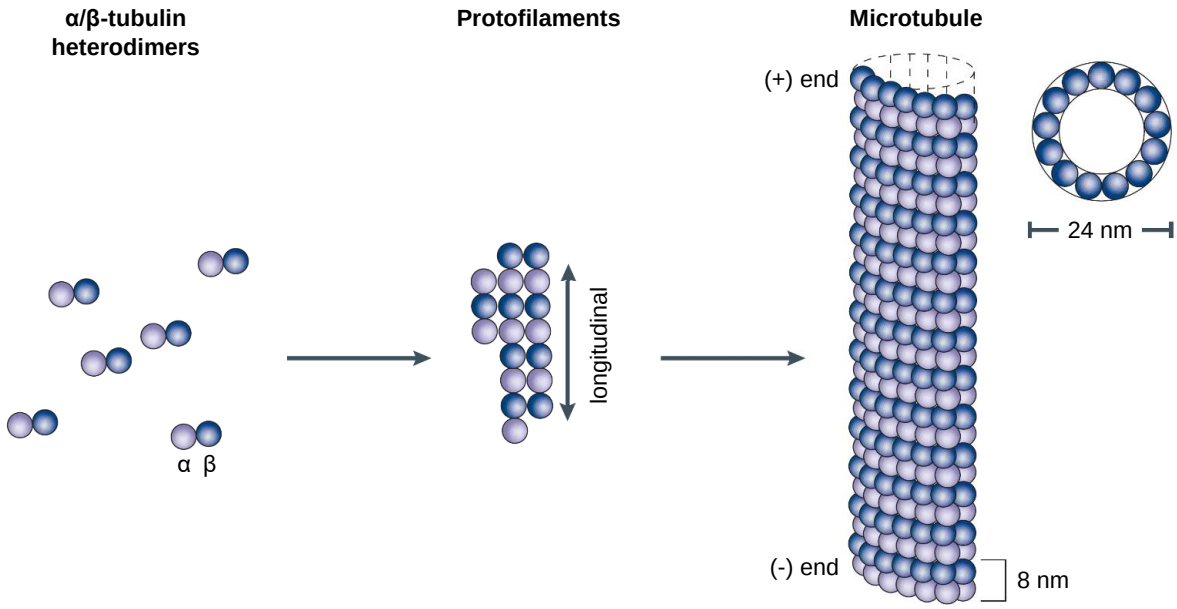

Figure 7: The structure of microtubules. Microtubules are polarized hollow tube-like structures composed of 13 parallel protofilaments. Each protofilament is formed by polymerization of α/β-tubulin heterodimers so that α- and β-tubulin subunits alternate along the longitudinal axis of the filament. The figure is partially adapted and modified by permission from Springer Customer Service Centre GmbH, Springer Nature, Nat Rev Cancer, Microtubules as a target for anticancer drugs, Jordan MA and Wilson L, © 2004.[201]

3.2 Microtubule dynamics – mode of action

The dynamic properties of microtubules are essential for their biological functions as they allow the rapid reorganization and the spatial and temporal differentiation of microtubules in accordance with the cell context.[208] Microtubule polymerization and thus microtubule assembly occurs on both ends of the growing structure in two phases: the formation of a short polymerization nucleus (nucleation) precedes the rapid polymer growth (elongation) by reversible, noncovalent addition of α/β-tubulin heterodimers.[201] Although both the α- and β-tubulin subunit possess a GTP binding region, GTP bound to α-tubulin at the dimer interface is never hydrolyzed or exchanged whereas these conversions are possible in the case of GTP bound to β-tubulin since it is able to bind both GTP and GDP.[205,208] During microtubule polymerization, GTP is hydrolyzed to GDP shortly after the GTP-bound β-tubulin subunit was integrated into the microtubule structure (Figure 8). In the case of rapid microtubule growth, which appears when microtubule polymerization is faster than microtubule depolymerization, a new GTP-bound β-tubulin subunit is incorporated into the microtubule polymer before the GTP of the previously integrated subunit is hydrolyzed leading to accumulation of GTP at the microtubule tip which is referred to as a GTP cap.[202,210,211] In contrast, a slow rate of GTP-bound β-tubulin subunit addition to the microtubule polymer offers enough time for hydrolysis of GTP of the previously integrated subunit into GDP leading to the loss of the GTP cap due

to GDP accumulation at the microtubule tip.[202] Since GTP hydrolysis triggers conformational changes of tubulin causing a reduction in the binding affinity of the subunits within the polymer, the protofilament is turned into a curved shape leading to the dissociation of α/β-tubulin heterodimers and thus microtubule depolymerization.[202,210] Since the microtubule (+)-end is terminated by β-tubulin subunits allowing the formation of a GTP cap, it grows and shrinks more rapidly than the microtubule (-)-end, which is terminated by α-tubulin subunits and embedded in the MTOC.[210,212] In addition to dynamic instability, microtubules display another dynamic behavior called treadmilling – a process by which α/β-tubulin heterodimers are released from the microtubule (-)-end and simultaneously added at the microtubule (+) end of the same microtubule.[213-215] Dynamic instability and treadmilling are not mutually exclusive activities as they even occur in some microtubule populations at the same time depending on intracellular conditions.[216,217]

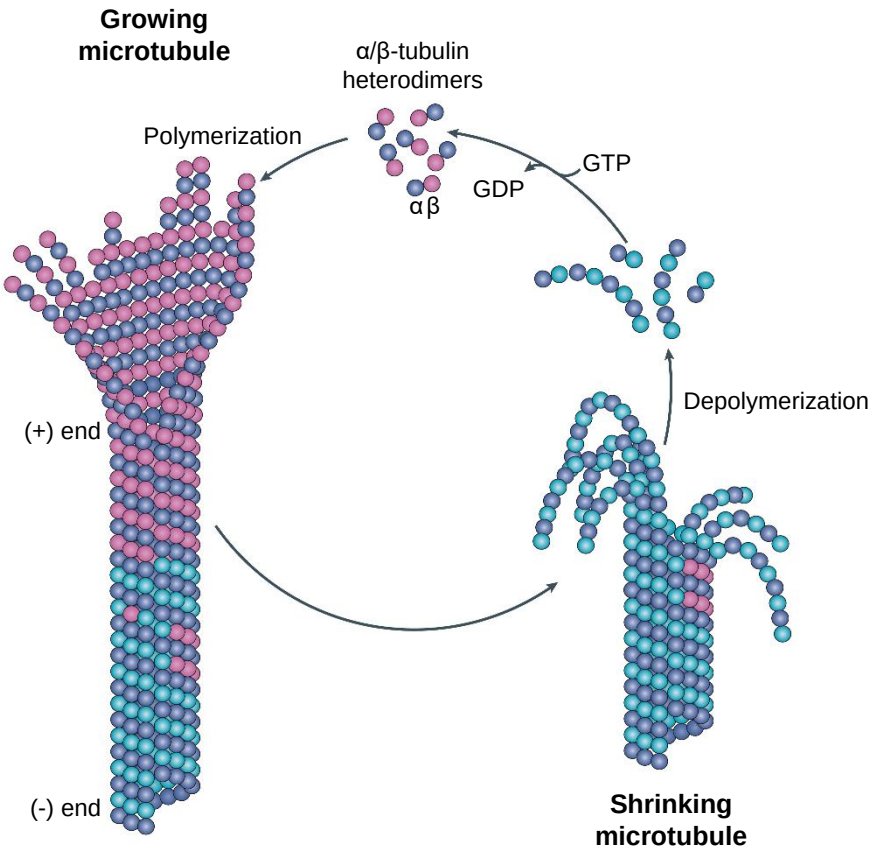

Figure 8: The dynamic instability of microtubules. The dynamic instability of microtubules is defined by their ability to undergo cycles of rapid growth (enabled by polymerization) and shrinkage (enabled by depolymerization). Microtubule polymerization is mediated *via* reversible and noncovalent addition of α/β-tubulin heterodimers to the growing microtubule, a process mediated by the hydrolysis and exchange of GTP into GDP. A slow rate of α/β-tubulin heterodimer addition leads to conformational changes of tubulin that results in a reduction of binding affinity of the tubulin subunits causing the protofilaments to turn into a curved shape and thus microtubule depolymerization. The figure is partially adapted and modified by permission from Springer Customer Service Centre GmbH, Springer Nature, Nat Rev Mol Cell Biol., Control of microtubule organization and dynamics: two ends in the limelight, Akhmanova A and Steinmetz MO, © 2015.[203]

3.3 The regulation of microtubule dynamics

Microtubule dynamics and activities are critical for the different cellular functions of microtubules and are therefore finely regulated by the expression level of different tubulin isoforms, posttranslational modifications and the activity of microtubule-associated proteins (MAPs).[201,206,218,219] To date, six α-tubulin and seven β-tubulin isoforms with various expression levels in different cells and tissues have been identified in humans.[202] According to posttranslational modifications including acetylation, detyrosination/tyrosination, palmitoylation, phosphorylation, polyglutamylation and polyglycylation, each isotype can further be divided into different subtypes.[206,219] Although microtubule dynamics can be directly affected by the tubulin isotypes and their posttranslational modifications, the main modulators of microtubule dynamics are MAPs and other microtubule-interacting proteins.[206] The large family of MAPs regulate microtubule dynamics either by favoring microtubule polymerization and thus stabilization, which is mediated by MAPs such as MAP4, Tau, survivin, EB1, dynactin 1, TOGp and FHIT, or by sequestering α/β-tubulin heterodimers and promoting microtubule depolymerization and thus destabilization, which is mediated by MAPs such as stathmin, the kinesin-13 family and MCAK.[206,220-225]

4 Microtubule-targeting agents (MTAs)

The involvement of microtubules in mitosis and cell division makes them an important target for anticancer drugs. In fact, microtubule-targeting agents (MTAs) are the most frequently used chemotherapeutic drugs. All therapeutically applied MTAs represent natural products – produced by a large number of plants and animals – or semisynthetic derivatives thereof.[31,201] MTAs are classified according to their action on the microtubule system into two main groups: microtubule-stabilizing and -destabilizing agents. Microtubule-destabilizing drugs, including the plant-derived *Vinca* alkaloids (from *Catharanthus roseus*) including vincristine, vinblastine, vinorelbine, vindesine and vinflunine as the main representatives, destabilize microtubules either by inhibiting microtubule polymerization or by depolymerizing existing ones and are currently used against hematological and lymphatic cancers and several solid tumors such as breast cancer.[226-228] Moreover, the plant-derived compound colchicine (from *Colchicum autumnale*) belongs to the class of microtubule-destabilizing drugs as well. However, colchicine has yet found no significant use in cancer treatment, but it is commonly used for the therapy of gout and familial Mediterranean fever as well as other inflammatory diseases.[31,201,229] Microtubule-stabilizing drugs, comprising the plant-derived taxane paclitaxel (from the bark of *Taxus brevifolia*) and its semi-synthetic derivative docetaxel as well as the

bacterial epothilones (from the myxobacterium *Sorangium cellulosum*), stabilize microtubules either by initiating microtubule polymerization or by hyper-stabilizing existing ones and are currently used against solid tumors including breast, ovarian and lung cancer.[230-234] Due to the clinical success of MTAs, a large variety of experimental drugs such as combretastatins, discodermolide, laulimalides, spongistatin and tubulysins are currently in preclinical and/or clinical trials.[31,201] Apart from the fact that some MTAs bind more or less to soluble tubulin, the aforementioned compounds bind directly with high affinity to β-tubulin along microtubules.[201,235] In the case of microtubule-destabilizing drugs, binding to the β-subunit of microtubules occurs either at the *Vinca*-binding domain located at the microtubule (+)-end or the colchicine-binding domain located within the microtubule lattice, whereas the binding of microtubule-stabilizing drugs occurs at the taxane-binding domain located on the inside surface of the microtubule (Figure 9).[31,201]

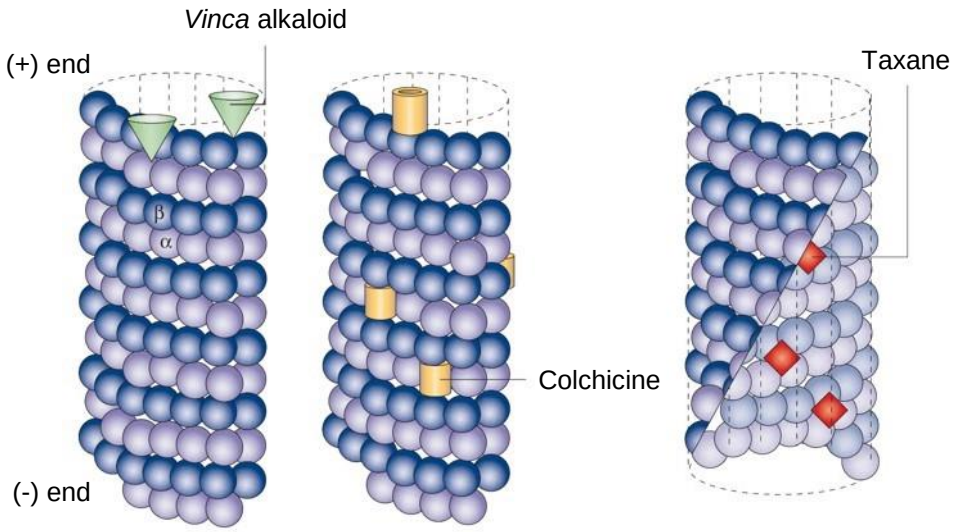

Figure 9: MTA-binding domains on microtubules. The microtubule-destabilizing *Vinca* alkaloids bind to microtubule β-tubulin at the *Vinca*-binding domain located at the microtubule (+)-end, whereas colchicine binds to the β-subunit of microtubules at the colchicine-binding domain located within the microtubule lattice. The microtubule-stabilizing taxanes bind to microtubule β-tubulin at the taxane-binding domain located on the inside surface of the microtubule. The figure is partially adapted and modified by permission from Springer Customer Service Centre GmbH, Springer Nature, Nat Rev Cancer, Microtubules as a target for anticancer drugs, Jordan MA and Wilson L, © 2004.[201]

4.1 The use of MTAs as anticancer drugs

The anti-cancer effects of MTAs have frequently been associated with the inhibition of mitosis causing the reduction of cancer cell proliferation and finally cell death induction through apoptosis.[235] However, other notable effects of MTAs including the induction of mitosis-independent cell death, the inhibition of metastasis and tumor angiogenesis as well as their

vascular disruptive activity strongly suggest that mitosis is not a key “target” of MTAs.[31] For instance, the microtubule-destabilizing drug spongistatin and paclitaxel have been shown to induce the phosphorylation and thus inactivation of Bcl-2, a mitosis-independent acting anti-apoptotic modulator of the Bcl-2 (B-cell lymphoma/leukemia-2) family.[236-239] Furthermore, in the case of paclitaxel it was demonstrated that it is also able to cleave Bcl-2 converting it from an anti-apoptotic into a pro-apoptotic modulator.[238] In addition, it was reported that both spongistatin and paclitaxel were able to interfere with cancer metastasis. While spongistatin reduced the expression of the matrix metalloproteinase 9 (MMP-9), a major gelatinase cleaving and degrading ECM microstructures, leading to reduced metastasis formation, paclitaxel caused the inhibition of MMP-2 and MMP-9 exocytosis from cytoplasmic vesicles and thus inhibition of invasion.[237,240-242] Besides their influence on cancer cells, paclitaxel and other microtubule-stabilizing agents as well as spongistatin and other microtubule-destabilizing agents also show anti-angiogenic effects in endothelial cells of the tumor vasculature both in classical *in vitro* and *in vivo* assays.[32-34,237,243] Moreover, the microtubule-destabilizing drug combretastatin A-4 3-*O*-phosphate (CA4P) currently undergoes advanced clinical testing, since it represents a very potent vascular disrupting agent (VDA), meaning that it induces a rapid collapse of the existing tumor vasculature without significantly harming normal tissues, causing the blockade of blood flow and tumor necrosis.[30,31,244]

4.2 The use of MTAs as anti-inflammatory drugs

The lead compound of MTAs as anti-inflammatory drugs is colchicine (COL). Beside its common use for the therapy of gout and familial Mediterranean fever, COL has also been used or is recommended (off-label) for the therapy of rheumatic diseases such as vasculitis and neutrophilic dermatoses as well as for cardiovascular diseases such as pericarditis, coronary artery disease and secondary atrial fibrillation.[229,245] The anti-inflammatory effect of COL is mainly mediated by interfering with cellular functions of leukocytes such as the chemotactic recruitment of neutrophils as well as neutrophil rolling and transendothelial migration.[246-249] For instance, COL reduced the monosodium urate crystals (MSU)-induced chemotactic recruitment of neutrophils in gout, which is on the one hand enabled by suppression of the MSU-induced activation of caspase-1, the enzymatic component of the nucleotide-binding oligomerization domain (NOD-like) receptor family pyrin 3 (NALP3) inflammasome, leading to reduced IL-1β as well as TNFα and IL-6 levels of macrophages.[246] On the other hand, in neutrophils the MSU-induced loss of the myeloid inhibitory C-type lectin-like receptor (MICL), an inhibitory receptor downregulated by several pro-inflammatory cytokines, is inhibited by COL.[249] Moreover, it was demonstrated that the IL-1- and TNFα-induced rolling of neutrophils on the vascular surface is reduced by COL due to changes in the distribution of E-selectin on

endothelial cells and a decreased expression of L-selectin on the surface of neutrophils.[247] In addition, recent studies suggest that COL reduces the transendothelial migration of neutrophils, since it inhibits their deformability and motility crucial for the process of extravasation.[248] Besides its well-studied use as an anti-cancer drug, studies of the last two decades recommended paclitaxel at low-doses for the use of non-cancer diseases such as fibrotic diseases, inflammation, critical limb ischemia and coronary artery restenosis.[250-255] To date, the molecular mechanism underlying the anti-inflammatory effect of paclitaxel is not fully understood. However, it was reported that paclitaxel clearly inhibits the chemotaxis of neutrophils triggered by endotoxin-activated serum.[256] Moreover, paclitaxel was demonstrated to attenuate TNFα- and thrombin-induced permeability of the endothelium by enhancing endothelial integrity and paracellular gap junction formation.[257,258] In addition, the decreased transendothelial migration of leukocytes due to paclitaxel treatment was attributed to an enhanced endothelial integrity as well.[259] In contrast to colchicine and paclitaxel, the possibility that also *Vinca* alkaloids could act as anti-inflammatory agents has not been investigated so far, most likely because of their inflammation-associated side effects. Nonetheless, one study revealed that vinblastine diminished the adhesion of untreated neutrophils to IL-1- and TNFα-activated endothelial cells, however, the underlying mechanisms remained unexplained.[247]

4.3 Major drawbacks of MTAs

Besides their sufficient supply, which represents a general drawback of all natural compounds, the major drawback of MTAs is the occurrence of side effects and – in the case of cancer treatment – resistance influencing their clinical success. The principle side effects of MTAs are peripheral neuropathy and reversible myelosuppression (also known as bone marrow suppression).[260,261] However, the causes of neurotoxicity have not been fully elucidated, but undoubtedly involve the impact of MTAs on neuronal microtubules.[262] Resistance of MTAs can occur at several stages including primarily cellular efflux of the drug, ineffective interaction with microtubules and deficient induction of apoptosis.[263] Cellular efflux of the drug is often caused by an overexpression of ABC-transporters – the membrane efflux pumps of the ATP-binding cassette (ABC) family. P-glycoprotein, the most prominent ABC protein, actively effluxes *Vinca* alkaloids and taxanes and is responsible for the classical multidrug-resistant phenotype. Moreover, *Vinca* alkaloids are substrates for the multidrug resistance-associated protein 1 (MRP1), taxanes are transported by MRP2 and MRP7 and epothilone B is a substrate for MRP7.[263-266] Unfortunately, attempts to avoid drugs resistance by combining MTAs with ABC protein inhibitors have been failed.[267] In addition, various factors such as the expression of different tubulin isoforms, post-translational modifications as well as the expression of MAPs participate in the regulation of microtubule dynamics and activities (see I.3.3) and several

studies indicate that changes in the expression level of the involved modulators correlate with the resistance to *Vinca* alkaloids, paclitaxel and other MTAs.[268-270]

5 Pretubulysin

Due to the major drawbacks of the currently used MTAs, the search for new classes of MTAs is still ongoing. In this context, the bacterial tubulysins produced by diverse myxobacterial strains including *Archangium gephyra* Ar 315, *Angiococcus disciformis* An d48, and *Cystobacter* sp. SBCb004 have recently been described as highly potent MTAs.[271] These compounds are linear tetrapeptides that bind at the *Vinca*-binding domain of β-tubulin causing microtubule destabilization.[271-274] Tubulysins have shown to be highly cytotoxic against different tumor cell lines, including multi drug resistant cells, whereby the potency of growth inhibition exceeds that of other tubulin modifiers such as epothilone, vinblastine and paclitaxel by 20- to 1000-fold.[243,271,272] But, as with other natural compounds, a crucial drawback of tubulysins is their sufficient supply. Fortunately, this obstacle has been overcome by the synthesis of simplified tubulysin derivatives including pretubulysin.[275-278]

Pretubulysin (PT), a biosynthetic precursor of tubulysin D (from *Angiococcus disciformis*), is a linear tetrapeptide consisting of *N*-methyl-(*R*)-pipecolic acid (Mep), *L*-isoleucine (Ile), the novel amino acid tubuvaline (Tuv) and a chain-extended analogue of phenylalanine, referred to as tubuphenylalanine (Tup) (Figure 10).[275,279] In contrast to tubulysins, PT is chemically accessible and can be synthesized in the gram scale.[279] Its profound anti-tumor activities such as cytotoxicity against various tumor cell lines or inhibition of tumor cell migration have been proven *in vitro* as well as *in vivo*.[279-282] Beyond its direct effect on tumor cells, these studies revealed that PT strongly influences endothelial cells. For instance, it was demonstrated that PT attenuates tumor angiogenesis in a murine subcutaneous tumor model that was also confirmed in several *in vitro* test systems such as endothelial migration or tube formation assays.[281] Moreover, it was reported that PT also exerts strong vascular-disruptive actions in A-Mel-3 amelanotic melanoma tumors as well as *in vitro* on primary endothelial cells.[283] Interestingly, PT was recommended as a promising agent for the treatment of metastatic cancer, since it strongly decreased the formation of metastasis induced by 4T1 mouse breast cancer cells *in vivo*.[282]

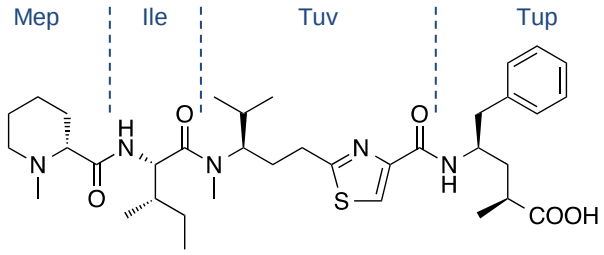

Figure 10: The chemical structure of pretubulysin (PT). PT is a linear tetrapeptide consisting of *N*-methyl-(*R*)-pipecolic acid (Mep), *L*-isoleucine (Ile), the novel amino acid tubuvaline (Tuv) and a chain-extended analogue of phenylalanine, referred to as tubuphenylalanine (Tup). The figure is partially adapted and modified from Schwenk *et al.*, 2017.[284]

6 Aim of the study

6.1 Characterization of the influence of PT on tumor-endothelial cell interactions

Both the well described anti-angiogenic and vascular-disruptive effects of MTAs such as PT depend on their influence on endothelial cells of the tumor vasculature. However, the impact of MTAs on endothelial cells of the non-tumor vasculature, with respect to its role in the rolling, adhesion and transendothelial migration of hematogenically disseminating tumor cells (see I.2.2.1 to I.2.2.3), has been widely neglected. Beside its cytotoxicity against tumor cells and its influence on the tumor vasculature, PT was shown to exert profound anti-metastatic activities and thus is recommended as a promising chemotherapeutic drug. However, the influence of PT on interactions between tumor cells and the non-tumor vasculature has not been investigated so far. Hence, one aim of the present study was to analyze the impact of PT on tumor-endothelial cell interactions *in vitro* in order to gain insights into the mechanism underlying the anti-metastatic effect of PT. Therefore, we performed cell adhesion and transendothelial migration assays as well as immunocytochemistry and microscopical analyses using the breast cancer cell line MDA-MB-231 and primary human umbilical vein endothelial cells (HUVECs). Moreover, the role of potentially involved cell adhesion molecules including ICAM-1, VCAM-1, N-cadherin, E-selectin and galectin-3 as well as of the chemokine system CXCL12/CXCR4 was elucidated to identify the mechanisms underlying the influence of PT on tumor-endothelial cell interactions.

The presented figures (Figures 12 to 21 except of Figure 16) and also parts of the text content belonging to this topic were adapted and partially modified according to the rules of the Creative Commons Attribution 3.0 License (CC BY 3.0) applied by Oncotarget from Schwenk *et al.*, 2017, whereby authors retain ownership of the copyright for their article.[284]

6.2 Characterization of the influence of PT and other MTAs on leukocyte-endothelial cell interactions

Besides colchicine (COL), currently the leading compound of MTAs as anti-inflammatory drugs, the potential use of other MTAs such as *Vinca* alkaloids or taxanes for the treatment of inflammatory diseases has been largely neglected (see I.4.2). Moreover, the few studies that exist often focused on the impact of MTAs on leukocytes such as neutrophils and macrophages but not on the endothelium, which plays a pivotal role in the rolling, firm adhesion and extravasation of leukocytes in the process of inflammation (see I.2.2.1 to I.2.2.3). Since COL represents an effective modulator of inflammatory processes, we therefore hypothesized that other MTAs including PT might also affect the process of inflammation. Thus, one aim of the present study was to characterize the impact of PT on leukocyte-endothelial cell interactions. Therefore, intravital microscopy of postcapillary venules of the mouse cremaster muscle were performed to investigate the anti-inflammatory potential of PT *in vivo*. Moreover, the influence of PT and that of vincristine (VIN), colchicine (COL) and paclitaxel (PAC) on leukocyte-endothelial cell interactions was analyzed *in vitro* in order to get insights into the underlying mechanism, focusing on the impact of the compounds on endothelial cells. Therefore, we performed cell adhesion assays using the monocyte-like cell line THP-1 and human dermal microvascular endothelial cells (HMEC-1). Moreover, the role of the potentially involved cell adhesion molecules ICAM-1 and VCAM-1 was elucidated using flow cytometric analysis and the effect of the compounds on the NFκB promotor activity was investigated to identify the underlying signaling pathway that might be affected by MTAs.

II MATERIALS AND METHODS

1. Materials

1.1 Compounds

Pretubulysin was synthesized as described previously and kindly provided by the group of Prof. Dr. Uli Kazmaier (Institute of Organic Chemistry, Saarland University, Saarbrücken, Germany).[279] Vincristine (AG-CN2-0446), colchicine (AG-CN2-0048) and paclitaxel (AG-CN2-0045) were purchased from Biomol GmbH (Hamburg, Germany).

Pretubulysin, vincristine, colchicine and paclitaxel were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM and stored at -20 °C. For experiments, each compound was freshly diluted in growth medium without exceeding a final DMSO concentration of 0.1 %. The exact concentrations are described in the respective passages.

1.2 Biochemicals, dyes, inhibitors and cell culture reagents

Biochemicals, dyes, inhibitors and cell culture reagents used in this study are listed in the following tables (Table 2 to 4).

Table 2: Biochemicals and dyes

Reagent	Supplier
1,4-Dithiothreitol (DTT)	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
2-Mercaptoethanol	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Ammonium persulfate (APS)	Carl Roth GmbH, Karlsruhe, Germany
Bovine serum albumin (BSA)	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Bovine serum albumin (BSA, fraction V)	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Calcium chloride dihydrate, $CaCl_2 \cdot 2\ H_2O$	Carl Roth GmbH, Karlsruhe, Germany
CellTracker™ Green CMFDA dye	Life Technologies GmbH, Darmstadt, Germany
Crystal violet	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
CXCL12 (SDF-1α)	PeproTech GmbH, Hamburg, Germany
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Disodium hydrogen phosphate dihydrate, $Na_2HPO_4 \cdot 2\ H_2O$	Carl Roth GmbH, Karlsruhe, Germany

Table 2: Continue biochemicals and dyes

Reagent	Supplier
FluorSave™ Reagent	Merck KGaA, Darmstadt, Germany
Formaldehyde, 16 % ultrapure	Polysciences Europe GmbH, Hirschberg (Bergstraße), Germany
Glycerol	Carl Roth GmbH, Karlsruhe, Germany
Glycine PUFFERAN® ≥ 99 %, p.a.	Carl Roth GmbH, Karlsruhe, Germany
HOECHST 33342	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Hydrogen peroxide, H_2O_2 (30 % solution)	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Luminol	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Magnesium chloride hexahydrate, $MgCl_2 \cdot 6\ H_2O$	Merck KGaA, Darmstadt, Germany
Nonidet™ P40	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
p-Coumaric acid ≥ 98.0 %	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
peqGOLD dNTP-Set	VWR International GmbH, Darmstadt, Germany
peqGOLD Protein Marker IV, 10–170 kDa	VWR International GmbH, Darmstadt, Germany
Potassium chloride, KCl	Merck KGaA, Darmstadt, Germany
Potassium dihydrogen phosphate, KH_2PO_4	Carl Roth GmbH, Karlsruhe, Germany
Powdered milk (low fat, blotting grade)	Carl Roth GmbH, Karlsruhe, Germany
Propidium iodide	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Pryonin Y	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Random Primer 6	New England BioLabs GmbH, Frankfurt am Main, Germany
RNase AWAY®	Molecular BioProducts Inc., San Diego, CA, USA
RNase-Free DNase Set	Qiagen GmbH, Hilden, Germany
Roti-Histofix 4 %	Carl Roth GmbH, Karlsruhe, Germany
Rotiphorese® Gel 30	Carl Roth GmbH, Karlsruhe, Germany
Sodium chloride, NaCl	Carl Roth GmbH, Karlsruhe, Germany
Sodium deoxycholate ≥ 97 %	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Sodium dodecyl sulfate (SDS)	Carl Roth GmbH, Karlsruhe, Germany
Staurosporine	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany

Table 2: Continue biochemicals and dyes

Reagent	Supplier
Substrate Reagent Pack for ELISA	R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany
SuperScript™ II Reverse Transcriptase	Thermo Fisher Scientific GmbH, Dreieich, Germany
SYBR Green PCR Master Mix	Life Technologies GmbH, Darmstadt, Germany
Tetramethylethylenediamine (TEMED)	Bio-Rad Laboratories GmbH, Munich, Germany
TRIS PUFFERAN® ≥99.3 %	Carl Roth GmbH, Karlsruhe, Germany
Trisodium citrate dihydrate	Carl Roth GmbH, Karlsruhe, Germany
Triton® X-100	Merck KGaA, Darmstadt, Germany
Tumor necrosis factor-α (TNFα)	PeproTech GmbH, Hamburg, Germany
Tween® 20	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany

Table 3: Inhibitors

Reagent	Supplier
cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail	Roche Diagnostics Deutschland GmbH, Mannheim, Germany
Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Plerixafor (AMD3100)	Santa Cruz Biotechnology, Heidelberg, Germany
Recombinant RNasin® Ribonuclease Inhibitor	Promega GmbH, Mannheim, Germany
Sodium fluoride, NaF	Merck KGaA, Darmstadt, Germany
Sodium orthovanadate, Na_3VO_4	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany

Table 4: Cell culture reagents

Reagent	Supplier
Amphotericin B (250 µg/ml)	PAN-Biotech GmbH, Aidenbach, Germany
Collagen G	Biochrom AG, Berlin, Germany
Dulbecco's modified eagle medium (DMEM), high glucose	GE Healthcare Life Sciences, Freiburg, Germany

Table 4: Continue cell culture reagents

Reagent	Supplier
EASY Endothelial cell growth medium (ECGM)	PELOBiotech GmbH, Planegg/Martinsried, Germany
Ethylenediaminetetraacetic acid (EDTA)	Biochrom AG, Berlin, Germany
Fetal Calf Serum, Superior	Biochrom AG, Berlin, Germany
Fibronectin from human plasma	Merck KGaA, Darmstadt, Germany
HyClone RPMI-1640 medium	GE Healthcare Life Sciences, Freiburg, Germany
HyClone™ HyQTase Cell Detachment Reagent	GE Healthcare Life Sciences, Freiburg, Germany
Medium 199	GE Healthcare Life Sciences, Freiburg, Germany
Penicillin-Streptomycin (P: 10,000 U/ml, S: 10 mg/ml)	PAN-Biotech GmbH, Aidenbach, Germany
Trypsin	Biochrom AG, Berlin, Germany

1.3 Buffers, solutions and media

Buffers, solutions and media used in cell culture or for *in vitro* assays are listed in the following tables (Table 5 to 7).

Table 5: Buffers and solutions used in cell culture

Buffer/Solution	Composition
CellTracker™ Green solution	5 µM CellTracker™ Green CMFDA dye, in serum-free DMEM or RPMI
PBS (pH 7.4)	137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4 \cdot 2\ H_2O$, 2 mM KH_2PO_4, in dist. water
PBS+ (pH 7.4)	137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4 \cdot 2\ H_2O$, 1.5 mM KH_2PO_4, 0.5 mM $MgCl_2 \cdot 6\ H_2O$, 0.7 mM $CaCl_2 \cdot 2\ H_2O$, in dist. water
PBSA (pH 7.4)	0.2 % BSA, in PBS
Trypsin/EDTA solution	0.05 % Trypsin, 0.02 % EDTA, in PBS

Table 6: Cell culture media

Medium	Composition
DMEM	10 % FCS Superior, 1 % penicillin, 1 % streptomycin, in DMEM (high glucose)
ECGM	10 % FCS Superior, 2.5 % supplement Mix, 1 % penicillin, 1 % streptomycin, 1 % amphotericin B, in EASY ECGM
Freezing medium	10 % DMSO, in FCS
M199 + 0.1 % BSA	0.1 % BSA, in Medium 199
M199 + 20 % FCS	20 % FCS, in Medium 199
RPMI	10 % FCS Superior, 1 % penicillin, 1 % streptomycin, in HyClone RPMI-1640
Stopping medium	10 % FCS Superior, 1 % penicillin, 1 % streptomycin, in Medium 199

Table 7: Buffers and solution used for *in vitro* assays

Buffer/Solution	Composition
1x sample buffer	125 mM Tris-HCl (pH 6.8), 20 % glycerol, 4 % SDS, 0.8 % DTT, 0.01 % Pryonin Y, in dist. water
5x sample buffer	312.5 mM Tris-HCl (pH 6.8), 50 % glycerol, 5 % SDS, 2 % DTT, 0.025 % Pryonin Y, in dist. water
Blotto-T (1 %/5 %)	1 %/5 % powdered milk, in TBS-T
BSA-T (1 %/5 %)	1 %/5 % BSA, in TBS-T
cOmplete™solution	1 tablet, in 1.5 ml dist. water
Crystal violet solution	0.5 % crystal violet, 20 % methanol, in dist. water
ECL solution	98.5 mM Tris-HCl (pH 8.5), 1 % luminol solution, 0.4 % p-coumaric acid solution, 0.02 % H_2O_2, in dist. water
Electrophoresis buffer	192 mM glycine, 25 mM Tris, 0.1 % SDS, in dist. water
HFS-PI solution	0.1 % Triton X-100, 0.1 % sodium citrate, 50 µg/ml propidium iodide, in PBS
Luminol solution	250 mM luminol, in DMSO
Lysis buffer	2 % cOmplete™ solution, 1 mM NaF, 1 mM PMSF, 1 mM Na_3VO_4, in RIPA buffer
Methanol/ethanol solution (2:1)	2 volumes methanol, 1 volume ethanol
p-Coumaric acid solution	90 mM p-coumaric acid, in DMSO
Reagent diluent	1 % BSA (fraction V), in PBS

Table 7: Continue buffers and solution used for *in vitro* assays

Buffer/Solution	Composition
RIPA buffer	150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1 % Nonidet™ P40, 0.25 % sodium deoxycholate, 0.1 % SDS, in dist. water
Separation gel (10 %)	33.4 % Rotiphorese® Gel 30, 370 mM Tris-HCl (pH 8.8), 0.1 % SDS, 0.1 % TEMED, 0.05 % APS, in dist. water
Separation gel (15 %)	49.6 % Rotiphorese® Gel 30, 375 mM Tris-HCl (pH 8.8), 0.1 % SDS, 0.1 % TEMED, 0.05 % APS, in dist. water
Sodium citrate solution	50 % sodium citrate (0.1 M in dist. water), 50 % ethanol
Stacking gel (4 %)	17.3 % Rotiphorese® Gel 30, 125 mM Tris-HCl (pH 6.8), 0.1 % SDS, 0.2 % TEMED, 0.1 % APS, in dist. water
Stripping buffer	62.5 mM Tris, 2 % SDS, 0.8 % 2-mercaptoethanol, in dist. water
Tank buffer	192 mM glycine, 25 mM Tris, 10 % methanol, in dist. water
TBS-T (pH 7.4–7.5)	25 mM Tris, 150 mM NaCl, 0.05 % Tween® 20, in dist. water
Triton X-100 (0.2 %)	0.2 % Triton X-100, in PBS
Wash buffer for ELISA	0.05 % Tween® 20, in PBS

1.4 Commercial Kits

Kits used this study are listed in table 8.

Table 8: Kits

Product	Supplier
CellTiter-Blue® Cell Viability Assay	Promega GmbH, Mannheim, Germany
CytoTox 96® Non-Radioactive Cytotoxicity Assay	Promega GmbH, Mannheim, Germany
Dual-Luciferase® Reporter Assay System	Promega GmbH, Mannheim, Germany
HUVEC Nucleofector® Kit	Lonza Cologne GmbH, Cologne, Germany
MiraCLEAN® Endotoxin Removal Kit	Mirus Bio LLC, Madison, WI, USA
Mouse CXCL12/SDF-1 DuoSet ELISA	R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany
Pierce™ BCA Protein Assay Kit	Thermo Fisher Scientific GmbH, Dreieich, Germany
PureYield™ Plasmid Maxiprep System	Promega GmbH, Mannheim, Germany
RNeasy Mini Kit	Qiagen GmbH, Hilden, Germany

1.5 Antibodies

Antibodies used for western blot analysis, immunocytochemistry, flow cytometric analysis and blocking experiments are listed in the following tables (Table 9 to 14).

