# The role of β1 integrins in a novel *in vitro* blood-brain barrier model during inflammation

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# 1 Introduction

## 1.1 The blood-brain barrier

### 1.1.1 Function and structure

The blood-brain barrier (BBB) is essential for normal cerebral function by maintaining an optimal chemical environment for the central nervous system (CNS). It protects the CNS from abrupt changes in the blood metabolism, e.g. after meals and physical exercises or during pathological conditions, and regulates the supply with nutrients as well as the removal of metabolites (Hawkins *et al.,* 2006; Bernacki *et al.*, 2008). This barrier comprises of different cell types: microvascular endothelial cells, pericytes and astrocytes (figure 1). Endothelial cells and pericytes are ensheathed by a basement membrane consisting of collagen IV, fibronectin and laminin. Astrocytes surround this membrane (Francis *et al.*, 2003; Hawkins and Davis, 2005). Interaction of endothelial cells with adjacent cells by interendothelial tight junctions is thought to be the reason of the barrier function resulting in high electrical resistances of brain capillaries of about 2,000 Ω x $cm^2$ and low permeability (Stamatovic *et al.*, 2008). The special structure of the BBB allows only the diffusion of small lipophilic molecules, like oxygen, carbon dioxide or ethanol, and restricts the paracellular pathway for small hydrophilic solutes and macromolecules (Rubin and Staddon, 1999; Abbott, 2002).

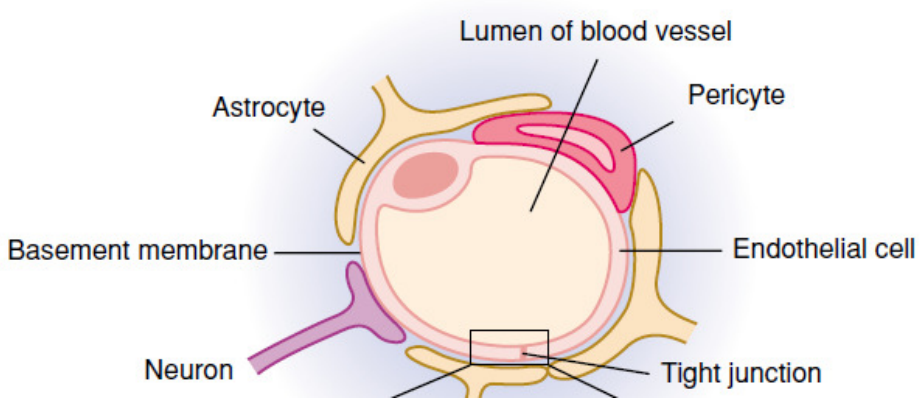


**Figure 1: Schematic cross-sectional representation of the BBB**

The BBB is formed mainly by the interaction of microvascular endothelial cells through tight junctions. Endothelial cells and pericytes are ensheathed by a basement membrane composed of collagen IV, laminin and fibronectin. Astrocytes surround this membrane and stay in close contact to endothelial cells and pericytes. The figure is based on Francis *et al.*, 2003.

### 1.1.2 Endothelial cells

Cerebral endothelial cells differ from those in the periphery. Mature brain endothelial cells are characterised by a low number of pinocytic vesicles, a lack of fenestrations and an increased mitochondrial content (Engelhardt, 2003; Hawkins and Davis, 2005; Bernacki *et al.*, 2008). In contrast to the highly permeable endothelial cells that lie outside the brain, cerebral endothelial cells are much more impermeable because of the formation of tight junctions (Wolburg *et al.*, 2009). Endothelial cells express several specific marker proteins, e.g. von Willebrand factor (vWF), γ-glutamyl transpeptidase and alkaline phosphatase. Cerebral endothelial cells are also marked by P-glycoprotein and multidrug-resistance-associated protein (Bernacki *et al.*, 2008).

Endothelial cells are polarised cells. Specialised transporters that convey nutrients into the brain, e.g. glucose, amino acids and vitamins, are expressed mainly at the luminal, blood-faced site. However, transporters for potential toxic molecules, like transmitter and metabolites, are found primarily on the abluminal site (Carson *et al.*, 2006).

Cerebral endothelial cells form different types of discontinuous, button-like junctions: tight junctions, adherens junctions and gap junctions. Whereas gap junctions are important for cell-cell communication, tight junctions and adherens junctions mediate cell-cell adhesion of adjacent endothelial cells and thereby contribute to the low paracellular permeability of the BBB (Hawkins and Davis, 2005). Moreover, endothelial cells express other adhesive proteins which are concentrated to the interendothelial cleft but are not specifically confined to the adherens junctions or tight junctions, like platelet endothelial cell adhesion molecule-1 (PECAM-1), S-endo 1 and endoglin (Bazzoni and Dejana, 2004).

#### 1.1.2.1 Tight junctions

Tight junctions are important to hold cerebral endothelial cells together and operate as barrier and fence. The barrier function of tight junctions results from the restriction of the permeability for small hydrophilic solutes and macromolecules. By limiting the movement of proteins and lipids from the luminal to the abluminal site, tight junctions act as a fence (Bednarczyk and Lukasiuk, 2011). Tight junctions are highly dynamic structures regulated in response to environmental conditions (Dejana *et al.*, 2009). Function and structure of these junctions are modulated by protein expression levels, post-translational modifications as well as protein-protein interactions (Bednarczyk and Lukasiuk, 2011).

Tight junctions comprise of transmembrane proteins and membrane-associated proteins (figure 2). The transmembrane protein occludin has been the first identified protein within tight junctions. It forms homophilic dimers with adjacent cells. Occludin-deficient mice do not

show any morphological differences in tight junction structure, indicating that occludin is not necessary for the formation of tight junctions (Wolburg and Lippoldt, 2002; Liu *et al.*, 2012).

Claudins, a family of 24 transmembrane proteins, represent the backbone of the tight junctions by forming homophilic dimers. Claudins regulate directly the permeability function of the BBB. Changes in claudin protein levels or substitutions of single amino acids affect the selectivity of the barrier (Bednarczyk and Lukasiuk, 2011). Cerebral endothelial cells express claudin-1, -2, -3, -5, -11 and -12. Claudin-5 is the major component of the tight junctions in the brain. Claudin-5-deficient animals show impaired BBB function and die within one day after birth although they do not have any morphological abnormalities in the brain (Dejana *et al.*, 2009; Bednarczyk and Lukasiuk, 2011).

Another group of transmembrane proteins within tight junctions are junctional adhesion proteins (JAM), which belong to the immunoglobulin (Ig) superfamily. They can be divided into two classes: the closely related molecules JAM-A, JAM-B and JAM-C; and the molecules coxsackie, adenovirus-receptor, endothelial cell-selective adhesion molecule and JAM-4 (Wolburg and Lippoldt, 2002; Sawada *et al.*, 2003; Bednarczyk and Lukasiuk, 2011). JAM-A and JAM-C are reported to maintain the stability of tight junctions and increase the electrical resistance in cells that do normally not express tight junctions (Wolburg and Lippoldt, 2002; Lui *et al.*, 2012).

The membrane-associated proteins involved in tight junction formation could be classified as membrane associated guanylate kinases (MAGUK) and non-MAGUK proteins. To the family of MAGUK belong *zona occludens* (ZO) -1, -2 and -3. They share three core regions: a SH3 domain, which binds signalling molecules and proteins of the cytoskeleton, a guanylate cyclase, mediating the binding to occludin, and three PDZ domains that are involved in the interaction with claudins and JAM (Wolburg and Lippoldt, 2002; Lui *et al.*, 2012). By anchoring tight junctions to the cytoskeleton and to signalling cascades, intracellular ZO proteins play an important role in the stability of tight junctions. The non-MAGUK proteins AF-6 and cingulin are associated with other tight junction proteins and involved in stabilising tight junction structure (Wolburg and Lippoldt, 2002).

#### 1.1.2.2 Adherens junctions

Adherens junctions mediate cell-cell adhesion, are involved in contact inhibition during cell growth or remodelling and initiate cell polarity (Hawkins and Davis, 2005). Furthermore, they regulate tight junction assembly as well as paracellular permeability and are important for endothelial cell signalling (Dejana *et al.*, 2009).

These junctions are mainly formed by transmembrane adhesion receptors of the cadherin family which mediate homophilic interaction between adjacent cells and form multimeric complexes at the cell borders (Bazzoni and Dejana, 2004; figure 2). Endothelial cells express the cell type specific vascular endothelial cadherin (VE-cadherin), which binds β-catenin and γ-catenin through its C-terminal region. These two proteins can bind α-catenin, which interacts with the adaptor proteins α-actinin and vinculin. These interactions result in anchoring the protein complex to the actin cytoskeleton and thus stabilise adherens junctions (figure 2). Another binding partner of VE-cadherin is catenin p120 (Rubin and Staddon, 1999; Hawkins and Davis, 2005). Besides its interaction with these adaptor proteins, VE-cadherin also binds signalling molecules, such as vascular endothelial growth factor receptor 2 and the receptor protein phosphatase VE-PTP (Bazzoni and Dejana, 2004). Besides this cadherin-catenin-complex, another protein complex is found in endothelial cells consisting of nectin, AF-6 and ponsin. This complex colocalises with cadherins and is thought to be involved in adherens junction organisation. However, the specific function of this complex is not fully understood to date (Bazzoni and Dejana, 2004).

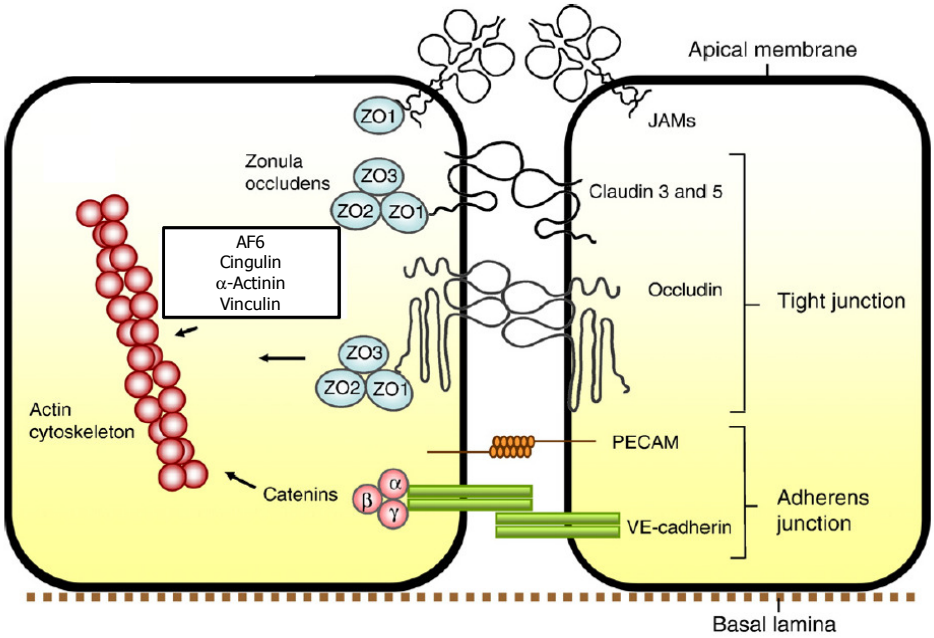


**Figure 2: Schematic representation of tight and adherens junctions at the BBB**

Tight junctions are composed of transmembrane proteins (e.g. occludin, JAMs and claudins) which are associated via cytoplasmic proteins (e.g. ZO, AF6, cingulin, α-actinin, vinculin) to the actin cytoskeleton. Adherens junctions are formed by the VE-cadherin-catenin complex. The figure is based on Abbott *et al.*, 2010.

#### 1.1.2.3 Gap Junctions

Gap junctions mediate interendothelial communication as well as communication of endothelial cells with astrocytes and pericytes. They are involved in several endothelial cell functions but do not control paracellular permeability (Dejana *et al.*, 2009). Gap junctions are formed by connexins which are organised in connexons. These are channels that facilitate the intracellular transport of ions and small molecules (Bazzoni and Dejana, 2004).

### 1.1.3 Astrocytes

Astrocytes can be divided into two types: fibrillary cells in the white matter and protoplasmic cells in the grey matter. The latter are characterised by a large nucleus and several cytoplasmic appendices which form cap-like structures at their ends. These so-called endfeet, marked by expression of the cytoskeleton intermediate filament glial fibrillary acidic protein, attach to neurons at one side and to blood vessels on the other side (Bernacki *et al.*, 2008). Astrocytes are important for neuronal function by influencing synapse formation and plasticity, regulating synaptic homeostasis of neurotransmitter and ions as well as controlling energetic and redox metabolism (Sá-Pereira *et al.*, 2012). Because astrocytes cover about 99% of the endothelium they affect also endothelial cell phenotype and expression of tight junctions. By secreting soluble factors, such as cytokines, steroids and growth factors, astrocytes play an important role in maintaining BBB structure and function (Abbott, 2002; Hawkins and Davis, 2005; Sá-Pereira *et al.*, 2012).

### 1.1.4 Pericytes

Pericytes are polymorphic, normally star-shaped cells which form long cytoplasmic appendices along the blood vessel. Secondary and tertiary processes branch off and encircle the blood vessel (Sá-Pereira *et al.*, 2012). Pericytes can be divided into two classes: one that is found at capillary straight parts and another that is located at capillary connections (Bernacki *et al.*, 2008). In contrast to endothelial cells, these cells have large vacuoles and lysosomes and are characterized by the expression of α-smooth muscle protein, platelet-derived growth factor receptor and pericytic aminopeptidase (Sá-Pereira *et al.*, 2012). Pericytes are important for brain homeostasis. They also influence the endothelium and the BBB because of the close contact to endothelial cells and the communication through gap junctions. They have been found to induce the formation of tight junctions, inhibit the expression of molecules that increase vascular permeability and promote endothelial survival (Dalkara *et al.*, 2011). Additionally, they are involved in the formation of the basement membrane by producing collagen IV, laminin and glycosaminoglycans and inducting

endothelial cells to secrete basement membrane components (Sá-Pereira *et al.*, 2012). By collaborating with astrocytes, pericytes are involved in the induction and maintenance of the BBB.

### 1.1.5 Basement membrane

The basement membrane is a thin, tightly interwoven network of extracellular matrix (ECM) proteins. Its major compounds are laminins, collagen IV, nidogens and proteoglycans (Korpos *et al.*, 2010). The endothelial basement membrane serves as a scaffold, which maintains the histological features of the vessels and bears mechanical forces like blood pressure. It also provides informational cues to endothelial cells, thus regulating their function, such as differentiation and proliferation during angiogenesis (Eble *et al.*, 2009). By storing and secreting growth factors, it modulates signalling pathways of endothelial cells and pericytes (Hermann *et al.*, 2012). The formation of the basement membrane is driven by self-assembly of laminins and collagen IV into two different three-dimensional networks which are bridged by nidogens, proteoglycans and fibulins. Fibulins bind strongly to laminins, nidogens and fibronectin which results in cross-linking the basement membrane components (Korpos *et al.*, 2010).