Table 9: Primary antibodies used for western blot analysis

Immunogen, Ref. number	Type	Supplier	Dilution	Solution
Anti-ICAM-1, sc-107	mouse, monoclonal	Santa Cruz Biotechnology, Heidelberg, Germany	1:500	5 % Blotto-T
Anti-N-cadherin, ab76057	rabbit, polyclonal	Abcam, Cambridge, UK	1:2,000	5 % BSA-T
Anti-p44/42 (Erk1/2), 9102	rabbit, polyclonal	Cell Signaling Technology Europe B.V., Frankfurt am Main, Germany	1:1,000	5 % Blotto-T
Anti-phospho-p44/42 (Erk1/2) (Thr202/ Tyr204), 9106	mouse, monoclonal	Cell Signaling Technology Europe B.V., Frankfurt am Main, Germany	1:2,000	5 % BSA-T
Anti-SDF-1 (CXCL12), sc-74271	mouse, monoclonal	Santa Cruz Biotechnology, Heidelberg, Germany	1:200	5 % Blotto-T
Anti-VCAM-1, sc-13160	mouse, monoclonal	Santa Cruz Biotechnology, Heidelberg, Germany	1:300	5 % Blotto-T
Anti-β-actin-peroxidase, A3854	mouse, monoclonal	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany	1:1 x 10^5	5 % BSA-T

Table 10: Secondary antibodies used for western blot analysis

Immunogen, Ref. number	Supplier	Dilution	Solution
Goat-anti-mouse IgG-HRP, sc-2005	Santa Cruz Biotechnology, Heidelberg, Germany	1:5,000	1 % Blotto-T/BSA-T
Goat-anti-rabbit IgG-HRP, sc-2004	Santa Cruz Biotechnology, Heidelberg, Germany	1:1,000	1 % Blotto-T/BSA-T

Table 11: Primary antibodies used for immunocytochemistry

Immunogen, Ref. number	Type	Supplier	Dilution	Solution
Anti-collagen I + II + III + IV, ab36064	rabbit, polyclonal	Abcam, Cambridge, UK	1:40	PBSA
Anti-PECAM-1, sc-53411	mouse, monoclonal	Santa Cruz Biotechnology, Heidelberg, Germany	1:400	PBSA
Anti-VE-cadherin, sc-9989	mouse, monoclonal	Santa Cruz Biotechnology, Heidelberg, Germany	1:400	PBSA

Table 12: Secondary antibodies used for immunocytochemistry

Immunogen, Ref. number	Supplier	Dilution	Solution
Alexa Fluor® 488-conjugated goat anti-rabbit IgG, A11008	Thermo Fisher Scientific GmbH, Dreieich, Germany	1:400	PBSA
Alexa Fluor® 633-conjugated goat anti-mouse IgG, A21050	Thermo Fisher Scientific GmbH, Dreieich, Germany	1:400	PBSA

Table 13: Antibodies used for flow cytometric analysis

Immunogen, Ref. number	Label	Type	Supplier	Dilution	Solution
Anti-CD106 (VCAM-1), 555647	PE	mouse, monoclonal	BD Biosciences, Heidelberg, Germany	1:20	PBSA
Anti-CD325 (N-cadherin), 561554	PE	mouse, monoclonal	BD Biosciences, Heidelberg, Germany	1:20	PBSA
Anti-CD54 (ICAM-1), MCA1615F	FITC	mouse, monoclonal	Bio-Rad Laboratories GmbH, Munich, Germany	1:33	PBS
Anti-CD62E (E-selectin), 551145	PE	mouse, monoclonal	BD Biosciences, Heidelberg, Germany	1:20	PBSA

Table 14: Antibodies used for blocking experiments

Immunogen, Ref. number	Type	Supplier
Anti-ICAM-1, sc-107	mouse, monoclonal	Santa Cruz Biotechnology, Heidelberg, Germany
Anti-VCAM-1, MA5-16429	mouse, monoclonal	Thermo Fisher Scientific GmbH, Dreieich, Germany

Table 14: Continue antibodies used for blocking experiments

Immunogen, Ref. number	Type	Supplier
Anti-β1-integrin, ab24693	mouse, monoclonal	Abcam, Cambridge, UK

1.6 Oligonucleotides for qRT-PCR

The primers used in this study for qRT-PCR were designed *via* the tool Primer-BLAST (NCBI, Bethasda, Maryland, USA) and were purchased from Eurofins Scientific SE (Luxemburg). The used primers are listed in table 15.

Table 15: Oligonucleotides

Gene name	Gene product	Direction	Sequence (5' → 3')
CDH2	Neural cadherin (N-cadherin)	forward	5'-CAG GAA AAG TGG CAA GTG GC-3'
CDH2	Neural cadherin (N-cadherin)	reverse	5'-AGG AAA AGG TCC CCT GGA GT-3'
CXCL12	C-X-C motif chemokine 12 (CXCL12)	forward	5'-GAA AGC CAT GTT GCC AGA GC-3'
CXCL12	C-X-C motif chemokine 12 (CXCL12)	reverse	5'-AGC TTC GGG TCA ATG CAC A-3'
CXCR4	C-X-C chemokine receptor type 4 (CXCR4)	forward	5'-GCT GTT GGC TGA AAA GGT GG-3'
CXCR4	C-X-C chemokine receptor type 4 (CXCR4)	reverse	5'-ATC TGC CTC ACT GAC GTT GG-3'
GAPDH	Glyceraldehyde-3-phos-phate dehydrogenase (GAPDH)	forward	5'-CCA CAT CGC TCA GAC ACC AT-3'
GAPDH	Glyceraldehyde-3-phos-phate dehydrogenase (GAPDH)	reverse	5'-TGA AGG GGT CAT TGA TGG CAA-3'
ICAM1	Intercellular adhesion molecule 1 (ICAM-1)	forward	5'-CTG CTC GGG GCT CTG TTC-3'
ICAM1	Intercellular adhesion molecule 1 (ICAM-1)	reverse	5'-AAC AAC TTG GGC TGG TCA CA-3'
LGALS3	Galectin-3	forward	5'-GCC TTC CAC TTT AAC CCA CG-3'
LGALS3	Galectin-3	reverse	5'-ACT GCA ACC TTG AAG TGG TCA-3'
SELE	E-selectin	forward	5'-AGA TGA GGA CTG CGT GGA GA-3'
SELE	E-selectin	reverse	5'-GTG GCC ACT GCA GGA TGT AT-3'
VCAM1	Vascular cell adhesion molecule 1 (VCAM-1)	forward	5'-CCA CAG TAA GGC AGG CTG TAA-3'

Table 15: Continue oligonucleotides

Gene name	Gene product	Direction	Sequence (5' → 3')
VCAM1	Vascular cell adhesion molecule 1 (VCAM-1)	reverse	5'-GCT GGA ACA GGT CAT GGT CA-3'

1.7 Plasmids

For Dual-Luciferase® reporter assays the vectors pGL4.32[*luc2P*/NF-κB-RE/Hygro] and pGL4.74[*hRluc*/TK] purchased from Promega GmbH (Mannheim, Germany) were used. While the pGL4.32[*luc2P*/NF-κB-RE/Hygro] vector contains five copies of an NFκB response element leading to the transcription of the luciferase reporter gene *luc2P* (*Photinus pyralis*), the pGL4.74[*hRluc*/TK] vector encodes the luciferase reporter gene *hRluc* (*Renilla reniformis*) leading to the constitutive expression of the reporter gene and was used as an internal control (Figure 11).

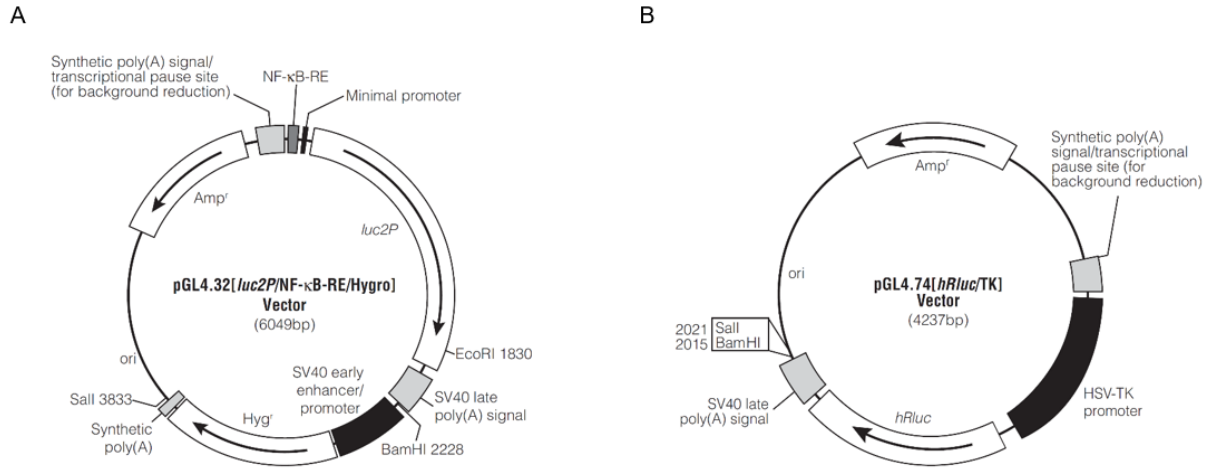

Figure 11: Plasmids used for Dual-Luciferase reporter assays. (A) The pGL4.32[*luc2P*/NF-κB-RE/Hygro] vector contains five copies of an NFκB response element, a minimal promoter, the *luc2p* reporter gene, an SV40 late poly(A) signal, an SV40 early enhancer/promoter, a synthetic hygromycin (Hyg^r) coding region, a synthetic poly(A) signal, a reporter vector primer 4 (RVprimer4)-binding region, a *CO*/E1-derived plasmid replication origin, a coding region for synthetic β-lactamase (Amp^r), a synthetic poly(A) signal/transcriptional pause site and a reporter vector primer 3 (RVprimer3)-binding region. **(B)** The pGL4.74[*hRluc*/TK] vector contains an HSV-TK promoter, the *hRluc* reporter gene, an SV40 late poly(A) signal, a reporter vector primer 4 (RVprimer4)-binding region, a *CO*/E1-derived plasmid replication origin, a coding region for synthetic β-lactamase (Amp^r), a synthetic poly(A) signal/transcriptional pause site and a reporter vector primer 3 (RVprimer3)-binding region. Figures and information adapted from Promega GmbH (https://www.promega.de/products/vectors).

1.8 Primary cells and cell lines

Primary cells and cell lines used in this study are listed in table 16.

Table 16: Primary cells and cell lines

Primary cells/ cell line	Description	Supplier	Reference number
CDC/EU.HMEC-1 (HMEC-1)	Derived by transfection of human dermal microvascular endothelial cells with a PBR-322-based plasmid containing the coding region for large T antigen, an SV40 A gene product[285]	American Type Culture Collection (ATCC), LGC Standards GmbH, Wesel, Germany	ATCC® CRL-3243™
HUVECs	Pooled primary human umbilical vein endothelial cells	PELOBiotech GmbH, Planegg/Martinsried, Germany	PB-CH-190-8013
MDA-MB-231	Derived from a single sample of pleural effusion of a 51-year-old woman with breast carcinoma[286,287]	Leibniz Institute for German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany	ACC-732
THP-1	Derived from the peripheral blood of a one-year-old boy suffering from acute monocytic leukemia (AML)[288]	Leibniz Institute for German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany	ACC-16

1.9 Technical equipment

Technical equipment used in this study is listed in table 17.

Table 17: Technical equipment

Device	Supplier
Autoclave: Systec VX-95	Systec GmbH, Linden, Germany
Blotter: Criterion™ Blotter	Bio-Rad Laboratories GmbH, Munich, Germany
Centrifuge: Heraeus™ Megafuge™ 16R	Thermo Fisher Scientific, Dreieich, Germany

Table 17: Continue technical equipment

Device	Supplier
Centrifuge: MICRO STAR 17R	VWR International GmbH, Darmstadt, Germany
Centrifuge: Mini Star silverline	VWR International GmbH, Darmstadt, Germany
Current source: PowerPacTM HC	Bio-Rad Laboratories GmbH, Munich, Germany
Electrophoresis system: Mini-PROTEAN® Tetra System	Bio-Rad Laboratories GmbH, Munich, Germany
Electroporator: Nucleofector 2b	Lonza Cologne GmbH, Cologne, Germany
Flow cytometer: FACSVerse™	BD Biosciences, Heidelberg, Germany
Fluorescence microscope: LSM 510	Carl Zeiss AG, Jena, Germany
Heatblock	VWR International GmbH, Darmstadt, Germany
Hemocytometer: Neubauer-improved	Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany
Incubator: HERACell 150i	Thermo Fisher Scientific, Dreieich, Germany
Inverted microscope: DM IL LED	Leica Microsystems, Wetzlar, Germany
Liquid nitrogen storage system: ARPEGE 110	Air Liquide S.A., Paris, France
Magnetic stirrer: IKA® RH basic 2	IKA-Werke GmbH & Co. KG, Staufen, Germany
Microplate reader: Infinite F200 pro	Tecan Trading AG, Männedorf, Switzerland
Microplate reader: Varioskan® Flash	Thermo Fisher Scientific, Dreieich, Germany
Nanophotometer: P 330	Implen GmbH, Munich, Germany
Orbital shaker: Sea Star	Heathrow Scientific, Vernon Hills, IL, USA
pH meter: FE20	Mettler-Toledo GmbH, Gießen, Germany
Pipette: Eppendorf Multipette® plus	Eppendorf AG, Hamburg, Germany
Pipettes: Eppendorf Research® plus	Eppendorf AG, Hamburg, Germany
Pipettor: Pipetus®	Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt, Germany
qRT-PCR system: StepOnePlus System™	Applied Biosystems GmbH, Darmstadt, Germany
Scale: Mettler-Toledo (0.1–200 mg)	Mettler-Toledo GmbH, Gießen, Germany
Scale: PIONEER™ (10–1000 g)	Ohaus Europe GmbH, Greifensee, Switzerland
Sterile bench: SAFE 2020	Thermo Fisher Scientific, Dreieich, Germany

Table 17: Continue technical equipment

Device	Supplier
Tube roller: stuart® SRT9D	Bibby Scientific Limited, Staffordshire, UK
Ultra pure water system: Astacus	MembraPure GmbH, Berlin, Germany
Ultrasonic cleaner: USC300TH	VWR International GmbH, Darmstadt, Germany
Vacuum pump: Integra Vacusafe	INTEGRA Biosciences GmbH, Biebertal, Germany
Vortexer: Vortex Genie® 2	Scientific Industries, Inc., Bohemia, NY, USA
Water bath: Julabo ED	JULABO GmbH, Seelbach, Germany
X-ray Film processor: Agfa CP 1000	AGFA, Cologne, Germany

1.10 Consumable materials

Consumable materials used in this study are listed in table 18.

Table 18: Consumable materials

Product	Supplier
8-well µ-slides: µ-Slide 8 well, ibiTreat	ibidi GmbH, Planegg/ Martinsried, Germany
Microplates for absorbance measurements: Nunc™ MicroWell™ 96-Well (269620)	Thermo Fisher Scientific GmbH, Dreieich, Germany
Cell culture dishes (10 cm)	Sarstedt AG & Co., Nümbrecht, Germany
Cell culture flasks (25 cm^2, 75 cm^2)	Greiner Bio-One GmbH, Frickenhausen, Germany
Cell culture plates (6-, 12-, 24-, 48-, 96-well)	Greiner Bio-One GmbH, Frickenhausen, Germany
Cell scraper	TPP Techno Plastic Products AG, Trasadingen, Switzerland
Centrifuge tubes (15 ml, 50 ml)	Greiner Bio-One GmbH, Frickenhausen, Germany
Cover slips	Helmut Saur, Reutlingen, Germany
Cryo vials	Greiner Bio-One GmbH, Frickenhausen, Germany
Eppendorf Combitips advanced®	Eppendorf AG, Hamburg, Germany
Low-binding tubes (1.5 ml)	Sarstedt AG & Co., Nümbrecht, Germany
Microplates for ELISA: Clear Polystyrene Microplates	R&D Systems GmbH, Wies-baden-Nordenstadt, Germany

Table 18: Continue consumable materials

Product	Supplier
Microplates for fluorescence measurements: Nunc™ MicroWell™ 96-Well (265301)	Thermo Fisher Scientific GmbH, Dreieich, Germany
Microplates for luminescence measurments: Corning® 96 well NBS™ Microplate	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Microplates for qRT-PCR: MicroAmp™ Fast Optical 96-Well Reaction Plate	Applied Biosystems GmbH, Darmstadt, Germany
Pipette tips (standard, 10 µl, 100 µl, 300 µl, 1000 µl)	Greiner Bio-One GmbH, Frickenhausen, Germany
Pipette tips with filter: ART® Aerosol Resistant Tips (10 µl)	Thermo Fisher Scientific GmbH, Dreieich, Germany
Pipette tips with filter: Biosphere® (200 µl, 1000 µl)	Sarstedt AG & Co., Nümbrecht, Germany
PVDF membran: Immun-Blot® PVDF	Bio-Rad Laboratories GmbH, Munich, Germany
Reaction tubes (0.2 ml, 1.5 ml, 2 ml)	Greiner Bio-One GmbH, Frickenhausen, Germany
Sealing tape for ELISA: StarSeal Sealing Tape Poly-olefin Film (E2796-9793)	STARLAB INTERNATIONAL GmbH, Hamburg, Germany
Sealing tape for qRT-PCR: StarSeal Sealing Tape Poly-olefin Film (E2796-9795)	STARLAB INTERNATIONAL GmbH, Hamburg, Germany
Serological pipettes (5 ml, 10 ml, 25 ml)	Greiner Bio-One GmbH, Frickenhausen, Germany
Transwell® Insert (polycarbonate membrane, diameter 6.5 mm, pore size 8 µm)	Corning, New York, USA
Whatman filter paper	Bio-Rad Laboratories GmbH, Munich, Germany
X-ray films: Super RX-N	FUJIFILM Europe GmbH, Düsseldorf, Germany

2. Cell culture

Primary cells and cell lines were cultured in a cell culture incubator under constant humidity at 37 °C with 5 % CO_2. Growth and morphology of all cells and cell lines were routinely observed by microscopy using the inverted microscope Leica DM IL LED (Leica Microsystems, Wetzlar, Germany). Unless otherwise stated, all buffers, solutions and media were pre-warmed at 37 °C before they were added to cells.

2.1 Endothelial cells

Unless otherwise stated, endothelial cells were seeded in culture flasks or plates coated for at least 30 min with collagen G (10 µg/ml) in PBS before use.

2.1.1 HUVECs – human umbilical vein endothelial cells

Primary human umbilical vein endothelial cells (HUVECs) were cultured in endothelial cell growth medium (ECGM) and were used for all assays studying the influence of PT and other MTAs on endothelial cell interactions with tumor cells and the underlying mechanisms except for adhesion assays with tumor cells.

2.1.2 HMEC-1 – human dermal microvascular endothelial cells

The immortalized human endothelial cell line CDC/EU.HMEC-1 (HMEC-1) was originally established by transfection of human dermal microvascular endothelial cells with a PBR-322-based plasmid containing the coding region for large T antigen, an SV40 A gene product.[285] HMECs were cultured in ECGM and were used for all assays studying the influence of PT and other MTAs on endothelial cell interactions with leukocytes and the underlying mechanisms.

2.2 Tumor cells and leukocytes

2.2.1 MDA-MB-231 – human epithelial breast carcinoma cell line

The human epithelial breast carcinoma cell line MDA-MB-231 was originally established from a single sample of pleural effusion of a 51-year-old woman with breast carcinoma.[286,287] MDA-MB-231 cells were cultured in Dulbecco's modified eagle medium (DMEM) and were exclusively used for adhesion assays.

2.2.2 THP-1 – human leukemia monocyte-like cell line

The human monocyte-like cell line THP-1 was originally established from the peripheral blood of a one-year-old boy suffering from acute monocytic leukemia (AML).[288] THP-1 cells were cultured in HyClone RPMI-1640 medium (RPMI) and were exclusively used for adhesion assays.

2.3 Passaging

For passaging confluent adherent cells (endothelial cells and MDA-MB-231 cells), cell culture medium was removed and the cells were carefully washed twice with phosphate-buffered saline (PBS, pH 7.4) before they were incubated with 2 ml of a Trypsin/EDTA solution at 37 °C for about 1 min. As soon as all cells were detached, the enzymatic reaction was stopped using stopping medium in the case of endothelial cells or DMEM in the case of MDA-MB-231 cells. Cells were harvested by centrifugation (300 g, 5 min, RT), the pellet was resuspended in culture medium and the cells were either transferred into a new cell culture flask (75 cm^2) or were seeded for experiments in cell culture dishes or plates. Endothelial cells were passaged three times a week in a ratio of 1:3 regarding the growth area while MDA-MB-231 cells were passaged twice a week in a ratio of 1:4 to 1:5 regarding the growth area.

For passaging the suspension cell line THP-1, cell number was determined using a hemocytometer and the cell concentration was adjusted to 3 x 10^5 cells per ml. For this purpose, the appropriate amount of cell suspension was centrifuged (300 g, 5 min, RT), the pellet was resuspended in culture medium and the cells were transferred into a new cell culture flask (25 or 75 cm^2). THP-1 cells were passaged three times a week.

2.4 Freezing and thawing of cells

Before freezing, confluent adherent cells were detached and suspension cells were removed as described above, harvested by centrifugation (300 g, 5 min, RT) and resuspended in pre-chilled freezing medium (2 ml per confluent 75 cm^2 flask or per 6 x 10^6 suspension cells). Cells were transferred into cryo vials and were frozen at -80 °C at least overnight before they were transferred into liquid nitrogen for long term storage.

For thawing, cryo vials were rapidly warmed to 37 °C in a water bath and the thawed cells were immediately transferred into culture medium (10 ml per cryo vial) to dilute the dimethyl sulfoxide (DMSO) containing freezing medium. Cells were harvested by centrifugation (300 g, 5 min, RT), the pellet was resuspended in culture medium and the cells were transferred into a 75 cm^2 (adherent cells) or 25 cm^2 (suspension cells) cell culture flask.

3. Cytotoxicity assays

To identify possible cytotoxic effects of PT, VIN, COL or PAC, the metabolic activity of cells was analyzed by the CellTiter-Blue® cell viability assay, the release of lactate dehydrogenase (LDH) was determined by the CytoTox 96® non-radioactive cytotoxicity assay and apoptosis (sub-diploid DNA content) was measured as described by Nicoletti *et al.*[289]

3.1 Cell viability assay

The metabolic activity of HMECs was analyzed by the CellTiter-Blue® cell viability assay (Promega GmbH, Mannheim, Germany). This assay is based on the principle that viable cells are able to reduce the indicator dye resazurin into fluorescent resorufin.

The assay was performed according to the manufacturer's instructions. HMECs were grown to confluence in 96-well cell culture plates and treated with PT, VIN, COL or PAC as described. CellTiter-Blue® reagent was added into the medium in a ratio of 1:10 for the last 4 hours of treatment and the reduction of resazurin into resorufin was determined by fluorescence measurements (ex: 579 nm, em: 584 nm) using an Infinite F200 pro microplate reader (Tecan Trading AG, Männedorf, Switzerland).

3.2 Cytotoxicity assay

The release of lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis, was determined by the CytoTox 96® non-radioactive cytotoxicity assay (Promega GmbH). This coupled enzymatic assay is based on the principle that LDH released in cell culture supernatants catalyzes the conversion of lactate into pyruvate leading to the reduction of oxidized nicotinamide adenine dinucleotide (NAD^+) into reduced NAD (NADH). The production of NADH results in the conversion of iodonitrotetrazolium violet (INT) into a red formazan product and NAD^+. The generation of the red formazan product is proportional to the amount of released LDH.

The assay was performed according to the manufacturer's instructions. HMECs were grown to confluence in 96-well cell culture plates and treated with PT, VIN, COL or PAC as described. For positive control, lysis solution was added into the medium of untreated cells in a ratio of 1:10 for the last 45 min of treatment. Then, 50 µl of medium from each well were transferred into a new 96-well cell culture plate, 50 µl of CytoTox 96® reagent was added to each sample

and the plate was incubated at room temperature for 30 min. The enzymatic reaction was stopped by adding 50 µl of stop solution and the absorbance of the red formazan product was measured at 490 nm using a Varioskan® Flash microplate reader (Thermo Fisher Scientific, Dreieich, Germany).

3.3 Cell apoptosis assay

The cell apoptosis assay described by Nicoletti *et al.* is based on the principle that cell apoptosis is accompanied by deoxyribonucleic acid (DNA) fragmentation due to chromatin cleavage at the linker regions between nucleosomes by endonucleases. The red-fluorescent dye propidium iodide (PI), which intercalates between DNA bases, is used for the detection of DNA fragments by flow cytometric analysis.[289]

HMECs were grown to confluence in 24-well cell culture plates and treated with PT, VIN, COL or PAC as described. Cell culture supernatants were collected and the remaining cells were washed twice with PBS. PBS wash fractions were added to the respective cell culture supernatants and the cells were incubated with a Trypsin/EDTA solution at 37 °C for a maximum of 3 min. As soon as all cells were detached, stopping medium was added to stop the enzymatic reaction. Detached cells were added to the cell culture supernatants including wash fractions and harvested by centrifugation (300 g, 10 min, 4 °C). Supernatants were removed, the cell pellets were resuspended in ice-cold PBS and the suspension was centrifuged (300 g, 10 min, 4 °C). The remaining cell pellets were permeabilized and stained using hypotonic fluorochrome solution (HFS) containing PI (HFS-PI solution) and incubated overnight at 4 °C protected from light. PI-staining was determined by flow cytometric analysis using a FACSVerse™ (BD Biosciences, Heidelberg, Germany).

4. Quantitative real-time polymerase chain reaction (qRT-PCR)

Quantitative real-time polymerase chain reaction (qRT-PCR) based on the $2^{-\Delta\Delta CT}$ method was used to quantify the messenger ribonucleic acid (mRNA) expression level of genes in PT-treated HUVECs. The initial step was, therefore, to transcribe isolated mRNA into complementary DNA (cDNA) *via* reverse transcriptase followed by qRT-PCR performed with the StepOnePlus System™ (Applied Biosystems GmbH, Darmstadt, Germany).

qRT-PCR is based on the principle that a fluorescent dye, SYBR-Green in this study, intercalates into double-stranded DNA (dsDNA) leading to an approximately 100-fold higher fluorescence signal. Thus, the measured fluorescence intensity correlates with the amplification of dsDNA. Since the initial amount of cDNA carrying the gene of interest correlates with the amount of its corresponding mRNA, calculation of the initial cDNA concentration on the basis of the measured fluorescence signal can be directly transferred to the initial mRNA expression of the gene. The $2^{-\Delta\Delta CT}$ method is used for relative quantification of the qRT-PCR and enables to analyze the normalized mRNA expression of the target gene in relation to an untreated control.[290]

All qRT-PCR experiments were kindly performed by Tanja Stehning. To prevent possible contaminations or RNA digestion by RNases, the workplace and equipment was cleaned with RNase AWAY® and filter tips were used during all work steps.

4.1 Sample preparation

HUVECs were grown to confluence in 6-well plates and treated with PT and TNFα as described. Then, cells were washed once with PBS, lysed with 350 µl of Buffer RLT (RNeasy Mini Kit, Qiagen GmbH, Hilden, Germany) containing 2-mercaptoethanol (1:100) and stored at -80 °C until used for RNA isolation.

4.2 RNA isolation and cDNA synthesis

Total RNA was isolated with an RNeasy Mini Kit (Qiagen GmbH) including on-column DNase digestion with an RNase-Free DNase Set (Qiagen GmbH) according to the manufacturer's protocol. Therefore, cell lysates (see II.4.1) were mixed with 70 % ethanol (1:1) and the mixture was transferred to an RNeasy spin column. After centrifugation (17,000 g, 15 s) the flow-through was discarded and the spin column membrane was washed by adding 700 µl of Buffer RW1 followed by centrifugation (17,000 g, 15 s). The flow-through was discarded and the spin column membrane was incubated with 80 µl of DNase I mix for 15 min at room temperature for DNA digestion. Then, 350 µl of Buffer RW1 were added followed by centrifugation (17,000 g, 15 s). The flow-through was discarded and the spin column membrane was washed by adding 500 µl of Buffer RPE followed by centrifugation (17,000 g, 15 s). The previous step was repeated except that the time for centrifugation was increased to 2 min. The spin column membrane was dried by centrifugation (17,000 g, 1 min) and RNA was eluted by adding 30 µl of RNase-free water to the membrane followed by centrifugation (17,000 g, 1 min). The

absorbance of the isolated RNA was measured at 260 nm using a P 330 nanophotometer (Implen GmbH, Munich, Germany) to determine the RNA concentration.

Isolated RNA was reversely transcribed into cDNA using SuperScript™ II Reverse Transcriptase (Thermo Fisher Scientific GmbH). Therefore, 1 µg of RNA was adjusted with RNase-free water to a total volume of 7 µl. 1 µl of Random Primer N6 (100 pmol) and 4 µl of deoxynucleoside triphosphates (dNTPs, 2 mM) were added. Secondary RNA structures were destroyed by incubation of the mixture at 70 °C for 10 min followed by cooling on ice for 2 min. Subsequently, 4 µl of 5x first-strand buffer, 2 µl of 100 mM DTT, 1 µl of RNAsin® and 1 µl of SuperScript™ II Reverse Transcriptase were added to the mixture followed by incubation at room temperature for 10 min. After incubation at 42 °C for 1 hour, 100 µl of RNase-free water was added to the mixture and the enzymatic reaction was stopped by incubation at 70 °C for 10 min. Synthesized cDNA was stored at -20 °C until used for qRT-PCR.

4.3 qRT-PCR performance

qRT-PCR based on the $2^{-\Delta\Delta CT}$ method was performed *via* the StepOnePlus™ System (Applied Biosystems GmbH) using the Power SYBR Green PCR Master Mix (Life Technologies GmbH, Darmstadt, Germany). Therefore, forward and reverse primers for the gene of interest listed in table 15 were diluted in RNase-free water in a ratio of 1:25 (corresponding to 4 pmol) and 1.25 µl of each diluted primer were added to 2.5 ng of synthesized cDNA adjusted with RNase-free water to a total volume of 2.5 µl. Then, 5 µl of Power SYBR Green PCR Master Mix was added to the mixture and the qRT-PCR reaction plate was covered with a plate sealer for optical assays. After centrifugation (300 g, 1 min), qRT-PCR was performed using the StepOnePlus™ System and the following thermocycler program (Table 19). In all qRT-PCR experiments, the threshold cycle for the gene of interest was normalized to that of the housekeeping gene *Gapdh*.

Table 19: Program for qRT-PCR

Reaction step	Temperature	Period	Number of cycles
Activation of DNA polymerase	95 °C	10 min	1
Denaturation Annealing/elongation	95 °C 60 °C	15 s 60 s	40
Melt curve stage	95 °C 60 °C 95 °C	15 s 60 s 15 s	1
Hold	4 °C	∞	1

5. Western blot analysis

Immunoblotting (western blot) was first devised by Towbin *et al.* in 1979 and is used for the identification of specific proteins out of a mixture of proteins extracted from cell lysates.[291] Western blot analysis is based on the transfer of proteins separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) onto a carrier membrane followed by detection of the protein of interest using specific antibodies.

5.1 Sample preparation

HUVECs were grown to confluence in 6-well plates and treated with PT and TNFα as described. Then, cells were washed once with PBS, lysed with lysis buffer (Table 7) and stored for at least 1 hour at -80 °C to promote cell destruction.

5.2 Protein quantification and sample adjustment

The total amount of protein in cell lysates was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific GmbH). The bicinchoninic acid assay (BCA) is based on the principle that peptide bonds are able to reduce Cu^{2+} to Cu^{+} ions in an alkaline environment by forming light blue complexes (biuret reaction). BCA is used in this assay to build purple-colored complexes with the Cu^{+} ions reduced in the first reaction leading to a strong increase in assay sensitivity. The complex quantity is determined by absorbance measurements at 562 nm and is proportional to the total protein amount.[292]

The assay was performed according to the manufacturer's instructions. Cell lysates were thawed on ice, collected with a cell scraper and incubated in an ultrasonic bath for 5 min. After centrifugation (17,000 g, 4 °C, 10 min), the supernatant was transferred into a new tube. For protein quantification the lysate was diluted with distilled water in a ratio of 1:10. Protein concentration was determined by linear regression using protein standards obtained using dilutions (0, 50, 100, 150, 200, 300, 400 and 500 µg/ml) of a 2 mg/ml bovine serum albumin (BSA) stock solution. Finally, 10 µl of cell lysate or standard were mixed with 190 µl of BCA working reagent and the mixture was incubated at 37 °C for 30 min before the absorbance was measured at 562 nm using an Infinite F200 pro microplate reader (Tecan Trading AG). Cell lysates were then mixed with 5x sample buffer and adjusted with 1x sample buffer to the

sample with the lowest concentration. Afterwards, samples were heated at 95 °C and stored at -20 °C until their usage for SDS-PAGE.

5.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is general a technique for the separation of proteins according to their electrophoretic mobility due to their molecular weight. In this study, discontinuous SDS-PAGE was performed using a vertical gel electrophoresis system (Mini-PROTEAN® Tetra System, Bio-Rad Laboratories GmbH, Munich, Germany) as described by Laemmli in 1970.[293] Thereby, the acrylamid (Rotiphorese® Gel 30, Carl Roth GmbH, Karlsruhe, Germany) concentration of the separation gel was adapted to the molecular weight of the proteins to be separated (15 % in the case of C-X-C motif chemokine 12 (CXCL12), 10 % in the case of other studied proteins). To determine the molecular weight of the separated proteins, a pre-stained protein marker (peqGOLD Protein Marker IV, 10–170 kDa, VWR International GmbH, Darmstadt, Germany) was used. Gel electrophoresis was performed at 80 V for 20 min and at 120 V for approximately 90 min as soon as the proteins accumulated in the stacking gel.

5.4 Immunoblotting

Proteins separated by SDS-PAGE were transferred onto a polyvinylidene difluoride (PVDF) membrane (Immun-Blot® PVDF, Bio-Rad Laboratories GmbH) in a vertical blotting system (Criterion™ Blotter, Bio-Rad Laboratories GmbH) by wet-tank electroblotting.[294] The transfer membrane was activated by incubation in methanol for approximately 1 min and equilibrated in tank buffer. Immunoblotting was performed at 100 V and 4 °C for 60 min.

5.5 Protein detection

In this study the immunological detection of proteins transferred onto membranes is based on the principle that the horseradish peroxidase (HRP) catalyzes the oxidation of luminol in the presence of hydrogen peroxide (H_2O_2) leading to a chemiluminescent signal. This signal can then be detected using radiographic films (Super RX-N, FUJIFILM Europe GmbH, Düsseldorf, Germany) developed in this study with an Agfa CP 1000 X-ray film processor (AGFA, Cologne, Germany).

After immunoblotting the membrane was incubated with a blocking buffer consisting of either 5 % non-fat milk powder (Blotto-T) or 5 % BSA (BSA-T) in Tris-buffered saline with Tween® 20 (TBS-T) for at least 1 hour at room temperature or overnight at 4 °C in order to block unspecific binding sites. Immunological detection of the protein of interest was then initiated by incubating the membrane with the respective primary antibody solution (Table 9) at 4 °C overnight. The membrane was washed three times for 10 min with TBS-T to remove unbound antibodies and was incubated with the respective HRP-conjugated secondary antibody solution (Table 10) for 2 hours at room temperature. Afterwards, the membrane was washed as described previously and the detection of HRP was initiated by incubating the membrane with enhanced chemiluminescence (ECL) solution for 2 min at room temperature protected from light. The target protein was finally detected as described above.

In all western blot experiments, β-actin was used as a loading control to ensure homogenous application of the samples. Therefore, the membrane was washed after detection of the protein of interest as described above followed by incubation with β-actin-peroxidase antibody solution (Table 9) for 1 hour at room temperature. The membrane was washed as described above and β-actin was detected. The intensity of protein bands was determined using the ImageJ gel analyzer tool (version 1.48v, National Institutes of Health, USA) followed by normalization of the target protein to β-actin.

5.6 Stripping of membranes

Since the proteins pERK1/2 and β-actin have a similar molecular weight of about 42 kDa, the membrane was stripped to avoid a possible crossover of remaining chemiluminescent signal from pERK1/2 antibodies before β-actin was detected. Therefore, the membrane was incubated with stripping buffer containing 2-mercaptoethanol at 50 °C for 30 min to remove all bound antibodies. Afterwards, the membrane was washed and blocked with the respective blocking buffer as described above before the detection of β-actin was performed.

6. Flow cytometric analysis

Flow cytometry is a widely used technique with various applications ranging from pure basic research up to extensive clinical research and diagnostics.[295-298] In this study, flow cytometry was used to analyze cell apoptosis (see II.3.3) and the surface expression of endothelial cell adhesion molecules. Flow cytometric analysis is based on the principle that cells are

transported *via* liquid flow enabling measurements of single cells. Therefore, flow cytometric systems are equipped with different lasers to examine diverse characteristics of the analyzed cell that is stained with fluorescence-labeled antibodies of interest. In this study, all measurements were performed using a FACSVerse™ (BD Biosciences).

To investigate the surface expression of endothelial cell adhesion molecules, HUVECs or HMECs were grown to confluence in 12-well cell culture plates and treated with PT, VIN, COL, PAC and TNFα as described. Cells were washed twice with PBS and were gently detached with 200 µl of HyClone™ HyQTase cell detachment reagent (GE Healthcare Life Sciences, Freiburg, Germany).

In the case of intercellular adhesion molecule 1 (ICAM-1), detached cells were mixed for fixation with 200 µl of 4 % formaldehyde (Polysciences Europe GmbH, Hirschberg an der Bergstraße, Germany) in PBS and incubated at room temperature for 10 min. The cell suspension was diluted with 1 ml of PBS and centrifuged at 300 g for 15 min. The supernatant was discarded and the cell pellet was resuspended in 100 µl of ICAM-1 antibody solution (Table 13). After incubation for 45 min protected from light, the mixture was diluted with 1 ml of PBS followed by centrifugation (300 g, 15 min). The supernatant was removed except of approximately 200 µl and the remaining solution was diluted by adding 100 µl of PBS. The pellet was resuspended by vortexing and 10,000 cells were analyzed *via* flow cytometry by measuring the median shift in fluorescence intensity.

In the case of other examined cell adhesion proteins, detached cells were mixed with 800 µl of ice-cold stopping medium to stop the enzymatic reaction. After centrifugation (300 g, 5 min, 4 °C), the supernatant was removed except of approximately 200 µl and the remaining solution was diluted by adding 1 ml of ice-cold PBS followed by centrifugation (300 g, 5 min, 4 °C). The supernatant was removed except of approximately 200 µl and the remaining solution was diluted by adding 1 ml of ice-cold PBS containing 0.2 % BSA (PBSA). After centrifugation (300 g, 5 min, 4 °C), the supernatant was completely discarded and the cell pellet was resuspended in 100 µl of the respective antibody solution (Table 13). After incubation on ice for 45 min protected from light, the mixture was diluted with 1 ml of ice-cold PBSA followed by centrifugation (300 g, 5 min, 4 °C). The supernatant was completely discarded and the pellet was resuspended in 1 ml of ice-cold PBSA followed by centrifugation (300 g, 5 min, 4 °C). The last step was repeated and the pellet was then resuspended in 300 µl of ice-cold PBSA. 10,000 cells were analyzed *via* flow cytometry by measuring the median shift in fluorescence intensity.

7. Enzyme-linked immunosorbent assay (ELISA)

The release of CXCL12 into cell culture supernatants of HUVECs was analyzed by enzyme-linked immunosorbent assay (ELISA). In this study, the secretion of CXCL12 was examined using the CXCL12/SDF-1 DuoSet ELISA kit (R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany). DuoSet ELISAs are based on the principle that a protein secreted in cell culture supernatants is linked to microplates using a capture antibody specific for the target protein followed by detection using a biotinylated detection antibody against the target protein. HRP-linked streptavidin, which binds with high affinity to biotin, is used to bind to the detection antibody. The build ELISA sandwich is finally detected by adding tetramethylbenzidine (TMB) substrate solution leading to the development of a blue color due to the oxidation of TMB catalyzed by HRP.[299] The enzymatic reaction is stopped by adding a 2 N sulfuric acid (H_2SO_4) turning the blue color to yellow. Blue or yellow color development is proportional to the amount of target protein and is detected by absorbance measurements.

HUVECs were grown to confluence in 6-well plates and treated with PT as described. Cell supernatants were collected, cleared by centrifugation (17,000 g, 10 min, 4 °C) and stored at -80 °C until used for ELISA performance. CXCL12/SDF-1 DuoSet ELISA (R&D Systems GmbH) was performed according to the manufacturer's instructions. Therefore, 96-well microplates suitable for ELISA were coated with capture antibody (2 µg/ml, 100 µl per well) in PBS overnight and washed three times with wash buffer (300 µl per well) before unspecific binding sites were blocked by incubating with reagent diluent (RD) for at least 1 hour at room temperature. Standards were obtained by stepwise diluting (1:1) a recombinant mouse stromal derived factor 1 (SDF-1) stock solution. The collected supernatants were diluted in RD in a ratio of 1:10. The microplate was washed as describe above before standards and the diluted supernatants were added (100 µl per well). After incubation at room temperature for 2 hours, the plate was washed as described above followed by incubation with detection antibody (0.4 µg/ml, 100 µl per well) in RD containing 2 % of heat inactivated normal goat serum (NGS) for 2 hours at room temperature. After washing as described above, the plate was incubated with streptavidin-HRP solution (100 µl per well) for 20 min at room temperature protected from light. Then, the plate was washed as described above, incubated with substrate solution (100 µl per well) for 20 min at room temperature and the enzymatic reaction was immediately stopped by adding 2 N H_2SO_4 (50 µl per well). CXCL12 secretion was determined by absorbance measurements at 450 nm with wavelength correction at 540 nm using an Infinite F200 pro microplate reader (Tecan Trading AG).