#### 1.1.5.1 Collagen IV

The principal component by weight of the basement membrane is collagen IV (Wiridjaja *et al.*, 2010). In humans, six different collagen IV genes encode the chains α1-α6, which are found in three trimeric combinations α1α1α2, α3α4α5 and α5α5α6 (Kuo *et al.*, 2012). Each collagen IV α chain consists of an N-terminal 7S domain, a core collagenous domain, which contain the characteristic Gly-X-Y repeats, and a C-terminal noncollagenous NC1 domain (Wiradjaja *et al.*, 2010). Collagen IV exists as a heterotrimeric helix where three α chains assemble covalently via their NC1 domains, with the central collagen domains coiled around one another (Wiradjaja *et al.*, 2010). These so-called protomers self-assemble and create a lattice network of collagen IV which provides structural stability to the basement membrane (Wiradjaja *et al.*, 2010).

#### 1.1.5.2 Laminins

Laminins are heterotrimeric glycoproteins composed of α, β and γ chains. They are often found in a cross-shaped structure consisting of three short arms formed by the N-terminal regions of the α, β and γ chain and a long arm, where the C-terminal regions of the three chains are associated in a triplehelix (Timpl and Brown, 1994). To date, 15 different laminin isoforms are known. Although all isoforms have been detected in basement membranes,

expression is tissue-specific (Wiradjaja *et al.*, 2010). In the endothelial basement membrane, two main laminin isoforms are found. Laminin-411 (composed of laminin α4, β1 and γ1, also known as laminin-8) is ubiquitously expressed by all endothelial cells and is strongly up-regulated by cytokines and growth factors. Laminin-511 (composed of laminin α5, β1 and γ1, also known as laminin-10) is mainly detectable in basement membranes of capillaries and post-capillary venules, where its expression is elevated only by strong pro-inflammatory cytokines, such as tumour necrosis factor α (Korpos *et al.*, 2010; Simon-Assmann *et al.*, 2011).

#### 1.1.5.3 Fibronectins

Fibronectins are glycoproteins comprising of three different types of modules termed type I, II and III repeats (Wierzbicka-Patynowski and Schwarzbauer, 2003, figure 3). Sets of adjacent modules form binding sites for collagen, fibrinogen, fibronectin itself and various glycosaminoglycans. Furthermore, fibronectins have two cell-binding sites by which they can interact with integrins: the RGD-sequence and the CS-I segment (Schwarzbauer and DeSimone, 2011, figure 3). Some integrins require the so-called synergy sequence for maximal interaction with fibronectins.

The primary gene transcript of fibronectin is alternatively spliced to generate multiple mRNAs. There are three sites for alternative splicing. The two type III repeats, EIIIA and EIIB, are included or omitted by alternative splicing, whereas the third region, the V-region, is included, excluded or partially included (Schwarzbauer and DeSimone, 2011, figure 3).

The molecule is synthesised as a monomer, followed by rapid dimerisation via disulfide bonds. Fibronectin is found as soluble plasma fibronectin and insoluble tissue fibronectin. The former is expressed by hepatocytes and secreted into the blood. Plasma fibronectin does not form fibrils even at extremely high concentrations (Singh *et al.*, 2010). However, it can diffuse into tissues and be incorporated into the fibrillar matrix of tissue fibronectin (Labat-Robert, 2012). Tissue fibronectin is expressed by various cell types, e.g. fibroblasts, astroglial cells, endothelial cells and epithelial cells (Ruoslahti, 1981). Fibronectin assembles through a cell-mediated process. In this process, cell-associated fibronectin is distributed diffusely over the cell surface. Binding of fibronectin to integrins leads to integrin clustering and converts fibronectin from an inactive to an active conformation facilitating fibronectin self-assemble and formation of fibrils (Singh *et al.*, 2010; Labat-Robert, 2012). The N-terminal 70 kDa fragment in the fibronectin molecule is essential for the fibrillogenesis (Wierzbicka-Patynowski and Schwarzbauer, 2003).

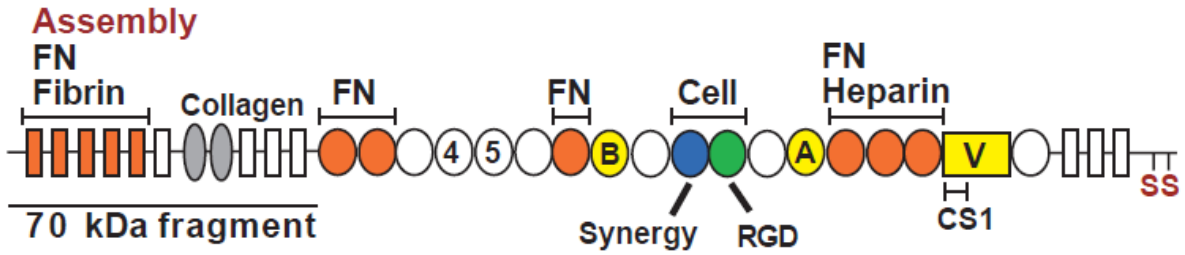


**Figure 3: Domain structure of fibronectin**

Fibronectin consists of three different types of modules termed type I (rectangle), type II (oval) and type III (circles) repeats. Sets of repeats constitute to binding sites for fibronectin, fibrin, collagen, cells and heparin, as indicated. The three alternatively spliced segments EIIIA, EIIIB and V-region are depicted in yellow. SS indicates the C-terminal cysteins that form fibronectin dimers. The figure is based on Wierzbicka-Patynowski and Schwarzbauer, 2003.

## 1.2 *In vitro* blood-brain barrier models

*In vitro* BBB models are suitable systems to study mechanistic effects of drug transport as well as general physiological and pathological processes at the BBB. An *in vitro* BBB model should meet the following requirements: restrictive paracellular permeability, determined by the transendothelial electrical resistance (TEER) or the permeability coefficient, physiologically realistic morphology and expression of functional influx and efflux transporters. Moreover, it should be easy to culture (Gumbleton and Audus, 2001).

*In vitro* models using primary cultures of brain microvascular endothelial cells (BMEC) present a close approximation to the *in vivo* situation. Primary BMEC originate mainly from rat, mouse, pig or cow, whereas the availability of human primary cells is restricted. Since human BMEC are derived from surgery, they cannot always be considered as healthy tissue (Wilhelm *et al.*, 2011). Beside their restricted availability, further disadvantages of primary BMEC are high costs and special technical skills that are necessary for the isolation of these cells. Furthermore, primary cells lose their specific characteristics in culture within limited passages.

These problems led to the development of immortalised endothelial cell lines generated by expression of simian virus 40 large T antigen (SV40-LT), E1A adenovirus gene, human papilloma E6E7 gene and Rous sarcoma virus or by incorporating human telomerase into the cell genome (Takeshita and Ransohoff, 2012). The only commercially available immortalised BMEC are the murine cell lines bEND3 as well as bEND5. Yet, they exhibit TEERs not greater than 60 Ω x $cm^2$ indicating only a limited barrier function (Gumbleton and Audus, 2001). The best studied human brain endothelial cell lines are human cerebral microvascular endothelial cells (hCMEC/D3) and transfected human brain microvascular

endothelial cells (THBMECs). hCMEC/D3 were generated by transducing primary human endothelial cells with lentiviral vectors incorporating human telomerase and SV40-LT (Weksler *et al.*, 2005). They have been used for many signalling studies and as a system to analyse drug transport (Takeshita and Ransohoff, 2012). THBMECs were isolated from adult human brain microvessels and immortalised by transfection with SV40-LT (Stins *et al.*, 2001). They are characterised by expression of tight junction proteins and a variety of endothelial-specific transporters. The TEERs of *in vitro* models generated on the basis of THBMECs range from 100 - 120 Ω x $cm^2$ (Callahan *et al.*, 2004; Man *et al.*, 2008). However, endothelial cell lines in general lack contact inhibition and lose their physical and morphological BBB characteristics at high passages or super-confluence (Takeshita and Ransohoff, 2012). Two recently established conditionally immortalised human brain endothelial cell lines seem to be a promising option to overcome these problems. The cell lines TY08 and HBMEC/ciβ were generated by transfection of a temperature-sensitive SV40-LT (Sano *et al.*, 2010; Kamiichi *et al.*, 2012). At 33°C, SV40-LT inhibits the tumour suppressor proteins, protein 53 and retinoblastoma proteins, which results in continuous cell proliferation. At 37°C, SV40-LT is inactivated and cells differentiate to endothelial cells.

Since other cell types are also involved in the formation and maintenance of the BBB function *in vitro* co-culture models were established, representing a closer reproduction of the *in vivo* conditions. The application of astrocytes conditioned media leads to enhanced expression of tight junction proteins, increased TEER and lower permeability indicating an improved barrier function (Rubin *et al.*, 1991; Siddharthan *et al.*, 2007). Co-culture BBB models, which bring endothelial cells and astrocytes in direct contact, mimic the *in vivo* BBB characteristics and result in strongly enhanced barrier tightness (Gaillard *et al.*, 2001; Cohen-Kashi Malina *et al.*, 2009). Besides astrocytes, pericytes, neurons and microglia were also used in co-culture models with endothelial cells which had been shown to improve the barrier characteristics of the *in vitro* models (Cestelli *et al.*, 2001; Kim *et al.*, 2006; Al Ahmad *et al.*, 2009; Nishioku *et al.*, 2010; Sumi *et al.*, 2010).

There is increasing evidence that shear stress affects morphology of endothelial cells as well as endothelial-leukocyte interactions. This understanding led to the development of dynamic *in vitro* BBB models. Dynamic models that mimic pulsatile blood flow show much higher TEERs than static models and represent the closest approximation of the *in vivo* conditions (Santaguida *et al.*, 2006; Neuhaus *et al.*, 2006; Cucullo *et al.*, 2008; Man *et al.*, 2009).

## 1.3 The blood-brain barrier in diseases

### 1.3.1 Inflammation

The CNS parenchyma is an immune-privileged region because it is devoid of antigen presenting cells, such as dentritic cells. It also lacks the constitutive expression of major histocompatibility complex (MHC) I and MHC II molecules as well as lymphatic vessels (Engelhardt and Ransohoff, 2005). Under physiological conditions only a few leukocytes circulate between the blood and the brain to control normal CNS function, a process known as immunosurveillance. Pathological conditions, such as viral infections, severe brain insults or chronic-inflammatory diseases, result in brain inflammation and enhanced transmigration of immune cells across the BBB into the CNS.

Brain inflammation is characterised by activation of microglia, astrocytes and endothelial cells which rapidly express cytokines, chemokines and prostaglandins. Following secretion of pro-inflammatory cytokines, e.g. tumour necrosis factor α (TNFα) and interleukin 1β (IL-1β), expression of endothelial adhesion molecules, such as selectins, vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) is enhanced (Simi *et al.*, 2007). Moreover, inflammatory diseases are often associated with alterations in the molecular composition of tight junctions or the functional state of tight junction proteins (Coisne and Engelhardt, 2011). Multiple sclerosis, for example, is an inflammatory demyelating disease which is characterised by accumulation of T lymphocytes and macrophages in the CNS and results in neuroinflammation following BBB disruption. In patients suffering from this disease, expression of the tight junctions proteins ZO-1, occludin, JAM-A as well as claudin-1 and claudin-3 is decreased (Wolburg and Lippoldt, 2002; Sawada *et al.*, 2003; Minagar *et al.*, 2003). Pro-inflammatory cytokines also influence the expression of matrix metalloproteinases (MMPs). These proteases cause an increase in permeability of the BBB by degrading ECM components in the endothelial basement membrane as well as tight junctions (Rosenberg, 2009). IL-1β, for example, has been reported to induce up-regulation of MMP-1, MMP-3, MMP-7 as well as MMP-9 (Sorokin, 2010). Proteolytic cleavage of ECM proteins by MMPs during inflammation results in the release of bioactive fragments. These so-called matrikines can regulate cell activities (Maquart *et al.*, 2004). Peptides generated from the laminin α5 chain, nidogen or collagen I have been described to act as chemoattractants for macrophages, polymorphonuclear leukocytes and neutrophils, respectively (Senior *et al.*, 1992; Adair-Kirk *et al.*, 2003; Korpos *et al.*, 2009). In addition, pro-inflammatory cytokines released during inflammation can

modulate the expression of ECM molecules and thereby influence the transmigration of immune cells into the CNS parenchyma (Sorokin, 2010).

### 1.3.2 Transmigration of leukocytes

During brain inflammation, circulating leukocytes transmigrate from the blood vessels across the endothelium into the inflamed CNS. This transendothelial migration of immune cells is a multi-step process (figure 4). In the first step, leukocytes make transient contact with the vascular endothelium. This so-called capture or tethering is mediated by interactions between selectins (L-selectin, P-selectin, E-selectin) and their highly glycosylated ligands. L-selectin is expressed by most lymphocytes, whereas P-selectin and E-selectin can be found on endothelial cells (Ley *et al.*, 2007). Initial contact between leukocytes and endothelial cells results in reduced velocity of the leukocytes (rolling). The immune cells roll slowly along the vessel and scan endothelial surfaces for luminal chemokines. Binding of chemokines to their receptors leads to activation of leukocyte integrins followed by leukocyte arrest and its firm adhesion to the endothelium. The most important molecules in this process are the leukocyte integrins αLβ2 (also known as LFA-1), αMβ2 (also known as Mac-1) and α4β1 (also known as VLA-4 or CD49e/Cd29) and their endothelial ligands ICAM-1, ICAM-2 and VCAM-1 (Engelhardt and Ransohoff, 2005). Leukocytes can transmigrate by either a paracellular or a trans-cellular route. Immune cells that use the paracellular route crawl along endothelial surfaces in search of interendothelial junctions, a process which is known as intravascular crawling. These cells extend protrusions that are enriched with chemokine receptors to inspect the abluminal environment and migrate between two endothelial cells (Man *et al.*, 2007). The transendothelial migration is mediated by heterophilic interactions between αLβ2 and ICAM-1 as well as ICAM-2 and homophilic interactions between PECAM-1, cluster of differentiation (CD) 99, JAM-A, JAM-B and JAM-C molecules. At least 90% of the transmigration events are paracellular (Muller, 2013). However, transcellular migration of T lymphocytes and neutrophils, leaving the tight junctions intact, has been observed in the CNS and other inflamed tissues. This process had also been detected in *in vitro* BBB models (Engelhardt and Wolburg, 2004; Ley *et al.*, 2007).