The measured levels of CXCL12 secretion were further normalized to the number of cells that were present in the respective wells when cell culture supernatants were collected. Therefore,

the cells were washed once with PBS and fixed by incubating with a methanol/ethanol solution (2:1) for 10 min at room temperature. Then, the cells were stained with crystal violet solution for 10 min at room temperature and unbound crystal violet was removed by washing with distilled water. The plate was dried overnight before crystal violet was reconstituted by incubation with 800 µl of sodium citrate solution for 30 min at room temperature. 60 µl of the stained solution was transferred into a 96-well cell culture plate and absorbance was measured at 540 nm using an Infinite F200 pro microplate reader (Tecan Trading AG). The amount of crystal violet after reconstitution is proportional to the initial number of cells.

8. Immunocytochemistry

HUVECs were grown to confluence on 8-well µ-slides and treated with PT as described. The cells were washed three times with PBS+ before they were fixed with 300 µl of Roti-Histofix 4 % for 10 min at room temperature. If immunocytochemistry was used to analyze the precise localization of cells in cell adhesion assays, cell adhesion assays were performed as described (see II.10.2) and the co-cultured cells were fixed. After fixation, cells were washed three times with 300 µl of PBS. If vascular endothelial cadherin (VE-cadherin) was analyzed with immunocytochemistry, cells were permeabilized by incubating with 300 µl of Triton X-100 (0.2 %) in PBS for 2 min at room temperature. Afterwards, cells were washed as described above. In all immunocytochemistry experiments, unspecific binding sites were blocked by incubating with 300 µl of PBSA for at least 20 min at room temperature followed by incubation with 230 µl of the respective (mixed) primary antibody solution (Table 11) for 2 hours at room temperature. Unbound antibodies were removed by washing the cells three times with PBS for 5 min each. Immunocytochemical detection of bound primary antibodies was enabled by incubating the cells with 230 µl of the respective fluorescence-labeled secondary antibody solution (Table 12) for 1 hour at room temperature protected from light. If collagen was analyzed *via* immunocytochemistry, HOECHST 33342 was added to the secondary antibody solution in a ratio of 1:10,000. The cells were washed three times with PBS for 10 min each and were finally embedded in FluorSave™ Reagent covered by coverslips. Immunocyto-chemical stainings were detected using the fluorescence microscope LSM 510 (Carl Zeiss AG, Jena, Germany).

9. Reporter gene assay

The reporter gene assay is a widely used and very sensitive method for studying cellular events associated with signal transduction and gene expression.[300] In this study, a Dual-Luciferase® reporter assay (Promega GmbH) was used to investigate the influence of microtubule-targeting agents (MTAs) on the NFκB promoter activity. Therefore, *Escherichia coli* (*E. coli*) were transformed by reporter plasmids followed by reproduction and preparation before they were used for the transfection of HMECs with subsequent performance of the reporter gene assay.

9.1 Transformation of *E. coli* with plasmid DNA

The transformation of competent *E. coli* cells (DH5α) by plasmid DNA (see II.1.7) was kindly performed by Jennifer Reis. Therefore, 1 µl of plasmid DNA was mixed with 200 µl of DH5α cells and incubated on ice for 30 min. After heat-shock at 42 °C for 2 min, the mixture was incubated on ice for 2 min before 1 ml of bacterial growth medium (YT medium) was added. The suspension was incubated for 30 min at 37 °C while shaking followed by streaking on YT agar plates containing ampicillin for selection. The transformed bacteria were grown on the plates overnight at 37 °C before a single colony was picked with a pipette tip for inoculation of a bacterial culture (250 ml).

9.2 Reproduction and preparation of plasmid DNA

The reproduction and preparation of plasmid DNA (see II.1.7) was kindly performed by Jennifer Reis. Therefore, transformed bacteria were cultured overnight at 37 °C while shaking in 250 ml of YT medium containing ampicillin for selection. Afterwards, plasmid DNA was prepared using the PureYield™ Plasmid Maxiprep System (Promega GmbH) according to the manufacturer's protocol. Therefore, bacterial cells were lysed enabling the isolation and purification of plasmid DNA. The concentration of the eluted plasmid DNA was determined by absorbance measurements at 260 nm using a P 330 nanophotometer (Implen GmbH).

Although plasmid DNA preparation using the PureYield™ Plasmid Maxiprep System includes a washing step for endotoxin removal, potentially remaining endotoxins were removed using the MiraCLEAN® Endotoxin Removal Kit (Mirus Bio LLC, Madison, WI, USA). Endotoxin removal was kindly performed by Mareike Lang according to the manufacturer's instructions. Therefore, plasmid DNA was incubated with EndoGO Reagent on ice for 5 min followed by

incubation at 50 °C for 30 min. Then the mixture was incubated on ice for 5 min and subsequently centrifuged (14,000 g, 1 min, RT). DNA was precipitated by adding 99.8 % ice-cold ethanol to the DNA-containing supernatant followed by incubation at room temperature for 30 min while inverting. DNA was harvested by centrifugation (17,000 g, 20 min, 4 °C), washed with 70 % ethanol and centrifuged (17,000 g, 20 min, 4 °C). The DNA-containing pellet was allowed to air dry and resuspended in DNase/RNase-free water. The next day, DNA concentration was determined by absorbance measurements at 260 nm using a P 330 nanophotometer (Implen GmbH).

9.3 Plasmid transfection and sample preparation

For reporter gene assays, HMEC-1 cells were transfected with purified reporter plasmids (see II.1.7 and II.9.2) using the HUVEC Nucleofector® Kit (Lonza Cologne GmbH, Cologne, Germany) that is based on electroporation, a widely used technique with various applications. In this study, electroporation was used for the transfection of cells since it leads to the formation of pores in the cell membrane due to an electric shock, thus enabling the cellular uptake of DNA.[301]

Transfection of cells was performed according to the manufacturer's instructions. Therefore, HMECs were detached as described earlier (see II.2.3) and the number of cells was determined using a hemocytometer. 1 x 10^6 cells were harvested by centrifugation (300 g, 5 min) followed by resuspension in 100 µl of Nucleofector® Solution. Then, 3.5 µg of the purified reporter plasmid pGL4.32[*luc2P*/NF-κB-RE/Hygro] and 1.4 µg of the purified reporter plasmid pGL4.74[*hRluc*/TK] (see II.1.7 and II.9.2) were added to the suspension and the mixture was immediately transferred into a Nucleofector® Cuvette for subsequent electroporation. Cells were co-transfected with both reporter plasmids using the Nucleofector® Program A-034 for HUVECs (Nucleofector 2b, Lonza Cologne GmbH). Immediately after electroporation, 350 µl of ECGM were added to the suspension and the cells were seeded onto 48-well cell culture plates (1.25 x 10^5 cells per well) prefilled with ECGM (500 µl per well). The medium was changed after 4–5 hours to remove all of the remaining Nucleofector® Solution. Approximately 24 hours post transfection the co-transfected cells were treated with PT, VIN, COL, PAC and TNFα as described. The cells were finally washed once with PBS, lysed with 65 µl of passive lysis buffer (PLB, Promega GmbH) and stored at -80 °C until used for Dual-Luciferase® reporter assay.

9.4 Dual-Luciferase® reporter assay

Dual-Luciferase® reporter assays are based on the principle that two individual reporter enzymes are simultaneously expressed and measured within a single sample. Thereby, the gene coding for one of these reporter enzymes is coupled to a regulated promoter to study the promoter-regulated gene expression, while the gene coding for the other reporter enzyme is coupled to an unregulated promoter as an internal control. In this study, NFκB promoter activity was analyzed using the pGL4 series of *Photinus* (*Photinus pyralis*) and *Renilla* (*Renilla reniformis*) luciferase vectors (see II.1.7). *Photinus* luciferase catalyzes the oxidation of beetle luciferin into oxyluciferin that requires adenosine triphosphate (ATP), magnesium ions and oxygen leading to a bioluminescent signal (550–570 nm). *Renilla* luciferase catalyzes the oxidation of coelenterazine into coelenteramide that merely requires oxygen leading to a bioluminescent signal (480 nm) as well. Then, the activity of *Photinus* and *Renilla* luciferases is determined in Dual-Luciferase® reporter assays by luminescence measurements. The measured bioluminescent signal is proportional to the expression of the respective luciferase.

The assay was performed according to the manufacturer's instructions. Therefore, cell lysates were thawed for 15 min at room temperature with gentle shaking and were cleared by centrifugation (17,000 g, 1 min) before they were transferred into a 96-well microplate suitable for luminescence measurements (10 µl per well). Luciferase substrate solution LARII and Stop & Glo® Reagent were freshly prepared and separately transferred into two independent injectors of an Infinite F200 pro microplate reader (Tecan Trading-AG). After priming both injectors, 50 µl of LARII were injected and the activity of *Photinus* luciferase was analyzed by luminescence measurements. Then, *Photinus* luciferase activity was quenched and *Renilla* luciferase reaction was initiated by injecting 50 µl of Stop & Glo® Reagent followed by luminescence measurements to detect the activity of *Renilla* luciferase. All measurements were performed using a delay of 2 s and a read time of 10 s. The signal of *Photinus* luciferase was finally normalized to that of *Renilla* luciferase. Since the measured bioluminescent signals were proportional to the expression of the used luciferases, the normalized signal was proportional to the NFκB promoter activity.

10. Cell adhesion assay

In this study, the influence of MTAs on the adhesion of tumor cells (MDA-MB-231 cells) or leukocytes (THP-1 cells) to endothelial cells was determined by cell adhesion assays.

Furthermore, the adhesion of tumor cells to extracellular matrix proteins in the absence of endothelial cells was investigated.

10.1 Adhesion of tumor cells to extracellular matrix proteins

Cell adhesion assays with MDA-MB-231 cells and different extracellular matrix proteins were performed in order to figure out onto which component the cells preferentially attach. Therefore, MDA-MB-231 cells were grown to confluence in cell culture dishes (Ø 10 cm) and were washed once with PBS. Then the cells were stained by incubating with 5 ml of CellTracker™ Green solution (5 µM, in serum-free DMEM) at 37 °C for 30 min. Afterwards, the cells were washed twice with PBS and detached by incubating with 2 ml of a Trypsin/EDTA solution at 37 °C for a maximum of 3 min. The enzymatic reaction was stopped by adding 10 ml of DMEM and the detached cells were harvested by centrifugation (300 g, 5 min). Cells were resuspended in 5 ml of DMEM and the number of cells was determined using a hemocytometer. If the assay was performed to determine the concentration of anti-β1-integrin antibody (Table 14) required to block the adhesion of MDA-MB-231 cells to collagen, anti-β1-integrin antibody was added to the suspension (1, 5 or 10 µg/ml per 1 x 10^6 cells) and the mixture was incubated for 30 min at 37 °C in a water bath. Afterwards, the cells were centrifuged (300 g, 5 min) and unbound antibody was removed by washing the pellet with 5 ml of DMEM followed by centrifugation (300 g, 5 min). Cells were resuspended in 5 ml of DMEM and the number of cells was determined. In all experiments the cell concentration was adjusted with DMEM to 2 x 10^5 cells per ml for assay performance. Thereafter, 500 µl of the cell suspension was transferred into wells of a 24-well plate that were left uncoated or pre-coated with PBS, fibronectin (5 µg/ml) or collagen (10 µg/ml) for 2 hours. MDA-MB-231 cells were allowed to adhere for 10 min before non-adherent cells were removed by washing three times with 500 µl of PBS+. Cell adhesion was quantified by microscopic analysis using the inverted microscope Leica DM IL LED (Leica Microsystems) and by fluorescence measurements (ex: 485 nm; em: 535 nm) using an Infinite F200 pro microplate reader (Tecan Trading-AG).

10.2 Adhesion of tumor cells to endothelial cells

To analyze the influence of MTAs on the adhesion of tumor cells to endothelial cells, adhesion assays were performed, in which MDA-MB-231 cells were allowed to adhere to HUVECs treated with MTAs. Therefore, HUVECs were grown to confluence in 24-well cell culture plates or, if cell adhesion assays were performed to focus on the precise location of the tumor cells, in 8-well µ-slides (ibidi GmbH). HUVECs were treated with PT, VIN, COL, PAC and TNFα as

described. If cell adhesion assays were performed in order to investigate the role of ICAM-1 or VCAM-1 in the experimental set-up, respective antibodies (Table 14) were added to PT-treated HUVECs as indicated. Furthermore, MDA-MB-231 cells were grown to confluence in cell culture dishes (10 cm), stained with CellTracker™ Green and detached as described above. After centrifugation (300 g, 5 min), MDA-MB-231 cells were resuspended in 5 ml of DMEM and were left untreated or treated with anti-β1-integrin antibody (1 µg/ml per 1 x 10^6 cells, Table 14) for 30 min in a water bath. For the latter, unbound antibody was removed, and the cells were washed with 10 ml of DMEM, harvested by centrifugation and resuspended as described above. The number of cells was determined using a hemocytometer and the cell concentration was adjusted with DMEM to 2 x 10^5 cells per ml. Thereafter, the culture medium was removed from HUVECs and the stained MDA-MB-231 cells were added to the endothelial monolayer (24-well plate: 500 µl of cell suspension per well; 8-well µ-slide: 250 µl of cell suspension per well). MDA-MB-231 cells were allowed to adhere for 10 min before non-adherent cells were removed by washing three times with 500 µl (24-well plates) or 250 µl (8-well µ-slides) of PBS+. The adhesion was either directly quantified by fluorescence measurements (ex: 485 nm; em: 535 nm) using an Infinite F200 pro microplate reader (Tecan Trading-AG) or the cells were lysed with 100 µl of RIPA buffer and 60 µl of the lysates were transferred into 96-well microplates suitable for fluorescence measurements before quantification. If cell adhesion assays were performed in order to analyze the precise location of the tumor cells, cells were fixed after assay performance instead of fluorescence measurements and HUVEC boundaries were visualized by immunocytochemical staining of VE-cadherin (see II.8).

10.3 Adhesion of leukocytes to endothelial cells

To analyze the influence of MTAs on the adhesion of leukocytes to endothelial cells, cell adhesion assays were performed, in which THP-1 cells were allowed to adhere to HMECs treated with MTAs. Therefore, HMECs were grown to confluence in 24-well cell culture plates and treated with PT, VIN, COL, PAC and TNFα as described. Furthermore, THP-1 cells were grown in cell culture flasks (75 cm^2) and the number of cells was determined using a hemocytometer. The required amount of cells was harvested by centrifugation (300 g, 5 min) and the cells were stained by incubating with the appropriate volume (1 ml per 1 x 10^6 cells) of CellTracker™ Green solution (5 µM, in serum-free RPMI) for 30 min at 37 °C in a water bath. While staining, the cell suspension was inverted every 10 min. Afterwards, cells were centrifuged (300 g, 5 min) and the pellet was washed with 10 ml of serum-free RPMI followed by centrifugation (300 g, 5 min). The cells were resuspended in 5 ml of RPMI, the number of cells was determined and the cell concentration was adjusted with RPMI to 2 x 10^5 cells per ml. Thereafter, the culture medium was removed from HMECs and the stained THP-1 cells

were added to the HMEC monolayer (500 µl of cell suspension per well). The cells were allowed to adhere for 5 min before non-adherent cells were removed by washing three times with 500 µl of PBS+. The adhesion was directly quantified by fluorescence measurements (ex: 485 nm; em: 535 nm) using an Infinite F200 pro microplate reader (Tecan Trading-AG).

The measured adhesion levels were further normalized to the number of HMECs that were present after treatment with the compounds before the adhesion assay was performed. Therefore, MTA-treated HMECs were washed once with RPMI followed by two washing steps with PBS+ before the cells were fixed by incubating with a methanol/ethanol solution (2:1) for 10 min at room temperature. Then the cells were stained with crystal violet solution for 10 min at room temperature and unbound crystal violet was removed by washing with distilled water. The plate was dried overnight before crystal violet was reconstituted by incubation with 200 µl of 20 % acetic acid for 30 min at room temperature. 60 µl of the stained solution was transferred into a 96-well cell culture plate and absorbance was measured at 590 nm. The amount of crystal violet after reconstitution is proportional to the number of cells. The fixation and staining of HMECs with crystal violet required for the normalization of adhesion assays was kindly performed by Mareike Lang.

11. Endothelial transmigration assay

To analyze the influence of PT on the transmigration of tumor cells through an endothelial monolayer, transmigration assays were performed, in which MDA-MB-231 cells were allowed to transmigrate through an HUVEC monolayer treated with PT. Therefore, 1 x 10^5 HUVECs were seeded in a volume of 100 µl ECGM into the upper compartment of a Transwell® Insert (polycarbonate membrane, diameter 6.5 mm, pore size 8 µm; Corning, New York, USA) coated with collagen. 48 hours after seeding, HUVECs were treated with PT as described. Furthermore, MDA-MB-231 cells were grown to confluence in cell culture dishes (10 cm), stained with CellTracker™ Green and detached as described above (see II.10.1). After centrifugation (300 g, 5 min), MDA-MB-231 cells were resuspended in 5 ml of M199 containing 0.1 % BSA and were left untreated or treated with anti-β1-integrin antibody (1 µg/ml per 1 x 10^6 cells, Table 14) for 30 min in a water bath. For the latter, unbound antibody was removed, and the cells were washed with 10 ml of M199 containing 0.1 % BSA, harvested by centrifugation (300 g, 5 min) and resuspended as described above. The number of cells was determined and the cell concentration was adjusted with M199 containing 0.1 % BSA to 2 x 10^5 cells per ml. Thereafter, the culture medium was removed from HUVECs and the stained MDA-MB-231 cells were added to the HUVEC monolayer on the upper compartment (100 µl

of cell suspension per insert). In the lower compartment, 600 µl of serum-free M199 was used for negative control, while 600 µl of M199 containing 20 % fetal calf serum (FCS) was used as a chemoattractant for the tumor cells and thus for positive control. MDA-MB-231 cells were allowed to transmigrate through the endothelial monolayer for 24 hours. Afterwards, non-transmigrated tumor cells on the upper compartment were carefully removed with a cotton swab and the transmigrated cells were lysed by transferring the Transwell® Insert into 200 µl of RIPA buffer. 60 µl of the lysates were transferred into 96-well microplates suitable for fluorescence measurements and the transmigration of MDA-MB-231 cells was quantified by fluorescence measurements (ex: 485 nm; em: 535 nm) using an Infinite F200 pro microplate reader (Tecan Trading-AG).

12. Intravital microscopy

Intravital microscopy (IVM) is a powerful technique used to visualize biological processes in animals. Through the wide range of applications, IVM was utilized until today in several *in vivo* studies, which have addressed biological key questions in the proper physiological context in fields such as microbiology, tumor biology, immunology and recently in stem cell research.[302] In this study, IVM was used to analyze the influence of PT on leukocyte-endothelial cell interactions.

12.1 Animals

Experiments were carried out using male C57BL/6 mice (20-25 g in weight, 6-10 weeks in age). The mice were purchased from Charles River (Sulzfeld, Germany), were held under standard laboratory conditions and were allowed free access to animal chow and tap water. All experiments were performed according to German legislation regarding the protection of animals (number of the animal experiment request: Az. 55.2-1-54-2532-115-12).

12.2 Intravital microscopy procedure

Intravital microscopy was kindly performed by Matthias Fabritius from the group of PD Dr. Christoph Andreas Reichel (Department of Otorhinolaryngology, Head and Neck Surgery, Walter Brendel Centre of Experimental Medicine, clinical center at the University of Munich, Germany). In this study, the influence of PT on leukocyte rolling and adhesion onto the

endothelium as well as leukocyte transmigration through the endothelium was analyzed in the mouse cremaster muscle under inflammatory conditions as recently described (Reichel *et al.*, 2008).[303]

In brief, PT (1 mg/kg) reconstituted in DMSO was administered to male C57BL/6 mice by intra-arterial (i. a.) injections. 30 min after PT application, inflammatory conditions in the mouse cremaster muscle were established by intrascrotal injection of TNFα (25 µg/kg). 4 hours after TNFα administration, leukocyte rolling and firm adherence onto the endothelium and transmigration through the endothelium was determined by intravital microscopy of postcapillary venules of the mouse cremaster muscle.

13. Statistical analysis

Unless otherwise stated, all experiments were performed at least three times. The actual numbers of independently performed experiments (n) are stated in the respective figure legends. Different lot numbers/donors (in the case of experiments performed in HUVECs) or different passages (in the case of experiments performed in HMECs) were used for each experiment. Bar graph data are expressed as means ± standard error of the mean (SEM). The GraphPad Prism software version 5.04 (GraphPad Software, San Diego, CA, USA) was used for the performance of statistical analyses. For the evaluation of non-grouped experiments, unpaired *t*-test or one-way ANOVA followed by Tukey's post-hoc test were used, while two-way ANOVA followed by the Bonferroni post-hoc test was used for the evaluation of grouped experiments. $P \leq 0.05$ was considered as statistically significant.

III RESULTS

1. The influence of PT on tumor-endothelial cell interactions

1.1 The effect of PT on tumor cell adhesion and transmigration *in vitro*

The adhesion of tumor cells onto and their subsequent transmigration through the endothelium represent two essential steps in cancer cell dissemination.[41,42] Therefore, cell adhesion assays and transendothelial migration assays were performed to analyze the effect of pretubulysin (PT) on endothelial cells in both processes.

1.1.1 PT increases the adhesion of tumor cells onto an endothelial monolayer

To analyze the influence of PT on the adhesion of tumor cells onto an endothelial monolayer, cell adhesion assays (see II.10.2) were performed in which human umbilical vein endothelial cells (HUVECs) were treated with increasing concentrations of PT (1, 3, 10, 30, 100, 300 nM) for 6 or 24 hours before fluorescence-labeled MDA-MB-231 tumor cells were allowed to adhere for 10 minutes. Since tumor necrosis factor alpha (TNFα) is known to activate endothelial cells, this cytokine was used as positive control in the cell adhesion assays.[304,305] The treatment of HUVECs with PT for 6 (Figure 12A) or 24 (Figure 12B) hours resulted in a concentration-dependent increase of tumor cell adhesion onto the endothelial monolayer. Both assays were kindly performed by Tanja Stehning.

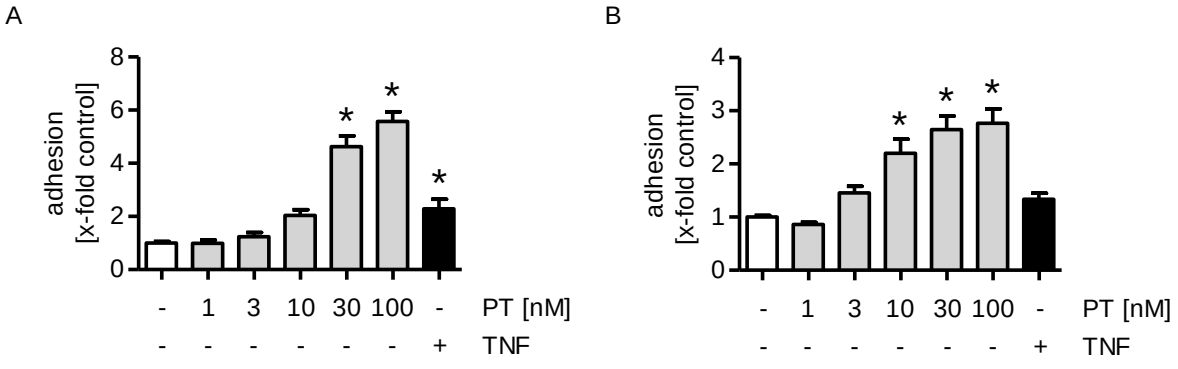

Figure 12: PT increases the adhesion of MDA-MB-231 tumor cells onto a HUVEC monolayer. Confluent HUVECs were treated with PT (1, 3, 10, 30, 100, 300 nM) or TNFα (10 ng/ml) for 6 hours **(A)** or 24 hours **(B)**. Fluorescence-labeled MDA-MB-231 tumor cells were allowed to adhere for 10 minutes and non-adherent cells were removed by washing. The amount of adherent tumor cells was quantified by fluorescence measurements using a microplate reader (Tecan Trading AG). Data are expressed as mean ± SEM. A: n=3, B: n=5. *p ≤ 0.05 versus negative control.

1.1.2 PT reduces the transmigration of tumor cells through an endothelial monolayer

To investigate the influence of PT on the transmigration of tumor cells through an endothelial monolayer, endothelial transmigration assays (see II.11) were performed. Therefore, HUVECs were grown to confluence in the upper compartment of Transwell® inserts and treated with increasing concentrations of PT (10, 30, 100 nM) for 24 hours before fluorescence-labeled MDA-MB-231 tumor cells were allowed to transmigrate through the endothelial monolayer for 24 hours. Fetal calf serum (FCS) in the lower compartment was used as a chemoattractant for the tumor cells and thus served as a positive control. Interestingly, although the adhesion of tumor cells onto an endothelial monolayer was enhanced upon PT treatment, the transendothelial migration of tumor cells was strongly decreased in a concentration-dependent manner when endothelial cells were treated with PT (Figure 13).

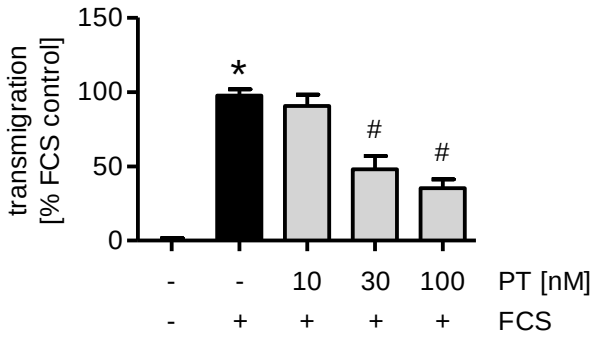

Figure 13: PT reduces the transmigration of MDA-MB-231 tumor cells through a HUVEC monolayer. HUVECs were grown to confluence in the upper compartment of Transwell® inserts (polycarbonate membrane, diameter 6.5 mm, pore size 8 µm) and treated with PT (10, 30, 100 nM) for 24 hours. Fluorescence-labeled MDA-MB-231 tumor cells were added into the upper compartment and were allowed to transmigrate for 24 hours. FCS (20 %) in the lower compartment was used as a positive control. The amount of transmigrated tumor cells on the lower part of the porous filter membrane was quantified by fluorescence measurements using a microplate reader (Tecan Trading AG). Data are expressed as mean ± SEM. n=4. *$p \leq 0.05$ versus negative control, $^{\#}p \leq 0.05$ versus FCS control.

1.2 The influence of PT on cell adhesion molecules typically relevant for tumor-endothelial cell interactions

We hypothesized that the enhanced adhesion of tumor cells onto an endothelial monolayer might be caused by an increased expression of endothelial cell adhesion molecules (CAMs). Endothelial CAMs that have been reported to be involved in tumor-endothelial cell interactions are the intercellular adhesion molecule 1 (ICAM-1), the vascular cell adhesion molecule 1 (VCAM-1), E-selectin, galectin-3 and neural cadherin (N-cadherin).[47] Hence, the effect of PT on the mRNA expression, the protein expression and the cell surface expression of these cell

adhesion molecules was investigated. Furthermore, cell adhesion assays were performed in which the role of potentially relevant cell adhesion molecules was clarified. The qRT-PCR experiments were kindly performed by Tanja Stehning.

1.2.1 The PT-induced expression of ICAM-1 is not linked to the PT-triggered tumor cell adhesion

qRT-PCR experiments, western blot analysis and flow cytometry were performed to analyze the impact of PT on the expression of ICAM-1 in HUVECs on the mRNA level, on the protein level and on the cell surface protein level, respectively. Therefore, HUVECs were treated with increasing concentrations of PT (qRT-PCR and western blot: 10, 30, 100 nM; flow cytometry: 30, 100 nM) for 12 (qRT-PCR) or 24 (western blot and flow cytometry) hours before the cells were analyzed (see II.4, II.5, II.6). TNFα is widely known to induce the expression of cell adhesion molecules in endothelial cells and served, therefore, as positive control. The treatment of HUVECs with PT resulted in a significant increase of ICAM-1 mRNA expression (Figure 14A), total protein expression (Figure 14B) and cell surface protein expression (Figure 14C).

To prove whether the effect of PT on the expression of ICAM-1 plays a role in the PT-evoked tumor cell adhesion, an ICAM-1 neutralizing/blocking antibody was used after PT treatment and before fluorescence-labeled MDA-MB-231 tumor cells were allowed to adhere for 10 minutes (see II.10.2). Despite the upregulation of ICAM-1 expression due to PT treatment, this cell adhesion molecule did not participate in the PT-triggered adhesion of tumor cells to an endothelial monolayer (Figure 14D).

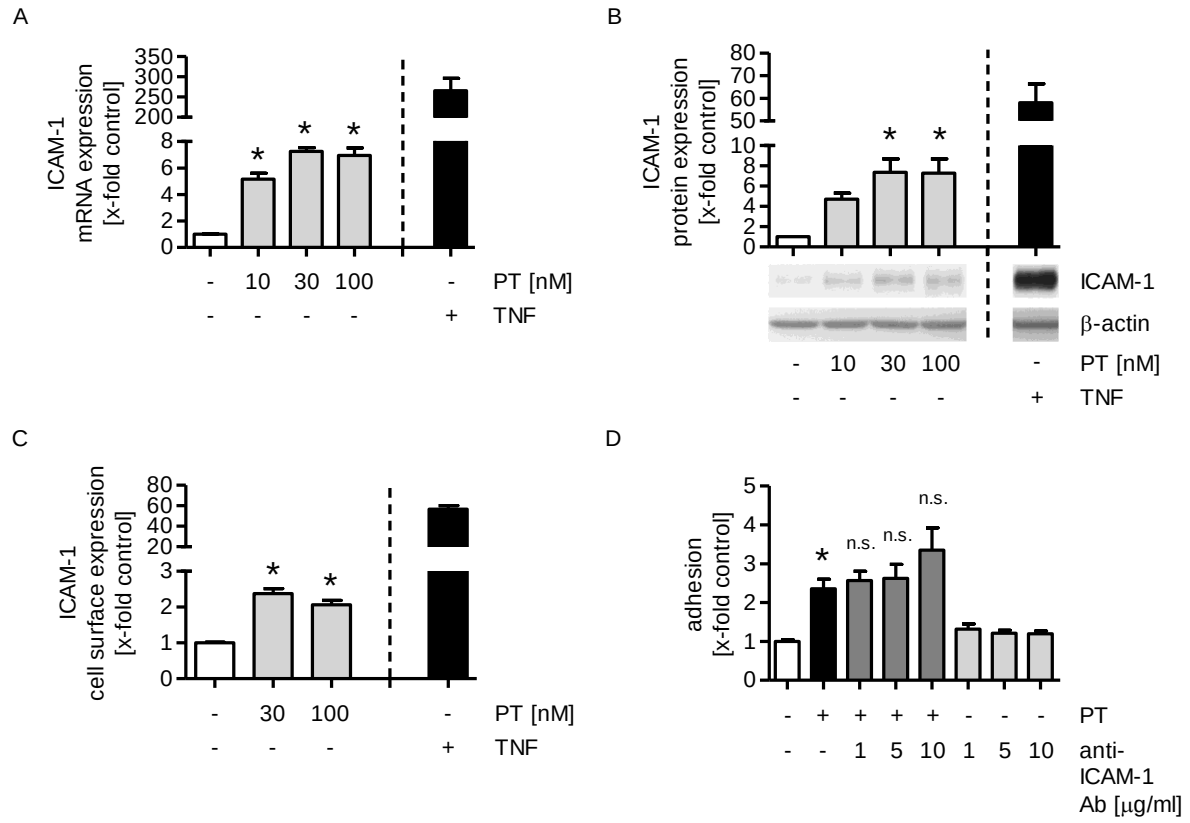

Figure 14: The enhanced expression of ICAM-1 is not responsible for the PT-evoked tumor cell adhesion. (A-C) HUVECs were grown to confluence and treated with PT (10, 30, 100 nM (A/B); 30, 100 nM (C)) or TNFα (10 ng/ml) for 12 (A) or 24 (B/C) hours. The mRNA expression of ICAM-1 (A) was analyzed by qRT-PCR experiments, the protein expression of ICAM-1 (B) was determined by western blot analysis and the cell surface expression of ICAM-1 (C) was analyzed by flow cytometry using a FACSVerse™ (BD Biosciences). **(D)** HUVECs were grown to confluence and treated with 30 nM PT for 24 hours. An ICAM-1 neutralizing antibody (1, 5, 10 µg/ml) was added for the last 30 minutes of PT treatment. Fluorescence-labeled MDA-MB-231 tumor cells were allowed to adhere for 10 minutes and non-adherent cells were removed by washing. The amount of adherent tumor cells was quantified by fluorescence measurements using a microplate reader (Tecan Trading AG). Data are expressed as mean ± SEM. A-C: n=3, D: n=5. *p ≤ 0.05 versus negative control, n.s. (not significant) ≤ 0.05 versus PT alone. TNFα was used as a positive control and was not included into the statistical analyses.

1.2.2 The PT-induced expression of VCAM-1 is not linked to the PT-evoked tumor cell adhesion

As with ICAM-1 the effect of PT on the expression of VCAM-1 in HUVECs was investigated on the mRNA level by qRT-PCR experiments, on the protein level *via* western blot analysis and on the cell surface protein level by flow cytometry. Therefore, HUVECs were treated with increasing concentrations of PT (qRT-PCR and western blot: 10, 30, 100 nM; flow cytometry: 30, 100 nM) for 12 (qRT-PCR) or 24 (western blot and flow cytometry) hours before the assays were performed (see II.4, II.5, II.6). TNFα served as a positive control to induce the expression of VCAM-1. The treatment of HUVECs with PT resulted in a significant increase of VCAM-1 mRNA expression (Figure 15A), total protein expression (Figure 15B) and cell surface protein expression (Figure 15C).

To prove whether the influence of PT on the expression of VCAM-1 plays a role in the PT-

triggered tumor cell adhesion, a VCAM-1 neutralizing/blocking antibody was used after PT treatment and before fluorescence-labeled MDA-MB-231 tumor cells were allowed to adhere for 10 minutes (see II.10.2). Despite the upregulation of VCAM-1 expression due to PT treatment, this cell adhesion molecule did not participate in the PT-evoked adhesion of tumor cells to an endothelial monolayer (Figure 15D).

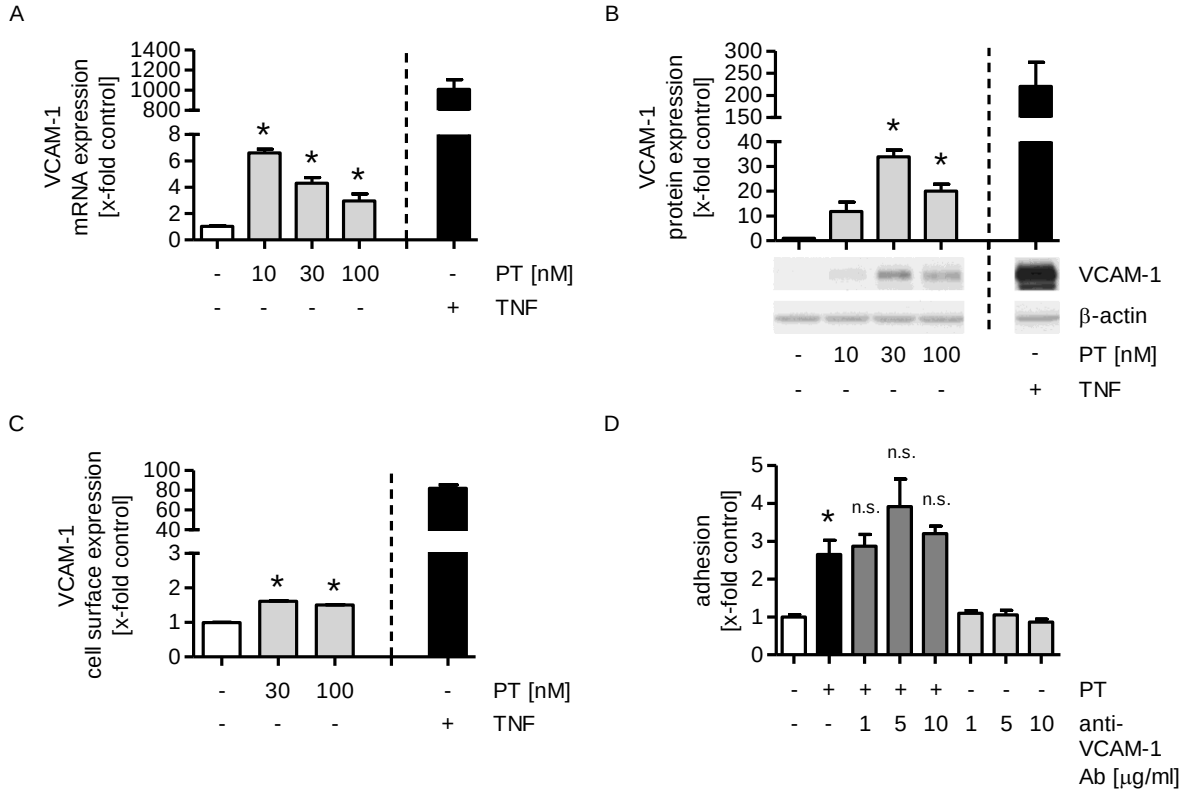

Figure 15: The enhanced expression of VCAM-1 is not responsible for the PT-triggered tumor cell adhesion. (A-C) HUVECs were grown to confluence and treated with PT (10, 30, 100 nM (A/B); 30, 100 nM (C)) or TNFα (10 ng/ml) for 12 (A) or 24 (B/C) hours. The mRNA expression of VCAM-1 (A) was analyzed by qRT-PCR experiments, the protein expression of VCAM-1 (B) was determined by western blot analysis and the cell surface expression of VCAM-1 (C) was analyzed by flow cytometry using a FACSVerse™ (BD Biosciences). **(D)** HUVECs were grown to confluence and treated with 30 nM PT for 24 hours. A VCAM-1 neutralizing antibody (1, 5, 10 µg/ml) was added for the last 30 minutes of PT treatment. Fluorescence-labeled MDA-MB-231 tumor cells were allowed to adhere for 10 minutes and non-adherent cells were removed by washing. The amount of adherent tumor cells was quantified by fluorescence measurements using a microplate reader (Tecan Trading AG). Data are expressed as mean ± SEM (n=3). *p ≤ 0.05 versus negative control, n.s. (not significant) ≤ 0.05 versus PT alone. TNFα was used as a positive control and was not included into the statistical analyses.

1.2.3 The PT-induced expression of N-cadherin is not linked to the PT-triggered tumor cell adhesion

As with ICAM-1 and VCAM-1, qRT-PCR experiments, western blot analysis and flow cytometry were performed to analyze the influence of PT on the expression of N-cadherin in HUVECs on the mRNA level, on the protein level and on the cell surface protein level, respectively.

Therefore, HUVECs were treated with increasing concentrations of PT (qRT-PCR and western blot: 10, 30, 100 nM; flow cytometry: 30, 100 nM) for 12 (qRT-PCR) or 24 (western blot and flow cytometry) hours before the assays were performed (see II.4, II.5, II.6), whereby western blot analysis was kindly performed by Tanja Stehning. The treatment of HUVECs with PT resulted in a concentration-dependent increase of N-cadherin mRNA expression (Figure 16A), total protein expression (Figure 16B) and cell surface protein expression (Figure 16C).

To prove whether the effect of PT on the expression of N-cadherin plays a role in the PT-evoked tumor cell adhesion, an N-cadherin neutralizing/blocking antibody was used after PT treatment and before fluorescence-labeled MDA-MB-231 tumor cells were allowed to adhere for 10 minutes (see II.10.2). Despite the upregulation of N-cadherin expression due to PT treatment, this cell adhesion molecule did not participate in the PT-triggered adhesion of tumor cells to an endothelial monolayer (Figure 16D).

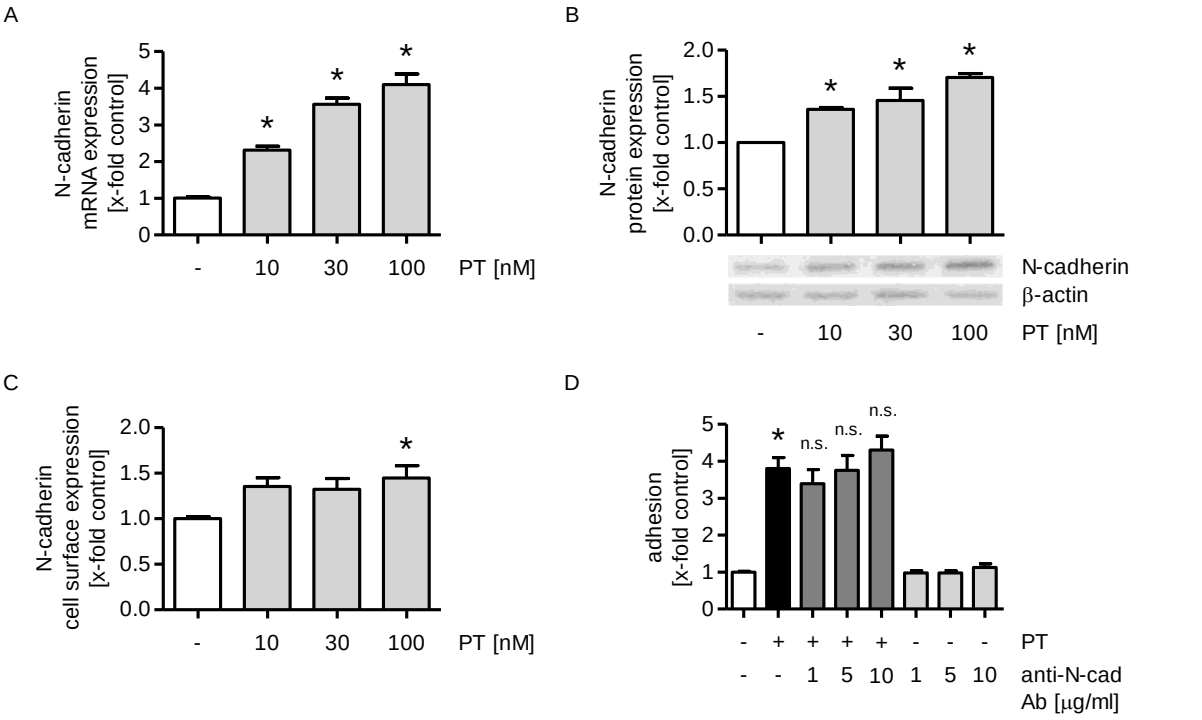

Figure 16: The enhanced expression of N-cadherin is not responsible for the PT-evoked tumor cell adhesion. (A-C) HUVECs were grown to confluence and treated with PT (10, 30, 100 nM) for 12 (A) or 24 (B/C) hours. The mRNA expression of N-cadherin (A) was analyzed by qRT-PCR experiments, the protein expression of N-cadherin (B) was determined by western blot analysis and the cell surface expression of N-cadherin (C) was analyzed by flow cytometry using a FACSVerse™ (BD Biosciences). **(D)** HUVECs were grown to confluence and treated with 30 nM PT for 24 hours. An N-cadherin neutralizing antibody (1, 5, 10 µg/ml) was added for the last 30 minutes of PT treatment. Fluorescence-labeled MDA-MB-231 tumor cells were allowed to adhere for 10 minutes and non-adherent cells were removed by washing. The amount of adherent tumor cells was quantified by fluorescence measurements using a microplate reader (Tecan Trading AG). Data are expressed as mean ± SEM (n=3). *p ≤ 0.05 versus control (A-C) or negative control (D), n.s. (not significant) ≤ 0.05 versus PT alone.

1.2.4 E-selectin and galectin-3 do not participate in the PT-evoked tumor cell adhesion

The impact of PT on the expression of E-selectin and galectin-3 in HUVECs was analyzed at the mRNA level by qRT-PCR experiments and in the case of E-selectin at the cell surface protein level by flow cytometry. Therefore, HUVECs were treated with increasing concentrations of PT (qRT-PCR: 10, 30, 100 nM; flow cytometry: 30, 100 nM) for 12 (qRT-PCR) or 4 (flow cytometry) hours before the assays were performed (see II.4, II.6). As with cell adhesion assays, TNFα served as a positive control when the influence of PT on E-selectin expression was investigated. The treatment of HUVECs with PT resulted in a significant increase of E-selectin mRNA expression (Figure 17A), whereas galectin-3 mRNA expression remained unaffected (Figure 17C). However, the cell surface expression of E-selectin was not influenced when HUVECs were treated with PT (Figure 17B). These findings indicate that neither E-selectin nor galectin-3 play a role in the PT-evoked adhesion of tumor cells to an endothelial monolayer.

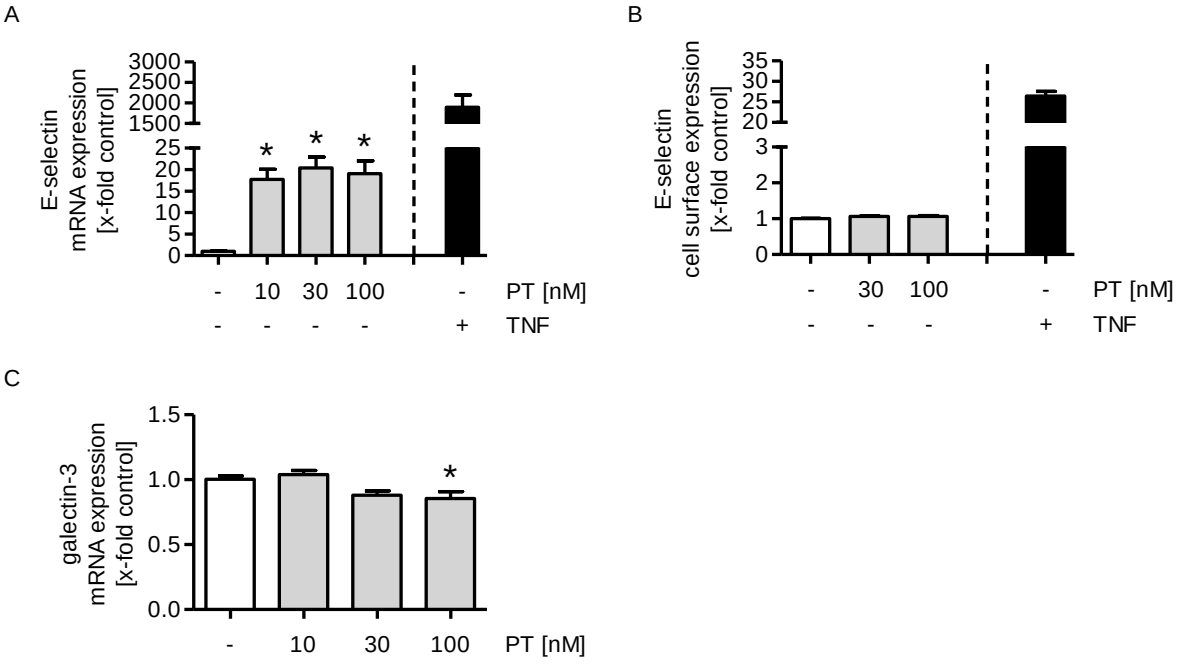

Figure 17: PT does not influence the surface expression of E-selectin and the mRNA expression of galectin-3. (A-C) HUVECs were grown to confluence and treated with PT (10, 30, 100 nM (A/C); 30, 100 nM (B)) or TNFα (10 ng/ml, A/B) for 12 (A/C) or 4 (B) hours. The mRNA expression of E-selectin/galectin-3 (A/C) was analyzed by qRT-PCR experiments and the cell surface expression of E-selectin (B) was analyzed by flow cytometry using a FACSVerse™ (BD Biosciences). Data are expressed as mean ± SEM (n=3). *p ≤ 0.05 versus negative control (A/B) or control (C). TNFα was used as a positive control and was not included into the statistical analyses.

1.3 The effect of PT on the chemokine system CXCL12/CXCR4

Since the chemokine system C-X-C motif chemokine 12/C-X-C chemokine receptor 4 (CXCL12/CXCR4) is substantially involved in the regulation of cancer cell dissemination,[306] the influence of PT on the mRNA expression and the protein expression of CXCL12 as well as its secretion into cell culture supernatants was investigated. Furthermore, cell adhesion assays were performed in which the role of CXCL12 was clarified. Here, qRT-PCR experiments were kindly performed by Tanja Stehning.

1.3.1 The PT-induced expression of CXCL12 is not linked to the PT-triggered tumor cell adhesion

qRT-PCR experiments and western blot analysis were performed to analyze the influence of PT on the expression of CXCL12 in HUVECs on the mRNA level and on the protein level, respectively. Moreover, the effect of PT on the endothelial secretion of CXCL12 into cell culture supernatants was determined by ELISA. Therefore, HUVECs were treated with increasing concentrations of PT (qRT-PCR: 10, 30, 100 nM; western blot and ELISA: 30, 100 nM) for 12 (qRT-PCR) or 24 (western blot and ELISA) hours before the assays were performed (see II.4, II.5, II.7). The treatment of HUVECs with PT resulted in a strong concentration-dependent increase of N-cadherin mRNA expression (Figure 18A), whereas its protein expression (Figure 18B) and its secretion in cell culture supernatants (Figure 18C) were only slightly induced when HUVECs were treated with PT.

Nonetheless, CXCL12 secreted from HUVECs might directly act on tumor cells. Therefore, cell adhesion assays (see II.10.2) were performed, in which the cell culture medium was removed and replaced by fresh medium or not after PT treatment before fluorescence-labeled MDA-MB-231 tumor cells were allowed to adhere for 10 minutes. Despite the slight upregulation of CXCL12 secretion due to PT treatment, no differences were observed between the two conditions in the cell adhesion assay (Figure 18D). Thus, the secretion of CXCL12 and cytokine secretion in general is not responsible for the PT-triggered adhesion of tumor cells to an endothelial monolayer.

To exclude a potential autocrine action of CXCL12 on HUVECs, additional cell adhesion assays (see II.10.2) were performed, in which the endothelial CXCL12 receptor CXCR4 was inhibited after PT treatment with plerixafor (AMD3100) before fluorescence-labeled MDA-MB-231 tumor cells were allowed to adhere for 10 minutes. However, the inhibition of CXCR4 did not affect the PT-evoked tumor cell adhesion (Figure 18E). Since the blocking of CXCR4 with AMD3100 was reported to inhibit the phosphorylation of extracellular signal-regulated kinases 1/2 (ERK1/2), the functionality of AMD3100 was confirmed by western blot analysis (see II.5)

in advance.[307] Therefore, HUVECs were treated with two concentrations of AMD3100 (0.5, 5 µg/ml) for 30 minutes and CXCL12 (500 ng/ml) was added for the last 5 minutes of AMD3100 treatment. The CXCL12-induced phosphorylation of ERK1/2 was completely abolished when HUVECs were treated with AMD3100 (Figure 18F). Taken together these data indicate that the chemokine system CXCL12/CXCR4 does not participate in the PT-triggered adhesion of tumor cells to an endothelial monolayer.

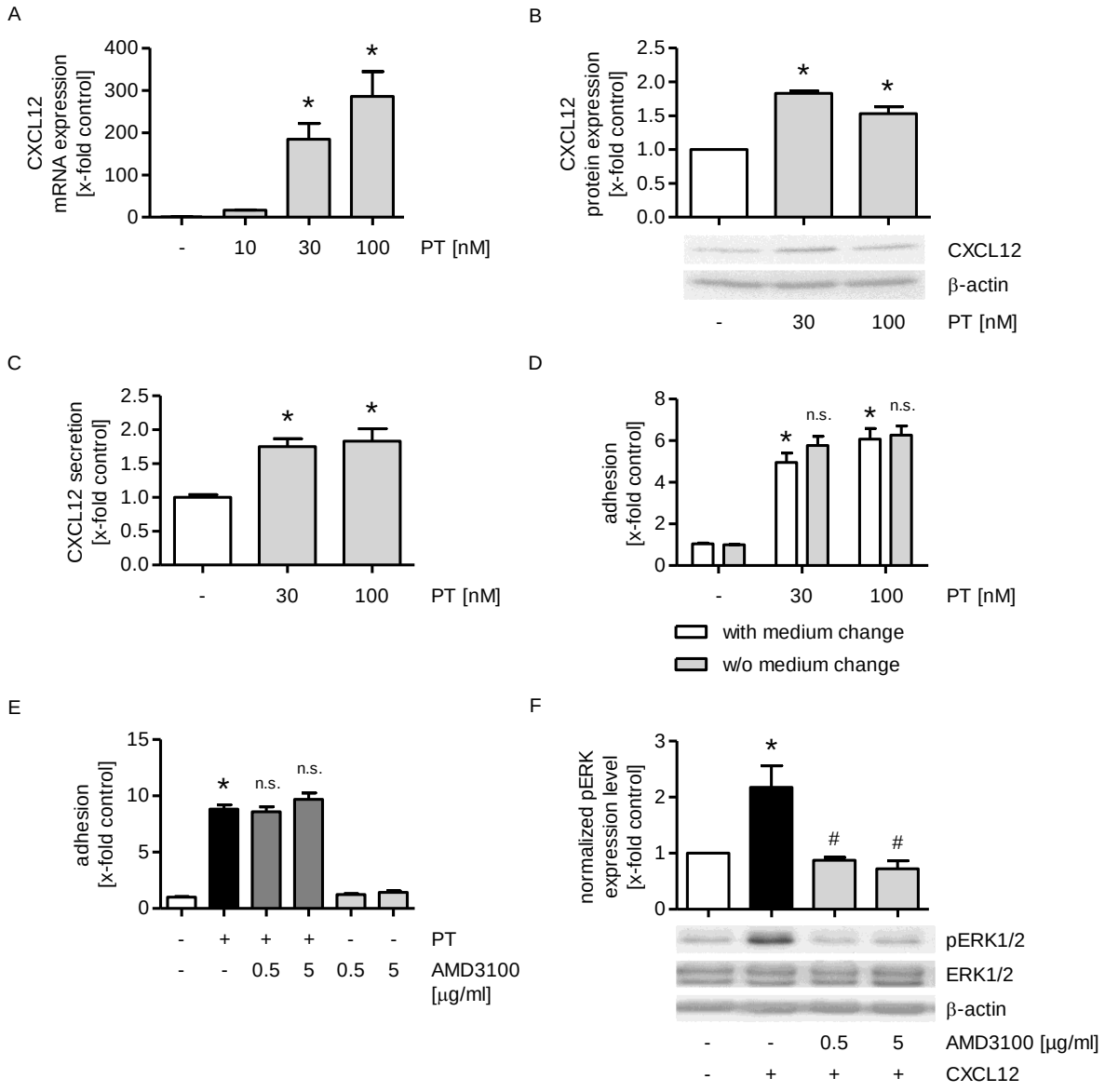

Figure 18: The enhanced expression of CXCL12 is not responsible for the PT-triggered tumor cell adhesion. **(A-C)** HUVECs were grown to confluence and treated with PT (10, 30, 100 nM (A); 30, 100 nM (B/C)) for 12 (A) or 24 (B/C) hours. The mRNA expression of CXCL12 (A) was analyzed by qRT-PCR experiments, the protein expression of CXCL12 (B) was determined by western blot analysis and the secretion of CXCL12 into cell culture supernatants (C) was analyzed by ELISA. **(D/E)** HUVECs were grown to confluence and treated with 30 nM PT for 24 hours. (D) The cell culture medium was removed and replaced by fresh medium (indicated as "with medium change") or not (indicated as "w/o medium change") before fluorescence-labeled MDA-MB-231 tumor cells were added. (E) AMD3100 (0.5, 5 µg/ml) was added for the last 30 minutes of PT treatment before fluorescence-labeled MDA-MB-231 tumor cells were added. (D/E) Tumor cells were allowed to adhere for 10 minutes and non-adherent cells were removed by washing. The amount of adherent tumor cells was quantified by fluorescence measurements using a microplate reader (Tecan Trading AG). **(F)** HUVECs were grown to confluence and treated with AMD3100

(0.5, 5 µg/ml) for 30 minutes. CXCL12 (500 ng/ml) was added for the last 5 minutes of AMD3100 treatment and the protein expression of phosphorylated ERK1/2 (pERK1/2) and ERK1/2 was determined by western blot analysis. Data are expressed as mean ± SEM. A-C/F: n=3, D: n=5, E: n=4. *p ≤ 0.05 versus control (A-E) or negative control (F), n.s. (not significant) ≤ 0.05 versus "with medium change" (D) or PT alone (E), #p ≤ 0.05 versus CXCL12 control (F).

1.4 The mechanism underlying the effects of PT on tumor-endothelial cell interactions

1.4.1 The PT-triggered tumor cell adhesion is mediated by the exposure of the extracellular matrix component collagen

PT was recently characterized as a vascular disrupting compound leading to the formation of gaps within the endothelial monolayer.[283] This characteristic was confirmed by phase-contrast images in which the dashed lines indicate the interendothelial gaps (Figure 19A, dashed lines indicate the gaps). Moreover, since the effects of PT on tumor cell adhesion onto endothelial cells were neither mediated *via* its influence on the cell adhesion molecules ICAM-1, VCAM-1, N-cadherin, E-selectin and galectin-3 nor *via* its influence on the chemokine system CXCL12/CXCR4, we hypothesized that there might be an indirect interaction between MDA-MB-231 tumor cells and endothelial cells. Thus, cell adhesion assays (see II.10.2) were performed, in which the precise location of MDA-MB-231 tumor cells on the endothelial monolayer was investigated. Therefore, fluorescence-labeled MDA-MB-231 tumor cells were added to PT-treated HUVECs and the endothelial cell boundaries were visualized by immunofluorescence staining (see II.8) of vascular endothelial cadherin (VE-cadherin). As presumed, tumor cells mainly adhered within the PT-induced gaps of the endothelial monolayer (Figures 19B and Figure 19C, dashed lines indicate the gaps) confirming the hypothesis of an indirect interaction between tumor and endothelial cells.

These findings led to the assumption that the PT-evoked tumor cell adhesion onto an endothelial monolayer might be caused by the interaction between tumor cells and extracellular matrix components that are exposed within the PT-induced interendothelial gaps. Therefore, cell adhesion assays (see II.10.1) were performed in which fluorescence-labeled MDA-MB-231 tumor cells were allowed to adhere on PBS-, fibronectin- or collagen-coated plastic in order to figure out on which extracellular matrix protein the cells preferentially attach. While the tumor cells show only a weak adherence to fibronectin, they strongly attached to collagen (Figure 19D). To prove whether collagen was present within the PT-triggered gaps of the endothelial monolayer, a HUVEC monolayer treated with PT was stained for collagen (see II.8). Here, endothelial cell boundaries were visualized by the staining of platelet endothelial cell adhesion molecule 1 (PECAM-1). Since collagen was present within the endothelial gaps due to PT treatment (Figure 19E, dashed line indicates the gap), we conclude that the PT-

evoked adhesion of tumor cells onto an endothelial monolayer is based on the exposure of the extracellular matrix component collagen.

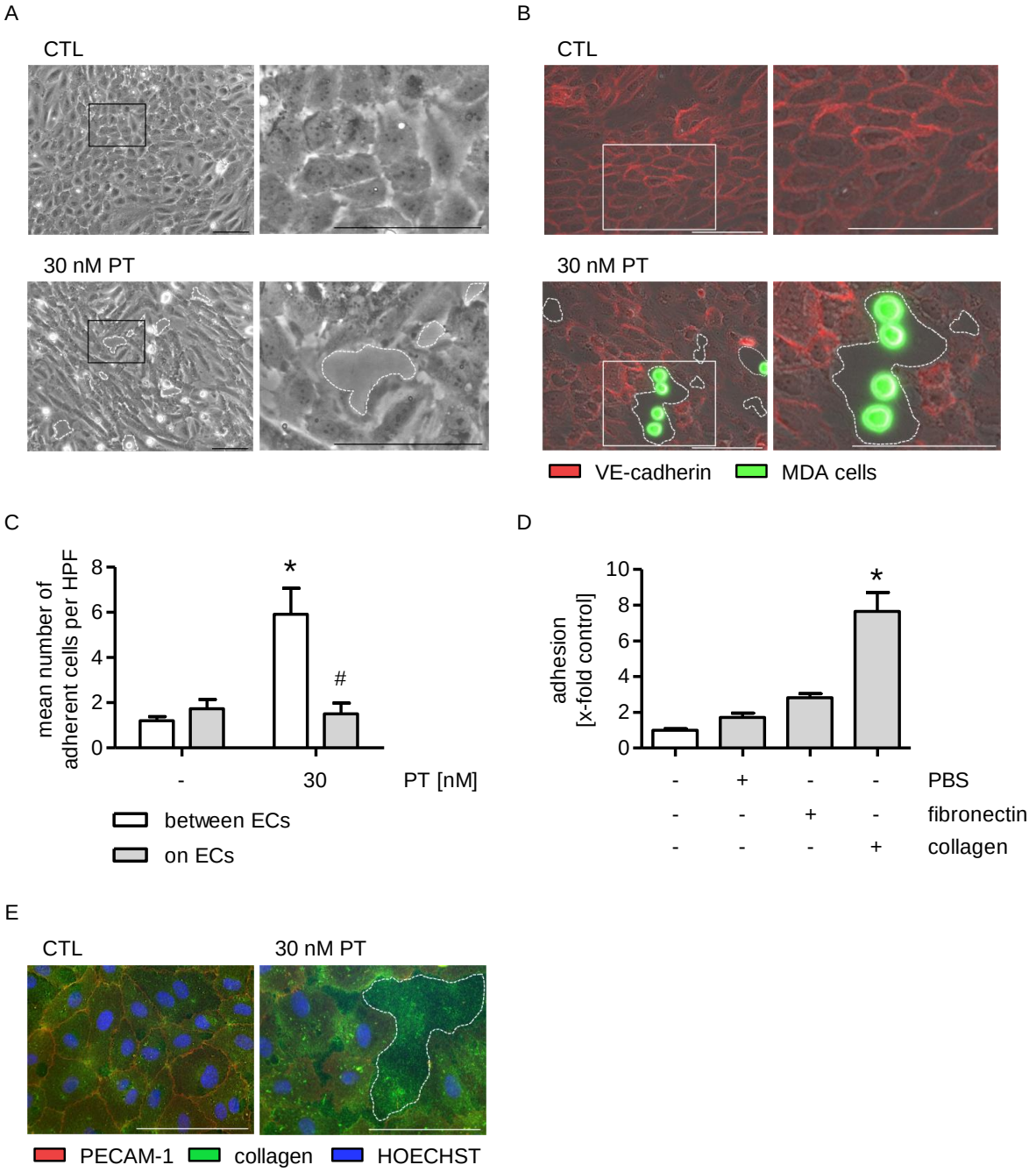

Figure 19: The PT-evoked tumor cell adhesion is based on the exposure of the extracellular matrix protein collagen within endothelial gaps. (A-C) HUVECs were grown to confluence and treated with 30 nM PT for 6 hours. (A) Culture medium was removed and the cells were washed with medium before phase-contrast images were captured using an inverted microscope (Leica DM IL LED, Leica Microsystems). (B/C) Fluorescence-labeled MDA-MB-231 tumor cells (green) were allowed to adhere for 10 minutes and non-adherent cells were removed by washing. After fixation and permeabilization, VE-cadherin (red) was visualized by immunocytochemistry. The localization of MDA-MB-231 tumor cells was analyzed by fluorescence microscopy using a fluorescence microscope (LSM 510, Carl Zeiss AG) (B). The mean number of tumor cells per high-power field (HPF) that adhered either within endothelial gaps (indicated as “between ECs”) or onto HUVECs (indicated as “on ECs”) was determined by microscopic analyses and cell counting. **(D)** Fluorescence-labeled MDA-MB-231 tumor cells were allowed to adhere onto uncoated, PBS-, fibronectin- (5 µg/ml) or collagen- (10 µg/ml) coated plastic for 10 minutes

and non-adherent cells were removed by washing. The amount of adherent tumor cells was quantified by fluorescence measurements using a microplate reader (Tecan Trading AG). **(E)** HUVECs were grown to confluence and treated with 30 nM PT for 6 hours. After fixation collagen (green) and PECAM-1 (red) were visualized by immunocytochemistry and fluorescence microscopy using a fluorescence microscope LSM 510 (Carl Zeiss AG). HOECHST was used for the staining of nuclei (blue). (C/D) Data are expressed as mean ± SEM. C: n=4, D: n=3. *p ≤ 0.05 versus control, #p ≤ 0.05 versus "30 nM PT between ECs". Microscopic images: Scale bar represents 100 µm. Dashed lines indicate interendothelial gaps. One representative image out of at least 3 independently performed experiments is shown. A: n=3, B/E: n=4.

1.4.2 Interactions of β1-integrins on tumor cells with extracellular collagen mediate the effects of PT on tumor cell adhesion and transmigration

The interactions between tumor cells and extracellular matrix components are mediated *via* β1-integrins on tumor cells.[95,96] To analyze the functional role of β1-integrins in the action of PT, cell adhesion assays (see II.10.2) and endothelial transmigration assays (see II.11) were performed, in which β1-integrins on fluorescence-labeled MDA-MB-231 tumor cells were blocked by a neutralizing antibody before they were added to an HUVEC monolayer treated with 30 nM PT. Both the PT-induced tumor cell adhesion onto an endothelial monolayer (Figure 20A) as well as the PT-evoked decrease in transendothelial migration of tumor cells (Figure 20B) were completely abolished upon the blocking of β1-integrins on MDA-MB-231 tumor cells. The functionality of the β1-integrin neutralizing antibody was confirmed by cell adhesion assays (see II.10.1), in which fluorescence-labeled MDA-MB-231 tumor cells were treated with increasing concentrations of the antibody (1, 5, 10 µg/ml). After 30 minutes the cells were allowed to adhere on collagen-coated plastic for 10 minutes. The adhesion of tumor cells onto collagen was completely abolished when the cells were treated with 1 µg/ml of the β1-integrin neutralizing antibody (Figure 20C). Taken together, these data indicate that the action of PT might be based on the trapping of tumor cells within endothelial gaps that is mediated *via* the interaction between collagen and β1-integrins on tumor cells.

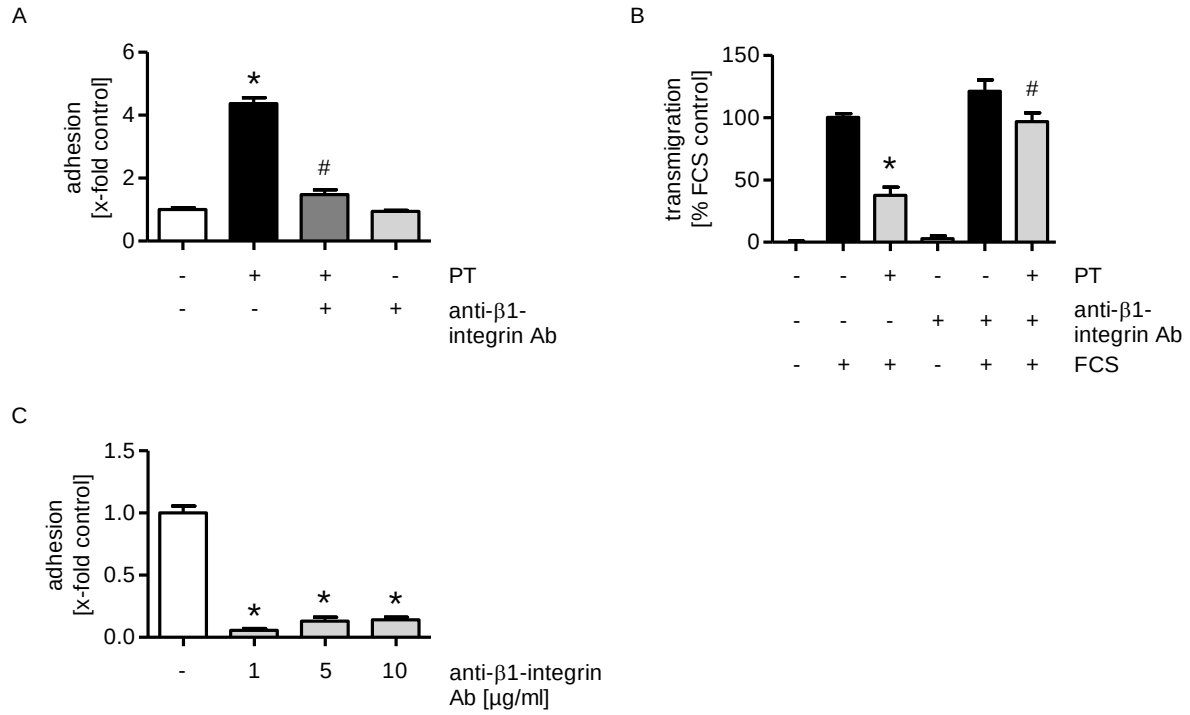

Figure 20: The effects of PT on tumor-endothelial cell interactions are based on the interaction between β1-integrins on tumor cells and collagen within interendothelial gaps. (A) HUVECs were grown to confluence and treated with 30 nM PT for 6 hours. Fluorescence-labeled MDA-MB-231 tumor cells were treated with a β1-integrin neutralizing antibody (1 µg/ml) for 30 minutes before they were added to the PT-treated HUVECs. The tumor cells were allowed to adhere for 10 minutes. Non-adherent cells were removed by washing and the amount of adherent tumor cells was quantified by fluorescence measurements using a microplate reader (Tecan Trading AG). **(B)** HUVECs were grown to confluence in the upper compartment of Transwell® inserts (polycarbonate membrane, diameter 6.5 mm, pore size 8 µm) and treated with 30 nM PT for 24 hours. Fluorescence-labeled MDA-MB-231 tumor cells were treated with a β1-integrin neutralizing antibody (1 µg/ml) for 30 minutes before they were added to the PT-treated HUVECs. The tumor cells were allowed to transmigrate for 24 hours. FCS (20 %) in the lower compartment was used as a positive control. The amount of transmigrated tumor cells on the lower part of the porous filter membrane was quantified by fluorescence measurements using a microplate reader (Tecan Trading AG). **(C)** Fluorescence-labeled MDA-MB-231 tumor cells were treated with a β1-integrin neutralizing antibody (1, 5, 10 µg/ml) for 30 minutes before they were added to plastic coated with collagen (10 µg/ml). The tumor cells were allowed to adhere for 10 minutes. Non-adherent cells were removed by washing and the amount of adherent tumor cells was quantified by fluorescence measurements using a microplate reader (Tecan Trading AG). (A-C) Data are expressed as mean ± SEM. A/C: n=3, B: n=5. *$p \leq 0.05$ versus control (A/C) or FCS alone (B), $^{\#}p \leq 0.05$ versus PT alone (A) or PT + FCS (B).

1.4.3 The reported effects of PT on tumor-endothelial cell interactions depend on its microtubule-destabilizing activity

To prove whether the reported effects of PT depend on its destabilizing action on the microtubule network, the influence of PT on endothelial cells in cell adhesion assays (see II.10.2) with tumor cells was compared to that of other MTAs. In this context, the functional role of β1-integrins in the action of the investigated MTAs was determined. Therefore, HUVECs were treated with the microtubule-destabilizing compounds PT (100 nM), (VIN, 100 nM) and colchicine (COL, 100 nM) or the microtubule-stabilizing agent paclitaxel (PAC, 100 nM). Phase-contrast images of the treated endothelial monolayers were obtained and β1-integrins on fluorescence-labeled MDA-MB-231 tumor cells were blocked by a neutralizing antibody

before they were allowed to adhere for 10 minutes. As with PT, the microtubule-destabilizing compounds VIN and COL led to the formation of interendothelial gaps, whereas the microtubule-stabilizing agent did not affect endothelial integrity (Figure 21A, dashed lines indicate the gaps). Similar results were obtained in cell adhesion assays: while the microtubule-destabilizing compounds PT, VIN and COL induced the adhesion of tumor cells onto an endothelial monolayer, tumor cell adhesion was not substantially affected by the microtubule-stabilizing agent paclitaxel (Figure 21B). Furthermore, tumor cell adhesion induced by the treatment of HUVECs with PT, VIN or COL was completely abolished when β1-integrins were blocked on tumor cells (Figure 21B). Hence, we conclude that the reported effects of PT on tumor-endothelial cell interactions depend on its microtubule-destabilizing activity.

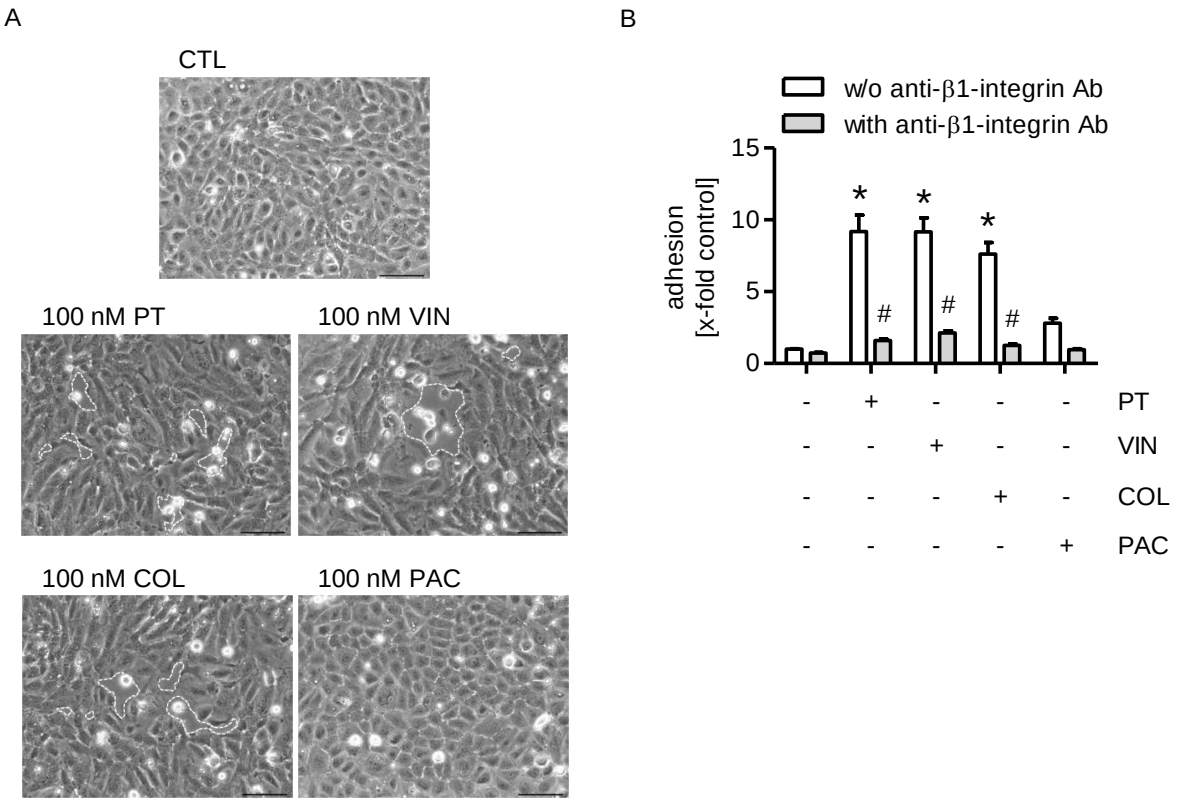

Figure 21: The effects of PT on tumor-endothelial cell interactions depend on its microtubule-destabilizing activity. HUVECs were grown to confluence and treated with 100 nM PT, VIN, COL or PAC for 6 hours. **(A)** Culture medium was removed and the cells were washed with medium before phase-contrast images were captured using an inverted microscope (Leica DM IL LED, Leica Microsystems). **(B)** Fluorescence-labeled MDA-MB-231 tumor cells were treated with a β1-integrin neutralizing antibody (1 µg/ml) for 30 minutes before they were added to treated HUVECs. The tumor cells were allowed to adhere for 10 minutes. Non-adherent cells were removed by washing and the amount of adherent tumor cells was quantified by fluorescence measurements using a microplate reader (Tecan Trading AG). (B) Data are expressed as mean ± SEM (n=3). *$p \leq 0.05$ versus control, $^{\#}p \leq 0.05$ versus "w/o anti-β1-integrin Ab". Microscopic images: Scale bar represents 100 µm. Dashed lines indicate interendothelial gaps. One representative image out of at least 3 independently performed experiments is shown.

2. The influence of PT and other MTAs on leukocyte-endothelial cell interactions

2.1 The effect of PT on leukocyte-endothelial cell interactions *in vivo*

Three essential steps in the recruitment of leukocytes to the site of inflammation are leukocyte rolling and adhesion onto as well as leukocyte transmigration through the endothelium. These processes were already described in the nineteenth century.[89] Today, intravital microscopy – evolved through the huge advances in optical microscopy techniques over the last decades – allows to visualize these cell interactions in living animals.[302]

2.1.1 PT reduces the adhesion and transmigration of leukocytes onto/through the TNFα-activated endothelium

In this study, the influence of PT on leukocyte-endothelial cell interactions under inflammatory conditions was investigated by intravital microscopy (see II.12) that was kindly performed by Matthias Fabritius from the group of PD Dr. Christoph Andreas Reichel (Department of Otorhinolaryngology, Head and Neck Surgery, Walter Brendel Centre of Experimental Medicine, Klinikum der Universität München, Munich, Germany) as recently described.[303] PT (1 mg/kg) was administered to male C57BL/6 mice by intra-arterial injection. Inflammatory conditions in the mouse cremaster muscle were established by intrascrotal injection of TNFα (25 µg/kg) 30 min after PT application. 4 hours after TNFα administration, leukocyte rolling and firm adherence onto and leukocyte transmigration through the endothelium was determined by intravital microscopy of postcapillary venules of the mouse cremaster muscle. While the rolling of leukocytes (Figure 22A) onto the endothelium was not reduced by PT, firm adhesion of leukocytes (Figure 22B) onto and their transmigration (Figure 22C) through the TNFα-activated endothelium were significantly decreased by the compound. Taken together these data clearly suggest that PT among its anti-tumoral and anti-metastatic activity also affects crucial steps in the process of acute inflammation.

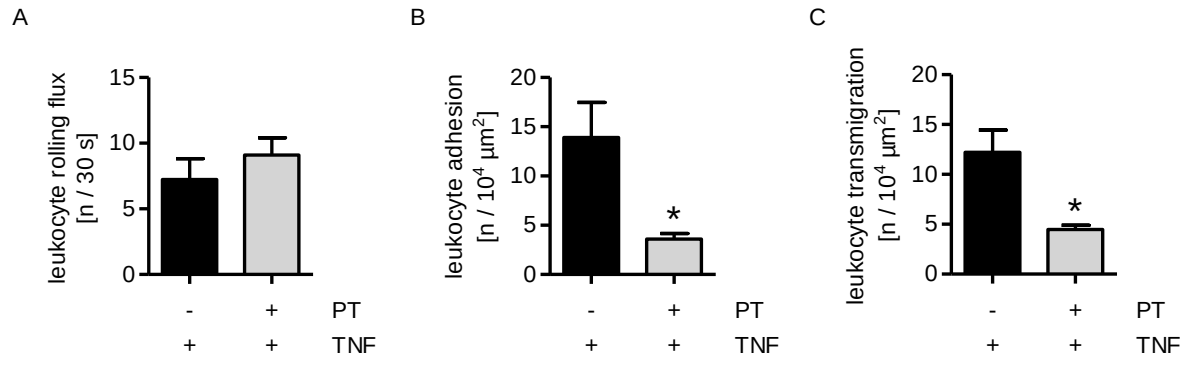

Figure 22: PT reduces the firm adhesion of leukocytes onto and the transmigration of leukocytes through the TNFα-activated endothelium *in vivo*. PT (1 mg/kg) was administered intraarterially to male C57BL/6 mice. 30 minutes after PT application, TNFα (25 µg/kg) was administered by intrascrotal application. Leukocyte rolling **(A)**, firm adhesion **(B)** and transmigration **(C)** onto/through the endothelium was analyzed by intravital microscopy 4 hours after TNFα induction. Data are expressed as mean ± SEM (n=4). *p ≤ 0.05 versus TNFα control.

2.2 The influence of PT and other MTAs on the viability of endothelial cells

To gain insights into the mechanism underling the anti-inflammatory potential of PT observed *in vivo*, the influence of PT on leukocyte-endothelial cell interactions was investigated *in vitro* by cell adhesion assays. Moreover, the influence of other MTAs (VIN, COL, PAC) on leukocyte-endothelial cell interactions was determined in this context, since their impact on the vascular endothelium during the process of acute inflammation is rarely investigated. Moreover, the few existing studies report contradictory results regarding the action of MTAs within the context of inflammation.[247,259,308-311] In a first approach, the influence of PT, VIN, COL and PAC on the viability of human microvascular endothelial cells (HMECs) was determined analyzing the metabolic activity, the release of lactate dehydrogenase (LDH) and the apoptotic cell rate.