Further investigations will be needed to unravel the detailed mechanism of transcellular transmigration but the involvement of vesiculo-vacuolar organelles (VVO) is suspected. VVO are small continuous membrane channels that might act as a gateway for the leukocyte through endothelial cells. First, the leukocyte extends membrane protrusions that penetrate into the endothelial cell. Ligation of ICAM-1 on endothelial cells results in translocation of ICAM-1 from the luminal surface to caveolae and F-actin-rich regions. Simultaneously,

caveolin-1 is transported to the abluminal plasma membrane (Ley *et al.*, 2007). These processes facilitate the formation of channels through, which leukocytes are able to migrate (Ley *et al.*, 2007; Muller, 2013). The transcellular migration occurs mainly at comparably thin endothelial cell layers.

Having passed the endothelial cells, leukocytes subsequently need to cross the endothelial basement membrane and the pericytes. In the endothelial basement membrane, regions with lower amounts of laminin-10 and collagen IV have been detected (Wang *et al.*, 2006a). The low-expression sites co-locate with gaps between pericytes and are therefore assumed to be targeted by transmigrating leukocytes. Ligation of PECAM-1 on leukocytes during transmigration results in activation of members of the β1, β2 and β3 integrin families (Newman and Newman, 2003). PECAM-1 is capable of inducing the mobilisation of the α6β1 integrin from intracellular stores to the cell surface of transmigrating immune cells. This integrin is the main laminin receptor on leukocytes and mediates the migration through the basement membrane (Dangerfield *et al.*, 2002; Ley *et al.*, 2007). Additionally, ligation of β2 leukocyte integrins results in increased expression as well as activation of α2β1 integrin on leukocytes, which is suggested to be involved in migration through the basement membrane (Werr *et al.*, 2000). Transmigration through the basement membrane may be facilitated by cell surface-expressed leukocyte proteases, which generate chemotactic fragments by selective cleava-ge of basement membrane constituents (Ley *et al.*, 2007). Antibody binding to α5β1, for example, was reported to induce the expression of genes for lytic enzymes, such as collagenase and stromelysin (Dejana *et al.*, 1993).

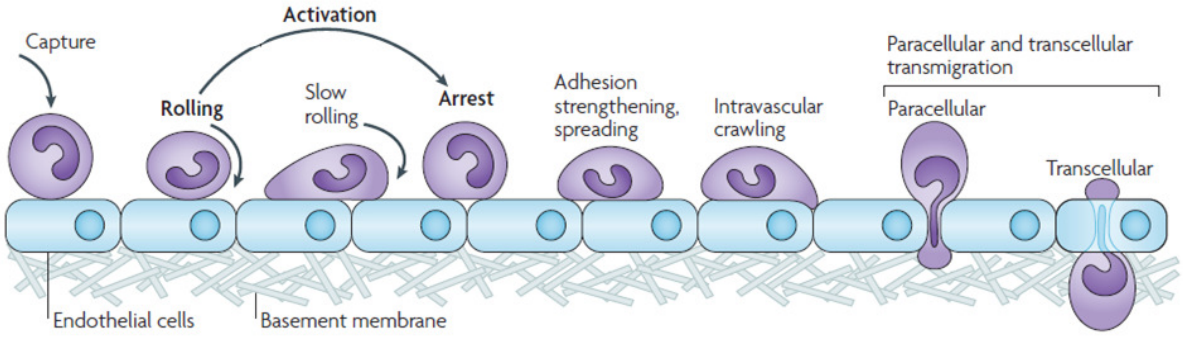


**Figure 4: Transendothelial migration of leukocytes**

Capture of leukocytes from the blood flow is mediated by selectins and results in rolling. Following binding of chemokines to their receptors, leukocyte integrins (e.g. αLβ2, αMβ2 and α4β1) were activated. Engagement of leukocyte integrins to their endothelial counterparts (e.g. ICAM-1 and VCAM-1) results in leukocyte arrest and adhesion to the endothelium. During intravascular crawling, attached leukocytes move along the vessel wall and search the surface for interendothelial junctions. Transmigration can then occur on the paracellular or transcellular route. The figure is based on Ley *et al.*, 2007.

## 1.4 Integrins

### 1.4.1 Classification and function

Integrins are cell surface receptors which mediate cell-cell and cell-ECM interactions (Hynes, 2002). These receptors are glycosylated heterodimers consisting of non-covalently associated α and β subunits. To date, 18 α and 8 β subunits are known, which assemble to 24 different integrin molecules. Integrin ligands include ECM proteins, like collagen, laminin and fibronectin, as well as soluble factors, such as vWF and fibrinogen. Furthermore, integrins can bind to adhesion receptors on other cells, e.g. cadherins and cell adhesion receptors of the Ig superfamily (Barczyk *et al.*, 2010).

With regard to their ligands, integrins are divided into four groups: collagen receptors, laminin receptors, integrins which recognise the amino acid sequence RGD, the most common binding motive for integrins, as well as the β2 and β7 integrins that are only expressed on lymphocytes (figure 5).

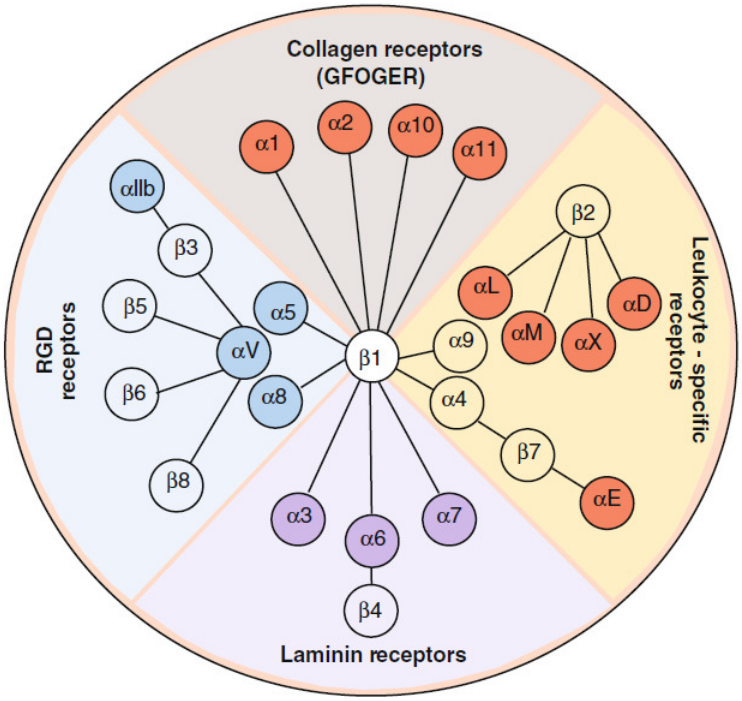


**Figure 5: Classification of the integrin receptor family**

Integrins are cell adhesion receptors which can be divided into four groups: collagen receptors, laminin receptors and integrins, which bind the RGD sequence which is present in various ECM proteins, e.g. in fibronectin and vitronectin. The last group contains integrins which are only expressed on lymphocytes. The figure is based on Barczyk *et al.*, 2010.

Integrins regulate many cellular processes including cell adhesion, spreading, migration, proliferation, differentiation, extravasation of lymphocytes, gene expression and apoptosis (van der Flier and Sonnenberg, 2001). Knockout of integrin subunits in mice cause a complete block in preimplantation, embryonic and perinatal lethality, severe developmental defects as well as impairment of physiological processes, such as inflammation, homeostasis, bone remodelling and angiogenesis (Hynes, 2002). Furthermore, integrin dysfunction is associated with many pathological events, such as chronic-inflammatory diseases and tumour growth (Hynes and Lander, 1992; Ben-Horin and Bank, 2004; Sampaio *et al.*, 2010).

### 1.4.2 Structure and conformation

Each integrin subunit consists of a large extracellular domain, a transmembrane domain and a short cytoplasmic domain (figure 6). The extracellular regions of both subunits form a head region, which binds the extracellular ligand, and a leg region. In the extracellular domain, three different metal ion binding sites have been indentified: the metal ion dependent adhesion site (MIDAS), the adjacent to the metal ion dependent adhesion site (ADMIDAS) and ligand induced metal binding site (LIMBS) which mediate the affinity state of the integrin molecule by binding divalent ions like $Mg^{2+}$, $Mn^{2+}$ and $Ca^{2+}$ (Gahmberg *et al.*, 2009). The transmembrane domains of both the α and β integrin subunit span the plasma membrane with a hydrophobic α helix. The cytoplasmic domains of the integrin molecule are much shorter than the extracellular domains and comprise of only 17 - 50 amino acids with exception of the β4 subunit, which contains more than 1000 amino acids. The cytoplasmic domains are important for dimerisation as well as signalling and regulate the activation state of the integrin molecule (McCall-Culbreath and Zutter, 2008). Moreover, the cytoplasmic domains mediate the interaction with the cytoskeleton. Whereas the α6β4 integrin interacts with intermediate filaments, all other integrins connect the ECM via adaptor proteins to the F-actin cytoskeleton (Hynes, 2002).

In general, integrins exist in two conformations (figure 6). The inactive conformation is characterised by a closed headpiece which results from tight association of the two subunits. Furthermore, the head region is turned towards the legs forming a V-like structure. In this conformation, integrins cannot bind their ligands. In contrast, in the active conformation the legs are extended and dissociated from each other. Moreover, the open headpiece facilitates the ligand binding. However, it has been shown that αvβ3 is capable of stably binding a ligand in a bent conformation (Adair *et al.*, 2005). Hence, the existence of intermediate conformations is now accepted (Mould and Humphries, 2004; Gahmberg *et al.*, 2009).

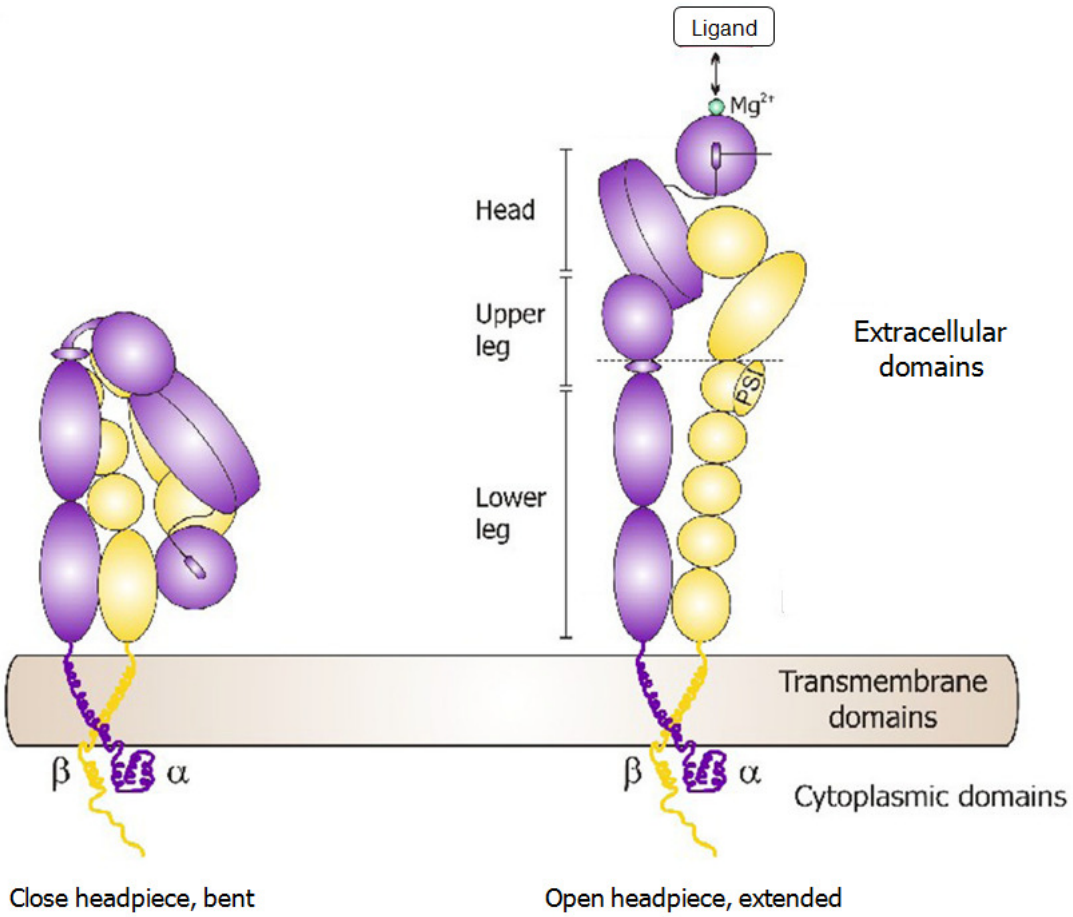


**Figure 6: Integrin structure and conformation**

An integrin molecule is composed of one α and one β chain. The subunits consist of a large extracellular domain, which can bind the ligand, a transmembrane domain and a short cytoplasmic domain. Integrins exists in at least two conformations: in a bent state with close headpiece and in an extended state with open headpiece. The figure is adapted from Gahmberg *et al.*, 2009.

### 1.4.3 Activation and signalling

Integrins are not constitutively active but expressed on the cell surface in an inactive state. This is of vital importance for their biological function. A good example is the integrin αIIbβ3 which is expressed on circulating platelets. Constitutively active αIIbβ3 would result in constitutive binding to fibrinogen and subsequent aggregation of platelets followed by thrombosis.

Activation of integrins is induced by two different events: an increase in integrin affinity or an enhancement of integrin avidity. Increased integrin affinity is caused by a conformational change in the integrin molecule following opening of the ligand binding site. Enhanced integrin avidity is achieved by clustering of multiple integrin molecules at the cell surface (Gahmberg *et al.*, 2009). A special feature of the integrin family is its ability to transfer signals inside and outside the cell. Due to this bidirectional signalling, integrins can be activated by

two different pathways. Binding of extracellular ligands, divalent cations or activating antibodies is known as outside-in activation (Takagi *et al.*, 2002). Inside-out activation primarily concerns leukocyte and thrombocyte integrins. Here, increased integrin affinity is achieved by intracellular signals, mainly caused by G protein-coupled receptors (e.g. chemokine receptors) or tyrosine kinase-coupled receptors which are transferred to cytoplasmic proteins like talin. These proteins interact with the cytoplasmic domains of the integrin molecule and induce conformational changes resulting in integrin activation (Springer and Wang, 2004).

Even though integrins lack any enzymatic activity they are essential for the activation of signalling cascades by interacting with several structural, adaptor and signalling proteins in focal adhesions. Focal adhesions are specialised structures which serve on the one hand as anchoring points, connecting the ECM to the cytoskeleton, and on the other hand as signalling platforms, transferring integrin-mediated signals. Following ligand binding, multiple integrins aggregate laterally in the plasma membrane and recruit several proteins, resulting in the formation of focal adhesions. Proteins, which are recruited into focal adhesions in the first place, are talin, paxillin and α-actinin, followed by proteins of the sarcoma (src) protein kinase family, which are able to induce activation of further kinases, e.g. mitogen-activated protein (MAP) kinases (van der Flier and Sonnenberg, 2001).