2.2.1 The metabolic activity of HMECs is not substantially affected by PT and other MTAs

The effect of PT, VIN, COL and PAC on the metabolic activity of HMECs was investigated using a CellTiter-Blue® Cell Viability Assay (Promega GmbH). Therefore, HMECs were treated with increasing concentrations of PT, VIN, COL and PAC (10, 30, 100, 300, 1000 nM) for 24 hours before the assay was performed (see II.3.1). While the endothelial metabolic activity was slightly reduced upon the treatment with the microtubule-stabilizing compounds PT (Figure 23A), VIN (Figure 23B) and COL (Figure 23C), the microtubule-stabilizing agent paclitaxel (Figure 23D) did not affect endothelial cell viability.

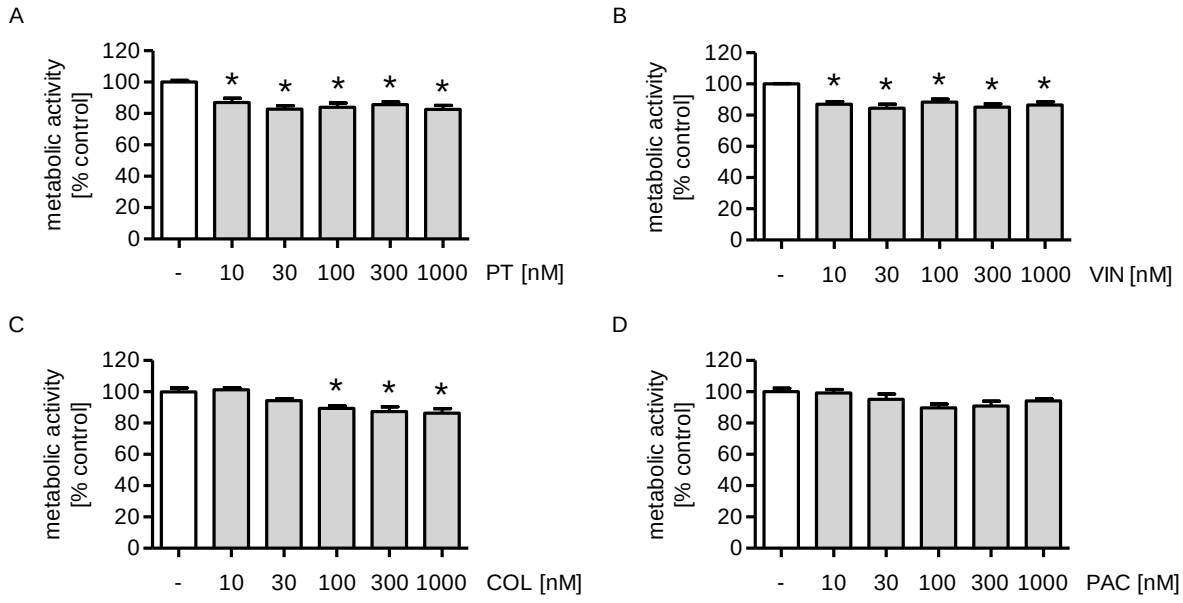

Figure 23: PT, VIN and COL slightly reduce the endothelial metabolic activity. HMECs were grown to confluence and treated with PT **(A)**, VIN **(B)**, COL **(C)** and PAC **(D)** (10, 30, 100, 300, 1000 nM) for 24 hours. CellTiter-Blue® reagent was added for the last 4 hours of treatment and metabolic activity was quantified by fluorescence measurements using a microplate reader (Tecan Trading AG). Data are expressed as mean ± SEM. A/C/D: n=3, B: n=6. *p ≤ 0.05 versus control.

2.2.2 LDH release of HMECs is not induced by PT and other MTAs

LDH is a stable cytosolic enzyme that is released upon cell lysis. A CytoTox 96® non-radioactive cytotoxicity assay (Promega GmbH) was therefore used to investigate the influence of PT, VIN, COL and PAC on the LDH release of HMECs.

HMECs were treated with increasing concentrations of PT, VIN, COL and PAC (10, 30, 100, 300, 1000 nM) for 24 hours before the assay was performed (see II.3.2). None of the compounds exhibited any effects on the release of LDH into cell culture supernatants (Figure 24).

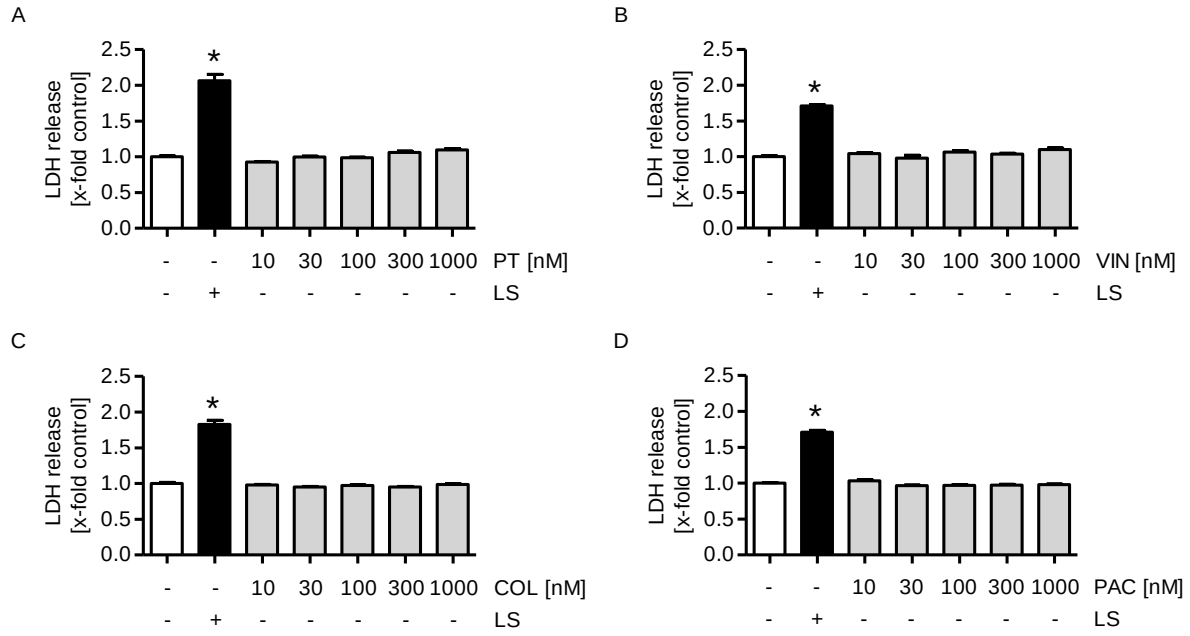

Figure 24: PT, VIN, COL and PAC did not affect the endothelial LDH release into cell culture supernatants. HMECs were grown to confluence and treated with PT **(A)**, VIN **(B)**, COL **(C)** and PAC **(D)** (10, 30, 100, 300, 1000 nM) for 24 hours. For positive control, lysis solution (LS) was added into the medium of untreated cells in a ratio of 1:10 for the last 45 min of compound treatment. CytoTox 96® reagent was mixed with the cell culture medium and the enzymatic reaction was stopped after 30 minutes using stop solution. The absorbance of the red formazan product was quantified by absorbance measurements using a microplate reader (Tecan Trading AG). Data are expressed as mean ± SEM (n=3). *p ≤ 0.05 versus negative control.

2.2.3 The apoptotic cell rate of HMECs is only slightly enhanced by PT and other MTAs

Cell apoptosis is accompanied by deoxyribonucleic acid (DNA) fragmentation due to chromatin cleavage. The effect of PT, VIN, COL and PAC on the apoptotic cell rate of HMECs was investigated as already described by flow cytometry using the DNA-intercalating fluorescent dye PI.[289] Therefore, HMECs were treated with increasing concentrations of PT, VIN, COL and PAC (10, 30, 100, 300, 1000 nM) for 24 hours before the cell apoptosis assay was performed (see II.3.3). While the apoptotic cell rate was slightly increased upon the treatment with PT (Figure 25A), VIN (Figure 25B) and PAC (Figure 25D), the treatment with COL (Figure 25C) did not affect apoptosis in endothelial cells. Together with the results received from investigating the metabolic activity and the release of LDH in cell culture supernatants, these data indicate that none of the compounds used at concentrations up to 1000 nM causes severe cytotoxic effects in HMECs.

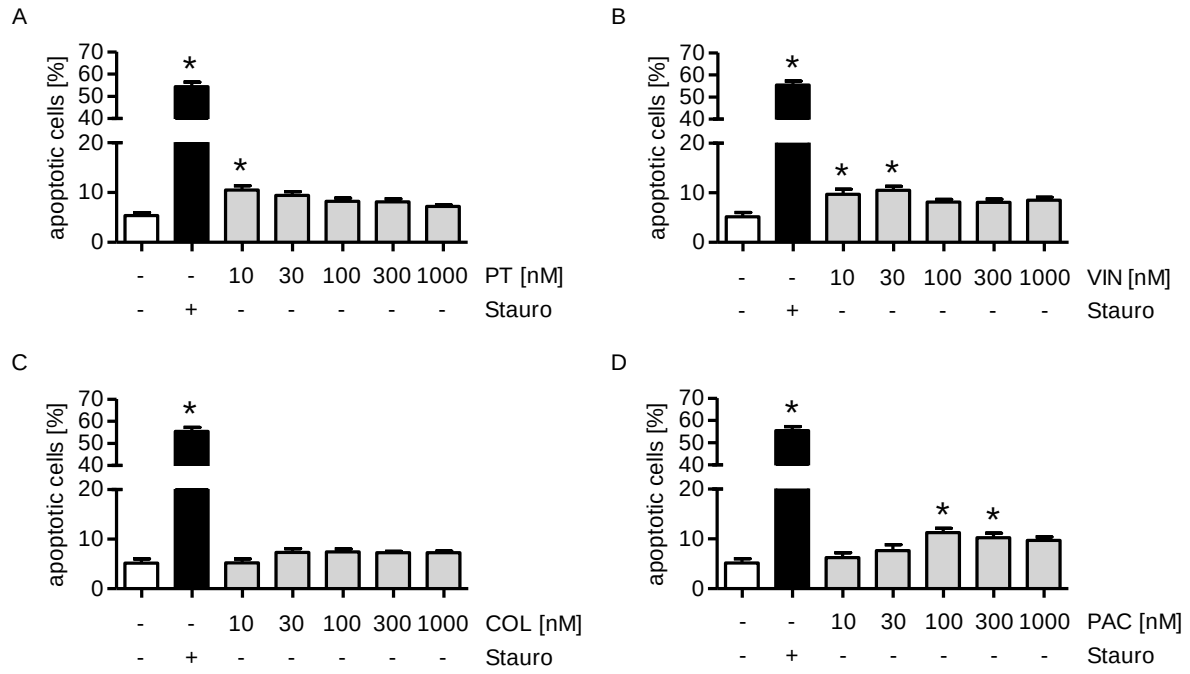

Figure 25: PT, VIN and PAC slightly enhance the endothelial apoptotic cell rate. HMECs were grown to confluence and treated with PT **(A)**, VIN **(B)**, COL **(C)** and PAC **(D)** (10, 30, 100, 300, 1000 nM) for 24 hours. For positive control, HMECs were treated with staurosporine (Stauro, 1µg/ml) for 24 hours. Live adherent cells and apoptotic cells in the cell culture medium were permeabilized and stained using hypotonic fluorochrome solution (HFS) containing PI (HFS-PI solution). PI-staining was quantified by fluorescence measurements using a microplate reader (Tecan Trading AG). Data are expressed as mean ± SEM (n=4). *p ≤ 0.05 versus negative control.

2.3 The effect of PT and other MTAs on leukocyte adhesion onto TNFα-activated endothelial cells *in vitro*

2.3.1 PT, VIN and COL decrease the adhesion of leukocytes onto TNFα-activated endothelial cells

Cell adhesion assays (see II.10.3) were performed, in which HMECs were pre-incubated with increasing concentrations of PT, VIN, COL or PAC (30, 100, 300, 1000 nM) for 30 minutes before TNFα (10 ng/ml) was added for 24 hours, to analyze the impact of PT, VIN, COL and PAC on the adhesion of leukocytes onto TNFα-activated endothelial cells *in vitro*. Fluorescence-labeled THP-1 cells were allowed to adhere for 5 minutes onto the treated endothelial cells. Since we could show that the treatment of HUVECs with PT, VIN and COL led to the formation of gaps within the endothelial monolayer (see III.1.4.3, Figure 21A) the amount of adherent THP-1 cells was normalized to the amount of HMECs after treatment with the compounds determined by crystal violet staining, which was kindly performed by Mareike Lang (see II.10.3). Thereby, we could exclude that effects of the compounds on leukocyte adhesion onto the activated HMEC monolayer were due to the reduced number of interacting endothelial cells caused by the vascular disruptive activity of the agents. While the treatment of HMECs with the microtubule-destabilizing compounds PT (Figure 26A), VIN (Figure 26B)

and COL (Figure 26C) resulted in a clear concentration-dependent decrease of leukocyte adhesion onto the endothelial monolayer, the microtubule-stabilizing compound PAC (Figure 26D) did not affect this process. These findings indicate that the influence of PT, VIN and COL on the adhesion of leukocytes onto activated endothelial cells depend on their microtubule-destabilizing activity.

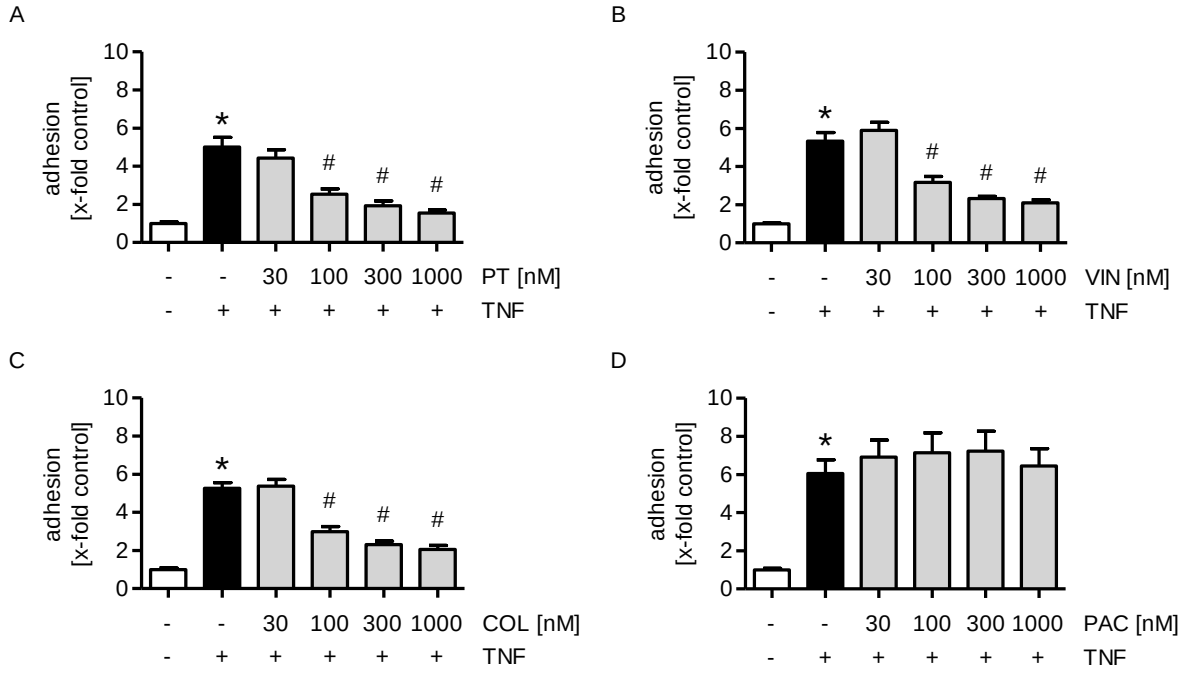

Figure 26: PT, VIN and COL decrease the adhesion of THP-1 cells onto a TNFα-activated HMEC monolayer. Confluent HMECs were pre-treated with PT **(A)**, VIN **(B)**, COL **(C)** and PAC **(D)** (30, 100, 300, 1000 nM) for 30 minutes before TNFα (10 ng/ml) was added for 24 hours. Fluorescence-labeled THP-1 cells were allowed to adhere for 5 minutes and non-adherent cells were removed by washing. The amount of adherent THP-1 cells was quantified by fluorescence measurements using a microplate reader (Tecan Trading AG) and normalized to the amount of HMECs after treatment (determined by crystal violet staining). Data are expressed as mean ± SEM (n=4). *p ≤ 0.05 versus negative control, #p ≤ 0.05 versus TNFα control.

2.4 The influence of PT and other MTAs on cell adhesion molecules typically relevant for leukocyte-endothelial cell interactions

As the firm adhesion of leukocytes onto the endothelium during the process of acute inflammation is mediated by CAMs, we analyzed the effects of PT, VIN, COL and PAC on the TNFα-induced expression of ICAM-1 and VCAM-1 on the surface of endothelial cells.[90,91]

2.4.1 The TNFα-induced surface expression of ICAM-1 and VCAM-1 is reduced by MTAs

To analyze the effect of PT, VIN, COL and PAC on the TNFα-induced cell surface expression of ICAM-1 and VCAM-1, HMECs were pre-incubated with increasing concentrations of PT, VIN, COL or PAC (30, 100, 300 nM in the case of ICAM-1 and 10, 30, 100, 300 nM in the case of VCAM-1) for 30 minutes before TNFα (10 ng/ml) was added for 24 hours. The protein expression of ICAM-1 and VCAM-1 on the endothelial cell surface was determined by flow cytometric analysis (see II.6). While the treatment of HMECs with the microtubule-destabilizing compounds PT (Figure 27A), VIN (Figure 27B) and COL (Figure 27C) resulted in a clear concentration-dependent decrease of the TNFα-induced ICAM-1 cell surface protein expression, the microtubule-stabilizing compound PAC (Figure 27D) did not affect this process. Moreover, the treatment of PT showed the strongest effects in the reduction of TNFα-induced endothelial cell surface expression of ICAM-1 compared to the treatment with VIN and COL (Figure 27). Thus, we assumed that the influence of PT, VIN and COL on the TNFα-induced endothelial cell surface expression of ICAM-1 might be involved in their action on leukocyte-endothelial cell interactions. While the treatment of HMECs with the microtubule-destabilizing compounds PT (Figure 28A), VIN (Figure 28B) and COL (Figure 28C) resulted in a strong concentration-dependent decrease of the TNFα-induced VCAM-1 cell surface protein expression, the microtubule-stabilizing compound PAC (Figure 28D) affected this process to a lesser extent. Furthermore, the treatment of PT and VIN showed the strongest effects in the reduction of TNFα-induced endothelial cell surface protein expression of VCAM-1 compared to the treatment with COL and PAC (Figure 28). These data indicate that the reduction of the TNFα-induced endothelial cell surface expression of VCAM-1 evoked by PT, VIN and COL might be involved in their action on leukocyte-endothelial cell interactions.

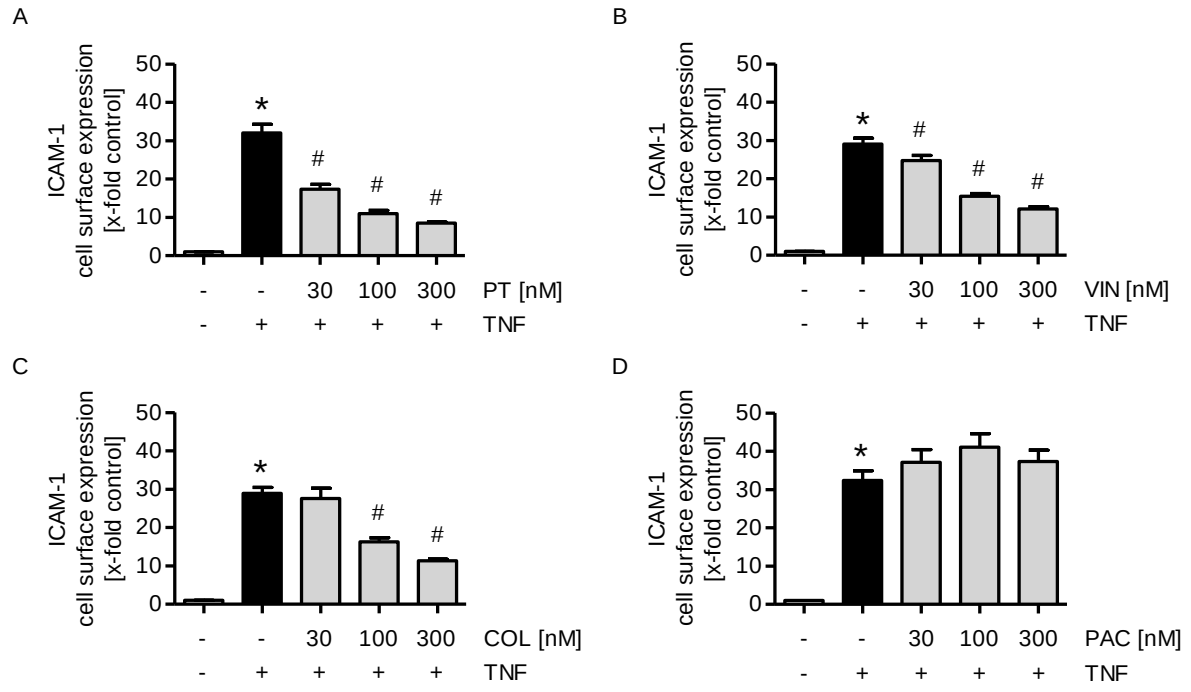

Figure 27: PT, VIN and COL decrease the TNFα-induced cell surface expression of ICAM-1 in HMECs. Confluent HMECs were pre-treated with PT **(A)**, VIN **(B)**, COL **(C)** and PAC **(D)** (30, 100, 300 nM) for 30 minutes before TNFα (10 ng/ml) was added for 24 hours. The cell surface expression of ICAM-1 was quantified by flow cytometry using a FACSVerse™ (BD Biosciences). Data are expressed as mean ± SEM (n=3). *p ≤ 0.05 versus negative control, #p ≤ 0.05 versus TNFα control.

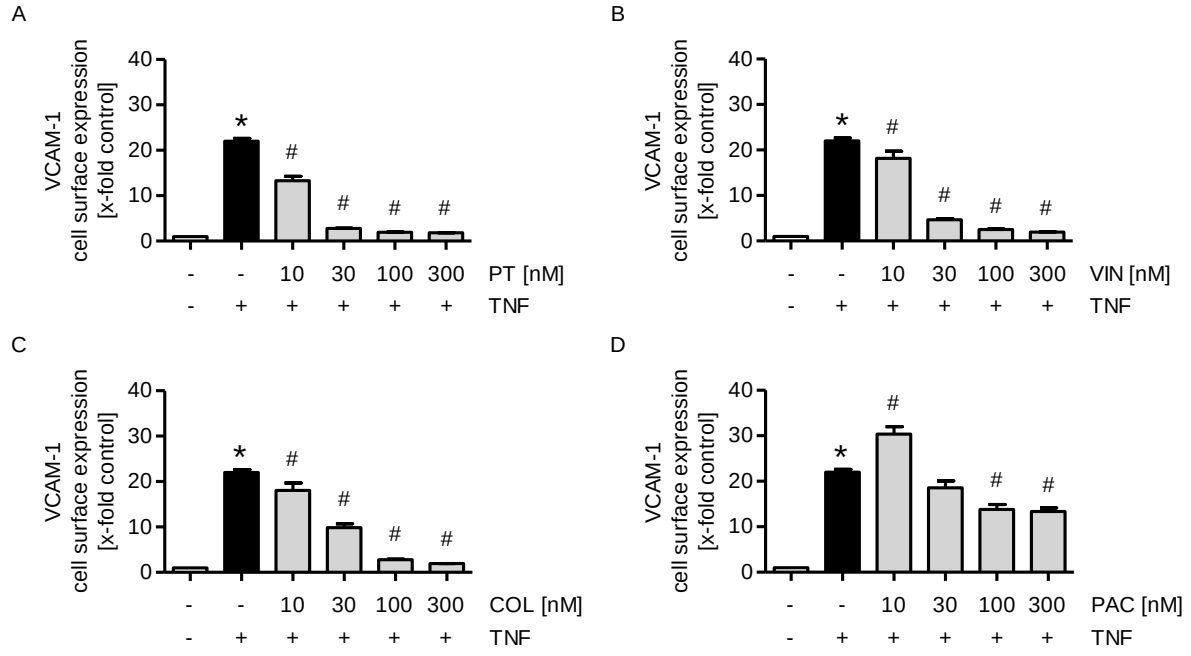

Figure 28: PT and other MTAs decrease the TNFα-induced cell surface expression of VCAM-1 in HMECs. Confluent HMECs were pre-treated with PT **(A)**, VIN **(B)**, COL **(C)** and PAC **(D)** (30, 100, 300, 1000 nM) for 30 minutes before TNFα (10 ng/ml) was added for 24 hours. The cell surface expression of VCAM-1 was quantified by flow cytometry using a FACSVerse™ (BD Biosciences). Data are expressed as mean ± SEM (n=4). *p ≤ 0.05 versus negative control, #p ≤ 0.05 versus TNFα control.

2.5 The effect of PT and other MTAs on the NFκB pathway

The pro-inflammatory transcription factor NFκB crucially regulates the TNFα-induced expression of ICAM-1, VCAM-1 and other endothelial cell adhesion molecules involved in the process of acute inflammation.[183,184] Therefore, the role of the NFκB signaling pathway in the action of PT, VIN, COL and PAC on leukocyte-endothelial cell interactions was examined.

2.5.1 PT, VIN and COL decrease the TNFα-induced NFκB promotor activity

The influence of the PT, VIN, COL and PAC on the TNFα-induced endothelial NFκB promotor activity was investigated using a Dual-Luciferase® reporter assay (Promega GmbH, see II.9) to analyze the impact of the compounds on the NFκB signaling pathway. Therefore, transfected HMECs were pre-incubated with increasing concentrations of PT, VIN, COL or PAC (30, 100, 300 nM) for 30 minutes before TNFα (10 ng/ml) was added for 6 hours. The effect of PT and other MTAs on the TNFα-induced NFκB promotor activity was quantified by luminescence measurements. While the treatment of HMECs with the microtubule-destabilizing compounds PT (Figure 29A), VIN (Figure 29B) and COL (Figure 29C) resulted in a slight decrease of the TNFα-induced NFκB promotor activity, this process was slightly enhanced by the microtubule-stabilizing compound PAC (Figure 29D). Moreover, in accordance with effects on TNFα-induced endothelial cell surface expression of ICAM-1 and VCAM-1, the treatment of PT showed the strongest effects in the reduction of TNFα-induced NFκB promotor activity compared to the treatment with the other MTAs (Figure 29). Taken together, these findings suggest that the reduction in the TNFα-induced expression of ICAM-1 and VCAM-1 on the endothelial surface caused by the treatment with the microtubule-destabilizing compounds PT, VIN and COL is at least in part mediated *via* their influence on the TNFα-induced NFκB promotor activity.

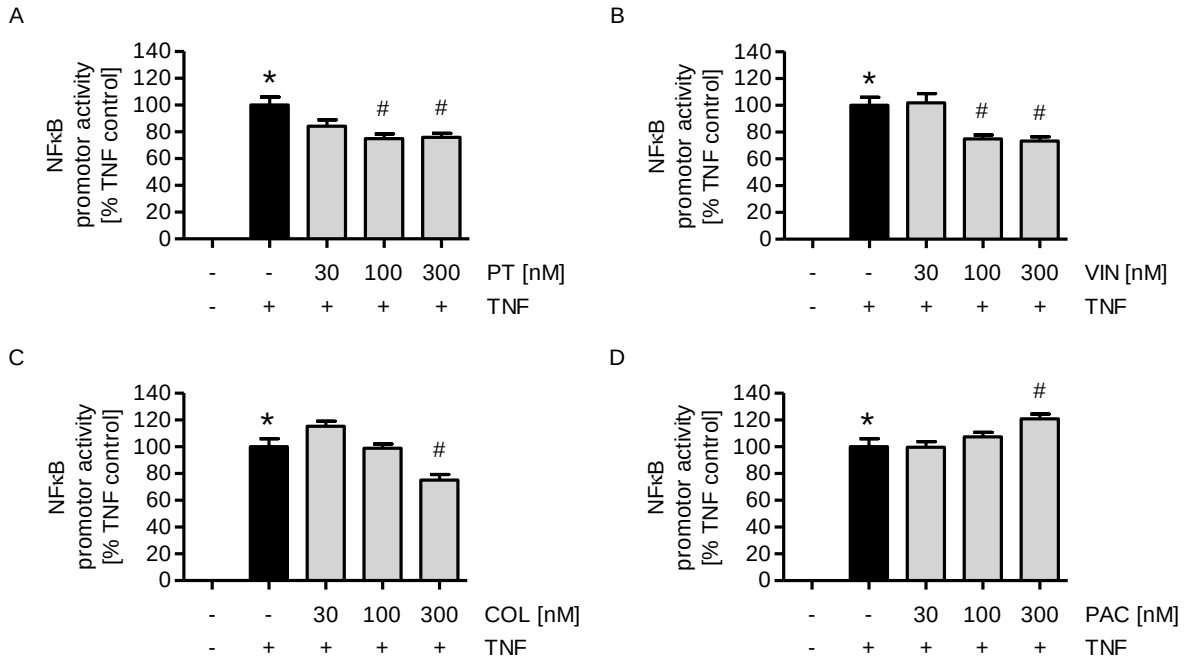

Figure 29: PT, VIN and COL decrease the TNFα-induced NFκB promotor activity in HMECs. HMECs were co-transfected with 3.5 µg of pGL4.32[luc2P/NF-κB-RE/Hygro] vector and 1.4 µg of pGL4.74[hRluc/TK] vector using a HUVEC Nucleofector® Kit (Lonza Cologne GmbH). 24 hours post transfection, HMECs were pre-treated with PT **(A)**, VIN **(B)**, COL **(C)** and PAC **(D)** (30, 100, 300 nM) for 30 minutes before TNFα (10 ng/ml) was added for 6 hours. The cells were lysed using passive lysis buffer (PLB). *Photinus* luciferase activity and *Renilla* luciferase activity were determined by luminescence measurements using a microplate reader (Tecan Trading AG). The signal of *Photinus* luciferase was normalized to that of *Renilla* luciferase. Data are expressed as mean ± SEM (n=4). *$p \leq 0.05$ versus negative control, $^{\#}p \leq 0.05$ versus TNFα control.

IV DISCUSSION

1 The role of MTAs in the treatment of cancer

MTAs, compounds that bind to the β-tubulin subunit of microtubules causing either microtubule-stabilization or -destabilization, are the most frequently used chemotherapeutic drugs until today.[31,312,313] Besides their well-known direct effects on tumor cells, such as induction of mitosis-dependent and -independent cell death, the influence of MTAs on endothelial cells is well studied regarding their anti-angiogenic and vascular-disruptive effects on the tumor microvasculature.[31,35-38] However, the impact of MTAs on endothelial cells of the non-tumor vasculature has been largely neglected, although these cells play an essential role in the metastatic dissemination of tumor cells – one of the hallmarks of cancer that is responsible for 90 % of cancer-related mortality.[40,52,53]

PT, a biosynthetic precursor of the myxobacterial tubulysins, was recently identified as a novel MTA showing profound anti-tumor activities demonstrated by its direct effect on tumor cells, its anti-metastatic effect as well as its influence on the tumor microvasculature observed *in vivo*.[275,279-283] Due to the gap in research regarding the influence of MTAs on endothelial cells of the non-tumor vasculature, one aim of the present study was, therefore, to analyze the impact of PT on the interaction of non-tumor endothelial cells and breast cancer cells *in vitro*, since we hypothesized that PT influences the metastatic process – at least in part – *via* a direct action on the healthy endothelium.

1.1 PT interferes with tumor-endothelial cell interactions

Endothelial cells of the non-tumor vasculature play a crucial role in metastasis formation of hematogenically disseminating tumor cells, since they are involved in the regulation of tumor cell adhesion onto and their transmigration through the endothelium during the process of cancer cell intra- and extravasation.[41-44] Since we hypothesized that the anti-metastatic effect of PT could be mediated *via* a direct action on the healthy endothelium influencing the previously described tumor-endothelial cell interactions, in the present study we analyzed the impact of PT on endothelial cells in cell adhesion and transendothelial migration assays with MDA-MB-231 breast cancer cells. This study revealed that the treatment of endothelial cells with PT strongly increased the adhesion of MDA breast cancer cells onto the endothelial monolayer. Surprisingly, although the adhesion of MDA cells onto endothelial cells was induced by the treatment with PT, the transendothelial migration of MDA cells through an endothelial monolayer was markedly reduced by the compound.

1.2 Elucidation of the mechanism responsible for the effect of PT on tumor-endothelial cell interactions

1.2.1 The impact of PT on endothelial cell adhesion molecules and the chemokine system CXCL12/CXCR4

Various studies have focused on the interaction between tumor cells and endothelial cells of the non-tumor vasculature, investigating the regulatory mechanisms underlying tumor cell intra- and extravasation.[137,314-317] Although the mechanisms by which tumor cells adhere to endothelial cells are not yet fully understood, it is very likely that they mimic mechanisms described for leukocyte-endothelial cell interactions.[70] Since several studies indicate that the CAMs E-selectin, N-cadherin, ICAM-1, VCAM-1 and galectin-3 expressed on endothelial cells are involved in either the rolling or firm adhesion of tumor cells onto the endothelium, we assumed that the PT-evoked tumor cell adhesion onto endothelial cells might be caused by an increased expression of these adhesion molecules.[47,68,71,82] While the treatment of endothelial cells with PT increased the mRNA expression, the protein expression as well as the cell surface protein expression of ICAM-1, VCAM-1 and N-cadherin, the protein expression of E-selectin was not affected on the endothelial cell surface and that of galectin-3 was not even influenced on the mRNA level.

Although the investigated endothelial CAMs have been reported to play a role in tumor-endothelial cell interactions, the impact of MTAs on the expression of the aforementioned proteins is rarely investigated. Nonetheless, in contrast to the PT-triggered increase in the endothelial surface protein expression of ICAM-1 presented in this work, the study of Cronstein *et al.* revealed that the treatment of endothelial cells with the microtubule-destabilizing agent colchicine did not affect the cell surface protein expression of ICAM-1.[247] The nuclear factor-κB (NFκB) is an important, ubiquitously expressed transcription factor involved in the regulation of the gene expression of CAMs, pro-inflammatory cytokines and chemokines.[183,184] While the influence of MTAs on the regulation of endothelial CAMs has been largely neglected, several studies have focused on their impact on the NFκB signaling cascade in various cancer cell types. In line with the PT-induced expression of endothelial ICAM-1 and VCAM-1, numerous studies have shown that the treatment of different cancer cells with microtubule-destabilizing drugs such as vincristine, vinblastine, colchicine and nocodazole caused a rapid and efficient activation of NFκB.[318-322]

However, despite the impact of PT on the expression of ICAM-1, VCAM-1 and N-cadherin, cell adhesion assays in which these proteins were blocked by neutralizing antibodies revealed that this effect is not linked to the PT-triggered tumor cell adhesion onto an endothelial monolayer.

Besides CAMs, several chemokine systems have also been suggested to play a crucial role in the determination of the metastatic pattern of various malignant neoplasias. Especially the chemokine receptor CXCR4 is highly expressed in different types of tumors such as breast

and prostate cancer, whereas its respective ligand, CXCL12 (also known as SDF-1α), is prominently expressed in organs that are preferential destinations for tumor metastasis.[82,154,306] For instance, Fernandis *et al.* demonstrated that CXCL12 induced the chemotactic migration of MDA-MB-231 breast cancer cells and their adhesion onto the extracellular matrix components fibronectin and collagen IV.[323] Moreover, it was also reported that CXCL12 promotes the transmigration of MDA-MB-231 cells through endothelial cells *in vitro* and that MDA-MB-231 breast cancer metastasis was blocked *in vivo* by silencing of CXCR4 expressed on the tumor cells.[159,324]

Thus, we hypothesized that the chemokine system CXCL12/CXCR4 might be involved in the PT-evoked tumor cell adhesion onto endothelial cells. Interestingly, while the mRNA expression of CXCL12 was strongly increased in PT-treated endothelial cells, the protein expression as well as the secretion of CXCL12 from endothelial cells was only marginally enhanced. One possible explanation for this might be that CXCL12 mRNA is not successfully transported to the ribosomes for protein synthesis, since it cannot be excluded that PT also affects microtubule-mediated transport processes caused by its impact on microtubule dynamics. This study furthermore revealed that the secretion of CXCL12, and cytokine secretion in general, is not obligatory for the PT-triggered tumor cell adhesion observed *in vitro*. In addition, it was reported by Song *et al.* that the treatment of endothelial cells with CXCL12 induced the adhesion of MDA-MB-231 breast cancer cells in a microfluidic vasculature model. Moreover, this effect was completely abrogated when the CXCR4 inhibitor AMD3100 was added to the endothelial cells, indicating that the enhanced tumor cell adhesion was due to CXCL12 signaling through CXCR4 on the vascular endothelium.[325] We therefore hypothesized that CXCL12 expressed by PT-treated endothelial cells might rather directly act on endothelial cells in an autocrine manner instead of an action on tumor cells. However, a potential autocrine effect of CXCL12 on endothelial cells was also excluded in this study. Nonetheless, the effect of PT on the endothelial secretion of CXCL12 could play a role in the anti-metastatic action of PT *in vivo*.

In summary, the present study revealed that the effects of PT on tumor-endothelial cell interactions *in vitro* are based neither on its influence on the cell adhesion molecules ICAM-1, VCAM-1 and N-cadherin nor on the chemokine system CXCL12/CXCR4.

1.2.2 The role of extracellular collagen and β1-integrins in the influence of PT and other MTAs on tumor-endothelial cell interactions

Besides the adhesion of tumor cells onto the endothelial monolayer, it was reported that tumor cells can also directly attach to ECM components of the underlying basal lamina.[96] Since PT was recently characterized as a vascular-disruptive compound causing the formation of

transient gaps within the endothelial monolayer, we hypothesized that the PT-evoked tumor cell adhesion might be rather mediated *via* an interaction of tumor cells with ECM components instead of a direct interaction with endothelial cells.[283] Indeed, we could demonstrate that MDA-MB-231 breast cancer cells predominantly adhere to the ECM within the PT-triggered interendothelial gaps. Moreover, we could clearly show in this study that MDA-MB-231 breast cancer cells strongly stuck to the ECM protein collagen, which was present within the PT-induced endothelial gaps.

The β1-integrin subfamily represents a major class of integrins that mediate cell interactions with ECM proteins such as collagen and is critically involved in the adhesion of tumor cells to the basal lamina underlying the endothelium.[95,96] For instance, it was demonstrated that the adhesion of MDA-MB-231 breast cancer cells to collagen is mediated *via* α2β1- and α3β1-integrins expressed on the tumor cells.[97,326-329] Thus, we examined the impact of β1-integrins in cell adhesion and transendothelial migration assays with MDA-MB-231 cells and PT-treated endothelial cells using a β1-integrin neutralizing antibody. In accordance with the previously described findings, both the PT-evoked increase in tumor cell adhesion within endothelial gaps as well as their reduced transmigration through the endothelial monolayer were completely abolished when β1-integrins were blocked on MDA-MB-231 cells.

Furthermore, to prove whether the reported effects of PT depend on its microtubule-destabilizing activity, the influence of PT on the adhesion of tumor cells onto endothelial cells was compared to that of other MTAs: the microtubule-destabilizing agents vincristine (VIN) and colchicine (COL) and the microtubule-stabilizing agent paclitaxel (PAC). As with PT, we could demonstrate that the treatment of endothelial cells with VIN and COL caused the formation of transient interendothelial gaps as well as an increase in breast cancer cell adhesion mediated by the exposure of extracellular collagen to tumoral β1-integrins. In stark contrast, PAC did not induce the formation of endothelial gaps and, consequently, did only slightly enhance the β1-integrin-mediated adhesion of tumor cells onto an endothelial monolayer. Hence, these results indicate that the observed effects of PT depend on its destabilizing action on the microtubule network.

Taken together, this study verified that the microtubule-destabilizing agent PT triggers the formation of interendothelial gaps leading to the exposure of the extracellular matrix protein collagen. Consequently, tumor cells are attached to the exposed collagen *via* β1-integrins, causing the inhibition of their transendothelial migration. Moreover, the effects of VIN and COL on tumor-endothelial cell interactions are consistent with that of PT, suggesting that these effects depend on their microtubule-destabilizing activity.

2 MTAs in the treatment of inflammation – old and new

Although the main function of MTAs is associated with their use as chemotherapeutic drugs, some MTAs such as COL are also commonly used for the treatment of inflammatory diseases. COL represents the lead compound regarding MTAs as anti-inflammatory drugs, since it is used for the therapy of gout and familial Mediterranean fever and at least recommended for the therapy of rheumatic and cardiovascular diseases.[229,245] While several studies have focused on its effect on cellular functions of leukocytes such as the chemotactic recruitment of neutrophils as well as neutrophil rolling on the vascular surface and their transendothelial migration, little is known about its direct influence on the endothelium.[246-249] In addition, studies of the last two decades recommended PAC for the therapy of non-cancer diseases such as inflammation, but the molecular mechanism underlying its anti-inflammatory action is not yet fully understood.[250-255] Moreover, research so far did not address *Vinca* alkaloids as possible anti-inflammatory agents, most likely because of their strong inflammation-associated side effects. Due to these aspects, the second aim of the current study was to analyze the impact of PT on leukocyte-endothelial cell interactions *in vivo* and to investigate the influence of PT, VIN, COL and PAC on leukocyte-endothelial cell interactions *in vitro*, since we hypothesized an anti-inflammatory effect of all of these compounds that is mediated – at least in part – through their direct action on the endothelium.

2.1 PT and other MTAs interfere with leukocyte-endothelial cell interactions

Three essential steps in the recruitment of leukocytes to the site of inflammation are leukocyte rolling on, adhesion onto as well as their transmigration through the endothelium, processes that were already described in the nineteenth century.[89] The visualization of these cell interactions in live animals is possible today by the use of intravital microscopy.[302] In the present study, the influence of PT on leukocyte-endothelial cell interactions under inflammatory conditions was investigated by intravital microscopy of postcapillary venules of the mouse cremaster muscle. While the rolling of leukocytes on the activated vascular surface was not affected by the compound, we could demonstrate that PT strongly decreased both the firm adhesion of leukocytes onto and their transmigration through the activated endothelium.

As with the influence of PT on tumor-endothelial cell interactions, we hypothesized that the anti-inflammatory effect of PT observed *in vivo* could be mediated *via* a direct action of the compound on the vascular endothelium. Moreover, the impact of frequently used MTAs on the vascular endothelium regarding leukocyte-endothelial cell interactions is rarely investigated

whereby the few existing studies revealed contradictory results.[247,259,308-311,330] We therefore investigate the influence of PT, VIN, COL and PAC on endothelial cells in *in vitro* cell adhesion assays using the monocyte-like cell line THP-1. In accordance to the results derived by intravital microscopy, treatment of activated endothelial cells with PT strongly reduced the adhesion of THP-1 cells. Similar effects were observed when activated endothelial cells were treated with VIN or COL whereas PAC did not affect the adhesion of THP-1 cells onto the endothelial monolayer. These findings are in line with some studies that focused on the anti-inflammatory potential of colchicine. For instance, it was demonstrated that the treatment of activated endothelial cells with colchicine attenuated the adhesion of neutrophils *in vitro*.[247,310] Moreover, in accordance with the decreased transendothelial migration of leukocytes due to PT treatment observed *in vivo*, it was demonstrated that the treatment of activated endothelial cells with the microtubule-destabilizing drugs demecolcine and nocodazole *reduced* the transendothelial migration of monocytes or lymphocytes *in vitro*.[331,332] In contrast, the study of Kielbassa *et al.* revealed that the treatment of endothelial cells with the microtubule-destabilizing drug colchicine *enhanced* the transendothelial migration of monocytes *in vitro*.[259] However, these findings are in line with some studies reporting that microtubule-stabilization due to the treatment with PAC is associated with enhanced endothelial integrity and a decrease in the transendothelial migration of leukocytes.[257-259]

In summary, the current study revealed that the treatment of endothelial cells PT substantially reduced the adhesion of leukocytes in the process of acute inflammation *in vitro* as well as *in vivo*. Consistent to PT, the adhesion of leukocytes onto an activated endothelial monolayer was decreased by the microtubule-destabilizing agents VIN and COL. In contrast to microtubule-destabilizing drugs, leukocyte-endothelial cell interactions were not affected by the microtubule-stabilizing compound PAC. Consequently, this clearly suggests that the effects of PT, VIN and COL depend on their destabilizing action on the microtubule network.

2.2 Elucidation of the mechanism responsible for the effect of PT and other MTAs on leukocyte-endothelial cell interactions

The rolling of leukocytes on the vascular surface in the process of acute inflammation is crucially regulated by endothelial E-selectin.[65-67] However, since it was verified by intravital microscopy that PT had no effect on the rolling of leukocytes on the activated endothelium, we assumed that the endothelial expression of E-selectin is not impaired in the presence of PT. In addition, the few studies that focused on the influence of colchicine on the surface expression of E-selectin on activated endothelial cells revealed conflicting results. For instance, the Perico *et al.* found that colchicine reduced the cell surface protein expression of

E-selectin on activated endothelial cells.[308] In contrast, Cronstein *et al.* demonstrated that colchicine did not affect the cell surface protein expression of E-selectin on activated endothelial cells but its distribution on the endothelial surface was altered in the presence of colchicine.[247]

Since ICAM-1 and VCAM-1 expressed on endothelial cells crucially regulate the firm adhesion of leukocytes onto the vascular surface, we hypothesized that the reduced leukocyte adhesion onto the activated endothelium due to PT, VIN or COL treatment might be caused by a decrease in the TNFα-induced expression of these adhesion molecules.[47,90,91] Thus, we analyzed the effect of PT, VIN, COL and PAC on the cell surface expression of ICAM-1 and VCAM-1 on activated endothelial cells. The treatment of endothelial cells with the microtubule-destabilizing agents PT, VIN and COL markedly decreased the TNFα-induced cell surface protein expression of ICAM-1 and VCAM-1, whereas the microtubule-stabilizing drug PAC did only slightly affect the cell surface protein expression of VCAM-1 and had no influence on the cell surface protein expression of ICAM-1. These findings are in line with the results presented by Perico *et al.*, Asahina *et al.* and Cuschieri *et al.* demonstrating that the treatment of endothelial cells with colchicine reduced the IL-1β-, TNFα- or LPS-induced cell surface protein expression of ICAM-1.[308-310] Moreover, it was also reported that the TNFα-induced cell surface protein expression of VCAM-1 was decreased in colchicine-treated endothelial cells.[309]

The TNFα-induced expression of ICAM-1 and VCAM-1 is typically mediated through activation of the nuclear factor-κB (NFκB) signaling cascade.[183,184] Thus, we hypothesized that the decrease in the TNFα-induced surface protein expression of ICAM-1 and VCAM-1 caused by treatment with the microtubule-destabilizing drugs PT, VIN and COL was due to a reduction of NFκB activation. This was proven by investigations into the influence of the used MTAs on the NFκB promotor activity of activated endothelial cells. In accordance with the compound-depended effects observed regarding the surface protein expression of ICAM-1 and VCAM-1, the present study revealed that the microtubule-destabilizing agents PT, VIN and COL were able to reduce the TNFα-induced NFκB promotor activity in endothelial cells, whereas treatment with the microtubule-stabilizing agent PAC did not affect this process. Although several studies showed that microtubule-destabilizing drugs cause a rapid and efficient activation of NFκB in various cancer cell types, little is known about the influence of MTAs on the NFκB signaling cascade in endothelial cells.[318-322] Nonetheless, in contrast to the current study, Cuschieri *et al.* could show that the LPS-induced activation of NFκB was not affected by colchicine in endothelial cells.[310] Moreover, it was reported that the TNFα- and IL-1-mediated activation of NFκB correlates with endothelial integrity, since it was able to induce the reorganization of the actin as well as the tubulin cytoskeleton leading to the formation of interendothelial gaps.[257,333] In line with these findings, paclitaxel was demonstrated to attenuate

TNFα- and thrombin-induced permeability of the endothelium by enhancing endothelial integrity and paracellular gap junction formation.[257,258] In addition, it was reported that the paclitaxel-triggered enhancement of endothelial integrity is associated with a decreased transendothelial migration of leukocytes.[259] Hence, it is therefore surprising that the NFκB promotor activity was decreased by the treatment of TNFα-activated endothelial cells with the microtubule-destabilizing drugs PT, VIN and COL in this study, since they also interfere with the endothelial integrity leading to the formation of interendothelial gaps.

Taken together, this study verified that the impact of the microtubule-destabilizing agents PT, VIN and COL on leukocyte-endothelial cell interactions are based on their influence on the cell surface protein expression of ICAM-1 and VCAM-1 in activated endothelial cells. These effects are – at least in part – associated with an impact on the activation of the transcription factor NFκB. In contrast to PT, VIN and COL, the microtubule-stabilizing drug paclitaxel did not affect leukocyte-endothelial cell interactions, although the TNFα-induced cell surface protein expression of VCAM-1 was slightly reduced by the compound, however, through an NFκB-independent mechanism.

3 Conclusion and future perspectives

3.1 Conclusion

The current study revealed that the microtubule-destabilizing agent PT triggers the formation of interendothelial gaps leading to the exposure of the extracellular matrix protein collagen. As a consequence, tumor cells are attached to the exposed collagen *via* β1-integrins, causing the inhibition of their transendothelial migration. These results provide – at least in part – an *in vitro* explanation for the anti-metastatic potential of PT observed *in vivo* and clearly highlights endothelial cells of the non-tumor vasculature as a promising target for the treatment of hematogenically disseminating tumors. Moreover, since the effects of VIN and COL regarding tumor-endothelial cell interactions are consistent with that of PT, this study indicates that both compounds might act as anti-metastatic agents affecting the non-tumor vasculature, furthermore highlighting the yet unknown but possible anticancer action of COL.

Moreover, in the present study we could demonstrate that the microtubule-destabilizing drugs PT, VIN and COL reduce the adhesion of leukocytes onto a TNFα-activated endothelial monolayer, whereas the microtubule-stabilizing agent PAC does not affect this process. While PT, VIN and COL induce the expression of ICAM-1 and VCAM-1 in quiescent endothelial cells, they strongly decrease the cell surface protein expression of these CAMs in TNFα-activated

endothelial cells. This effect is – at least in part – associated with a reduction in the TNFα-induced activation of NFκB caused by the microtubule-destabilizing compounds. Nonetheless, in contrast to the strong effects on the cell surface protein expression of ICAM-1 and VCAM-1, the influence of PT, VIN and COL on the NFκB promotor activity was rather low. Consequently, this suggests that these microtubule-destabilizing drugs might also affect other signaling pathways such as that of activator protein 1 (AP-1), mitogen-activated protein (MAP) kinases (MAPKs) and signal transducer and activators of transcription (STATs) that are involved in the regulation of endothelial activation.[334-337] Moreover, it is very likely that the effect of PT, VIN and COL on microtubule dynamics might interfere with the microtubule-mediated transport of CAMs to the vascular surface and thus decreasing their TNFα-induced cell surface expression. Finally, this study gives first insights about the anti-inflammatory potential of the microtubule-destabilizing agents PT, VIN and COL regarding their influence on endothelial cells.

3.2 Future perspectives

In this work, the influence of the novel MTA PT on endothelial cells of the non-tumor vasculature was analyzed regarding tumor-endothelial cell interactions in order to gain insights into the mechanisms underlying the anti-metastatic action of the compound observed *in vivo*. However, it would be interesting to investigate whether PT and other MTAs interfere with interactions between tumor cells and the healthy endothelium when tumor cells instead of endothelial cells were treated with the compounds. If such an interference could be observed, future research should examine, whether there is an additive effect regarding the influence of PT and other MTAs on tumor-endothelial cell interactions when both tumor cells and endothelial cells are treated with the drugs.

As the present study gives only first insights into the mechanisms underlying the anti-inflammatory potential of the microtubule-destabilizing agents PT, VIN and COL, several further experiments are needed to fully characterize the influence of these compounds on inflammatory processes of the vascular endothelium.

The first point that should be addressed in this context is to investigate the role of VIN, COL and PAC on leukocyte-endothelial cell interactions *in vivo* (rolling, adhesion, transmigration) by intravital microscopy, in order to figure out whether similar results can be observed for VIN and COL compared to PT. In addition, while it was demonstrated by intravital microscopy that the transendothelial migration of leukocytes was reduced by PT under inflammatory conditions *in vivo*, the effect of PT on this crucial step in the inflammatory cascade was so far not

investigated *in vitro*. Thus, the impact of PT, VIN, COL and PAC on the transendothelial migration of leukocytes through an activated endothelial monolayer should be investigated *in vitro* using a classical boyden chamber assay. Furthermore, the anti-inflammatory potential of PT, VIN and COL should be also demonstrated in other *in vivo* assays such as a peritonitis or enteritis model.

In addition, further experiments are needed to elucidate the mechanism underlying the anti-inflammatory effect of PT, VIN and COL. In this context, it would be of advantage to investigate the influence of PT, VIN, COL and PAC on the total protein expression and the gene expression of ICAM-1 and VCAM-1 in order to prove whether the decrease in the TNFα-induced cell surface protein expression is caused by a reduction in gene transcription and protein translation or whether it should be assumed that the intracellular transport of these molecules is affected by the influence of MTAs on the microtubule network. Moreover, since PT reduced the transendothelial migration of leukocytes through an activated endothelial monolayer, future experiments should also focus on the impact of PT, VIN, COL and PAC on cell adhesion molecules such as PECAM-1, CD99 and JAMs involved in this step of the inflammatory cascade. Since the TNFα-induced activation of NFκB was reduced, albeit only slightly, by all of these compounds, their influence on members of the signaling cascade upstream of the NFκB promotor activity such as IKK activation, IκBα degradation and p65 nuclear translocation should be analyzed. As mentioned previously, the differences in intensity of the effects of PT, VIN and COL on the cell surface protein expression of ICAM-1 and VCAM-1 compared to that of their impact on NFκB activation suggests that other signaling pathways such as AP-1, MAPKs or STATs could be affected by the compounds. Therefore, further reporter assays are needed to address this point.

Furthermore, it would be of great interest to examine whether PT, VIN, COL or PAC interfere with leukocyte-endothelial cell interactions when leukocytes instead of endothelial cells were treated with the compounds, since PT was presented to both endothelial cells and leukocytes during intravital microscopy. Indeed, first data of our group showed that the adhesion of primary monocytes onto an activated endothelial monolayer was reduced when monocytes and not endothelial cells were treated with PT. In addition, it would be interesting to investigate whether this effect is caused by alterations of cell adhesion molecules such as LFA-1 and VLA-4 expressed on monocytes due to PT treatment. Here again, first data revealed that PT actually decreased the cell surface protein expression of LFA-1 and VLA-1 in primary monocytes.

Since chemokines and pro-inflammatory cytokines such as TNFα, IL-1β, IL-6, IL-8, CXCL12, CX3CL1 or MCP-1 secreted by either endothelial cells or leukocytes are crucially involved in regulating the inflammatory response, the influence of PT, VIN, COL and PAC on the protein expression and on the secretion level of these molecules should be examined in further

experiments. Consequently, the impact of PT, VIN, COL and PAC on the underlying signaling pathways including COX-1, AP-1, SP-1, MAPKs, STATs, DUSP-1 and eNOS should also be investigated in future experiments.

V SUMMARY

Pretubulysin (PT), a biosynthetic precursor of the myxobacterial compound tubulysin D, was recently identified as a novel microtubule-targeting agent (MTA) causing microtubule destabilization. MTAs are the most frequently used chemotherapeutic drugs. They are well studied regarding their direct cytotoxic effects against various tumors as well as for their anti-angiogenic and vascular-disrupting action addressing endothelial cells of the tumor vasculature. However, the impact of MTAs on endothelial cells of the non-tumor vasculature has been largely neglected, although tumor cell interactions with the healthy endothelium play a crucial role in the process of cancer metastasis. Besides their use as potent anti-cancer drugs, some MTAs such as colchicine are traditionally used or recommended for the therapy of inflammatory diseases. Here, too, the role of endothelial cells has been largely neglected, although the endothelium is crucially involved in regulating the process of inflammation.

In the present study, the impact of PT on tumor-endothelial cell interactions was therefore analyzed in vitro to gain insights into the mechanism underlying its anti-metastatic effect that was recently confirmed in vivo. In the second part of this work, the influence of PT and other MTAs, namely the microtubule-destabilizing compounds vincristine (VIN) and colchicine (COL) and the microtubule-stabilizing drug paclitaxel (PAC), on leukocyte-endothelial cell interactions was investigated in vitro and in vivo (only PT). It is important to mention that in all in vitro experiments solely endothelial cells and not tumor cells or leukocytes were treated with the MTAs to strictly focus on the role of the endothelium in the action of these compounds.

The impact of PT on tumor-endothelial cell interactions was analyzed in vitro by cell adhesion and transendothelial migration assays as well as immunocytochemistry using the breast cancer cell line MDA-MB-231 and primary human umbilical vein endothelial cells (HUVECs). The treatment of HUVECs with PT increased the adhesion of MDA cells onto the endothelial monolayer, whereas their transendothelial migration was reduced by the compound. Thereafter, the influence of PT on the endothelial cell adhesion molecules (CAMs) E-selectin, N-cadherin, ICAM-1, VCAM-1 and galectin-3 and on the CXCL12/CXCR4 chemokine system was examined, since they might be involved in the PT-triggered tumor cell adhesion. Interestingly, although PT induced the upregulation of ICAM-1, VCAM-1, N-cadherin and CXCL12, cell adhesion assays using neutralizing antibodies or the CXCL12 inhibitor AMD3100 revealed that all these molecules were dispensable for the PT-evoked tumor cell adhesion. As PT induces the formation of interendothelial gaps and MDA cells might adhere onto components of the underlying extracellular matrix (ECM), the precise location of MDA cells attached to the PT-treated endothelial monolayer was investigated. Instead of a direct interaction between tumor and endothelial cells, this work showed that MDA cells preferred to adhere to the ECM component collagen that was exposed within PT-triggered endothelial gaps. Both the PT-evoked increase in tumor cell adhesion onto and the decrease in trans-

endothelial migration were completely abolished when β1-integrins were blocked on MDA cells. Similar results were obtained when endothelial cells were treated with VIN and COL but not PAC, indicating that the observed effects of PT depend on its microtubule-destabilizing activity.

The impact of PT, VIN, COL and PAC on leukocyte-endothelial cell interactions was analyzed in vivo (only PT) by intravital microscopy of the mouse cremaster muscle and in vitro by cell adhesion assays using the monocyte-like cell line THP-1 and TNFα-activated human dermal microvascular endothelial cells (HMEC-1). While PT did not affect the rolling of leukocytes on the endothelium, their firm adhesion onto and transmigration through the activated endothelium was reduced by PT in vivo. In accordance, the treatment of HMEC-1 with PT, VIN and COL decreased the TNFα-induced adhesion of THP-1 cells onto the endothelial monolayer, whereas PAC had no influence on this process. Thereafter, the influence of PT, VIN, COL and PAC on endothelial ICAM-1 and VCAM-1 was examined, since these molecules are substantially involved in the firm adhesion of leukocytes onto the endothelium. The cell surface protein expression of ICAM-1 and VCAM-1 was reduced by PT, VIN and COL in activated endothelial cells, whereas PAC did only slightly affect the TNFα-induced upregulation of VCAM-1. As the pro-inflammatory transcription factor NFκB plays a crucial role in the TNFα-induced expression of these CAMs, the impact of the MTAs on the NFκB promotor activity was investigated. While PT, VIN and COL decreased the activation of NFκB in activated endothelial cells, PAC did not affect this process. However, in contrast to the strong effects regarding the cell surface protein expression of ICAM-1 and VCAM-1, the effects of PT, VIN and COL on the NFκB activity was rather low. Thus, the used MTAs might also affect other relevant signaling pathways and/or the intracellular transport of CAMs might be influenced by the impact of the MTAs on the microtubule network.

Taken together, the current study provides – at least in part – an explanation for the anti-metastatic potential of PT and gives first insights into the use of PT and VIN as anti-inflammatory drugs. Moreover, this work highlights the endothelium as an attractive target for the development of new anti-cancer and anti-inflammatory drugs.

VI ZUSAMMENFASSUNG

Das vaskuläre Endothel besteht aus einer einzigen, dünnen Schicht von Endothelzellen, die sich als innerste Wandschicht von Blutgefäßen über den gesamten Blutkreislauf vom Herzen bis zu den kleinsten Kapillaren erstrecken. Neben seiner Funktion als selektive Barriere, spielt das vaskuläre Endothel eine zentrale Rolle in der Modulation des Gefäßtonus und damit des Blutflusses, der Regulation der Gefäßbildung, der Koordination von Thrombozyten- und Leukozyten-Interaktionen und damit der Regulation von Gerinnungs- und Entzündungsreaktionen. Eine akute Entzündung ist im Allgemeinen ein nützliches, streng reguliertes Ereignis, das für die Beseitigung von Infektionen oder Verletzungen und somit die Wiederherstellung der Homöostase sorgt. Eine Entzündungsreaktion schließt jedoch auch das rechtzeitige Einleiten einer Entzündungsauflösung und einer Reparaturphase ein, um das Ausbilden einer chronisch-pathologischen Reaktion zu verhindern, die durch eine ständige Extravasation von Leukozyten gekennzeichnet ist, was wiederum zu Gewebeschäden und sogar Krebs führen kann. Wie gesunde Zellen und Gewebe, müssen auch Tumoren über Blutgefäße mit Nährstoffen und Sauerstoff versorgt werden und geben über diese Stoffwechselprodukte und Kohlendioxid ab. Ein funktionierendes Tumor-Gefäßsystem ist jedoch nicht nur für das Überleben und Wachstums eines Tumors, sondern auch für die Metastasierung unerlässlich, sodass es einen vielversprechenden therapeutischen Angriffsort darstellt.

Mikrotubuli sind hochdynamische, polarisierte, röhrenförmige Strukturen aus meist 13 parallelen Protofilamenten, die durch Polymerisation von α/β-Tubulin-Heterodimeren gebildet werden. Sie spielen bei einer Vielzahl von zellulären Prozessen, wie beispielsweise der Ausbildung und Erhaltung der Zellform, dem intrazellulären Transport, der Signaltransduktion, der Mitose und der Zellpolarisation und -migration eine wesentliche Rolle. Das dynamische Verhalten von Mikrotubuli wird durch ihre Fähigkeit definiert, Zyklen von schnellem Wachstum (Polymerisation) und Schrumpfung (Depolymerisation) zu durchlaufen.

Die Beteiligung von Mikrotubuli an Zellteilung und Mitose macht sie zu einem wichtigen Angriffspunkt für antitumorale Wirkstoffe. Tatsächlich sind Tubulin-Antagonisten (MTA, microtubule-targeting agent) die am häufigsten verwendeten Chemotherapeutika. MTAs werden nach ihrer Wirkung auf Mikrotubuli in zwei Hauptgruppen eingeteilt. Mikrotubuli-destabilisierende Wirkstoffe, mit den pflanzlichen Vinca-Alkaloiden (aus *Catharanthus roseus*) als Hauptvertreter, destabilisieren Mikrotubuli entweder durch eine Polymerisationsinhibierung oder durch die Depolymerisation bereits bestehender Mikrotubuli. Sie werden derzeit gegen hämatologische und lymphatische Krebserkrankungen und verschiedene solide Tumore, wie beispielsweise Brustkrebs, eingesetzt. Mikrotubuli-stabilisierende Substanzen, mit den pflanzlichen Taxanen (aus der Rinde von *Taxus brevifolia*) sowie den bakteriellen Epothilonen (aus dem *Myxobakterium Sorangium cellulosum*) als Hauptvertreter, stabilisieren Mikrotubuli

entweder durch Polymerisationsinitiierung oder durch Hyperstabilisierung bereits bestehender Mikrotubuli. Sie werden gegen solide Tumore, wie Brust-, Eierstock- und Lungenkrebs, eingesetzt. Darüber hinaus hat der pflanzliche, Mikrotubuli-destabilisierende Wirkstoff Colchicin (aus *Colchicum autumnale*) bisher keinen signifikanten Nutzen in der Krebsbehandlung gezeigt, wird aber häufig zur Therapie von Gicht und familiärem Mittelmeerfieber sowie anderen entzündlichen Erkrankungen eingesetzt. Aufgrund der wesentlichen Nachteile der derzeit verwendeten MTAs, insbesondere aufgrund ihrer Nebenwirkungen und der Entwicklung von Resistenzen, ist die Suche nach neuen MTA-Klassen nach wie vor sehr wichtig.

Prätubulysin (PT), eine biosynthetische Vorstufe des von Myxobakterien produzierten Tubulysins D, wurde kürzlich als neuer Tubulin-Antagonist identifiziert, der eine Destabilisierung der Mikrotubuli bewirkt. Wie viele andere MTAs, wurde PT hinsichtlich seines direkten zytotoxischen Effekts auf Tumorzellen sowie seiner antiangiogenen und gefäßzerstörenden Wirkung auf Endothelzellen des Tumorgefäßsystems bereits gut untersucht. Der Einfluss von PT und anderen MTAs auf Endothelzellen des nicht-tumoralen Gefäßsystems wurde jedoch weitgehend vernachlässigt, obwohl Tumorzell-Interaktionen mit dem gesunden Endothel eine entscheidende Rolle im Prozess der Krebsmetastasierung spielen. Für PT konnte in diesem Zusammenhang allerdings schon gezeigt werden, dass es die Bildung von Metastasen in vivo stark reduziert. Wie bereits erwähnt, werden manche MTAs, wie zum Beispiel Colchicin, zur Therapie bestimmter entzündungsassoziierter Krankheiten eingesetzt. Jedoch wurde auch hier die Rolle des Endothels weitgehend vernachlässigt, obwohl es entscheidend an der Regulierung des Entzündungsprozesses beteiligt ist.

In der vorliegenden Studie wurde daher der Einfluss von PT auf Tumor-Endothelzell-Interaktionen in vitro analysiert, um Einblicke in den Mechanismus zu gewinnen, der der vor kurzem in vivo bestätigten, antimetastatischen Wirkung von PT zugrunde liegt. Im zweiten Teil dieser Arbeit wurde der Einfluss von PT und anderen MTAs, nämlich den Mikrotubuli-destabilisierenden Wirkstoffen Vincristin (VIN) und Colchicin (COL) und dem Mikrotubuli-stabilisierenden Wirkstoff Paclitaxel (PAC), auf Leukozyten-Endothelzell-Interaktionen in vitro und in vivo (nur PT) untersucht. Es ist wichtig zu erwähnen, dass in allen in vitro Experimenten ausschließlich Endothelzellen und nicht Tumorzellen oder Leukozyten mit den verwendeten MTAs behandelt wurden, um strikt auf die Rolle des Endothels bei der Wirkung dieser Substanzen zu fokussieren.

Der Einfluss von PT auf Tumor-Endothelzell-Interaktionen wurde in vitro mittels Zelladhäsions- und transendothelialen Migrations-Assays sowie Immunzytochemie und Mikroskopie analysiert. Hierbei wurde mit der Brustkrebs-Zelllinie MDA-MB-231 und primären humanen Endothelzellen aus der Nabelschnurvene (HUVECs, human umbilical vein endothelial cells)

gearbeitet. Die Behandlung von HUVECs mit PT erhöhte die Adhäsion von MDA-Zellen an die Endothelzellschicht, wohingegen ihre transendotheliale Migration durch diesen Wirkstoff reduziert wurde.

Obwohl die Mechanismen, mit denen Tumorzellen an Endothelzellen haften, noch nicht vollständig verstanden sind, ist es sehr wahrscheinlich, dass sie die für Leukozyten-Endothelzell-Interaktionen beschriebenen Mechanismen nutzen. Mehrere Studien konnten zeigen, dass die auf Endothelzellen exprimierten Zelladhäsionsmoleküle (CAMs, cell adhesion molecules) E-Selektin, N-Cadherin, ICAM-1, VCAM-1 und Galectin-3 entweder am Rollen von Tumorzellen auf oder deren festen Adhäsion am Endothel beteiligt sind. Daher wurde der Einfluss von PT auf die Genexpression (mRNA), die Gesamtproteinmenge und die Proteinexpression auf der Zelloberfläche dieser endothelialen CAMs mittels qRT-PCR, Western Blot und Durchflusszytometrie untersucht. Obwohl PT die Hochregulierung von ICAM-1, VCAM-1 und N-Cadherin induzierte, zeigten Zelladhäsions-Assays, bei denen neutralisierende Antikörper verwendet wurden, interessanterweise, dass diese Moleküle für die durch PT ausgelöste Tumorzelladhäsion verzichtbar waren. Neben CAMs spielen diverse Chemokinsysteme bei der Bestimmung des metastatischen Musters verschiedener maligner Neoplasien eine entscheidende Rolle. Insbesondere der Chemokinrezeptor CXCR4 ist in zahlreichen Tumorarten wie Brust- und Prostatakrebs stark exprimiert, während sein entsprechender Ligand CXCL12 (auch bekannt als SDF-1α) besonders von den Organen sezerniert wird, die als bevorzugte Zielorte für die Bildung von Metastasen bekannt sind. Daher wurde der Einfluss von PT auf die Genexpression, die Gesamtproteinmenge und die Sezernierung von endothelialem CXCL12 mittels qRT-PCR, Western Blot und ELISA analysiert. Interessanterweise war die mRNA-Expression von CXCL12 in PT-behandelten Endothelzellen stark erhöht, während die Gesamtproteinmenge sowie die Sekretion von CXCL12 aus dem Endothel nur marginal gesteigert wurde. Es ergab sich außerdem, dass die Sekretion von CXCL12 und die Sekretion von Zytokinen im Allgemeinen für die PT-induzierte Tumorzelladhäsion in vitro nicht relevant sind. Eine mögliche autokrine Wirkung von CXCL12 auf Endothelzellen wurde mithilfe des CXCR4-Inhibitors AMD3100 ebenfalls ausgeschlossen.

Da PT die Bildung von transienten Lücken in der Endothelzellschicht induziert und Tumorzellen an Komponenten der darunter liegenden extrazellulären Matrix (ECM, extracellular matrix) haften können, wurde die genaue Position der MDA-Zellen, die an der Endothelzellschicht adhärierten, mittels Immunzytochemie und Mikroskopie untersucht. Statt einer direkten Interaktion zwischen Tumor- und Endothelzellen zeigte diese Studie, dass MDA-Zellen bevorzugt an der ECM-Komponente Kollagen, die in den durch PT hervorgerufenen endothelialen Lücken freigelegt wurde, hafteten. β1-Integrine stellen eine wichtige Klasse innerhalb der Familie der Integrine dar, die Zellinteraktionen mit ECM-Komponenten, wie

beispielsweise Kollagen, vermitteln. Es wurde gezeigt, dass die Adhäsion von MDA-MB-231 Brustkrebszellen an Kollagen über α2β1- und α3β1-Integrine auf den Tumorzellen vermittelt wird. In dieser Arbeit wurde mithilfe eines neutralisierenden Antikörpers untersucht, ob der Einfluss von PT auf die Zelladhäsion an und die transendotheliale Migration von MDA-Zellen durch eine Endothelzellschicht von β1-Integrinen auf der Oberfläche von MDA-Zellen abhängig ist. Wurden β1-Integrine auf den MDA-Zellen blockiert, wurde sowohl der durch PT induzierte Anstieg der Tumorzelladhäsion als auch die Abnahme der Tumorzelltransmigration vollständig aufgehoben. Vergleichbare Ergebnisse wurden erzielt, wenn Endothelzellen mit VIN oder COL, die ebenfalls die Bildung von transienten Lücken in der Endothelzellschicht induzieren, behandelt wurden. Die Behandlung von Endothelzellen mit PAC, das keine endothelialen Lücken hervorruft, erhöhte die Tumorzelladhäsion nur geringfügig. Dies deutet darauf hin, dass die beobachteten Effekte von PT von seiner destabilisierenden Wirkung auf das Mikrotubuli-Netzwerk abhängig waren.

Drei wesentliche Schritte bei der Rekrutierung von Leukozyten an den Ort der Entzündung, die bereits im 19. Jahrhundert beschrieben wurden, sind das Rollen von Leukozyten auf, die feste Adhäsion von Leukozyten an sowie deren transendotheliale Migration durch das Endothel. Daher wurde zunächst der Einfluss von PT auf diese drei Schritte der Leukozyten-Endothelzell-Interaktionen in vivo mittels Intravitalmikroskopie des Maus-Kremastermuskels untersucht. Während PT das Rollen der Leukozyten auf dem Endothel nicht beeinflusste, wurden ihre feste Adhäsion an und ihre transendotheliale Migration durch das aktivierte Endothel durch PT reduziert. Anschließend wurde der Einfluss von PT, VIN, COL und PAC auf Leukozyten-Endothelzell-Interaktionen in vitro mittels Zelladhäsions-Assays analysiert. Hierbei wurde mit der Monozyten-ähnlichen Zelllinie THP-1 und TNFα-aktivierten humanen mikrovaskulären Endothelzellen (HMEC-1, human dermal microvascular endothelial cells) gearbeitet. Entsprechend des entzündungshemmenden Effekts von PT in vivo, verringerte die Behandlung von HMECs mit PT, VIN und COL die TNFα-induzierte Adhäsion von THP-1 an die Endothelzellschicht, wohingegen PAC keinen Einfluss auf diesen Prozess hatte.

Das Rollen von Leukozyten auf dem Endothel wird bei akuten Entzündungen im Wesentlichen durch die Interaktion von Selektinen mit ihren Liganden reguliert. Da jedoch mittels Intravitalmikroskopie nachgewiesen wurde, dass PT keinen Einfluss auf das Rollen von Leukozyten auf dem aktivierten Endothel hat, nahmen wir an, dass die endotheliale Expression von Selektinen oder Selektinliganden in Gegenwart von PT nicht beeinträchtigt wird. Die feste Adhäsion von Leukozyten an das Endothel wird vor allem durch die endotheliale Expression der CAMs ICAM-1 und VCAM-1 beeinflusst. Daher wurde der Einfluss von PT, VIN, COL und PAC auf die Proteinexpression dieser CAMs auf der Zelloberfläche von aktivierten Endothelzellen mittels Durchflusszytometrie untersucht. Während die Proteinexpression von

ICAM-1 und VCAM-1 an der Zelloberfläche TNFα-aktivierter HMECs durch PT, VIN und COL reduziert wurde, wurde die TNFα-induzierte Hochregulierung von VCAM-1 nur geringfügig durch PAC beeinflusst. Die TNFα-induzierte Expression von ICAM-1 und VCAM-1 wird typischerweise durch die Aktivierung der NFκB-Signalkaskade vermittelt. Es war daher naheliegend, dass der Rückgang der TNFα-induzierten Oberflächenproteinexpression von ICAM-1 und VCAM-1 durch die Behandlung mit PT, VIN und COL auf eine Reduktion der NFκB-Aktivierung zurückzuführen ist. Daher wurde der Einfluss der verwendeten MTAs auf die Promotoraktivität von NFκB mittels Reportergen-Assays untersucht. Während PT, VIN und COL die Aktivierung von NFκB in Endothelzellen verminderten, hatte PAC hierauf keinen Einfluss.

Zusammengefasst ergab die vorliegende Studie, dass PT die Bildung von Lücken innerhalb der Endothelzellschicht auslöst, die zur Exposition der ECM-Komponenten Kollagen führen. Folglich können Tumorzellen über β1-Integrine an das freiliegende Kollagen haften, wodurch ihre Transmigration durch das Endothel gehemmt wird. Diese Ergebnisse liefern – zumindest teilweise – eine Erklärung für das in vivo beobachtete antimetastatische Potenzial von PT. Darüber hinaus wird durch die Ergebnisse klar, dass Endothelzellen des nicht-tumoralen, also gesunden Gefäßsystems ein vielversprechendes Angriffsziel für die Behandlung von hämatogen streuenden Tumoren darstellen. Ferner zeigt diese Arbeit, dass die Auswirkungen von VIN und COL auf Tumor-Endothelzell-Interaktionen mit denen von PT übereinstimmen, wohingegen PAC diese Interaktionen kaum beeinflusste. Das deutet darauf hin, dass die antimetastatische Wirkung von PT, VIN und COL auf das gesunde Endothel von ihrem Mikrotubuli-destabilisierenden Effekt abhängig ist und weist außerdem auf eine bisher unbekannte antimetastatische Eigenschaft von COL hin. Darüber hinaus konnten wir in der vorliegenden Studie zeigen, dass PT, VIN und COL die Adhäsion von Leukozyten an das TNFα-aktivierte Endothel in vitro und in vivo (nur PT) reduzieren, während PAC diesen Prozess nicht beeinflusst. Diese Effekte werden sehr wahrscheinlich durch eine verminderte Oberflächenproteinexpression von ICAM-1 und VCAM-1 auf dem aktivierten Endothel hervorgerufen, die – zumindest teilweise – auf eine reduzierte Aktivierung des NFκB-Signalwegs zurückzuführen ist. Im Gegensatz zu den starken Effekten bezüglich der Expression von ICAM-1 und VCAM-1, waren die Effekte von PT, VIN und COL auf die Aktivität von NFκB nämlich eher gering. Das deutet darauf hin, dass die verwendeten MTAs möglicherweise auch andere relevante Signalwege beeinflussen und/oder der intrazelluläre Transport von CAMs durch den Einfluss der MTAs auf das Mikrotubuli-Netzwerk beeinflusst werden könnte. Somit bringt diese Arbeit erste Erkenntnisse über den Einsatz von PT und VIN als entzündungshemmende Substanzen und hebt den bisher wenig untersuchten Effekt von COL auf das Endothel hervor.

VII REFERENCES

1 Sumpio, B. E., Riley, J. T. & Dardik, A. Cells in focus: endothelial cell. *The international journal of biochemistry & cell biology* **34**, 1508-1512 (2002).

2 Rajendran, P. *et al.* The vascular endothelium and human diseases. *International journal of biological sciences* **9**, 1057-1069, doi:10.7150/ijbs.7502 (2013).

3 Behrendt, D. & Ganz, P. Endothelial function. From vascular biology to clinical applications. *The American journal of cardiology* **90**, 40L-48L (2002).

4 Cines, D. B. *et al.* Endothelial cells in physiology and in the pathophysiology of vascular disorders. *Blood* **91**, 3527-3561 (1998).

5 Ziche, M., Donnini, S. & Morbidelli, L. Development of new drugs in angiogenesis. *Current drug targets* **5**, 485-493 (2004).

6 Tomasoni, L. *et al.* Effects of treatment strategy on endothelial function. *Autoimmunity reviews* **9**, 840-844, doi:10.1016/j.autrev.2010.07.017 (2010).

7 Boisrame-Helms, J., Kremer, H., Schini-Kerth, V. & Meziani, F. Endothelial dysfunction in sepsis. *Current vascular pharmacology* **11**, 150-160 (2013).

8 Yang, O., Li, J. & Kong, J. The Endothelium as a Target for the Treatment of Heart Failure. *Cell biochemistry and biophysics* **72**, 751-756, doi:10.1007/s12013-015-0526-7 (2015).

9 Medzhitov, R. Origin and physiological roles of inflammation. *Nature* **454**, 428-435, doi:10.1038/nature07201 (2008).

10 Serhan, C. N., Chiang, N. & Van Dyke, T. E. Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. *Nature reviews. Immunology* **8**, 349-361, doi:10.1038/nri2294 (2008).

11 Levy, B. D., Clish, C. B., Schmidt, B., Gronert, K. & Serhan, C. N. Lipid mediator class switching during acute inflammation: signals in resolution. *Nature immunology* **2**, 612-619, doi:10.1038/89759 (2001).

12 Bannenberg, G. L. *et al.* Molecular Circuits of Resolution: Formation and Actions of Resolvins and Protectins. *The Journal of Immunology* **174**, 4345-4355, doi:10.4049/jimmunol.174.7.4345 (2005).