### 1.4.4 Integrins in the central nervous system

Within the CNS, integrins are expressed on several cell types including neurons, glial cells, meningeal cells, endothelial cells and infiltrating leukocytes (Milner and Campbell, 2002). They are involved in neuronal development, angiogenesis, inflammation as well as building and maintaining synaptic structures. Moreover, integrins are thought to be important for maintaining the BBB integrity. Del Zoppo and colleagues suggest a model, in which the structure of the BBB is influenced by horizontal and vertical components (del Zoppo and Milner, 2006; del Zoppo *et al.*, 2006). The tight junctions and adherens junctions, formed by adjacent endothelial cells, constitute the horizontal components. The vertical components consist of matrix adhesion complexes, formed between integrins and dystroglycans on endothelial cells and astrocytes, which anchor the cells to the ECM proteins in the basement membrane. Studies, demonstrating that alterations in the expression of integrins coincide with the leakiness of the BBB, support this model. Furthermore, in transgenic mice the absence of specific integrins leads to the breakdown of the cerebral vasculature (del Zoppo and Milner, 2006). A recent study reported that blocking of endothelial β1 integrins results in reduction of claudin-5 expression as well as decrease in the permeability of the BBB *in vitro*

and *in vivo*, indicating a role for integrins in maintaining the integrity of the BBB (Osada *et al.*, 2011).

### 1.4.5 Integrins in inflammation

Integrins play an important role in transendothelial migration of leukocytes during inflammation (see chapter 1.3.2). Leukocytes express mainly two subtypes of the integrin family: β1 and the β2 integrins. The latter, which are exclusively expressed by leukocytes, are the best studied leukocyte integrins. The integrins αLβ2 and αMβ2 mediate leukocyte arrest, firm adhesion and spreading as well as paracellular transmigration by interacting with endothelial ICAM-1 and ICAM-2. In the inflammatory disease multiple sclerosis, the expression of β2 integrins is increased, and knock down of the integrins αLβ2 and αMβ2 as well as αXβ2 reduces the incidence and the severity of multiple sclerosis (Hu *et al.*, 2010). Among the β1 integrins expressed on leukocytes, the α4β1 integrin is the best investigated. It binds to VCAM-1, which is up-regulated by endothelial cells during inflammation, and contributes to arrest and firm adhesion of leukocytes. The monoclonal antibody Natalizumab, which is used in multiple sclerosis therapy, binds to α4β1 integrin and prevents the initial contact between lymphocytes and endothelium (Engelhardt and Kappos, 2008). However, data obtained by *in vitro* transmigration assays indicate a role for other β1 integrins than α4β1 integrin in the transmigration process. Transmigration of leukocytes is reduced significantly by the use of function-blocking antibodies raised against the integrin subunits α2, α3, α5 and β1 (Roth *et al.*, 1995). Furthermore, several studies show increased expression of the α1β1 integrin on lymphocytes, indicating a role for this integrin in the progression of many chronic-inflammatory diseases, such as multiple sclerosis, arthritis, atherosclerosis and psoriasis (Bank *et al.*, 2002; Ben-Horin and Bank, 2004; Ben-Horin *et al.*, 2007; Mandel *et al.*, 2009). In addition, expression of α5β1 integrin is enhanced on monocytes and lymphocytes during inflammation (Sampaio *et al.*, 2010; Li *et al.*, 2011).

Little is known about the importance of endothelial integrins during inflammatory processes. Nevertheless, there are some hints that integrins, expressed by endothelial cells, also play a significant role during inflammation. In cerebral endothelial cells from patients with multiple sclerosis, reduced expression of the α6 and β1 integrin subunit was observed. On the contrary, expression of the α1 integrin subunit was enhanced both in active and chronic-inactive lesions (Sobel *et al.*, 1998). *In vitro* studies revealed an influence of TNFα on the activity of the endothelial α5β1 integrin without altering the total expression of this protein (Sun *et al.*, 2010). Furthermore, blocking of α5β1 integrin revealed a role for this molecule in leukocyte adhesion.

## 1.5 Aim of the work

The BBB is a specialised layer consisting of endothelial cells, pericytes and astrocytes, which restricts the diffusion for small hydrophilic solutes and macromolecules as well as the transmigration of leukocytes into the CNS. During inflammation, pro-inflammatory cytokines induce changes in the expression of adhesion proteins and MMPs as well as the composition of tight junctions resulting in BBB leakage and an increase in infiltrating leukocytes. The process of transendothelial migration is characterised by expression and activation of leukocyte integrins. However, less is known about the impact of integrins, expressed on the endothelium, during these processes.

The aim of this study was to investigate the role of endothelial β1 integrins in an *in vitro* BBB model during inflammation. For this purpose an appropriate *in vitro* BBB model, characterised by high electrical resistance and low permeability, needed to be established. This *in vitro* BBB model was extended to an inflammatory model by the application of suitable pro-inflammatory cytokines. The functionality of the model as well as the role of endothelial β1 integrins under inflammatory conditions was evaluated.

To analyse whether the function of β1 integrins is altered during inflammation, adhesion assays with cytokine-stimulated endothelial cells on different proteins of the extracellular matrix was carried out. Moreover, analysis of integrin expression and activity on the inflamed endothelium needed to reveal whether these molecules are involved in inflammatory processes at the BBB. To gain insight in the localisation of integrin subunits during these conditions indirect, immunofluorescence microscopy was performed. Since complexes of integrins and proteins of the basement membrane were suggested to stabilise the BBB, the physical properties of the *in vitro* model were evaluated in the presence of function-blocking antibodies directed against β1 integrins. Furthermore, transmigration assays with leukocytes across the endothelium after inhibition of endothelial integrins needed to clarify whether these receptors participate in the transendothelial migration of leukocytes under physiological and inflammatory conditions.

# 2 Materials and Methods

## 2.1 Materials

### 2.1.1 Equipment

| | | |
|---|---|---|
| agarose gel electrophoresis | model B2 | Owl Separation System, USA |
| Camera | Nikon D90 | Nikon, Japan |
| cell separator | QuadroMACS™ Separator | Miltenyi Biotec, Bergisch Gladbach |
| Centrifuges | Heraeus Biofuge pico | ThermoElectron, Langenselbold |
| | Heraeus Biofuge fresco | ThermoElectron, Langenselbold |
| | Heraeus Megafuge 1.0R | ThermoElectron, Langenselbold |
| ELISA Reader | Sunrise | Tecan, Crailsheim |
| Endohm chamber | Endohm-12 | World Precision Instruments, USA |
| flow cytometer | FACSCalibur™ | BD Biosciences, Heidelberg |
| Imager | Versadoc 4000™ MP | BioRad, Munich |
| impedance meter | CellZscope® | NanaAnalytics, Münster |
| magnetic stirrer | type RH B2 | IKA Werke, Staufen |
| microplate reader | Synergy HT | Bio-Tek Instruments Inc., USA |
| Microscopes | Nikon TMS-F | Nikon, Japan |
| | Diavert | Leica, Bensheim |
| | Axiovert 200 Fluorescence | Zeiss, Jena |
| | Leica TCS SP2 | Leica, Bensheim |
| PCR cycler | iCycler | BioRad, Munich |
| | MyiQ™ (Single Color Real Time Detection System) | BioRad, Munich |
| pH meter | Model 646 digital | Knick, Berlin |
| Photometer | Biophotometer UV | Eppendorf, Hamburg |
| power supply | Power PAC 200 | BioRad, Munich |
| Scales | Adventurer (d=0.0001 g) | Ohaus Corp., USA |
| | CP622 (d=0.01g) | Sartorius AG, Göttingen |
| SDS-PAGE system | Mini Protean® System | BioRad, Munich |
| Shakers | Model 3013 | GFL, Burgwedel |
| | Stuart Orbital S150 | Rhys International Ltd., UK |
| Thermomixer | Compact | Eppendorf, Hamburg |

| | | |
|---|---|---|
| volt-ohm-meter | Millicell® ERS-2 | Millipore, USA |
| Vortex | Mixer Genie® 2 | Scientific Industries, USA |

### 2.1.2 Chemicals and consumables

Chemicals were purchased from Carl Roth (Karlsruhe), Sigma-Aldrich (Steinheim), Merck (Darmstadt) and Applichem (Darmstadt), unless stated otherwise. Consumables were obtained from Corning Inc. (Corning, USA), Nunc Inc. (Naperville, USA), Schott (Mainz), Carl Roth (Karlsruhe), Eppendorf (Hamburg), BD Bioscience (Heidelberg), Sarstedt (Nümbrecht), Miltenyi Biotec (Bergisch Gladbach) and Whatman (Maidstone, UK).

### 2.1.3 Reagents

| | |
|---|---|
| 6x DNA loading dye | Fermentas, St. Leon-Rot |
| BCA™ protein assay reagent A and B | Pierce, USA |
| calcein AM | Life Technologies, Darmstadt |
| CD 14 MicroBeads, human | Miltenyi Biotec, Bergisch Gladbach |
| CD 3 MicroBeads, human | Miltenyi Biotec, Bergisch Gladbach |
| collagen, type I | Biochrom AG, Berlin |
| collagen, type IV | Sigma-Aldrich, Steinheim |
| Cytokines | Immunotools, Friesoythe |
| DMEM:F12 | Lonza, USA |
| fetal bovine serum | Thermo Fisher Scientific, Schwerte |
| fibronectin | Applichem, Darmstadt |
| Ficoll Plaque™ Plus | GE Healthcare, Munich |
| Gel Mount™ | Sigma-Aldrich, Steinheim |
| gelatine from porcine skin, type A | Sigma-Aldrich, Steinheim |
| gelatine agarose | Sigma-Aldrich, Steinheim |
| GelRed™ Nucleic Acid Stain | Biotium Inc., USA |
| Hoechst 33342 | Sigma-Aldrich, Steinheim |
| immersion oil | Zeiss, Jena |
| laminin-1 | Sigma-Aldrich, Steinheim |

| | |
|---|---|
| L-glutamine | PAN-Biotech, Aidenbach |
| penicillin/ streptomycin | PAN-Biotech, Aidenbach |
| phenylmethanesulfonyl fluoride (PMSF) | Sigma-Aldrich, Steinheim |
| poly-L-lysine | Sigma-Aldrich, Steinheim |
| protease inhibitor cocktail (PIC) | Sigma-Aldrich, Steinheim |
| RPMI 1640 without phenol red | Biochrom AG, Berlin |
| sodium fluorescein salt | Fluka, Steinheim |
| tryptan blue solution (0.4%) | Sigma-Aldrich, Steinheim |

### 2.1.4 Cells

The endothelial cell line human brain microvascular endothelial cells (THBMECs) originating from primary cells, which were isolated from adult human brain microvascular endothelium and immortalised by a plasmid containing SV-40LT, was a gift of Dr. Kwang Sik Kim (The Johns Hopkins University, Baltimore, USA; Stins *et al.*, 2001).

### 2.1.5 Primer

All primers used for quantitative and qualitative polymerase chain reactions are shown in table 6 (appendix).

### 2.1.6 Antibodies

All used primary antibodies, secondary antibodies and isotype controls are listed in table 1.

**Table 1: Primary antibodies, secondary antibodies and isotype controls**

Block: function-blocking, FC: flow cytometry, IF: immunofluorescence microscopy, MACS: magnetic cell separation, WB: western blotting, POD: peroxidase

| Primary antibodies | | | |
|---|---|---|---|
| **Name** | **Source** | **Host** | **Dilution** |
| anti-CD 14, PE | Immunotools | Mouse | FC 1:20 |
| anti-CD 16, FITC | Immunotools | Mouse | FC 1:20 |
| anti-CD 19, FITC | Immunotools | Mouse | FC 1:20 |
| anti-CD 3, FITC | Immunotools | Mouse | FC 1:20 |
| anti-CD 4, PE | Immunotools | Mouse | FC 1:20 |
| anti-CD106, FITC | BD Bioscience | Mouse | FC 1:40 |
| anti-CD29 | BD | Mouse | IF 1:100 |
| anti-CD29, clone 12G10 | AbD Serotec | Mouse | FC 1:100; IF 1:200 |
| anti-CD29, Clone P5D2 | R&D Systems | Mouse | Block 1:50 |
| anti-CD29, FITC | Immunotech | Mouse | FC 1:20 |

| | | | |
|---|---|---|---|
| anti-CD49a, PE | BD Bioscience | Mouse | FC 1:40 |
| anti-CD49b, FITC | BD Bioscience | Mouse | FC 1:40 |
| anti-CD49c, PE | BD Bioscience | Mouse | FC 1:20 |
| anti-CD49e | BioLegend Inc. | Mouse | Block 1:100 |
| anti-CD49e, PE | BD Bioscience | Mouse | FC 1:20 |
| anti-CD49f | BioLegend Inc. | Mouse | Block 1:100 |
| anti-CD49f, FITC | Chemicon | Mouse | Flow 1:40 |
| anti-CD54, PE | BD Bioscience | Mouse | FC 1:20 |
| anti-claudin-5 | Abcam | Rabbit | WB 1:750; IF 1:100 |
| anti-collagen IV | Acris | Mouse | WB 1:1000 |
| anti-fibronectin | Sigma-Aldrich | Mouse | WB 1:1000; IF 1:200 |
| anti-laminin β1 | Acris | Rabbit | WB 1:1000 |
| anti-occludin | BD Bioscience | Mouse | WB 1:750; IF 1:100 |
| anti-ZO-1 | BD Bioscience | Mouse | WB 1:750; IF 1:200 |
| anti-α-tubulin | Abcam | Mouse | WB 1:5000 |

**Secondary antibodies**

| Name | Source | Host | Dilution |
|---|---|---|---|
| anti-mouse IgG, POD | Jackson Immunoresearch | Rat | WB 1:5000 |
| anti-mouse, Alexa Fluor® 488 | Molecular Probes | Goat | IF 1:750 |
| anti-mouse, Alexa Fluor® 555 | Molecular Probes | Goat | FC 1:750 |
| anti-rabbit IgG, POD | Jackson Immunoresearch | Goat | WB 1:5000 |
| anti-rabbit, Alexa Fluor® 488 | Molecular Probes | Goat | IF 1:750 |
| anti-rabbit, Alexa Fluor® 594 | Molecular Probes | Goat | IF 1:750 |

**Isotype controls**

| Name | Source | Dilution |
|---|---|---|
| mouse IgG1 κ | BioLegend Inc. | FC 1:100 |
| mouse IgG1 κ LEAF™ | BioLegend Inc. | Block1:100 |
| mouse IgG1 κ PE | BioLegend Inc. | FC 1:100 |

### 2.1.7 Protein and DNA Markers

| | |
|---|---|
| Precision Plus Protein™ Standards Dual Color | BioRad, Munich |
| Recombinant human MMP-2, NS0-derived | R&D Systems, USA |
| Recombinant human MMP-9, NS0-derived | R&D Systems, USA |
| Quick Load® 100bp DNA Ladder | New England BioLabs, UK |

### 2.1.8 Kits

| | |
|---|---|
| GoScript™ Reverse Transcription System | Promega, Mannheim |
| GoTaq qPCR® Master Mix | Promega, Mannheim |
| Human IL-1β ELISA MAX™ Deluxe | BioLegend Inc., USA |

| | |
|---|---|
| Mini ELISA Development Kit (Human IL-6, IL-8, TNFα) | Preprotech, Hamburg |
| Quick-RNA™ Mini Prep | Zymo Research Cor., USA |
| SuperSignal® West Femto Maximum Sensitivity Substrate | Thermo Scientific Inc, USA |
| SuperSignal® West Pico Chemiluminicent Substrate | Thermo Scientific Inc, USA |

### 2.1.9 Buffers, Solutions and Media

Commonly used buffers and solutions were prepared using double-distilled water. If necessary, solutions were autoclaved at 121°C for 20 min at 1 bar. Thermolabile components were filter-sterilized (0.22 µm) and added after autoclaving. The pH was adjusted using HCl or NaOH. Buffers, solutions, media are listed at the end of each method. Cell culture media were purchased from Lonza (Switzerland) and Biochrom AG (Berlin).

### 2.1.10 Software and Databases

| | |
|---|---|
| Axiovision (version 8.0.1) | Zeiss, Jena |
| BioRad iQ™ (version 2.0) | BioRad, Munich |
| CellQuest Pro (version 5.2.1) | Becton Dickinson and Company, USA |
| CellZscope® software | NanoAnalytics, Münster |
| Primer-BLAST | http://www.ncbi.nlm.nih.gov/tools/primer-blast/ |
| PUBMED | http://www.ncbi.nlm.nih.gov/ |

## 2.2 Methods

### 2.2.1 Cell biology

THBMECs were isolated from adult human brain microvascular endothelium, transfected and immortalised by a plasmid containing SV-40LT (Stins *et al.*, 2001). Cells were grown in DMEM:F12 1:1 medium supplemented with penicillin/streptomycin (100 U/ml), L-glutamine (440 mg/l) and heat-inactivated fetal bovine serum (10%).

Cells were grown on plastic and passaged all 3-4 days. THBMECs were detached with PBS/ETDA and pelleted at 170 x g for 3 min. For this study, cells were used at passages 19-24. Cytokine stimulation was performed in serum-free medium.

To set up the *in vitro* BBB model, 2 x $10^5$ THBMECs were seeded in 12mm Transwell® inserts with 3.0 µm pore polycarbonate membrane (Corning Inc., USA) which had been coated with a mixture of 10 µg/ml fibronectin and 10 µg/ml collagen IV in PBS for 1 h at 37°C shortly before seeding the cells. Cells were grown to confluence for 10-15 days with medium change all 1-3 days. For cytokine stimulation, growth medium containing 10 ng/ml cytokines were added to the upper compartment.

Growth Medium
DMEM:F12 1:1
fetal bovine serum (10%)
penicillin/streptomycin (100 U/ml)
L-glutamine (440 mg/l)

Stimulation Medium
DMEM:F12 1:1
penicillin/streptomycin (100 U/ml)
L-glutamine (440 mg/l)

PBS/EDTA solution
0.05% (w/v) EDTA
in PBS w/o $Mg^{2+}$, $Ca^{2+}$

#### 2.2.1.1 Determination of the transendothelial electrical resistance

To monitor the barrier tightness of the endothelial cell layer, TEER was measured using an Endohm-12 chamber (World Precision Instruments, USA) and a volt-ohm-meter (Millipore, USA). Due to two annular electrodes in the upper and lower compartment, the specific electrical resistance of the cell monolayer was defined. After subtraction of the blank filter's TEER, the value was multiplied by the filter area. Each approach was performed in duplicate.

To measure direct effects of function-blocking antibodies on the tightness of the endothelial cell layer, the automatic cell monitoring system cellZscope® (nanoAnalytics GmbH, Münster) was used according to the manufacturer's instructions. Data analysis was performed with the cellZscope® software (nanoAnalytics GmbH, Münster).

#### 2.2.1.2 Permeability assay

Another method to control barrier tightness is to determine the paracellular permeability coefficient (Deli *et al.*, 2005). The lower compartment of the transwell was filled with 1.5 ml RPMI medium without phenol red. In the upper compartment 0.5 ml of a 10 µg/ml sodium fluorescein solution was added. After 20 and 40 min, the insert was transferred into another well filled with 1.5 ml RPMI without phenol red. Fluorescence of aliquots, taken after 20, 40

and 60 min, was determined in a fluorescence multiplate reader at excitation 485/20 nm and emission 528/20 nm. By means of a standard curve the abluminal concentration was calculated. All approaches were performed in duplicate. The calculation of the permeability coefficient (Pe in cm/s) is described below (Deli *et al.*, 2005).

Calculation of the amount of sodium fluorescein in the upper compartment

$$\text{n lum } 20\text{min} = \left(\text{V lum } \cdot 10\frac{\mu g}{ml}\right) - (\text{V ablum} \cdot \text{c ablum } 20 \text{ min})$$

$$\text{n lum } 40\text{min} = \left(\text{V lum } \cdot 10\frac{\mu g}{ml}\right) - (\text{V ablum } \cdot (\text{c ablum } 20 \text{ min} + \text{c ablum } 40 \text{ min}))$$

$$\text{n lum } 60\text{min} = \left(\text{V lum } \cdot 10\frac{\mu g}{ml}\right) - (\text{V ablum } \cdot (\text{c ablum } 20 \text{ min} + \text{c ablum } 40 \text{ min} + \text{c ablum } 60 \text{ min}))$$

Calculation of the concentration of sodium fluorescein in the upper compartment

$$\text{c ablum } 20 \text{ min} = \text{n lum } 20\text{min} \cdot 2$$

$$\text{c ablum } 40 \text{ min} = \text{n lum } 40\text{min} \cdot 2$$

$$\text{c ablum } 60 \text{ min} = \text{n lum } 60\text{min} \cdot 2$$

Calculation of the flow-through of sodium fluorescein

$$\text{flow} - \text{through } 20 \text{ min} = \frac{\text{c ablum } 20\text{min} \cdot \text{V ablum}}{\text{c lum } 20 \text{ min}}$$

$$\text{flow} - \text{through } 40 \text{ min} = \text{flow} - \text{through } 20 \text{ min} + \frac{\text{c ablum } 40\text{min} \cdot \text{V ablum}}{\text{c lum } 40 \text{ min}}$$

$$\text{flow} - \text{through } 60 \text{ min} = \text{flow} - \text{through } 40 \text{ min} + \frac{\text{c ablum } 60\text{min} \cdot \text{V ablum}}{\text{c lum } 60 \text{ min}}$$

Calculation of the permeability coefficient

The flow-through of the filters is plotted against the time and the slope can be calculated.

$$\frac{1}{\text{slope endothel}} = \frac{1}{\text{slope cells}} - \frac{1}{\text{slope blank}}$$

$$\text{Pe} = \text{slope endothel } \cdot \text{area of the membrane}$$

Legend:

c ablum: abluminal concentration in the lower compartment

c lum: luminal concentration in the upper compartment

n lum: absolute amount of sodium fluorescein in the upper compartment

V ablum: volume in the lower compartment (1.5 ml)

V lum: volume in the upper compartment (0.5 ml)

#### 2.2.1.3 Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh heparinised whole blood of healthy donors by density centrifugation. For this purpose, blood was diluted 1:1 with PBS. 10 ml Ficoll Plaque$^{TM}$ Plus was covered with 40 ml blood solution and centrifuged at 400 x g with the break off for 30 min at room temperature. Serum was aspirated and the white interphase, containing the PBMCs, was transferred into a new tube. Cells were washed in 40 ml PBS and centrifuged at 400 x g for 7 min. Wash steps were repeated at least twice until the supernatant was clear. PBMCs were used either directly for transmigration experiments (see chapter 2.2.1.4), labelled with calcein AM (see chapter 2.2.1.5) or separated into monocytes and T lymphocytes in further steps by magnetic cell separation (see chapter 2.2.1.6.).

#### 2.2.1.4 Fluorescence labelling of peripheral blood mononuclear cells

PBMCs were labelled with an acetomethoxy derivate of calcein (calcein AM; Life Technologies, Darmstadt) at a concentration of 10 µM in serum-free medium for 15 min. Calcein AM is transported through the cellular membrane into cells. After transport into the cell, intracellular esterases remove the acetomethoxy group and the remaining molecule gives out strong green fluorescence. As dead cells lack active esterases, only living cells will be labelled. Fluorescence was measured in a fluorescence plate reader at excitation 485/20 nm and emission 528/20 nm. For quantification, a standard curve with a defined amount of fluorescently labelled cells was prepared. Fluorescence of transmigrated cells was correlated to the standard curve.

#### 2.2.1.5 Magnetic cell separation

Magnetic cell separation (MACS) was performed to separate PBMCs into CD3+ T lymphocytes and CD14+ monocytes. For this purpose, magnetic microbeads coupled with specific antibodies were incubated with $10^7$ - $10^8$ PBMCs. This mixture of cells and micro-beads was loaded onto a magnetic column, what resulted in the isolation of the target cells which had bound to the antibody. Unbound cells were removed by several washes with MACS buffer. After removal of the magnetic field, target cells were eluted. MACS was performed with QuadroMACS$^{TM}$ separator, LS columns and CD3 MicroBeads or CD14 MicroBeads in accordance to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach).

MACS buffer
0.5% (w/v) BSA
2 mM EDTA
in PBS pH 7.2

### 2.2.1.6 Transmigration assay

Transmigration assay was performed with THBMEC layer, exhibiting a TEER of at least 150 Ω x $cm^2$, based on Callahan *et al.*, 2004. For transmigration, $10^6$ calcein AM labelled PBMCs were suspended in 0.5 ml of transendothelial migration buffer (TEM buffer), added to the upper compartment and allowed to transmigrate at 37° C for the indicated times. The lower compartment was filled with 1.5 ml TEM buffer. After incubation, migrated and non-migrated cells were resuspended by pipetting them up and down, and the bottom of the membrane was washed thoroughly.

For quantification, aliquots of 100 µl cell suspension were measured in a fluorescence plate reader at excitation 485/20 nm and emission 528/20 nm. The amount of transmigrated cells was calculated by means of a standard curve. Each approach was performed in duplicate.

For qualitative analysis, transmigration was performed with unlabelled PBMCs. At least ten filters were pooled for each condition tested. Cells from the upper and the lower compartment were examined for expression of CD3, CD14, CD16 and CD19 by flow cytometry.

For functional studies of integrins during transmigration, THBMEC layer was incubated with function-blocking antibodies directed against integrin subunits for 30 min. Subsequently, cells were washed with PBS and $10^6$ calcein AM labelled PBMCs in TEM buffer were added to the upper compartment and allowed to transmigrate at 37°C for the indicated times.

TEM buffer
RPMI medium without phenol red
1% (w/v) BSA
L-glutamine (440 mg/l)
penicillin/ streptomycin (100U/ml)

### 2.2.1.7 Flow cytometry

5 x $10^5$ THBMECs, grown in 6 cm dishes for three days, were detached with PBS/EDTA and centrifuged at 170 x g for 3 min. To reduce unspecific binding, cells were incubated in 1% BSA in PBS for 30 min. Cells were washed in FACS Flow™ (BD Bioscience, Heidelberg). Then, 5 x $10^5$ cells were resuspended in 200 µl FACS Flow™ containing the respective concentration of primary antibody and incubated for 45 min on ice. Subsequently, cells were

washed twice and incubated with the secondary antibody in the dark for another 45 min on ice. Cells were washed twice in FACS Flow™. For controls, cells were incubated with an isotype control antibody. Labelled cells were measured at a FACSCalibur™ (BD Bioscience, Heidelberg). Analysis was performed using CellQuest™ Pro (Version 5.2.1, BD Bioscience, Heidelberg).

FACS-Flow™ (BD Biosciences, Heidelberg)
0.1% (w/v) BSA
0.03% (w/v) $NaN_3$
in PBS

### 2.2.1.8 Adhesion assay

For quantification of cell matrix adhesion, 96-well plates were coated with fibronectin, collagen IV, collagen I and laminin-1 (10 µg/ml in PBS) for 1 h at 37°C. Non-specific binding was blocked with 1% BSA in PBS for 1 h at 37°C. Cells were incubated in serum-free medium for at least 1 h. 5 x $10^4$ cells in 100 µl serum-free medium were added to each well. After 1 h of incubation at 37°C, non-adherent cells were removed by washing three times with PBS. Attached cells were fixed and stained with 0.1% crystal violet. Following intensive washing with distilled water, plates were solubilised in Triton X-100 and photometrically measured at 570 nm. Each approach was performed at least in triplicate.

Blocking solution
1% (w/v) BSA
in PBS

Fixation/permeabilisation solution
4% (w/v) paraformaldehyde
0.25% (w/v) saponin
in PBS

Solubilisation solution
0.1% (v/v)Triton X-100

Staining solution
0.1% (w/v) crystal violet

### 2.2.1.9 Immunofluorescence analysis

1 x $10^4$ THBMECs (subconfluent state) or 2 x $10^4$ THBMECs (confluent state) were seeded onto 8-well Permanox™ slides (Nunc, Wiesbaden), coated with fibronectin and collagen IV (10 µg/ml in PBS each), and cultivated for 24 h and 72 h, respectively. Subsequently, cells were washed and incubated with cytokines for 72 h. Cells were washed with PBS, fixed and permeabilised for 15 min at room temperature. After blocking for 30 min with 1% BSA in PBS, cells were incubated with the respective antibody at 4°C overnight. Then, cells were accurately washed with PBS and PBS/Triton X-100. Afterwards, cells were incubated in the dark with the secondary antibody for 1 h and accurately washed again. Slides were analysed on a Zeiss Axiovert 200 microscope. Images were taken with an Axiocam at a magnification

of 64x and processed with Axiovision software (AxioVs40V; Zeiss, Jena, Germany). Images were further processed using Adobe Photoshop (version 8.0.1). Images that were meant to be compared one with another were acquired using identical settings of exposure and processing.