13 Serhan, C. N. Resolution phase of inflammation: novel endogenous anti-inflammatory and proresolving lipid mediators and pathways. *Annual review of immunology* **25**, 101-137, doi:10.1146/annurev.immunol.25.022106.141647 (2007).

14 Strieter, R. M., Gomperts, B. N. & Keane, M. P. The role of CXC chemokines in pulmonary fibrosis. *The Journal of clinical investigation* **117**, 549-556, doi:10.1172/JCI30562 (2007).

15 Nathan, C. & Ding, A. Nonresolving inflammation. *Cell* **140**, 871-882, doi:10.1016/j.cell.2010.02.029 (2010).

16 Calderon, E. & Lockey, R. F. A possible role for adhesion molecules in asthma. *The Journal of allergy and clinical immunology* **90**, 852-865 (1992).

17 Hurlimann, D. *et al.* Anti-tumor necrosis factor-alpha treatment improves endothelial function in patients with rheumatoid arthritis. *Circulation* **106**, 2184-2187 (2002).

18 Correale, J. & Villa, A. The blood-brain-barrier in multiple sclerosis: functional roles and therapeutic targeting. *Autoimmunity* **40**, 148-160, doi:10.1080/08916930601183522 (2007).

19 Cromer, W. E., Mathis, J. M., Granger, D. N., Chaitanya, G. V. & Alexander, J. S. Role of the endothelium in inflammatory bowel diseases. *World journal of gastroenterology* **17**, 578-593, doi:10.3748/wjg.v17.i5.578 (2011).

20 Hanahan, D. & Folkman, J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* **86**, 353-364 (1996).

21 Ferrara, N. Vascular endothelial growth factor. *Arterioscler Thromb Vasc Biol* **29**, 789-791, doi:10.1161/ATVBAHA.108.179663 (2009).

22 Mac Gabhann, F. & Popel, A. S. Systems biology of vascular endothelial growth factors. *Microcirculation* **15**, 715-738, doi:10.1080/10739680802095964 (2008).

23 Carmeliet, P. VEGF as a key mediator of angiogenesis in cancer. *Oncology* **69 Suppl 3**, 4-10, doi:10.1159/000088478 (2005).

24 Baeriswyl, V. & Christofori, G. The angiogenic switch in carcinogenesis. *Semin Cancer Biol* **19**, 329-337, doi:10.1016/j.semcancer.2009.05.003 (2009).

25 Qian, B. Z. & Pollard, J. W. Macrophage diversity enhances tumor progression and metastasis. *Cell* **141**, 39-51, doi:10.1016/j.cell.2010.03.014 (2010).

26 Zumsteg, A. & Christofori, G. Corrupt policemen: inflammatory cells promote tumor angiogenesis. *Current opinion in oncology* **21**, 60-70, doi:10.1097/CCO.0b013e32831bed7e (2009).

27 Murdoch, C., Muthana, M., Coffelt, S. B. & Lewis, C. E. The role of myeloid cells in the promotion of tumour angiogenesis. *Nat Rev Cancer* **8**, 618-631, doi:10.1038/nrc2444 (2008).

28 De Palma, M., Murdoch, C., Venneri, M. A., Naldini, L. & Lewis, C. E. Tie2-expressing monocytes: regulation of tumor angiogenesis and therapeutic implications. *Trends in immunology* **28**, 519-524, doi:10.1016/j.it.2007.09.004 (2007).

29 Ferrara, N. Pathways mediating VEGF-independent tumor angiogenesis. *Cytokine & growth factor reviews* **21**, 21-26, doi:10.1016/j.cytogfr.2009.11.003 (2010).

30 Tozer, G. M., Kanthou, C. & Baguley, B. C. Disrupting tumour blood vessels. *Nat Rev Cancer* **5**, 423-435, doi:10.1038/nrc1628 (2005).

31 Fürst, R. & Vollmar, A. M. A new perspective on old drugs: non-mitotic actions of tubulin-binding drugs play a major role in cancer treatment. *Pharmazie* **68**, 478-483 (2013).

32 Belotti, D. *et al.* The microtubule-affecting drug paclitaxel has antiangiogenic activity. *Clin Cancer Res* **2**, 1843-1849 (1996).

33 Vacca, A. *et al.* Antiangiogenesis is produced by nontoxic doses of vinblastine. *Blood* **94**, 4143-4155 (1999).

34 Bijman, M. N., van Nieuw Amerongen, G. P., Laurens, N., van Hinsbergh, V. W. & Boven, E. Microtubule-targeting agents inhibit angiogenesis at subtoxic concentrations, a process associated with inhibition of Rac1 and Cdc42 activity and changes in the endothelial cytoskeleton. *Molecular cancer therapeutics* **5**, 2348-2357, doi:10.1158/1535-7163.MCT-06-0242 (2006).

35 Dark, G. G. *et al.* Combretastatin A-4, an agent that displays potent and selective toxicity toward tumor vasculature. *Cancer Res* **57**, 1829-1834 (1997).

36 Griffon-Etienne, G., Boucher, Y., Brekken, C., Suit, H. D. & Jain, R. K. Taxane-induced apoptosis decompresses blood vessels and lowers interstitial fluid pressure in solid tumors: clinical implications. *Cancer Res* **59**, 3776-3782 (1999).

37 Davis, P. D. *et al.* ZD6126: a novel vascular-targeting agent that causes selective destruction of tumor vasculature. *Cancer Res* **62**, 7247-7253 (2002).

38 Kruczynski, A. *et al.* Anti-angiogenic, vascular-disrupting and anti-metastatic activities of vinflunine, the latest vinca alkaloid in clinical development. *Eur J Cancer* **42**, 2821-2832, doi:10.1016/j.ejca.2006.06.019 (2006).

39 Siemann, D. W. The unique characteristics of tumor vasculature and preclinical evidence for its selective disruption by Tumor-Vascular Disrupting Agents. *Cancer treatment reviews* **37**, 63-74, doi:10.1016/j.ctrv.2010.05.001 (2011).

40 Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144**, 646-674, doi:10.1016/j.cell.2011.02.013 (2011).

41 Nguyen, D. X., Bos, P. D. & Massagué, J. Metastasis: from dissemination to organ-specific colonization. *Nat Rev Cancer* **9**, 274-284, doi:10.1038/nrc2622 (2009).

42 Orr, F. W., Wang, H. H., Lafrenie, R. M., Scherbarth, S. & Nance, D. M. Interactions between cancer cells and the endothelium in metastasis. *J Pathol* **190**, 310-329, doi:10.1002/(SICI)1096-9896(200002)190:3<310::AID-PATH525>3.0.CO;2-P (2000).

43 Iiizumi, M., Mohinta, S., Bandyopadhyay, S. & Watabe, K. Tumor-endothelial cell interactions: therapeutic potential. *Microvasc Res* **74**, 114-120, doi:10.1016/j.mvr.2007.04.002 (2007).

44 Spano, D., Heck, C., De Antonellis, P., Christofori, G. & Zollo, M. Molecular networks that regulate cancer metastasis. *Semin Cancer Biol* **22**, 234-249, doi:10.1016/j.semcancer.2012.03.006 (2012).

45 Vicente-Manzanares, M. & Sanchez-Madrid, F. Role of the cytoskeleton during leukocyte responses. *Nature reviews. Immunology* **4**, 110-122, doi:10.1038/nri1268 (2004).

46 Krishnamoorthy, S. & Honn, K. V. Inflammation and disease progression. *Cancer metastasis reviews* **25**, 481-491, doi:10.1007/s10555-006-9016-0 (2006).

47 Strell, C. & Entschladen, F. Extravasation of leukocytes in comparison to tumor cells. *Cell Commun Signal* **6**, 10, doi:10.1186/1478-811X-6-10 (2008).

48 Constantin, G. *et al.* Chemokines trigger immediate beta2 integrin affinity and mobility changes: differential regulation and roles in lymphocyte arrest under flow. *Immunity* **13**, 759-769 (2000).

49 Laudanna, C., Kim, J. Y., Constantin, G. & Butcher, E. Rapid leukocyte integrin activation by chemokines. *Immunological reviews* **186**, 37-46 (2002).

50 Arnaout, M. A., Mahalingam, B. & Xiong, J. P. Integrin structure, allostery, and bidirectional signaling. *Annual review of cell and developmental biology* **21**, 381-410, doi:10.1146/annurev.cellbio.21.090704.151217 (2005).

51 Muller, W. A. Leukocyte-endothelial cell interactions in the inflammatory response. *Laboratory investigation; a journal of technical methods and pathology* **82**, 521-533 (2002).

52 Fidler, I. J. Metastasis: quantitative analysis of distribution and fate of tumor emboli labeled with 125 I-5-iodo-2'-deoxyuridine. *Journal of the National Cancer Institute* **45**, 773-782 (1970).

53 Joyce, J. A. & Pollard, J. W. Microenvironmental regulation of metastasis. *Nat Rev Cancer* **9**, 239-252, doi:10.1038/nrc2618 (2009).

54 Ito, S. *et al.* Real-time observation of micrometastasis formation in the living mouse liver using a green fluorescent protein gene-tagged rat tongue carcinoma cell line. *International journal of cancer* **93**, 212-217, doi:10.1002/ijc.1318 (2001).

55 Ferjancic, S. *et al.* VCAM-1 and VAP-1 recruit myeloid cells that promote pulmonary metastasis in mice. *Blood* **121**, 3289-3297, doi:10.1182/blood-2012-08-449819 (2013).

56 Kienast, Y. *et al.* Real-time imaging reveals the single steps of brain metastasis formation. *Nature medicine* **16**, 116-122, doi:10.1038/nm.2072 (2010).

57 Friedl, P. & Gilmour, D. Collective cell migration in morphogenesis, regeneration and cancer. *Nature reviews. Molecular cell biology* **10**, 445-457, doi:10.1038/nrm2720 (2009).

58 Sahai, E. Illuminating the metastatic process. *Nat Rev Cancer* **7**, 737-749, doi:10.1038/nrc2229 (2007).

59 Chiang, A. C. & Massague, J. Molecular basis of metastasis. *The New England journal of medicine* **359**, 2814-2823, doi:10.1056/NEJMra0805239 (2008).

60 Fidler, I. J. The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. *Nat Rev Cancer* **3**, 453-458, doi:10.1038/nrc1098 (2003).

61 Aird, W. C. Phenotypic heterogeneity of the endothelium: I. Structure, function, and mechanisms. *Circulation research* **100**, 158-173, doi:10.1161/01.RES.0000255691.76142.4a (2007).

62 Liu, L. & Kubes, P. Molecular mechanisms of leukocyte recruitment: organ-specific mechanisms of action. *Thrombosis and haemostasis* **89**, 213-220 (2003).

63 Wagner, J. G. & Roth, R. A. Neutrophil migration mechanisms, with an emphasis on the pulmonary vasculature. *Pharmacological reviews* **52**, 349-374 (2000).

64 Doerschuk, C. M., Beyers, N., Coxson, H. O., Wiggs, B. & Hogg, J. C. Comparison of neutrophil and capillary diameters and their relation to neutrophil sequestration in the lung. *Journal of applied physiology* **74**, 3040-3045, doi:10.1152/jappl.1993.74.6.3040 (1993).

65 Carlos, T. M. & Harlan, J. M. Leukocyte-endothelial adhesion molecules. *Blood* **84**, 2068-2101 (1994).

66 Etzioni, A., Doerschuk, C. M. & Harlan, J. M. Of man and mouse: leukocyte and endothelial adhesion molecule deficiencies. *Blood* **94**, 3281-3288 (1999).

67 Vestweber, D. & Blanks, J. E. Mechanisms that regulate the function of the selectins and their ligands. *Physiological reviews* **79**, 181-213, doi:10.1152/physrev.1999.79.1.181 (1999).

68 Strell, C., Lang, K., Niggemann, B., Zaenker, K. S. & Entschladen, F. Surface molecules regulating rolling and adhesion to endothelium of neutrophil granulocytes

and MDA-MB-468 breast carcinoma cells and their interaction. *Cellular and molecular life sciences : CMLS* **64**, 3306-3316, doi:10.1007/s00018-007-7402-6 (2007).

69 Henderson, R. B. *et al.* The Use of Lymphocyte Function–Associated Antigen (Lfa)-1–Deficient Mice to Determine the Role of Lfa-1, Mac-1, and α4 Integrin in the Inflammatory Response of Neutrophils. *The Journal of Experimental Medicine* **194**, 219-226, doi:10.1084/jem.194.2.219 (2001).

70 Miles, F. L., Pruitt, F. L., van Golen, K. L. & Cooper, C. R. Stepping out of the flow: capillary extravasation in cancer metastasis. *Clinical & experimental metastasis* **25**, 305-324, doi:10.1007/s10585-007-9098-2 (2008).

71 Witz, I. P. The involvement of selectins and their ligands in tumor-progression. *Immunology letters* **104**, 89-93, doi:10.1016/j.imlet.2005.11.008 (2006).

72 Kim, Y. J. & Varki, A. Perspectives on the significance of altered glycosylation of glycoproteins in cancer. *Glycoconjugate journal* **14**, 569-576 (1997).

73 Kannagi, R., Izawa, M., Koike, T., Miyazaki, K. & Kimura, N. Carbohydrate-mediated cell adhesion in cancer metastasis and angiogenesis. *Cancer science* **95**, 377-384 (2004).

74 Aigner, S. *et al.* CD24 mediates rolling of breast carcinoma cells on P-selectin. *The FASEB Journal* **12**, 1241-1251, doi:10.1096/fasebj.12.12.1241 (1998).

75 Schindelmann, S. *et al.* Expression profiling of mammary carcinoma cell lines: correlation of in vitro invasiveness with expression of CD24. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine* **23**, 139-145, doi:10.1159/000064030 (2002).

76 Goetz, D. J. *et al.* A human colon carcinoma cell line exhibits adhesive interactions with P-selectin under fluid flow via a PSGL-1-independent mechanism. *The American journal of pathology* **149**, 1661-1673 (1996).

77 McCarty, O. J., Mousa, S. A., Bray, P. F. & Konstantopoulos, K. Immobilized platelets support human colon carcinoma cell tethering, rolling, and firm adhesion under dynamic flow conditions. *Blood* **96**, 1789-1797 (2000).

78 Weichert, W. *et al.* Cytoplasmic CD24 expression in colorectal cancer independently correlates with shortened patient survival. *Clin Cancer Res* **11**, 6574-6581, doi:10.1158/1078-0432.CCR-05-0606 (2005).

79 Konno, A., Hoshino, Y., Terashima, S., Motoki, R. & Kawaguchi, T. Carbohydrate expression profile of colorectal cancer cells is relevant to metastatic pattern and prognosis. *Clinical & experimental metastasis* **19**, 61-70 (2002).

80 Ura, H. *et al.* Close correlation between increased sialyl-Lewisx expression and metastasis in human gastric carcinoma. *World journal of surgery* **21**, 773-776 (1997).

81 Steplewska-Mazur, K., Gabriel, A., Zajecki, W., Wylezol, M. & Gluck, M. Breast cancer progression and expression of blood group-related tumor-associated antigens. *Hybridoma* **19**, 129-133, doi:10.1089/02724570050031167 (2000).

82 Dittmar, T., Heyder, C., Gloria-Maercker, E., Hatzmann, W. & Zanker, K. S. Adhesion molecules and chemokines: the navigation system for circulating tumor (stem) cells to metastasize in an organ-specific manner. *Clinical & experimental metastasis* **25**, 11-32, doi:10.1007/s10585-007-9095-5 (2008).

83 ten Kate, M. *et al.* Influence of proinflammatory cytokines on the adhesion of human colon carcinoma cells to lung microvascular endothelium. *International journal of cancer* **112**, 943-950, doi:10.1002/ijc.20506 (2004).

84 Khatib, A.-M. *et al.* Characterization of the Host Proinflammatory Response to Tumor Cells during the Initial Stages of Liver Metastasis. *The American journal of pathology* **167**, 749-759, doi:10.1016/s0002-9440(10)62048-2 (2005).

85 van Grevenstein, W. M., Hofland, L. J., Jeekel, J. & van Eijck, C. H. The expression of adhesion molecules and the influence of inflammatory cytokines on the adhesion of human pancreatic carcinoma cells to mesothelial monolayers. *Pancreas* **32**, 396-402, doi:10.1097/01.mpa.0000220865.80034.2a (2006).

86 ten Kate, M., Hofland, L. J., van Koetsveld, P. M., Jeekel, J. & van Eijck, C. H. Pro-inflammatory cytokines affect pancreatic carcinoma cell. Endothelial cell interactions. *JOP : Journal of the pancreas* **7**, 454-464 (2006).

87 Campbell, J. J., Qin, S., Bacon, K. B., Mackay, C. R. & Butcher, E. C. Biology of chemokine and classical chemoattractant receptors: differential requirements for adhesion-triggering versus chemotactic responses in lymphoid cells. *J Cell Biol* **134**, 255-266 (1996).

88 Campbell, J. J. *et al.* Chemokines and the arrest of lymphocytes rolling under flow conditions. *Science* **279**, 381-384 (1998).

89 Ley, K., Laudanna, C., Cybulsky, M. I. & Nourshargh, S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nature reviews. Immunology* **7**, 678-689, doi:10.1038/nri2156 (2007).

90 Pober, J. S. & Cotran, R. S. The role of endothelial cells in inflammation. *Transplantation* **50**, 537-544 (1990).

91 Luo, B. H., Carman, C. V. & Springer, T. A. Structural basis of integrin regulation and signaling. *Annual review of immunology* **25**, 619-647, doi:10.1146/annurev.immunol.25.022106.141618 (2007).

92 La, M. *et al.* A Novel Biological Activity for Galectin-1. *The American journal of pathology* **163**, 1505-1515, doi:10.1016/s0002-9440(10)63507-9 (2003).

93 Nieminen, J., St-Pierre, C., Bhaumik, P., Poirier, F. & Sato, S. Role of Galectin-3 in Leukocyte Recruitment in a Murine Model of Lung Infection by Streptococcus pneumoniae. *The Journal of Immunology* **180**, 2466-2473, doi:10.4049/jimmunol.180.4.2466 (2008).

94 Zhu, C. *et al.* The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nature immunology* **6**, 1245-1252, doi:10.1038/ni1271 (2005).

95 Humphries, J. D., Byron, A. & Humphries, M. J. Integrin ligands at a glance. *J Cell Sci* **119**, 3901-3903, doi:10.1242/jcs.03098 (2006).

96 Aoudjit, F. & Vuori, K. Integrin signaling in cancer cell survival and chemoresistance. *Chemother Res Pract* **2012**, 283181, doi:10.1155/2012/283181 (2012).

97 Aoudjit, F. & Vuori, K. Integrin signaling inhibits paclitaxel-induced apoptosis in breast cancer cells. *Oncogene* **20**, 4995-5004, doi:10.1038/sj.onc.1204554 (2001).

98 Okahara, H., Yagita, H., Miyake, K. & Okumura, K. Involvement of very late activation antigen 4 (VLA-4) and vascular cell adhesion molecule 1 (VCAM-1) in tumor necrosis factor alpha enhancement of experimental metastasis. *Cancer Res* **54**, 3233-3236 (1994).

99 Garofalo, A. *et al.* Involvement of the very late antigen 4 integrin on melanoma in interleukin 1-augmented experimental metastases. *Cancer Res* **55**, 414-419 (1995).

100 Stroeken, P. J., van Rijthoven, E. A., van der Valk, M. A. & Roos, E. Targeted disruption of the beta1 integrin gene in a lymphoma cell line greatly reduces metastatic capacity. *Cancer Res* **58**, 1569-1577 (1998).

101 Klemke, M., Weschenfelder, T., Konstandin, M. H. & Samstag, Y. High affinity interaction of integrin alpha4beta1 (VLA-4) and vascular cell adhesion molecule 1 (VCAM-1) enhances migration of human melanoma cells across activated endothelial cell layers. *Journal of cellular physiology* **212**, 368-374, doi:10.1002/jcp.21029 (2007).

102 Liang, S. & Dong, C. Integrin VLA-4 enhances sialyl-Lewisx/a-negative melanoma adhesion to and extravasation through the endothelium under low flow conditions. *American journal of physiology. Cell physiology* **295**, C701-707, doi:10.1152/ajpcell.00245.2008 (2008).

103 Nangia-Makker, P. *et al.* Galectin-3 and L1 retrotransposons in human breast carcinomas. *Breast cancer research and treatment* **49**, 171-183 (1998).

104 Liu, F. T. & Rabinovich, G. A. Galectins as modulators of tumour progression. *Nat Rev Cancer* **5**, 29-41, doi:10.1038/nrc1527 (2005).

105 Yu, L. G. *et al.* Galectin-3 interaction with Thomsen-Friedenreich disaccharide on cancer-associated MUC1 causes increased cancer cell endothelial adhesion. *The Journal of biological chemistry* **282**, 773-781, doi:10.1074/jbc.M606862200 (2007).

106 Yu, L. G. The oncofetal Thomsen-Friedenreich carbohydrate antigen in cancer progression. *Glycoconjugate journal* **24**, 411-420, doi:10.1007/s10719-007-9034-3 (2007).

107 Fonseca, I. *et al.* Simple mucin-type carbohydrate antigens (T, Tn and sialosyl-Tn) in

mucoepidermoid carcinoma of the salivary glands. *Histopathology* **25**, 537-543 (1994).
108 Springer, G. F., Desai, P. R., Ghazizadeh, M. & Tegtmeyer, H. T/Tn pancarcinoma autoantigens: fundamental, diagnostic, and prognostic aspects. *Cancer detection and prevention* **19**, 173-182 (1995).
109 Glinsky, V. V., Huflejt, M. E., Glinsky, G. V., Deutscher, S. L. & Quinn, T. P. Effects of Thomsen-Friedenreich antigen-specific peptide P-30 on beta-galactoside-mediated homotypic aggregation and adhesion to the endothelium of MDA-MB-435 human breast carcinoma cells. *Cancer Res* **60**, 2584-2588 (2000).
110 Glinsky, V. V. *et al.* The role of Thomsen-Friedenreich antigen in adhesion of human breast and prostate cancer cells to the endothelium. *Cancer Res* **61**, 4851-4857 (2001).
111 Khaldoyanidi, S. K. *et al.* MDA-MB-435 human breast carcinoma cell homo- and heterotypic adhesion under flow conditions is mediated in part by Thomsen-Friedenreich antigen-galectin-3 interactions. *The Journal of biological chemistry* **278**, 4127-4134, doi:10.1074/jbc.M209590200 (2003).
112 Sarafian, V. *et al.* Expression of Lamp-1 and Lamp-2 and their interactions with galectin-3 in human tumor cells. *International journal of cancer* **75**, 105-111 (1998).
113 Boukerche, H. *et al.* Platelet-melanoma cell interaction is mediated by the glycoprotein IIb-IIIa complex. *Blood* **74**, 658-663 (1989).
114 Nierodzik, M. L., Klepfish, A. & Karpatkin, S. Role of platelets, thrombin, integrin IIb-IIIa, fibronectin and von Willebrand factor on tumor adhesion in vitro and metastasis in vivo. *Thrombosis and haemostasis* **74**, 282-290 (1995).
115 Felding-Habermann, B., Habermann, R., Saldivar, E. & Ruggeri, Z. M. Role of beta3 integrins in melanoma cell adhesion to activated platelets under flow. *The Journal of biological chemistry* **271**, 5892-5900 (1996).
116 Felding-Habermann, B. *et al.* Integrin activation controls metastasis in human breast cancer. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 1853-1858, doi:10.1073/pnas.98.4.1853 (2001).
117 Dardik, R., Savion, N., Kaufmann, Y. & Varon, D. Thrombin promotes platelet-mediated melanoma cell adhesion to endothelial cells under flow conditions: role of platelet glycoproteins P-selectin and GPIIb-IIIA. *British journal of cancer* **77**, 2069-2075 (1998).
118 Petri, B. & Bixel, M. G. Molecular events during leukocyte diapedesis. *The FEBS journal* **273**, 4399-4407, doi:10.1111/j.1742-4658.2006.05439.x (2006).
119 Muller, W. A., Ratti, C. M., McDonnell, S. L. & Cohn, Z. A. A human endothelial cell-restricted, externally disposed plasmalemmal protein enriched in intercellular junctions. *J Exp Med* **170**, 399-414 (1989).
120 Newman, P. J. *et al.* PECAM-1 (CD31) cloning and relation to adhesion molecules of the immunoglobulin gene superfamily. *Science* **247**, 1219-1222 (1990).
121 Schenkel, A. R., Mamdouh, Z., Chen, X., Liebman, R. M. & Muller, W. A. CD99 plays a major role in the migration of monocytes through endothelial junctions. *Nature immunology* **3**, 143-150, doi:10.1038/ni749 (2002).
122 Muller, W. A., Weigl, S. A., Deng, X. & Phillips, D. M. PECAM-1 is required for transendothelial migration of leukocytes. *J Exp Med* **178**, 449-460 (1993).
123 Liao, F. *et al.* Migration of monocytes across endothelium and passage through extracellular matrix involve separate molecular domains of PECAM-1. *J Exp Med* **182**, 1337-1343 (1995).
124 Muller, W. A. The role of PECAM-1 (CD31) in leukocyte emigration: studies in vitro and in vivo. *Journal of leukocyte biology* **57**, 523-528 (1995).
125 Bird, I. N., Spragg, J. H., Ager, A. & Matthews, N. Studies of lymphocyte transendothelial migration: analysis of migrated cell phenotypes with regard to CD31 (PECAM-1), CD45RA and CD45RO. *Immunology* **80**, 553-560 (1993).
126 Lou, O., Alcaide, P., Luscinskas, F. W. & Muller, W. A. CD99 Is a Key Mediator of the Transendothelial Migration of Neutrophils. *The Journal of Immunology* **178**, 1136-1143, doi:10.4049/jimmunol.178.2.1136 (2007).
127 Bixel, M. G. *et al.* A CD99-related antigen on endothelial cells mediates neutrophil but not lymphocyte extravasation in vivo. *Blood* **109**, 5327-5336, doi:10.1182/blood-2006-08-043109 (2007).

128 Vestweber, D. Adhesion and signaling molecules controlling the transmigration of leukocytes through endothelium. *Immunological reviews* **218**, 178-196, doi:10.1111/j.1600-065X.2007.00533.x (2007).

129 Ostermann, G., Weber, K. S., Zernecke, A., Schroder, A. & Weber, C. JAM-1 is a ligand of the beta(2) integrin LFA-1 involved in transendothelial migration of leukocytes. *Nature immunology* **3**, 151-158, doi:10.1038/ni755 (2002).

130 Engelhardt, B. & Wolburg, H. Mini-review: Transendothelial migration of leukocytes: through the front door or around the side of the house? *European journal of immunology* **34**, 2955-2963, doi:10.1002/eji.200425327 (2004).

131 Heyder, C. *et al.* Realtime visualization of tumor cell/endothelial cell interactions during transmigration across the endothelial barrier. *Journal of cancer research and clinical oncology* **128**, 533-538, doi:10.1007/s00432-002-0377-7 (2002).

132 Haidari, M. *et al.* Integrin alpha2beta1 mediates tyrosine phosphorylation of vascular endothelial cadherin induced by invasive breast cancer cells. *The Journal of biological chemistry* **287**, 32981-32992, doi:10.1074/jbc.M112.395905 (2012).

133 Tichet, M. *et al.* Tumour-derived SPARC drives vascular permeability and extravasation through endothelial VCAM1 signalling to promote metastasis. *Nature communications* **6**, 6993, doi:10.1038/ncomms7993 (2015).

134 Zhang, P. *et al.* Mutant B-Raf(V600E) Promotes Melanoma Paracellular Transmigration by Inducing Thrombin-mediated Endothelial Junction Breakdown. *The Journal of biological chemistry* **291**, 2087-2106, doi:10.1074/jbc.M115.696419 (2016).

135 Dejana, E. Endothelial cell-cell junctions: happy together. *Nature reviews. Molecular cell biology* **5**, 261-270, doi:10.1038/nrm1357 (2004).

136 Reymond, N., d'Agua, B. B. & Ridley, A. J. Crossing the endothelial barrier during metastasis. *Nat Rev Cancer* **13**, 858-870, doi:10.1038/nrc3628 (2013).

137 Weis, S., Cui, J., Barnes, L. & Cheresh, D. Endothelial barrier disruption by VEGF-mediated Src activity potentiates tumor cell extravasation and metastasis. *J Cell Biol* **167**, 223-229, doi:10.1083/jcb.200408130 (2004).

138 Rousseau, S., Houle, F., Landry, J. & Huot, J. p38 MAP kinase activation by vascular endothelial growth factor mediates actin reorganization and cell migration in human endothelial cells. *Oncogene* **15**, 2169-2177, doi:10.1038/sj.onc.1201380 (1997).

139 Bogatcheva, N. V. & Verin, A. D. The role of cytoskeleton in the regulation of vascular endothelial barrier function. *Microvasc Res* **76**, 202-207, doi:10.1016/j.mvr.2008.06.003 (2008).

140 Li, Y. H. & Zhu, C. A modified Boyden chamber assay for tumor cell transendothelial migration in vitro. *Clinical & experimental metastasis* **17**, 423-429 (1999).

141 Mueller, B. M., Reisfeld, R. A., Edgington, T. S. & Ruf, W. Expression of tissue factor by melanoma cells promotes efficient hematogenous metastasis. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 11832-11836 (1992).

142 Rabiet, M. J. *et al.* Thrombin-Induced Increase in Endothelial Permeability Is Associated With Changes in Cell-to-Cell Junction Organization. *Arteriosclerosis, Thrombosis, and Vascular Biology* **16**, 488-496, doi:10.1161/01.atv.16.3.488 (1996).

143 Luster, A. D. Chemokines--chemotactic cytokines that mediate inflammation. *The New England journal of medicine* **338**, 436-445, doi:10.1056/NEJM199802123380706 (1998).

144 Murphy, P. M. The molecular biology of leukocyte chemoattractant receptors. *Annual review of immunology* **12**, 593-633, doi:10.1146/annurev.iy.12.040194.003113 (1994).

145 Premack, B. A. & Schall, T. J. Chemokine receptors: gateways to inflammation and infection. *Nature medicine* **2**, 1174-1178 (1996).

146 Baggiolini, M., Dewald, B. & Moser, B. Interleukin-8 and related chemotactic cytokines--CXC and CC chemokines. *Advances in immunology* **55**, 97-179 (1994).

147 Garcia-Zepeda, E. A. *et al.* Human monocyte chemoattractant protein (MCP)-4 is a novel CC chemokine with activities on monocytes, eosinophils, and basophils induced in allergic and nonallergic inflammation that signals through the CC chemokine receptors (CCR)-2 and -3. *Journal of immunology* **157**, 5613-5626 (1996).

148 Garcia-Zepeda, E. A. *et al.* Human eotaxin is a specific chemoattractant for eosinophil cells and provides a new mechanism to explain tissue eosinophilia. *Nature medicine* **2**, 449-456 (1996).
149 Kaplanski, G. *et al.* Interleukin-1 induces interleukin-8 secretion from endothelial cells by a juxtacrine mechanism. *Blood* **84**, 4242-4248 (1994).
150 Middleton, J. *et al.* Transcytosis and surface presentation of IL-8 by venular endothelial cells. *Cell* **91**, 385-395 (1997).
151 Chollet-Martin, S. *et al.* High levels of interleukin-8 in the blood and alveolar spaces of patients with pneumonia and adult respiratory distress syndrome. *Infection and immunity* **61**, 4553-4559 (1993).
152 Reinecker, H. C. *et al.* Monocyte-chemoattractant protein 1 gene expression in intestinal epithelial cells and inflammatory bowel disease mucosa. *Gastroenterology* **108**, 40-50 (1995).
153 Grimm, M. C. & Doe, W. F. Chemokines in Inflammatory Bowel Disease Mucosa: Expression of RANTES, Macrophage Inflammatory Protein (MIP)-1alpha, MIP-1beta, and gamma-Interferon-Inducible Protein-10 by Macrophages, Lymphocytes, Endothelial Cells, and Granulomas. *Inflammatory bowel diseases* **2**, 88-96 (1996).
154 Kakinuma, T. & Hwang, S. T. Chemokines, chemokine receptors, and cancer metastasis. *Journal of leukocyte biology* **79**, 639-651, doi:10.1189/jlb.1105633 (2006).
155 Rollins, B. J. Inflammatory chemokines in cancer growth and progression. *Eur J Cancer* **42**, 760-767, doi:10.1016/j.ejca.2006.01.002 (2006).
156 Marchesi, F. *et al.* Increased survival, proliferation, and migration in metastatic human pancreatic tumor cells expressing functional CXCR4. *Cancer Res* **64**, 8420-8427, doi:10.1158/0008-5472.CAN-04-1343 (2004).
157 Campbell, A. S., Albo, D., Kimsey, T. F., White, S. L. & Wang, T. N. Macrophage inflammatory protein-3alpha promotes pancreatic cancer cell invasion. *The Journal of surgical research* **123**, 96-101, doi:10.1016/j.jss.2004.07.013 (2005).
158 Jones, D. H. *et al.* Regulation of cancer cell migration and bone metastasis by RANKL. *Nature* **440**, 692-696, doi:10.1038/nature04524 (2006).
159 Lee, B. C., Lee, T. H., Avraham, S. & Avraham, H. K. Involvement of the chemokine receptor CXCR4 and its ligand stromal cell-derived factor 1alpha in breast cancer cell migration through human brain microvascular endothelial cells. *Molecular cancer research : MCR* **2**, 327-338 (2004).
160 Burger, J. A. & Kipps, T. J. CXCR4: a key receptor in the crosstalk between tumor cells and their microenvironment. *Blood* **107**, 1761-1767, doi:10.1182/blood-2005-08-3182 (2006).
161 Kang, H. *et al.* Stromal cell derived factor-1: its influence on invasiveness and migration of breast cancer cells in vitro, and its association with prognosis and survival in human breast cancer. *Breast cancer research : BCR* **7**, R402-410, doi:10.1186/bcr1022 (2005).
162 Mochizuki, H. *et al.* Interaction of ligand-receptor system between stromal-cell-derived factor-1 and CXC chemokine receptor 4 in human prostate cancer: a possible predictor of metastasis. *Biochemical and biophysical research communications* **320**, 656-663, doi:10.1016/j.bbrc.2004.06.013 (2004).
163 Wang, J. *et al.* Diverse signaling pathways through the SDF-1/CXCR4 chemokine axis in prostate cancer cell lines leads to altered patterns of cytokine secretion and angiogenesis. *Cellular signalling* **17**, 1578-1592, doi:10.1016/j.cellsig.2005.03.022 (2005).
164 Brueckmann, M. & Borggrefe, M. Therapeutic potential of fractalkine: a novel approach to metastatic colon cancer. *Gut* **56**, 314-316, doi:10.1136/gut.2006.103317 (2007).
165 Kollmar, O. *et al.* Liver resection-associated macrophage inflammatory protein-2 stimulates engraftment but not growth of colorectal metastasis at extrahepatic sites. *The Journal of surgical research* **145**, 295-302, doi:10.1016/j.jss.2007.02.010 (2008).
166 Schimanski, C. C. *et al.* Effect of chemokine receptors CXCR4 and CCR7 on the metastatic behavior of human colorectal cancer. *Clin Cancer Res* **11**, 1743-1750, doi:10.1158/1078-0432.CCR-04-1195 (2005).

167 Kim, J. *et al.* Chemokine receptor CXCR4 expression in patients with melanoma and colorectal cancer liver metastases and the association with disease outcome. *Annals of surgery* **244**, 113-120, doi:10.1097/01.sla.0000217690.65909.9c (2006).

168 Longo-Imedio, M. I., Longo, N., Trevino, I., Lazaro, P. & Sanchez-Mateos, P. Clinical significance of CXCR3 and CXCR4 expression in primary melanoma. *International journal of cancer* **117**, 861-865, doi:10.1002/ijc.21269 (2005).

169 Muller, A. *et al.* Involvement of chemokine receptors in breast cancer metastasis. *Nature* **410**, 50-56, doi:10.1038/35065016 (2001).

170 Li, Y. M. *et al.* Upregulation of CXCR4 is essential for HER2-mediated tumor metastasis. *Cancer Cell* **6**, 459-469, doi:10.1016/j.ccr.2004.09.027 (2004).

171 Dittmar, T. *et al.* Induction of cancer cell migration by epidermal growth factor is initiated by specific phosphorylation of tyrosine 1248 of c-erbB-2 receptor via EGFR. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **16**, 1823-1825, doi:10.1096/fj.02-0096fje (2002).

172 Sun, Y. X. *et al.* Skeletal localization and neutralization of the SDF-1(CXCL12)/CXCR4 axis blocks prostate cancer metastasis and growth in osseous sites in vivo. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* **20**, 318-329, doi:10.1359/JBMR.041109 (2005).

173 Zeelenberg, I. S., Ruuls-Van Stalle, L. & Roos, E. The chemokine receptor CXCR4 is required for outgrowth of colon carcinoma micrometastases. *Cancer Res* **63**, 3833-3839 (2003).

174 Kim, J. *et al.* Chemokine receptor CXCR4 expression in colorectal cancer patients increases the risk for recurrence and for poor survival. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **23**, 2744-2753, doi:10.1200/JCO.2005.07.078 (2005).

175 Murakami, T. *et al.* Expression of CXC chemokine receptor-4 enhances the pulmonary metastatic potential of murine B16 melanoma cells. *Cancer Res* **62**, 7328-7334 (2002).

176 Cardones, A. R., Murakami, T. & Hwang, S. T. CXCR4 enhances adhesion of B16 tumor cells to endothelial cells in vitro and in vivo via beta(1) integrin. *Cancer Res* **63**, 6751-6757 (2003).

177 Sun, Y. X. *et al.* Expression and activation of alpha v beta 3 integrins by SDF-1/CXC12 increases the aggressiveness of prostate cancer cells. *The Prostate* **67**, 61-73, doi:10.1002/pros.20500 (2007).

178 Kukreja, P., Abdel-Mageed, A. B., Mondal, D., Liu, K. & Agrawal, K. C. Up-regulation of CXCR4 expression in PC-3 cells by stromal-derived factor-1alpha (CXCL12) increases endothelial adhesion and transendothelial migration: role of MEK/ERK signaling pathway-dependent NF-kappaB activation. *Cancer Res* **65**, 9891-9898, doi:10.1158/0008-5472.CAN-05-1293 (2005).

179 Engl, T. *et al.* CXCR4 chemokine receptor mediates prostate tumor cell adhesion through alpha5 and beta3 integrins. *Neoplasia* **8**, 290-301, doi:10.1593/neo.05694 (2006).

180 Kawada, K. *et al.* Chemokine receptor CXCR3 promotes colon cancer metastasis to lymph nodes. *Oncogene* **26**, 4679-4688, doi:10.1038/sj.onc.1210267 (2007).

181 Robledo, M. M. *et al.* Expression of functional chemokine receptors CXCR3 and CXCR4 on human melanoma cells. *The Journal of biological chemistry* **276**, 45098-45105, doi:10.1074/jbc.M106912200 (2001).

182 Kawada, K. *et al.* Pivotal role of CXCR3 in melanoma cell metastasis to lymph nodes. *Cancer Res* **64**, 4010-4017, doi:10.1158/0008-5472.CAN-03-1757 (2004).

183 Ghosh, S. & Hayden, M. S. New regulators of NF-kappaB in inflammation. *Nature reviews. Immunology* **8**, 837-848, doi:10.1038/nri2423 (2008).

184 Mankan, A. K., Lawless, M. W., Gray, S. G., Kelleher, D. & McManus, R. NF-kappaB regulation: the nuclear response. *Journal of cellular and molecular medicine* **13**, 631-643, doi:10.1111/j.1582-4934.2009.00632.x (2009).

185 Lawrence, T. The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harbor perspectives in biology* **1**, a001651, doi:10.1101/cshperspect.a001651 (2009).

186 Sprague, A. H. & Khalil, R. A. Inflammatory cytokines in vascular dysfunction and

vascular disease. *Biochemical pharmacology* **78**, 539-552, doi:10.1016/j.bcp.2009.04.029 (2009).

187 Blaschke, F., Bruemmer, D. & Law, R. E. Egr-1 is a major vascular pathogenic transcription factor in atherosclerosis and restenosis. *Reviews in endocrine & metabolic disorders* **5**, 249-254, doi:10.1023/B:REMD.0000032413.88756.ee (2004).