PBS
137 mM NaCl
2.7 mM KCl
8 mM $Na_2HPO_2.2H_2O$
1.8 mM $KH_2PO_4$
adjust to pH 7.4

Fixation/ permeabilisation solution
4% (w/v) paraformaldehyde
0.25% (w/v) saponin
in PBS

PBS/Triton X-100
0.1% (v/v) Triton X-100
in PBS pH 7.4

Fixation solution
ice cold methanol

Permeabilisation solution
0.2 % (v/v) Triton X-100
in PBS pH 7.4

## 2.2.2 Biochemistry

### 2.2.2.1 Cell solubilisation

To prepare THBMEC lysates, cultured cells were washed with ice-cold PBS, scraped in solubilisation buffer and incubated for 60 min at 4°C. Lysates were centrifuged at 16,000 x g for 15 min at 4°C. Protein concentration of the supernatant was quantified using the bicinchoninic acid (BCA) protein assay. Lysates were boiled in the presence of Laemmli sample buffer at 95°C for 5 min and stored at -20°C or used directly for gel electrophoresis (see chapter 2.2.2.5).

Solubilisation buffer
20 mM Hepes/NaOH pH 7.5
150 mM NaCl
1 mM $MgCl_2$
1 mM $CaCl_2$
1% (v/v) triton X-100
0.1 mM $NaVO_4$
1 mM PMSF
0.1% (v/v) protease inhibitor cocktail (PIC)
25 mM NaF

Laemmli sample buffer
250 mM Tris/HCl pH 6.8
25% glycerol
7.5% SDS
0.25 mg/ml bromphenol blue
12.5 % β-mercaptoethanol

#### 2.2.2.2 Matrix protein extraction

To extract matrix proteins synthesised from endothelial cell monolayers, cells were washed with ice-cold PBS, scraped in carbamide buffer and incubated for 120 min at 4°C. The extracts were centrifuged at 16,000 x g for 15 min at 4°C. The supernatant was transferred into a new tube, quantified using BCA protein assay (see chapter 2.2.2.4) and boiled in the presence of Laemmli sample buffer at 95°C for 5 min. Lysates were stored at -20°C or used directly for SDS-PAGE.

Carbamide buffer
0.1 M Tris/HCl 7.5
10 mM EDTA
2 M $CO(NH_2)_2$
10 mM $Na_2S_2O_5$
1 mM PMSF
0.1% (v/v) PIC

#### 2.2.2.3 Concentration of cell culture supernatants

THBMECs, seeded at 5 x $10^5$ cells in 10 cm dishes, were grown to confluence and then incubated in serum-free medium for 3 days. 4 ml of cell culture supernatant was centrifuged at 250 x g for 4 min to remove debris. The supernatant was transferred onto Vivaspin 4 ml devices (Satorius Stedim Biotech GmbH, Göttingen) and centrifuged at 3,300 x g for 10 - 15 min. Subsequently, the membrane was washed accurately with the remaining 100 µl medium to remove all proteins from the membrane. Then medium was boiled in the presence of Laemmli sample buffer at 95°C for 5 min, stored at -20°C or used directly for gel electrophoresis (see chapter 2.2.2.5).

#### 2.2.2.4 Protein quantification

BCA protein assay is a detergent-compatible formulation based on bicinchoninic acid for the colourimetric detection and quantification of total protein (Smith *et al.*, 1985). A fresh protein standard was prepared by diluting the 2 mg/ml BSA stock standard (Pierce, USA) with water in serial dilution in a 96-well plate, reaching a volume of 20 µl. The samples were diluted 1:10 in water. BCA™ protein assay reagent A and B (Pierce, USA) were mixed together in a ratio of 1:50, and 180 µl of this mixture was added to standard and samples. The samples were shortly mixed, and the plate was subsequently incubated for 30 min at room temperature. Absorptions were determined at 570 nm in an ELISA plate reader. Protein concentrations were calculated using a standard curve.

### 2.2.2.5 SDS polyacrylamide gel electrophoresis

Denaturing sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a commonly used method to separate proteins on polyacrylamide gels according to their size (Laemmli, 1970). Solutions A, B and C were used to prepare separating and stacking gel (table 2). Protein samples, which were supplemented and boiled with Laemmli sample buffer, were loaded onto the gels. Gel electrophoresis was performed in SDS-PAGE running buffer for 1.5 -2.5 h at 100-150 V. Gels were then applied to western blotting.

| Solution A (Rotiphorese Gel 30) | Solution B | Solution C |
|---|---|---|
| 30% (w/v) acrylamide | 1.5 M Tris/HCl pH 8.8 | 0.5 M Tris/HCl pH 6.8 |
| 0.8% (w/v) bis-acrylamide | 0.4% (w/v) SDS | 0.4% (w/v) SDS |

SDS-PAGE running buffer (10x)
192 mM Tris/HCl pH 7.3
25 mM glycine
0.1% (w/v) SDS

**Table 2: Preparation of separating and stacking gel**

| Solution | Separating gel | | | Stacking gel |
|---|---|---|---|---|
| | 6% | 7.5% | 12.5% | 4% |
| Solution A | 1.8 ml | 2.25 ml | 3.75 ml | 0.4 ml |
| Solution B | 2.25 ml | 2.25 ml | 2.25 ml | - |
| Solution C | - | - | - | 0.75 ml |
| Water | 4.95 ml | 4.5 ml | 3.0 ml | 1.85 ml |
| APS | 45 µl | 45 µl | 45 µl | 18 µl |
| TEMED | 4.5 µl | 4.5 µl | 4.5 µl | 5 µl |

### 2.2.2.6 Western blotting

For western blotting, cells were lysed in solubilisation buffer. Supernatants were denatured by boiling with Laemmli's sample buffer. Samples were separated by 6%, 7.5% or 10% SDS-PAGE under reducing conditions. Separated proteins were transferred to nitrocellulose membranes for 1 h in blotting buffer with a constant amperage of 0.25 A. The protein transfer to the membrane was verified by staining with Ponceau S solution, followed by the decolouration with 0.1% acetic acid solution and tris-buffered saline (TBS). Membranes were subsequently blocked for 1 h. The blots were incubated with suitable primary antibody overnight at 4°C. After incubation with the appropriate horseradish peroxidase-conjugated secondary antibody, proteins were detected with Supersignal West Pico or Femto reagents

(Thermo Scientific Inc, USA) and signals were visualized using the Versadoc 4000 MP imaging system (BioRad, Munich).

TBS 1x (Abcam)
20 mM Tris/HCl pH 7.6
140 mM NaCl

TBS 1x (BD)
10 mM Tris/HCl pH 7.5
100 mM NaCl

TBS/Tween-20
0.1% (v/v) Tween-20
in TBS

Blocking buffer
5% (w/v) BSA
in TBS

Ponceau S staining solution (5x)
2% (w/v) ponceau S
30% (v/v) trichloroacetic acid
30% (v/v) sulfosalicylic acid

Blotting buffer (10x)
1 M Tris/HCl pH 8.3
1.92 M glycine
10% (v/v) ethanol

#### 2.2.2.7 Enzyme-linked Immunosorbent Assay

Enzyme-linked Immunosorbent Assay (ELISA) was performed to measure cytokine secretion into cell culture medium. Conditioned cell culture media were centrifuged at 250 g for 4 min to remove debris. The supernatant was used directly or stored at -80°C.

A human IL-1β ELISA was performed with pre-coated plates in accordance to the manufacturer's instructions (BioLegend Inc., USA). For human IL-6, IL-8 and TNFα ELISA Development Kit purchased by Preprotech (Hamburg), 96well-plates (Maxisorp, Nunc, USA) were coated with 1 - 1.5 µg/ml capture antibody over night at room temperature. Wells were then washed three times in washing buffer and blocked in blocking solution for 1 h at room temperature. After three washes, wells were incubated with cytokine standard in different concentrations and the cell culture supernatants, respectively, on a plate shaker for 2 h at room temperature. Following further washing steps, antigen/antibody complex was incubated with the detection antibody for another 2 h. Wells were again washed three times and avidin peroxidase was added to each well for 1 h. After washing intensively, peroxidase reaction was initiated by adding ABTS substrate solution. Absorptions were determined at 405 nm in an ELISA plate reader for 60 min every 5 min. Each approach was performed in triplicate.

Washing buffer
0.05% (v/v) Tween-20
in PBS

Blocking buffer
1% (w/v) BSA
in PBS

ABTS substrate solution
1 tablet ABTS (10 mg, Sigma-Aldrich, Steinheim)
in 0.05M phosphate-citrate buffer pH 5.0
30% $H_2O_2$ (added immediately prior to use)

#### 2.2.2.8 Zymography

5 x $10^5$ THBMECs, seeded in 6 cm dishes, were grown to confluence for 3 days and then incubated in the presence of cytokines (10 ng/ml in serum-free medium) for 24 h and 48 h. 2 ml of cell culture supernatant was centrifuged at 250 x g for 3 min to remove debris. Supernatant was transferred onto 70 µl gelatine agarose and incubated on a spinning wheel for 2 h at 4°C. Subsequently, suspension was centrifuged at 400 x g for 2 min. Gelatine agarose was washed three times with PBS, resuspended in 30 µl Laemmli sample buffer and boiled at 95°C for 5 min. Samples were loaded onto the polyacrylamide gels containing gelatine (table 3). Gel electrophoresis was performed in SDS-PAGE running buffer for 3 h at 100 V on ice. Gel was incubated in washing buffer for 1 h, washed twice in distilled water and incubated at 37°C for 16 h. After incubation time, gel was stained in coomassie brilliant blue solution for 30 min at room temperature and decolourised with distilled water. Gel was documented using a Nikon D90 camera. Images were further processed using Adobe Photoshop (version 8.0.1).

Washing buffer
2.5% (v/v) Triton X-100

Incubation buffer
50 mM Tris/HCl pH 7.5
200 mM NaCl
0.02 Brij 35
5 mM $CaCl_2$

**Table 3: Preparation of zymography gels**

| Solution | Separating gel | Stacking gel |
|---|---|---|
| Solution A | 3 ml | 0.4 ml |
| Solution B | 1.8 ml | - |
| Solution C | - | 0.75 ml |
| Water | 3.75 ml | 1.85 ml |
| Gelatine | 450 µl | - |
| APS | 50 µl | 18 µl |
| TEMED | 5 µl | 5 µl |

## 2.2.3 Molecular biology

### 2.2.3.1 RNA isolation

RNA was extracted from cells with Quick-RNA™ Mini Prep in accordance to the manufacturer's instructions (Zymo Research Inc., USA). Quality of the RNA was verified by the presence of the 28S and 18S rRNA on agarose gels and an $OD_{260}/OD_{280}$ ratio in the range of 1.9-2.1. RNA samples were stored at -80°C or used directly for cDNA synthesis.

### 2.2.3.2 cDNA synthesis

Synthesis of cDNA from 1 µg RNA was performed with GoScript™ Reverse Transcription system in accordance to the manufacturer's instructions (Promega, Mannheim).

### 2.2.3.3 Polymerase Chain Reaction

To analyse the usability of different primers for quantitative Real-Time polymerase-chain reaction (qRT-PCR) a PCR with a temperature-gradient was performed. The following PCR mix was added to each well of the PCR 8-stripe tube (Eppendorf, Hamburg).

PCR mix

6.5 µl BioMix 2x (Bioline GmbH, Luckenwalde)
0.25 µl primer sense (10 µM)
0.25 µl primer antisense (10 µM)
0.5 µg cDNA
ad 12.5 µl $H_2O$

Gradient PCR was run in an iCylcer system (BioRad, Munich) with the following programm:

| | | | |
|---|---|---|---|
| Cycle 1 (1x): | Step 1: | 94°C | 2 min |
| Cycle 2 (30x): | Step 1: | 94°C | 20 s |
| | Step 2: | 55°C-65°C | 60 s |
| | Step 3: | 72°C | 2 min |
| Cycle 3 (1x): | Step 1: | 72°C | 10 min |
| Cycle 4 (1x): | Step 1: | 4°C | 10 min |

PCR products were supplemented with 6x DNA loading dye solution (Fermentas, St. Leon-Rot), stored at -20°C or used directly for agarose gel electrophoresis.

#### 2.2.3.4 Agarose gel electrophoresis

Agarose gel electrophoresis was performed to validate RNA quality and to check primer usability. Samples were supplemented with 6x loading dye solution (Fermentas, St. Leon-Rot) and loaded on 2% agarose gels, which were prepared with 1:10,000 GelRed Nucleic Acid Stain (Biotium Inc., USA) in Tris-acetate-EDTA (TAE) buffer. Quick load® 100 bp DNA ladder (New England Biolabs, UK) was used as size standard. Gels were run in agarose gel electrophoresis chambers at 100 V for approximately 1 h in TAE buffer. Bands were visualised by the Versadoc 4000 MP imaging system (BioRad, Munich).

TAE-Buffer (1x)
40 mM Tris
1 mM EDTA
40 mM acetic acid

#### 2.2.3.5 Quantitative Real-Time-PCR

For qRT-PCR, GoTaq® qPCR Master Mix (Promega, Mannheim) was used. For all samples, a master mix was prepared, and 18 µl of this mixture were added to one well of the 96 well-PCR plate (Sarstedt, Nümbrecht). 2 µl of the 1:20 diluted cDNA was added and mixed gently. Each approach was performed in quadruplicate.
The qRT-PCR was run in an iCycler MyiQ™ Single Colour Real-Time Detection System (BioRad, Munich) with the following programm:

| | | | |
|---|---|---|---|
| Cycle 1 (1x): | Step 1: | 95°C | 2 min |
| Cycle 2 (33x): | Step 1: | 95°C | 15 s |
| | Step 2: | 62°C | 30 s |
| | Step 3: | 72°C | 35 s |
| | Data collection and real-time analysis were performed. | | |
| Cycle 3 (36x): | Step 1: | 60°C-95°C | 30 s |
| | Set point temperature was increased after cycle 2 by 1°C. Melt curve data collection and analysis were performed using BioRad iQ™5 standard edition optical system software (version 2.0; BioRad, Munich). | | |

Master mix

10 µl GoTaq® qPCR Master Mix 2x (Promega, Mannheim)

0.75 µl primer sense (10µM)

0.75 µl primer antisense (10µM)

6.5 µl $H_2O$

### 2.2.4 Statistical analysis

Statistical differences between two groups were calculated by means of the student's t-test, if data was normally distributed. Otherwise, significances were determined by Mann-Whitney U-test. All experiments were performed at least three times.

# 3 Results

## 3.1 Establishment of an *in vitro* blood-brain barrier model

One main goal of this study was the establishment of an *in vitro* BBB model which resembles the *in vivo* barrier as closely as possible with regard to its specific morphological, physical and functional characteristics.

### 3.1.1 Verification of the endothelial cell specific protein von Willebrand factor

For the establishment of an *in vitro* BBB model, the cell line THBMECs was used, derived from adult human brain microvascular endothelium and transfected as well as immortalised by a plasmid containing SV-40LT, kindly provided by Dr. Kwang Sik Kim (The Johns Hopkins University, Baltimore, USA; Stins *et al.*, 2001). To evaluate if this cell line still fulfils the most important characteristics of endothelial cells, the expression of the endothelial cell specific glycoprotein vWF was investigated. Therefore, total RNA was isolated from THBMECs, grown on dishes coated with a mixture of fibronectin and collagen IV for 3 days, and re-transcribed into cDNA. PCR analysis revealed that THBMECs expressed the vWF mRNA. The fibroblastic cell line CHO served as a negative control (figure 7A). Furthermore, supernatant from THBMEC cultures was concentrated and subjected to SDS-PAGE. Subsequently, western blotting with a vWF-specific antibody was performed. It was shown that the vWF protein was also synthesised and secreted by THBMECs (figure 7B). Human normal plasma as well as a vWF concentrate, kindly provided by Dr. Brite Fuchs (Octapharma R&D Molecular Biochemistry Department Berlin), served as positive controls. The mature protein of 225 kDa as well as the vWF precursor protein of 309 kDa was detected in THBMEC conditioned medium (figure 7B).

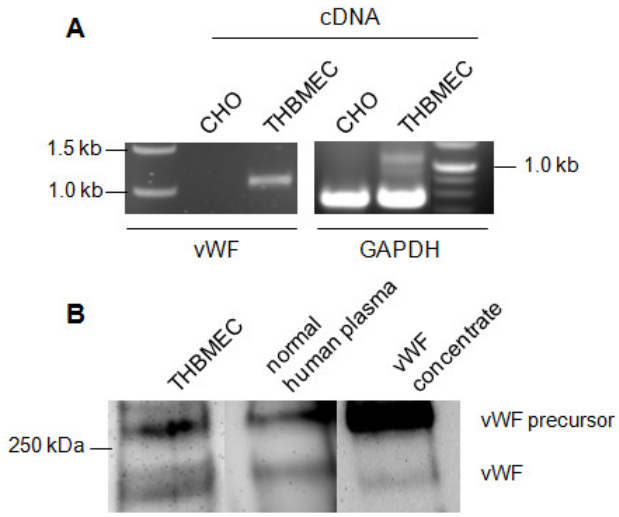


**Figure 7: Expression and secretion of von Willebrand factor**

Total RNA was extracted from confluent CHO cells as well as THBMECs, and cDNA was synthesised. PCR was performed with primers specific for vWF and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Following agarose gel electrophoresis, bands were visualised using the Versadoc 4000-MP imaging system. CHO cells served as negative control. B) Cell culture supernatant from confluent THBMECs was concentrated using Vivaspin 4ml (Sartorius AG, Göttingen). Proteins were separated by SDS-PAGE and subjected to western blotting using an HRP-anti-vWF antibody. Bands were visualised using the Versadoc 4000-MP imaging system. Human normal plasma as well as a vWF concentrate served as positive controls. The vWF precursor protein (309 kDa) and the vWF mature protein (225 kDa) could be detected.

### 3.1.2 Morphological validation

During the process of establishment of the *in vitro* BBB model different parameters, e.g. cell number, material of the insert membrane as well as composition of the medium and the underlying matrix, were varied and the optimal conditions were determined. To set up the *in vitro* BBB model, 2 x $10^5$ THBMECs were seeded onto 12mm Transwell® inserts with 3.0 µm pore polycarbonate membrane (Corning Inc., USA), which had been coated with a mixture of 10 µg/ml fibronectin and 10 µg/ml collagen IV. Cells were grown to confluence in DMEM:F12 1:1 medium supplemented with L-glutamine, fetal bovine serum and penicillin/streptomycin for 10-15 days.

To evaluate the morphological features of the *in vitro* BBB model, the expression and localisation of the tight junction proteins claudin-5, occludin and ZO-1, which are important for developing and maintaining the BBB function, were investigated. THBMECs were grown to confluence on fibronectin and collagen IV coated dishes, and total RNA as well as protein lysates were extracted. Analyses of RNA-derived cDNA by PCR and of cell lysates by western blotting revealed that all mentioned proteins were expressed at the mRNA and protein level (figure 8A). Using immunofluorescence microscopy it was shown that localisation of claudin-5, occludin and ZO-1 was restricted mainly to cell-cell junctions as

typical for brain endothelial cells (figure 8B). Furthermore, the F-actin cytoskeleton was organised in cortical rings and only a few F-actin stress fibres were detected (data not shown).

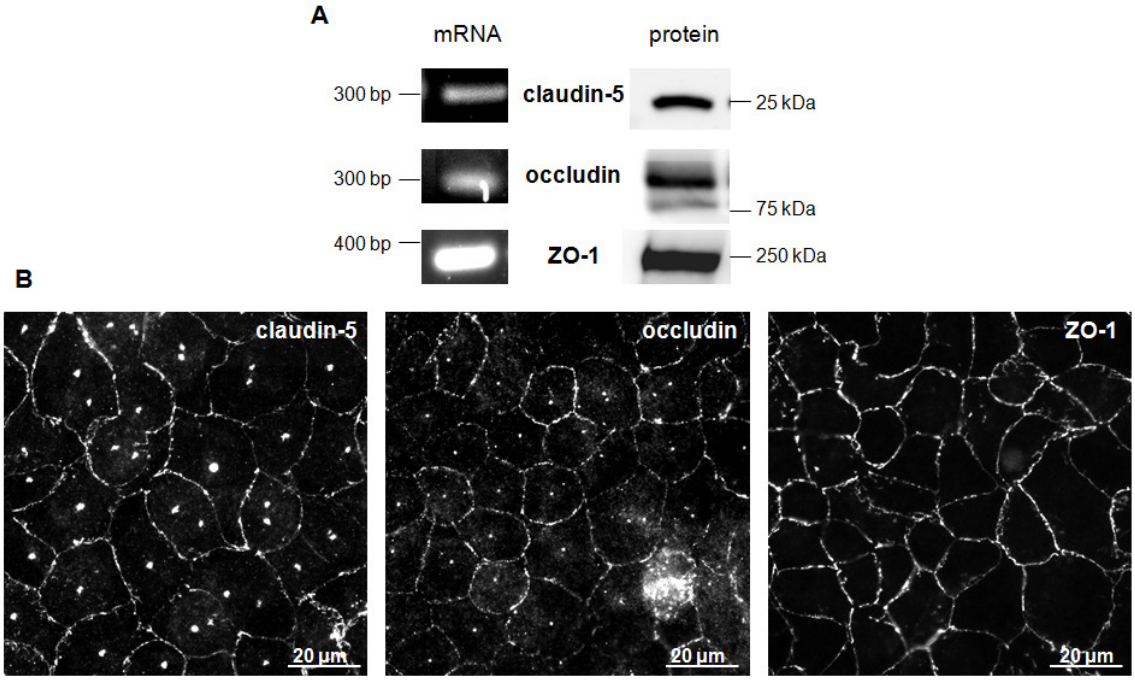


**Figure 8: Expression and localisation of tight junction proteins**

5 x $10^5$ THBMECs were seeded and incubated for 3 days in growth medium. For mRNA expression, total RNA was extracted, cDNA was synthesised, and PCR was performed with primers specific for claudin-5, occludin and ZO-1. Following agarose gel electrophoresis, bands were detected with the Versadoc 4000-MP imaging system. For analysis of protein expression, cells were lysed, and extracted proteins were separated by SDS-PAGE and subjected to western blotting using antibodies raised against claudin-5, occludin and ZO-1. B) Confluent THBMECs were fixed and permeabilised with PFA/saponin or ice cold methanol/PBS-Triton X-100, blocked in 1% BSA and incubated with the appropriate primary and secondary antibodies. Images were taken on a Zeiss Axiovert 200 microscope with a magnification of 64x.

### 3.1.3 Physical validation

To monitor the barrier tightness of the *in vitro* BBB model, the TEER was determined. 2 x $10^5$ THBMECs were seeded onto 12mm Transwell® inserts. The TEER was measured every 2-4 days over a period of 17 days. A higher transendothelial resistance of the monolayer reflects a tighter barrier. The *in vitro* BBB model established in this work reached maximum values of 250 Ω x $cm^2$. Figure 9 shows the course of TEER over time (black bars). Only monolayers with a TEER of at least 150 Ω x $cm^2$ or higher were used for further experiments.

Besides a high electrical resistance, the BBB is characterised by a low permeability for water soluble substances due to the tight interaction of endothelial cells through interendothelial junctions. To ensure the comparability of the permeability of different *in vitro* BBB models,

a permeability coefficient for tracer substances, which do not bind to transporters, receptors or enzymes, needed to be determined. To evaluate the paracellular permeability of this model, the permeability coefficient of the paracellular permeability marker sodium fluorescein was determined. Here, the THBMEC layers that were also used for TEER measurements were incubated with sodium fluorescein, and the flow-through was analysed. It was observed that the paracellular permeability coefficient decreased with increasing TEER and reached minimum values of 1 x $10^{-6}$ cm/s over time (figure 9, blue bars).

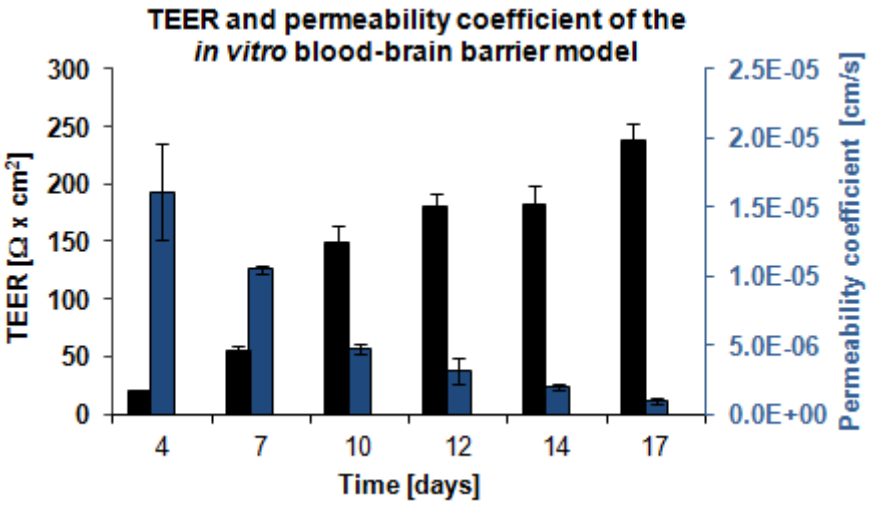


**Figure 9: Transendothelial resistance and permeability coefficient of the *in vitro* BBB model**

THBMECs were seeded in growth media at 2 x $10^5$ cells onto 12mm Transwell® inserts with 3.0 µm pore polycarbonate membrane (Corning Inc., USA) coated with 10 µg/ml fibronectin and 10 µg/ml collagen IV. TEER was measured by means of an Endohm-12 chamber (WPI, USA) and the volt-ohm-meter Millicell® ERS-2 (Millipore, USA). Permeability coefficient of 10 µg/ml sodium fluorescein was determined by permeability assay. All approaches were performed in duplicate. Three independent experiments were summarised.

### 3.1.4 Functional validation

Under physiological conditions, infiltration of immune cells into the central nervous system is kept at a low level. To validate the barrier function of the *in vitro* model, calcein AM labelled PBMCs, isolated from fresh whole blood of healthy donors, were added to Transwells® inserts, containing a THBMEC layer of high resistance or matrix coated blank filters, and allowed to transmigrate for the indicated times. figure 10 illustrates the barrier function of the *in vitro* model. Whereas only 5% of PBMC input crossed the cell monolayer after a transmigration period of 3 h, about 10% passed the layer after 10h and almost 25% of PBMCs migrated after 18 h. In contrast, approximately 25% of the input population

transmigrated across the blank filter after 3 h, about 40% after 10 h and 50% of PBMCs traversed the membrane after 18 h incubation time. Because of the quantification limit, PBMCs were allowed to transmigrate for 18 h in all further experiments.

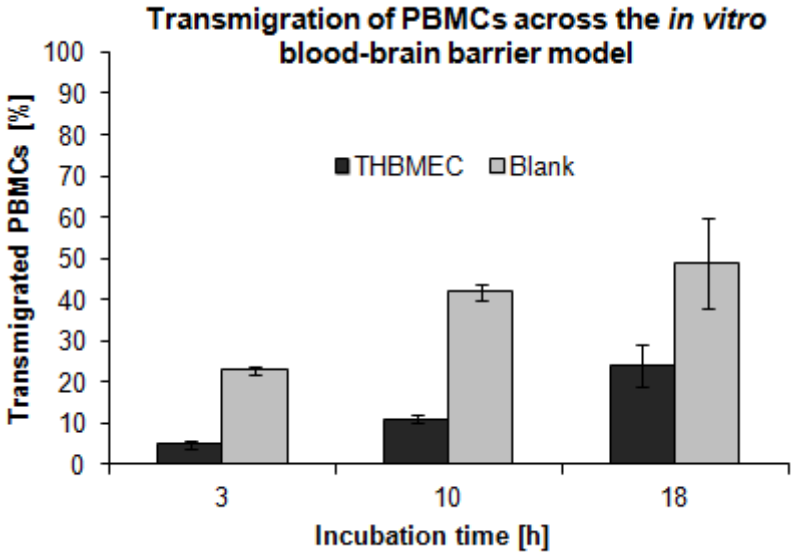


**Figure 10: Quantitative analysis of transmigration of PBMCs**

PBMCs were isolated from fresh heparinised blood of healthy donors by density centrifugation and labelled with calcein AM. Transmigration assays were performed with Transwell® inserts containing confluent THBMECs with a TEER of at least 150 Ω x $cm^2$ for the indicated times or matrix coated blank filters. Aliquots of transmigrated cells were measured in a fluorescence plate reader at 520 nm. The amount of transmigrated cells was calculated by means of a standard curve. All approaches were performed in duplicate. Three independent experiments were summarised.

To investigate which PBMC subpopulation transmigrated, freshly isolated unlabelled PBMCs were allowed to transmigrate for 18 h across the THBMEC layer. Subsequently, the input and the transmigrated population were analysed for expression of the proteins CD3, CD14, CD16 and CD19 by flow cytometry. CD3 is expressed by T lymphocytes. CD14 is a marker protein primarily for macrophages and monocytes. CD16 is found to be expressed on the surface of natural killer (NK) cells, neutrophil cells, monocytes and macrophages. CD19 is expressed by B lymphocytes. The PBMC input consisted of about 70% T lymphocytes, 10% monocytes and macrophages, 10% NK cells and neutrophils and only 5% B lymphocytes (figure 11). After 3 h, the transmigrated fraction comprised of about 5% T lymphocytes, 15% CD14+ monocytes and macrophages as well as 2% NK cells and neutrophils. Only 1% of the cells in the lower compartment tested positive for the B lymphocyte marker CD19 (figure 11). Surprisingly, a major part of the cells, which migrated for 3h, tested negative for all of the CD proteins investigated.

After 18 h, the composition of the migrated population had changed. The fractions comprised of about 40% T lymphocytes, 20-27% monocytes, macrophages, NK cells and neutrophils and 7% B lymphocytes (figure 11).