188 Aud, D. & Peng, S. L. Mechanisms of disease: Transcription factors in inflammatory arthritis. *Nature clinical practice. Rheumatology* **2**, 434-442, doi:10.1038/ncprheum0222 (2006).

189 Ware, L. B. & Matthay, M. A. The acute respiratory distress syndrome. *The New England journal of medicine* **342**, 1334-1349, doi:10.1056/NEJM200005043421806 (2000).

190 Abdel-Latif, M. M. *et al.* Helicobacter pylori extract induces nuclear factor-kappa B, activator protein-1, and cyclooxygenase-2 in esophageal epithelial cells. *Journal of gastrointestinal surgery : official journal of the Society for Surgery of the Alimentary Tract* **10**, 551-562, doi:10.1016/j.gassur.2005.09.003 (2006).

191 Scheidereit, C. IkappaB kinase complexes: gateways to NF-kappaB activation and transcription. *Oncogene* **25**, 6685-6705, doi:10.1038/sj.onc.1209934 (2006).

192 Hacker, H. & Karin, M. Regulation and function of IKK and IKK-related kinases. *Science's STKE : signal transduction knowledge environment* **2006**, re13, doi:10.1126/stke.3572006re13 (2006).

193 Hoffmann, A., Natoli, G. & Ghosh, G. Transcriptional regulation via the NF-kappaB signaling module. *Oncogene* **25**, 6706-6716, doi:10.1038/sj.onc.1209933 (2006).

194 Hayden, M. S. & Ghosh, S. Shared principles in NF-kappaB signaling. *Cell* **132**, 344-362, doi:10.1016/j.cell.2008.01.020 (2008).

195 Nolan, G. P. *et al.* The bcl-3 proto-oncogene encodes a nuclear I kappa B-like molecule that preferentially interacts with NF-kappa B p50 and p52 in a phosphorylation-dependent manner. *Molecular and cellular biology* **13**, 3557-3566 (1993).

196 Massoumi, R., Chmielarska, K., Hennecke, K., Pfeifer, A. & Fassler, R. Cyld inhibits tumor cell proliferation by blocking Bcl-3-dependent NF-kappaB signaling. *Cell* **125**, 665-677, doi:10.1016/j.cell.2006.03.041 (2006).

197 Fiorini, E. *et al.* Peptide-Induced Negative Selection of Thymocytes Activates Transcription of an NF-KB Inhibitor. *Molecular Cell* **9**, 637-648, doi:10.1016/s1097-2765(02)00469-0 (2002).

198 Kuwata, H. *et al.* IkappaBNS inhibits induction of a subset of Toll-like receptor-dependent genes and limits inflammation. *Immunity* **24**, 41-51, doi:10.1016/j.immuni.2005.11.004 (2006).

199 Liu, Y. C., Penninger, J. & Karin, M. Immunity by ubiquitylation: a reversible process of modification. *Nature reviews. Immunology* **5**, 941-952, doi:10.1038/nri1731 (2005).

200 Nogales, E. Structural insights into microtubule function. *Annual review of biochemistry* **69**, 277-302, doi:10.1146/annurev.biochem.69.1.277 (2000).

201 Jordan, M. A. & Wilson, L. Microtubules as a target for anticancer drugs. *Nat Rev Cancer* **4**, 253-265, doi:10.1038/nrc1317 (2004).

202 Stanton, R. A., Gernert, K. M., Nettles, J. H. & Aneja, R. Drugs that target dynamic microtubules: a new molecular perspective. *Medicinal research reviews* **31**, 443-481, doi:10.1002/med.20242 (2011).

203 Akhmanova, A. & Steinmetz, M. O. Control of microtubule organization and dynamics: two ends in the limelight. *Nature reviews. Molecular cell biology* **16**, 711-726, doi:10.1038/nrm4084 (2015).

204 Giannakakou, P., Sackett, D. & Fojo, T. Tubulin/microtubules: still a promising target for new chemotherapeutic agents. *Journal of the National Cancer Institute* **92**, 182-183 (2000).

205 Erickson, H. P. & O'Brien, E. T. Microtubule dynamic instability and GTP hydrolysis. *Annual review of biophysics and biomolecular structure* **21**, 145-166, doi:10.1146/annurev.bb.21.060192.001045 (1992).

206 Pasquier, E. & Kavallaris, M. Microtubules: a dynamic target in cancer therapy. *IUBMB life* **60**, 165-170, doi:10.1002/iub.25 (2008).

207 Brinkley, B. R. Microtubule organizing centers. *Annual review of cell biology* **1**, 145-172, doi:10.1146/annurev.cb.01.110185.001045 (1985).
208 Kirschner, M. W. & Mitchison, T. Microtubule dynamics. *Nature* **324**, 621, doi:10.1038/324621a0 (1986).
209 Burbank, K. S. & Mitchison, T. J. Microtubule dynamic instability. *Current biology : CB* **16**, R516-517, doi:10.1016/j.cub.2006.06.044 (2006).
210 Desai, A. & Mitchison, T. J. Microtubule polymerization dynamics. *Annual review of cell and developmental biology* **13**, 83-117, doi:10.1146/annurev.cellbio.13.1.83 (1997).
211 Valiron, O., Caudron, N. & Job, D. Microtubule dynamics. *Cellular and molecular life sciences : CMLS* **58**, 2069-2084, doi:10.1007/PL00000837 (2001).
212 Mitchison, T. & Kirschner, M. Dynamic instability of microtubule growth. *Nature* **312**, 237-242 (1984).
213 Margolis, R. L. & Wilson, L. Opposite end assembly and disassembly of microtubules at steady state in vitro. *Cell* **13**, 1-8 (1978).
214 Margolis, R. L. & Wilson, L. Microtubule treadmilling: what goes around comes around. *BioEssays : news and reviews in molecular, cellular and developmental biology* **20**, 830-836, doi:10.1002/(SICI)1521-1878(199810)20:10<830::AID-BIES8>3.0.CO;2-N (1998).
215 McIntosh, J. R., Grishchuk, E. L. & West, R. R. Chromosome-microtubule interactions during mitosis. *Annual review of cell and developmental biology* **18**, 193-219, doi:10.1146/annurev.cellbio.18.032002.132412 (2002).
216 Farrell, K. W. Phase dynamics at microtubule ends: the coexistence of microtubule length changes and treadmilling. *The Journal of Cell Biology* **104**, 1035-1046, doi:10.1083/jcb.104.4.1035 (1987).
217 Wilson, L., Panda, D. & Jordan, M. A. Modulation of microtubule dynamics by drugs: a paradigm for the actions of cellular regulators. *Cell structure and function* **24**, 329-335 (1999).
218 Honore, S., Pasquier, E. & Braguer, D. Understanding microtubule dynamics for improved cancer therapy. *Cellular and molecular life sciences : CMLS* **62**, 3039-3056, doi:10.1007/s00018-005-5330-x (2005).
219 Conde, C. & Caceres, A. Microtubule assembly, organization and dynamics in axons and dendrites. *Nature reviews. Neuroscience* **10**, 319-332, doi:10.1038/nrn2631 (2009).
220 Giodini, A. *et al.* Regulation of microtubule stability and mitotic progression by survivin. *Cancer Res* **62**, 2462-2467 (2002).
221 Ligon, L. A., Shelly, S. S., Tokito, M. & Holzbaur, E. L. The microtubule plus-end proteins EB1 and dynactin have differential effects on microtubule polymerization. *Molecular biology of the cell* **14**, 1405-1417, doi:10.1091/mbc.E02-03-0155 (2003).
222 Spittle, C., Charrasse, S., Larroque, C. & Cassimeris, L. The interaction of TOGp with microtubules and tubulin. *The Journal of biological chemistry* **275**, 20748-20753, doi:10.1074/jbc.M002597200 (2000).
223 Galmarini, C. M. *et al.* Drug resistance associated with loss of p53 involves extensive alterations in microtubule composition and dynamics. *British journal of cancer* **88**, 1793-1799, doi:10.1038/sj.bjc.6600960 (2003).
224 Cassimeris, L. The oncoprotein 18/stathmin family of microtubule destabilizers. *Current opinion in cell biology* **14**, 18-24 (2002).
225 Maney, T., Wagenbach, M. & Wordeman, L. Molecular dissection of the microtubule depolymerizing activity of mitotic centromere-associated kinesin. *The Journal of biological chemistry* **276**, 34753-34758, doi:10.1074/jbc.M106626200 (2001).
226 Johnson, I. S., Armstrong, J. G., Gorman, M. & Burnett, J. P., Jr. The Vinca Alkaloids: A New Class of Oncolytic Agents. *Cancer Res* **23**, 1390-1427 (1963).
227 Jordan, M. A., Thrower, D. & Wilson, L. Mechanism of inhibition of cell proliferation by Vinca alkaloids. *Cancer Res* **51**, 2212-2222 (1991).
228 Gupta, S. & Bhattacharyya, B. Antimicrotubular drugs binding to vinca domain of tubulin. *Molecular and cellular biochemistry* **253**, 41-47 (2003).
229 Slobodnick, A., Shah, B., Pillinger, M. H. & Krasnokutsky, S. Colchicine: old and new.

The American journal of medicine **128**, 461-470, doi:10.1016/j.amjmed.2014.12.010 (2015).

230 Francis, P. A., Kris, M. G., Rigas, J. R., Grant, S. C. & Miller, V. A. Paclitaxel (Taxol) and docetaxel (Taxotere): active chemotherapeutic agents in lung cancer. *Lung cancer* **12 Suppl 1**, S163-172 (1995).

231 Blagosklonny, M. V. & Fojo, T. Molecular effects of paclitaxel: myths and reality (a critical review). *International journal of cancer* **83**, 151-156 (1999).

232 Gerth, K., Bedorf, N., Hofle, G., Irschik, H. & Reichenbach, H. Epothilons A and B: antifungal and cytotoxic compounds from Sorangium cellulosum (Myxobacteria). Production, physico-chemical and biological properties. *J Antibiot (Tokyo)* **49**, 560-563 (1996).

233 Goodin, S. Novel cytotoxic agents: epothilones. *American journal of health-system pharmacy : AJHP : official journal of the American Society of Health-System Pharmacists* **65**, S10-15, doi:10.2146/ajhp080089 (2008).

234 Villanueva, C. *et al.* Ixabepilone: a new active chemotherapy in the treatment of breast cancer. *Women's health* **5**, 115-121, doi:10.2217/17455057.5.2.115 (2009).

235 Jordan, M. A. Mechanism of action of antitumor drugs that interact with microtubules and tubulin. *Current medicinal chemistry. Anti-cancer agents* **2**, 1-17 (2002).

236 Schneiders, U. M., Schyschka, L., Rudy, A. & Vollmar, A. M. BH3-only proteins Mcl-1 and Bim as well as endonuclease G are targeted in spongistatin 1-induced apoptosis in breast cancer cells. *Molecular cancer therapeutics* **8**, 2914-2925, doi:10.1158/1535-7163.MCT-08-1179 (2009).

237 Rothmeier, A. S. *et al.* The marine compound spongistatin 1 targets pancreatic tumor progression and metastasis. *International journal of cancer* **127**, 1096-1105, doi:10.1002/ijc.25241 (2010).

238 Blagosklonny, M. V., Chuman, Y., Bergan, R. C. & Fojo, T. Mitogen-activated protein kinase pathway is dispensable for microtubule-active drug-induced Raf-1/Bcl-2 phosphorylation and apoptosis in leukemia cells. *Leukemia* **13**, 1028-1036 (1999).

239 Antonsson, B. & Martinou, J. C. The Bcl-2 protein family. *Exp Cell Res* **256**, 50-57, doi:10.1006/excr.2000.4839 (2000).

240 Mehlen, P. & Puisieux, A. Metastasis: a question of life or death. *Nat Rev Cancer* **6**, 449-458, doi:10.1038/nrc1886 (2006).

241 Schnaeker, E. M. *et al.* Microtubule-dependent matrix metalloproteinase-2/matrix metalloproteinase-9 exocytosis: prerequisite in human melanoma cell invasion. *Cancer Res* **64**, 8924-8931, doi:10.1158/0008-5472.CAN-04-0324 (2004).

242 Geho, D. H., Bandle, R. W., Clair, T. & Liotta, L. A. Physiological mechanisms of tumor-cell invasion and migration. *Physiology (Bethesda)* **20**, 194-200, doi:10.1152/physiol.00009.2005 (2005).

243 Kaur, G. *et al.* Biological evaluation of tubulysin A: a potential anticancer and antiangiogenic natural product. *The Biochemical journal* **396**, 235-242, doi:10.1042/BJ20051735 (2006).

244 Kanthou, C. & Tozer, G. M. Microtubule depolymerizing vascular disrupting agents: novel therapeutic agents for oncology and other pathologies. *International journal of experimental pathology* **90**, 284-294, doi:10.1111/j.1365-2613.2009.00651.x (2009).

245 Leung, Y. Y., Yao Hui, L. L. & Kraus, V. B. Colchicine--Update on mechanisms of action and therapeutic uses. *Seminars in arthritis and rheumatism* **45**, 341-350, doi:10.1016/j.semarthrit.2015.06.013 (2015).

246 Martinon, F., Petrilli, V., Mayor, A., Tardivel, A. & Tschopp, J. Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* **440**, 237-241, doi:10.1038/nature04516 (2006).

247 Cronstein, B. N. *et al.* Colchicine alters the quantitative and qualitative display of selectins on endothelial cells and neutrophils. *The Journal of clinical investigation* **96**, 994-1002, doi:10.1172/JCI118147 (1995).

248 Paschke, S. *et al.* Technical advance: Inhibition of neutrophil chemotaxis by colchicine is modulated through viscoelastic properties of subcellular compartments. *Journal of leukocyte biology* **94**, 1091-1096, doi:10.1189/jlb.1012510 (2013).

249 Gagne, V. *et al.* Modulation of monosodium urate crystal-induced responses in neutrophils by the myeloid inhibitory C-type lectin-like receptor: potential therapeutic implications. *Arthritis research & therapy* **15**, R73, doi:10.1186/ar4250 (2013).

250 Zhou, J., Zhong, D. W., Wang, Q. W., Miao, X. Y. & Xu, X. D. Paclitaxel ameliorates fibrosis in hepatic stellate cells via inhibition of TGF-beta/Smad activity. *World journal of gastroenterology* **16**, 3330-3334 (2010).

251 Sun, L. *et al.* Low-dose paclitaxel ameliorates fibrosis in the remnant kidney model by down-regulating miR-192. *J Pathol* **225**, 364-377, doi:10.1002/path.2961 (2011).

252 Mirzapoiazova, T. *et al.* Suppression of endotoxin-induced inflammation by taxol. *The European respiratory journal* **30**, 429-435, doi:10.1183/09031936.00154206 (2007).

253 Tepe, G. *et al.* Local delivery of paclitaxel to inhibit restenosis during angioplasty of the leg. *The New England journal of medicine* **358**, 689-699, doi:10.1056/NEJMoa0706356 (2008).

254 Stone, G. W. *et al.* A polymer-based, paclitaxel-eluting stent in patients with coronary artery disease. *The New England journal of medicine* **350**, 221-231, doi:10.1056/NEJMoa032441 (2004).

255 Zhang, D. *et al.* Low-dose paclitaxel ameliorates renal fibrosis in rat UUO model by inhibition of TGF-beta/Smad activity. *Laboratory investigation; a journal of technical methods and pathology* **90**, 436-447, doi:10.1038/labinvest.2009.149 (2010).

256 Roberts, R. L., Nath, J., Friedman, M. M. & Gallin, J. I. Effects of taxol on human neutrophils. *Journal of immunology* **129**, 2134-2141 (1982).

257 Petrache, I., Birukova, A., Ramirez, S. I., Garcia, J. G. & Verin, A. D. The role of the microtubules in tumor necrosis factor-alpha-induced endothelial cell permeability. *American journal of respiratory cell and molecular biology* **28**, 574-581, doi:10.1165/rcmb.2002-0075OC (2003).

258 Birukova, A. A. *et al.* Microtubule disassembly induces cytoskeletal remodeling and lung vascular barrier dysfunction: role of Rho-dependent mechanisms. *Journal of cellular physiology* **201**, 55-70, doi:10.1002/jcp.20055 (2004).

259 Kielbassa, K., Schmitz, C. & Gerke, V. Disruption of endothelial microfilaments selectively reduces the transendothelial migration of monocytes. *Exp Cell Res* **243**, 129-141, doi:10.1006/excr.1998.4133 (1998).

260 Gidding, C. E., Kellie, S. J., Kamps, W. A. & de Graaf, S. S. Vincristine revisited. *Critical reviews in oncology/hematology* **29**, 267-287 (1999).

261 Markman, M. Managing taxane toxicities. *Supportive care in cancer : official journal of the Multinational Association of Supportive Care in Cancer* **11**, 144-147, doi:10.1007/s00520-002-0405-9 (2003).

262 Carlson, K. & Ocean, A. J. Peripheral neuropathy with microtubule-targeting agents: occurrence and management approach. *Clinical breast cancer* **11**, 73-81, doi:10.1016/j.clbc.2011.03.006 (2011).

263 Dumontet, C. & Jordan, M. A. Microtubule-binding agents: a dynamic field of cancer therapeutics. *Nature reviews. Drug discovery* **9**, 790-803, doi:10.1038/nrd3253 (2010).

264 Breuninger, L. M. *et al.* Expression of multidrug resistance-associated protein in NIH/3T3 cells confers multidrug resistance associated with increased drug efflux and altered intracellular drug distribution. *Cancer Res* **55**, 5342-5347 (1995).

265 Huisman, M. T., Chhatta, A. A., van Tellingen, O., Beijnen, J. H. & Schinkel, A. H. MRP2 (ABCC2) transports taxanes and confers paclitaxel resistance and both processes are stimulated by probenecid. *International journal of cancer* **116**, 824-829, doi:10.1002/ijc.21013 (2005).

266 Hopper-Borge, E., Chen, Z. S., Shchaveleva, I., Belinsky, M. G. & Kruh, G. D. Analysis of the drug resistance profile of multidrug resistance protein 7 (ABCC10): resistance to docetaxel. *Cancer Res* **64**, 4927-4930, doi:10.1158/0008-5472.CAN-03-3111 (2004).

267 Anderson, H. L. *et al.* Assessment of pharmacodynamic vascular response in a phase I trial of combretastatin A4 phosphate. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **21**, 2823-2830, doi:10.1200/JCO.2003.05.186 (2003).

268 Kavallaris, M. *et al.* Multiple microtubule alterations are associated with Vinca alkaloid

resistance in human leukemia cells. *Cancer Res* **61**, 5803-5809 (2001).
269 Sève, P. & Dumontet, C. Is class III β-tubulin a predictive factor in patients receiving tubulin-binding agents? *The Lancet Oncology* **9**, 168-175, doi:10.1016/s1470-2045(08)70029-9 (2008).
270 Dumontet, C., Jordan, M. A. & Lee, F. F. Ixabepilone: targeting betaIII-tubulin expression in taxane-resistant malignancies. *Molecular cancer therapeutics* **8**, 17-25, doi:10.1158/1535-7163.MCT-08-0986 (2009).
271 Sasse, F., Steinmetz, H., Heil, J., Höfle, G. & Reichenbach, H. Tubulysins, new cytostatic peptides from myxobacteria acting on microtubuli. Production, isolation, physico-chemical and biological properties. *J Antibiot (Tokyo)* **53**, 879-885 (2000).
272 Steinmetz, H. *et al.* Isolation, crystal and solution structure determination, and biosynthesis of tubulysins--powerful inhibitors of tubulin polymerization from myxobacteria. *Angew Chem Int Ed Engl* **43**, 4888-4892, doi:10.1002/anie.200460147 (2004).
273 Khalil, M. W., Sasse, F., Lünsdorf, H., Elnakady, Y. A. & Reichenbach, H. Mechanism of action of tubulysin, an antimitotic peptide from myxobacteria. *Chembiochem* **7**, 678-683, doi:10.1002/cbic.200500421 (2006).
274 Chai, Y. *et al.* Discovery of 23 natural tubulysins from Angiococcus disciformis An d48 and Cystobacter SBCb004. *Chem Biol* **17**, 296-309, doi:10.1016/j.chembiol.2010.01.016 (2010).
275 Kazmaier, U., Ullrich, A. & Hoffmann, J. Synthetic approaches towards tubulysins and derivatives thereof. *Open Nat Prod J* **6**, 12-30 (2013).
276 Murray, B. C., Peterson, M. T. & Fecik, R. A. Chemistry and biology of tubulysins: antimitotic tetrapeptides with activity against drug resistant cancers. *Nat Prod Rep* **32**, 654-662, doi:10.1039/c4np00036f (2015).
277 Nicolaou, K. C. *et al.* Total Synthesis and Biological Evaluation of Natural and Designed Tubulysins. *J Am Chem Soc* **138**, 1698-1708, doi:10.1021/jacs.5b12557 (2016).
278 Sandmann, A., Sasse, F. & Müller, R. Identification and analysis of the core biosynthetic machinery of tubulysin, a potent cytotoxin with potential anticancer activity. *Chem Biol* **11**, 1071-1079, doi:10.1016/j.chembiol.2004.05.014 (2004).
279 Ullrich, A. *et al.* Pretubulysin, a potent and chemically accessible tubulysin precursor from Angiococcus disciformis. *Angew Chem Int Ed Engl* **48**, 4422-4425, doi:10.1002/anie.200900406 (2009).
280 Herrmann, J. *et al.* Pretubulysin: from hypothetical biosynthetic intermediate to potential lead in tumor therapy. *PLoS One* **7**, e37416, doi:10.1371/journal.pone.0037416 (2012).
281 Rath, S. *et al.* Anti-angiogenic effects of the tubulysin precursor pretubulysin and of simplified pretubulysin derivatives. *Br J Pharmacol* **167**, 1048-1061, doi:10.1111/j.1476-5381.2012.02037.x (2012).
282 Braig, S. *et al.* Pretubulysin: a new option for the treatment of metastatic cancer. *Cell Death Dis* **5**, e1001, doi:10.1038/cddis.2013.510 (2014).
283 Kretzschmann, V. K. *et al.* Novel tubulin antagonist pretubulysin displays antivascular properties in vitro and in vivo. *Arterioscler Thromb Vasc Biol* **34**, 294-303, doi:10.1161/ATVBAHA.113.302155 (2014).
284 Schwenk, R. *et al.* The pretubulysin-induced exposure of collagen is caused by endothelial cell retraction that results in an increased adhesion and decreased transmigration of tumor cells. *Oncotarget* **8**, 77622-77633, doi:10.18632/oncotarget.20746 (2017).
285 Ades, E. W. *et al.* HMEC-1: Establishment of an Immortalized Human Microvascular Endothelial Cell Line. *Journal of Investigative Dermatology* **99**, 683-690, doi:10.1111/1523-1747.ep12613748 (1992).
286 Cailleau, R., Olive, M. & Cruciger, Q. V. Long-term human breast carcinoma cell lines of metastatic origin: preliminary characterization. *In vitro* **14**, 911-915 (1978).
287 Cailleau, R., Young, R., Olive, M. & Reeves, W. J., Jr. Breast tumor cell lines from pleural effusions. *Journal of the National Cancer Institute* **53**, 661-674 (1974).
288 Tsuchiya, S. *et al.* Establishment and characterization of a human acute monocytic

leukemia cell line (THP-1). *International journal of cancer* **26**, 171-176 (1980).
289 Nicoletti, I., Migliorati, G., Pagliacci, M. C., Grignani, F. & Riccardi, C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *Journal of immunological methods* **139**, 271-279 (1991).
290 Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**, 402-408, doi:10.1006/meth.2001.1262 (2001).
291 Towbin, H., Staehelin, T. & Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 4350-4354 (1979).
292 Smith, P. K. *et al.* Measurement of protein using bicinchoninic acid. *Analytical biochemistry* **150**, 76-85 (1985).
293 Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685 (1970).
294 Kurien, B. T. & Scofield, R. H. Protein blotting: a review. *Journal of immunological methods* **274**, 1-15, doi:10.1016/s0022-1759(02)00523-9 (2003).
295 Laerum, O. D. & Farsund, T. Clinical application of flow cytometry: a review. *Cytometry* **2**, 1-13, doi:10.1002/cyto.990020102 (1981).
296 Knapp, W., Strobl, H. & Majdic, O. Flow cytometric analysis of cell-surface and intracellular antigens in leukemia diagnosis. *Cytometry* **18**, 187-198, doi:10.1002/cyto.990180402 (1994).
297 Majumdar, M. K. *et al.* Characterization and functionality of cell surface molecules on human mesenchymal stem cells. *Journal of biomedical science* **10**, 228-241, doi:10.1159/000068710 (2003).
298 Jayat, C. & Ratinaud, M. H. Cell cycle analysis by flow cytometry: principles and applications. *Biology of the cell* **78**, 15-25 (1993).
299 Mesulam, M. M. Tetramethyl benzidine for horseradish peroxidase neurohistochemistry: a non-carcinogenic blue reaction product with superior sensitivity for visualizing neural afferents and efferents. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society* **26**, 106-117, doi:10.1177/26.2.24068 (1978).
300 Naylor, L. H. Reporter gene technology: the future looks bright. *Biochemical pharmacology* **58**, 749-757 (1999).
301 Potter, H. Electroporation in biology: methods, applications, and instrumentation. *Analytical biochemistry* **174**, 361-373 (1988).
302 Weigert, R., Sramkova, M., Parente, L., Amornphimoltham, P. & Masedunskas, A. Intravital microscopy: a novel tool to study cell biology in living animals. *Histochemistry and cell biology* **133**, 481-491, doi:10.1007/s00418-010-0692-z (2010).
303 Reichel, C. A. *et al.* Gelatinases mediate neutrophil recruitment in vivo: evidence for stimulus specificity and a critical role in collagen IV remodeling. *Journal of leukocyte biology* **83**, 864-874, doi:10.1189/jlb.1007666 (2008).
304 Mackay, F., Loetscher, H., Stueber, D., Gehr, G. & Lesslauer, W. Tumor necrosis factor alpha (TNF-alpha)-induced cell adhesion to human endothelial cells is under dominant control of one TNF receptor type, TNF-R55. *J Exp Med* **177**, 1277-1286 (1993).
305 Coussens, L. M. & Werb, Z. Inflammation and cancer. *Nature* **420**, 860-867, doi:10.1038/nature01322 (2002).
306 Cojoc, M. *et al.* Emerging targets in cancer management: role of the CXCL12/CXCR4 axis. *Onco Targets Ther* **6**, 1347-1361, doi:10.2147/OTT.S36109 (2013).
307 Kawaguchi, A. *et al.* Inhibition of the SDF-1alpha-CXCR4 axis by the CXCR4 antagonist AMD3100 suppresses the migration of cultured cells from ATL patients and murine lymphoblastoid cells from HTLV-I Tax transgenic mice. *Blood* **114**, 2961-2968, doi:10.1182/blood-2008-11-189308 (2009).
308 Perico, N. *et al.* Colchicine interferes with L-selectin and leukocyte function-associated antigen-1 expression on human T lymphocytes and inhibits T cell activation. *Journal of the American Society of Nephrology : JASN* **7**, 594-601 (1996).

309 Asahina, A., Tada, Y., Nakamura, K. & Tamaki, K. Colchicine and griseofulvin inhibit VCAM-1 expression on human vascular endothelial cells - evidence for the association of VCAM-1 expression with microtubules. *Journal of dermatological science* **25**, 1-9 (2001).
310 Cuschieri, J., Gourlay, D., Garcia, I., Jelacic, S. & Maier, R. V. Modulation of endotoxin-induced endothelial activity by microtubule depolymerization. *The Journal of trauma* **54**, 104-112; discussion 112-103, doi:10.1097/01.TA.0000046322.77061.65 (2003).
311 Wood, S. C., Tang, X. & Tesfamariam, B. Paclitaxel potentiates inflammatory cytokine-induced prothrombotic molecules in endothelial cells. *Journal of cardiovascular pharmacology* **55**, 276-285, doi:10.1097/FJC.0b013e3181d263f7 (2010).
312 Altmann, K. H. & Gertsch, J. Anticancer drugs from nature--natural products as a unique source of new microtubule-stabilizing agents. *Nat Prod Rep* **24**, 327-357, doi:10.1039/b515619j (2007).
313 Singh, P., Rathinasamy, K., Mohan, R. & Panda, D. Microtubule assembly dynamics: an attractive target for anticancer drugs. *IUBMB life* **60**, 368-375, doi:10.1002/iub.42 (2008).
314 Bezuhly, M. *et al.* Role of activated protein C and its receptor in inhibition of tumor metastasis. *Blood* **113**, 3371-3374, doi:10.1182/blood-2008-05-159434 (2009).
315 Khuon, S. *et al.* Myosin light chain kinase mediates transcellular intravasation of breast cancer cells through the underlying endothelial cells: a three-dimensional FRET study. *J Cell Sci* **123**, 431-440, doi:10.1242/jcs.053793 (2010).
316 Wolf, M. J. *et al.* Endothelial CCR2 signaling induced by colon carcinoma cells enables extravasation via the JAK2-Stat5 and p38MAPK pathway. *Cancer Cell* **22**, 91-105, doi:10.1016/j.ccr.2012.05.023 (2012).
317 Soto, M. S., Serres, S., Anthony, D. C. & Sibson, N. R. Functional role of endothelial adhesion molecules in the early stages of brain metastasis. *Neuro Oncol* **16**, 540-551, doi:10.1093/neuonc/not222 (2014).
318 Rosette, C. & Karin, M. Cytoskeletal control of gene expression: depolymerization of microtubules activates NF-kappa B. *J Cell Biol* **128**, 1111-1119 (1995).
319 Das, K. C. & White, C. W. Activation of NF-kappaB by antineoplastic agents. Role of protein kinase C. *The Journal of biological chemistry* **272**, 14914-14920 (1997).
320 Jung, Y. J., Isaacs, J. S., Lee, S., Trepel, J. & Neckers, L. Microtubule disruption utilizes an NFkappa B-dependent pathway to stabilize HIF-1alpha protein. *The Journal of biological chemistry* **278**, 7445-7452, doi:10.1074/jbc.M209804200 (2003).
321 Huang, Y. *et al.* Regulation of Vinca alkaloid-induced apoptosis by NF-kappaB/IkappaB pathway in human tumor cells. *Molecular cancer therapeutics* **3**, 271-277 (2004).
322 Mistry, P., Deacon, K., Mistry, S., Blank, J. & Patel, R. NF-kappaB promotes survival during mitotic cell cycle arrest. *The Journal of biological chemistry* **279**, 1482-1490, doi:10.1074/jbc.M310413200 (2004).
323 Fernandis, A. Z., Prasad, A., Band, H., Klosel, R. & Ganju, R. K. Regulation of CXCR4-mediated chemotaxis and chemoinvasion of breast cancer cells. *Oncogene* **23**, 157-167, doi:10.1038/sj.onc.1206910 (2004).
324 Liang, Z. *et al.* Silencing of CXCR4 blocks breast cancer metastasis. *Cancer Res* **65**, 967-971 (2005).
325 Song, J. W. *et al.* Microfluidic endothelium for studying the intravascular adhesion of metastatic breast cancer cells. *PLoS One* **4**, e5756, doi:10.1371/journal.pone.0005756 (2009).
326 Gui, G. P., Puddefoot, J. R., Vinson, G. P., Wells, C. A. & Carpenter, R. In vitro regulation of human breast cancer cell adhesion and invasion via integrin receptors to the extracellular matrix. *The British journal of surgery* **82**, 1192-1196 (1995).
327 Lundstrom, A., Holmbom, J., Lindqvist, C. & Nordstrom, T. The role of alpha2 beta1 and alpha3 beta1 integrin receptors in the initial anchoring of MDA-MB-231 human breast cancer cells to cortical bone matrix. *Biochemical and biophysical research communications* **250**, 735-740, doi:10.1006/bbrc.1998.9389 (1998).
328 Ibaragi, S. *et al.* Induction of MMP-13 expression in bone-metastasizing cancer cells by type I collagen through integrin alpha1beta1 and alpha2beta1-p38 MAPK signaling.

Anticancer research **31**, 1307-1313 (2011).

329 Dudley, D. T. *et al.* A 3D matrix platform for the rapid generation of therapeutic anti-human carcinoma monoclonal antibodies. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 14882-14887, doi:10.1073/pnas.1410996111 (2014).

330 Kuijpers, T. W. *et al.* Cytokine-activated endothelial cells internalize E-selectin into a lysosomal compartment of vesiculotubular shape. A tubulin-driven process. *Journal of immunology* **152**, 5060-5069 (1994).

331 Mamdouh, Z., Mikhailov, A. & Muller, W. A. Transcellular migration of leukocytes is mediated by the endothelial lateral border recycling compartment. *J Exp Med* **206**, 2795-2808, doi:10.1084/jem.20082745 (2009).

332 Nakhaei-Nejad, M., Zhang, Q. X. & Murray, A. G. Endothelial IQGAP1 regulates efficient lymphocyte transendothelial migration. *European journal of immunology* **40**, 204-213, doi:10.1002/eji.200839214 (2010).

333 Pober, J. S. *et al.* Activation of cultured human endothelial cells by recombinant lymphotoxin: comparison with tumor necrosis factor and interleukin 1 species. *Journal of immunology* **138**, 3319-3324 (1987).

334 Pober, J. S. Endothelial activation: intracellular signaling pathways. *Arthritis research* **4 Suppl 3**, S109-116, doi:10.1186/ar576 (2002).

335 Sabio, G. & Davis, R. J. TNF and MAP kinase signalling pathways. *Seminars in immunology* **26**, 237-245, doi:10.1016/j.smim.2014.02.009 (2014).

336 Levy, D. E. & Darnell, J. E., Jr. Stats: transcriptional control and biological impact. *Nature reviews. Molecular cell biology* **3**, 651-662, doi:10.1038/nrm909 (2002).

337 O'Shea, J. J. & Plenge, R. JAK and STAT signaling molecules in immunoregulation and immune-mediated disease. *Immunity* **36**, 542-550, doi:10.1016/j.immuni.2012.03.014 (2012).

VIII APPENDIX

1 Declaration

Except where stated otherwise by reference or acknowledgment, the work presented was generated by myself under the supervision of my advisors during my doctoral studies. All contributions from colleagues are explicitly referenced in the thesis. The material listed below was obtained in the context of collaborative research:

Figure 12: PT increases the adhesion of MDA-MB-231 tumor cells onto a HUVEC monolayer, Tanja Stehning, Institute of Pharmaceutical Biology, Goethe University, assay performance (her contribution), data analysis and interpretation, figure preparation (my own contribution)

Figure 14: The enhanced expression of ICAM-1 is not responsible for the PT-evoked tumor cell adhesion, Tanja Stehning, Institute of Pharmaceutical Biology, Goethe University, qRT-PCR performance (her contribution), data analysis and interpretation, figure preparation (my own contribution)

Figure 15: The enhanced expression of VCAM-1 is not responsible for the PT-triggered tumor cell adhesion, Tanja Stehning, Institute of Pharmaceutical Biology, Goethe University, qRT-PCR performance (her contribution), data analysis and interpretation, figure preparation (my own contribution)

Figure 16: The enhanced expression of N-cadherin is not responsible for the PT-evoked tumor cell adhesion, Tanja Stehning, Institute of Pharmaceutical Biology, Goethe University, qRT-PCR and western blot performance (her contribution), data analysis and interpretation, figure preparation (my own contribution)

Figure 17: PT does not influence the surface expression of E-selectin and the mRNA expression of galectin-3, Tanja Stehning, Institute of Pharmaceutical Biology, Goethe University, qRT-PCR performance (her contribution), data analysis and interpretation, figure preparation (my own contribution)

Figure 18: The enhanced expression of CXCL12 is not responsible for the PT-triggered tumor cell adhesion, Tanja Stehning, Institute of Pharmaceutical Biology, Goethe University, qRT-PCR performance (her contribution), data analysis and interpretation, figure preparation (my own contribution)

Figure 22: PT reduces the firm adhesion of leukocytes onto and the transmigration of leukocytes through the TNFα-activated endothelium *in vivo*, Matthias Fabritius, Department of Otorhinolaryngology, Head and Neck Surgery, Walter Brendel Centre of Experimental

Medicine, Clinical center at the University of Munich, assay performance and data analysis (his contribution), data interpretation, figure preparation (my own contribution)

Figure 26: PT, VIN and COL decrease the adhesion of THP-1 cells onto a TNFα-activated HMEC monolayer, Mareike Lang, Institute of Pharmaceutical Biology, Goethe University, crystal violet staining (her contribution), cell adhesion assay performance, data analysis and interpretation, figure preparation (my own contribution)

Figure 29: PT, VIN and COL decrease the TNFα-induced NFκB promotor activity in HMECs, Jennifer Reis, Institute of Pharmaceutical Biology, Goethe University, transformation of *E. coli* with plasmid DNA, reproduction and preparation of plasmid DNA (her contribution), Mareike Lang, Institute of Pharmaceutical Biology, Goethe University, endotoxin removal from plasmid DNA (her contribution), plasmid transfection, sample preparation, reporter assay performance, data analysis and interpretation, figure preparation (my own contribution)

Whenever a figure, table or text is identical to a previous publication, it is stated explicitly in the thesis that copyright permission and/or co-author agreement has been obtained. The following parts of the thesis have been previously published:

Chapter I.2.1, Figure 1: Important steps in the process of inflammation

Chapter I.2.1, Figure 2: Important steps in the process of tumor metastasis

Chapter I.2.4, Figure 6: Crucial steps in the canonical NFκB signaling pathway

Chapter I.3.1, Figure 7: The structure of microtubules

Chapter I.3.2, Figure 8: The dynamic instability of microtubules

Chapter I.4, Figure 9: MTA-binding domains on microtubules

Chapter I.5, Figure 10: The chemical structure of pretubulysin (PT)

Chapter II.1.7, Figure 11: Plasmids used for Dual-Luciferase reporter assays

Chapter III.1.1.1, Figure 12: PT increases the adhesion of MDA-MB-231 tumor cells onto a HUVEC monolayer

Chapter III.1.1.2, Figure 13: PT reduces the transmigration of tumor cells through an endothelial monolayer

Chapter III.1.2.1, Figure 14: The enhanced expression of ICAM-1 is not responsible for the PT-evoked tumor cell adhesion

Chapter III.1.2.2, Figure 15: The enhanced expression of VCAM-1 is not responsible for the PT-triggered tumor cell adhesion

Chapter III.1.2.4, Figure 17: PT does not influence the surface expression of E-selectin and the mRNA expression of galectin-3

Chapter III.1.3.1, Figure 18: The enhanced expression of CXCL12 is not responsible for the PT-triggered tumor cell adhesion

Chapter III.1.4.1, Figure 19: The PT-evoked tumor cell adhesion is based on the exposure of the extracellular matrix protein collagen within endothelial gaps

Chapter III.1.4.2, Figure 20: The effects of PT on tumor-endothelial cell interactions are based on the interaction between β1-integrins on tumor cells and collagen within interendothelial gaps

Chapter III.1.4.3, Figure 21: The effects of PT on tumor-endothelial cell interactions depend on its microtubule-destabilizing activity

2 Publications

2.1 Articles

Schwenk R, Stehning T, Bischoff I, Ullrich A, Kazmaier U, Fürst R. *The pretubulysin-induced exposure of collagen is caused by endothelial cell retraction that results in an increased adhesion and decreased transmigration of tumor cells*. Oncotarget 2017; 8:77622-77633.

2.2 Poster presentations

Schwenk R, Stehning T, Bischoff I, Ullrich A, Kazmaier U, Fürst R. *The anti-metastatic properties of the tubulin-binding agent pretubulysin could be based on the trapping of tumor cells to the endothelium.* 82nd Annual Meeting of the German Society for Experimental and Clinical Pharmacology and Toxicology (DGPT), 18th Annual Meeting of the Clinical Pharmacology (VKliPha) in cooperation with the AGAH, February 29 - March 3, 2016, Berlin, Germany.

Schwenk R, Stehning T, Bischoff I, Ullrich A, Kazmaier U, Fürst R. *The anti-metastatic properties of the tubulin-binding agent pretubulysin could be based on the trapping of tumor cells to the endothelium.* DphG-Annual Meeting 2016, October 5-7, 2016, Munich, German