The enhancement of the percentage of monocytes in the migrated population both after 3 h and after 18 h indicated that monocytes had the most pronounced migratory potential. However, with prolonged time of transmigration other PBMC subtypes also crossed the *in vitro* BBB model, namely T lymphocytes, NK cells and neutrophils. The small amount of B lymphocytes in the fraction of transmigrated cells suggested that they persisted in the upper compartment.

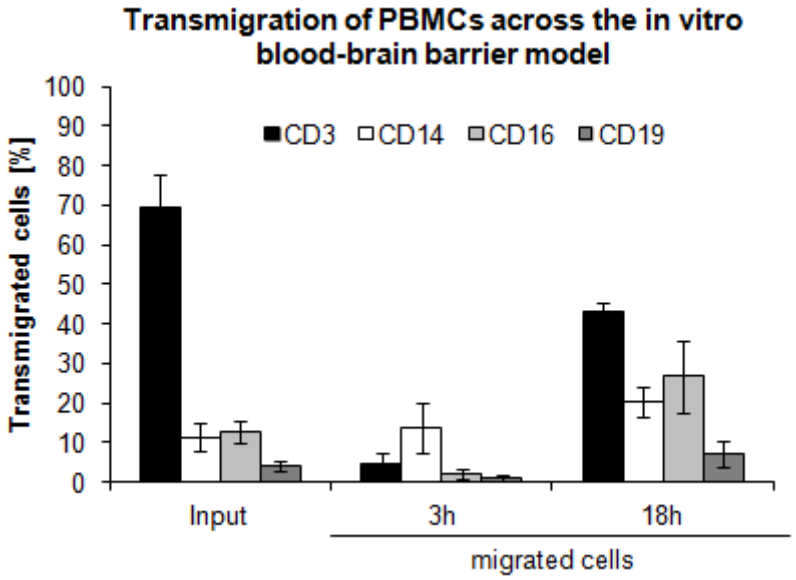


**Figure 11: Qualitative analysis of PBMCs after transmigration**

PBMCs were isolated from fresh whole heparinised blood of healthy donors by density centrifugation and allowed to transmigrate across confluent THBMECs with TEER of at least 150 Ω x $cm^2$ for 3 h or 18 h. Cells in the lower compartment of at least 10 transwells were pooled and analysed for CD3, CD14, CD16 and CD19 expression by flow cytometry. Four independent experiments were summarised.

The data presented demonstrated that this *in vitro* BBB model met physical and functional requirements of the BBB and therefore was a useful tool to investigate the role of β1 integrins at the BBB in further experiments.

### 3.1.5 Characterisation of integrins expressed in THBMECs

To investigate the role of β1 integrins at the BBB, THBMECs had to be characterised in respect of integrin expression and cell matrix adhesion behaviour.

#### 3.1.5.1 Integrin expression

In order to examine mRNA expression of integrins, total RNA was isolated from confluent THBMECs and applied for cDNA synthesis. PCR with primers specific for several integrin subunits was performed and analysed by agarose gel electrophoresis. The mRNA specific for α1, α2, α3, α5, α6 and β1 integrin was detected in THBMECs (figure 12A). Furthermore, surface expression of these integrin subunits was analysed by flow cytometry using appropriate antibodies. Surprisingly, the α1 integrin subunit was detected at the protein level neither by flow cytometry (figure 12B) nor by western blotting (data not shown). However, THBMECs expressed α2, α3, α5, α6 and β1 integrin subunits at the cell surface. Moreover, αv and β3 integrin subunits were detected at the surface of THBMECs (figure 12 B).

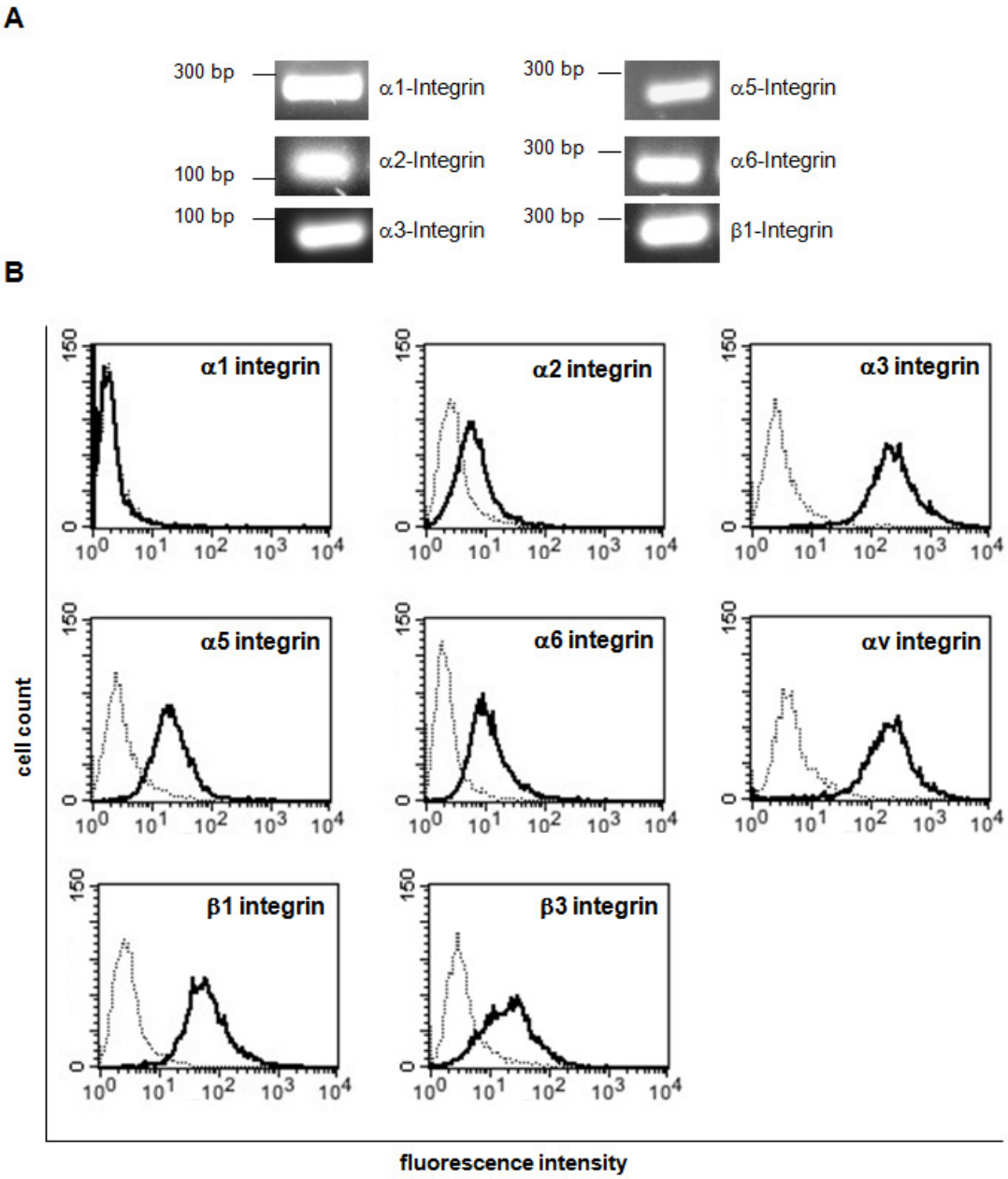


**Figure 12: Integrin expression in THBMECs**

A) 5 x $10^5$ THBMECs were grown for 3 days in growth medium. For mRNA expression, total RNA was extracted, cDNA was synthesised, and PCR was performed with primers specific for different integrins. Following agarose gel electrophoresis, bands were detected with the Versadoc 4000-MP imaging system. B) Confluent THBMECs were analysed for integrin expression by flow cytometry using appropriate fluorophor-coupled antibodies ( ——). Cells, which were incubated with an isotype control antibody, served as negative control ( ·····).

These results indicated that THBMECs express functional α2β1, α3β1, α5β1, α6β1 and αvβ3 integrin at the cell surface.

#### 3.1.5.2 Integrin localisation

Localisation of integrin subunits α3, α5, α6 and β1 was analysed in subconfluent as well as in confluent THBMECs by indirect epifluorescence microscopy. In subconfluent THBMECs, α3 integrin subunit was distributed very diffusely over the cell and lightly enriched in cell protrusions (figure 13A, upper panel, left, arrows). In confluent THBMECs, α3 integrin subunit was mainly concentrated in cell-cell contacts (figure 13B, upper panel, left). The α5 integrin subunit accumulated perinuclearly and in focal contacts at the plasma membrane of THBMECs under subconfluent conditions (figure 13A, lower panel, left, arrowheads). In confluent cells, the α5 integrin subunit was distributed diffusely over the cell with accumulation at the cell borders to adjacent cells (figure 13B, lower panel, left). Subconfluent THBMECs showed a strong localisation of the α6 integrin subunit in the perinuclear region (figure 13A, upper panel, right). Moreover, it was located at plasma membrane ruffles (figure 13B, upper panel, right, arrows). In confluent THBMECs, α6 integrin was also located at the plasma membrane, mainly at cell-cell contacts (figure 13A, upper panel, right). The β1 integrin subunit was, besides its perinuclear localisation, also expressed at the lamellipodium of subconfluent THBMECs (figure 13B, lower panel, right, arrowheads). Distribution of the β1 integrin in confluent cells was similar to that of the α6 integrin subunit. It was distributed all over the plasma membrane with accumulation at the cell-cell contacts of adjacent cells (figure 13B, lower panel, right).

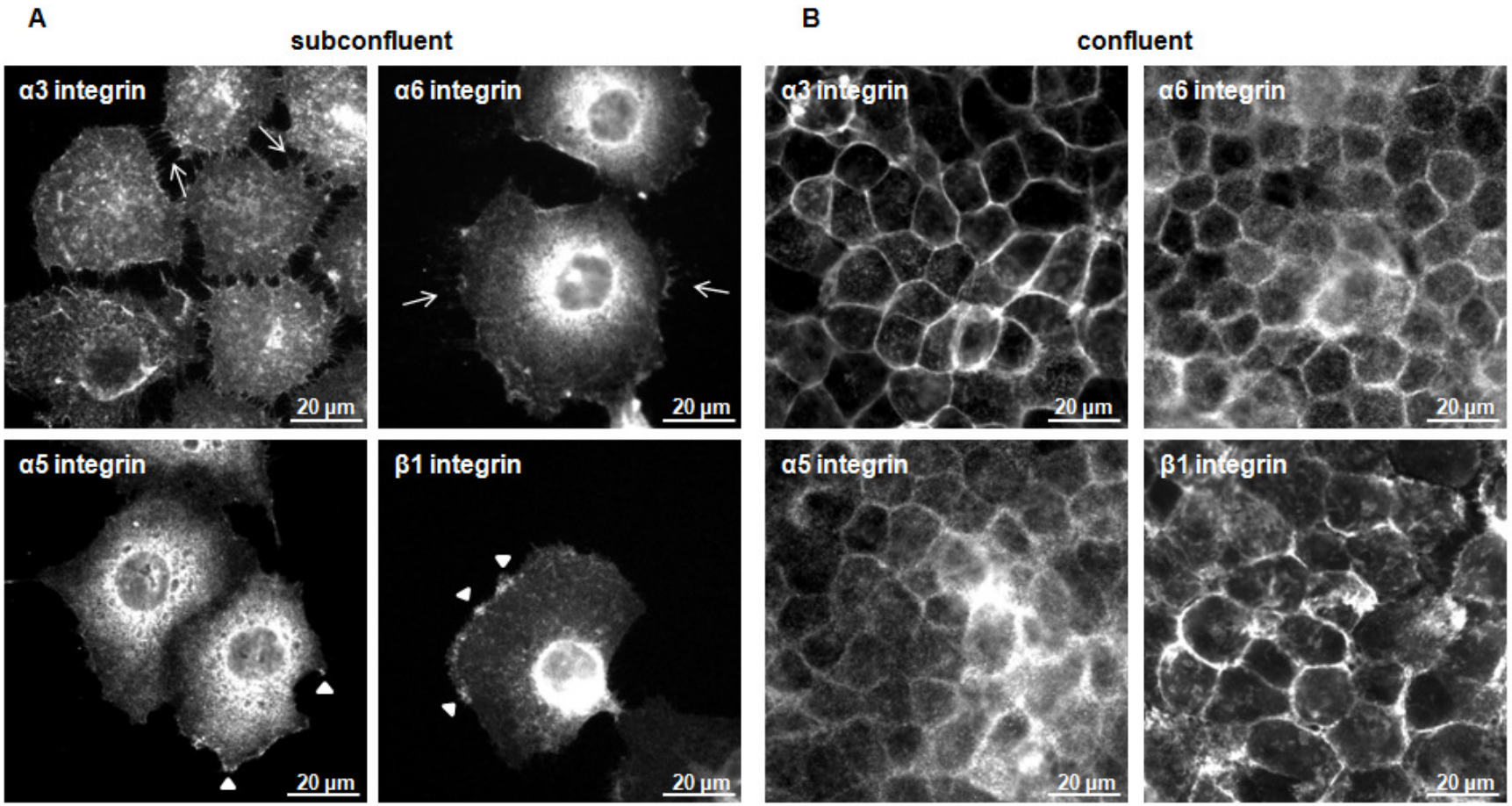


**Figure 13: Localisation of the integrins subunits α3, α5, α6 and β1 in subconfluent and confluent THBMECs**

Subconfluent (A) and confluent (B) THBMECs, grown onto a matrix of fibronectin and collagen IV, were treated with the respective cytokine for 72 h. Subsequently, cells were washed with PBS, fixed and permeabilised. BSA-treated cells were then incubated with the respective antibodies. Images were taken on a Zeiss Axiovert 200 microscope with a magnification of 64x. Arrows indicate membrane ruffles, arrowheads indicate focal contacts at the lamellipodium.

### 3.1.6 Matrix adhesion

B1 integrins are receptors that connect the surrounding ECM outside the cell with the cytoskeleton inside the cell. The combination of the β1 integrin subunit with a defined α integrin subunit determines ligand specificity and accordingly influences adhesion behaviour of cells. To investigate the adhesion of THBMECs to different matrix proteins, adhesion assays on fibronectin, collagen IV, collagen I, laminin-1 and poly-L-lysine were performed.

As depicted in figure 14, adhesion to fibronectin and collagen I was most pronounced. Cells spread on these matrix proteins within 1 h and revealed their typical endothelial morphology (figure 15). THBMECs adhered to laminin-1 to a lower extent (figure 14). Moreover, cells attached to collagen IV but were not able to spread on this protein (figure 15). Poly-L-lysine served as a control for integrin-independent attachment of cells. Attachment to poly-L-lysine resulted in a round, non-spread morphology of the cells (data not shown).

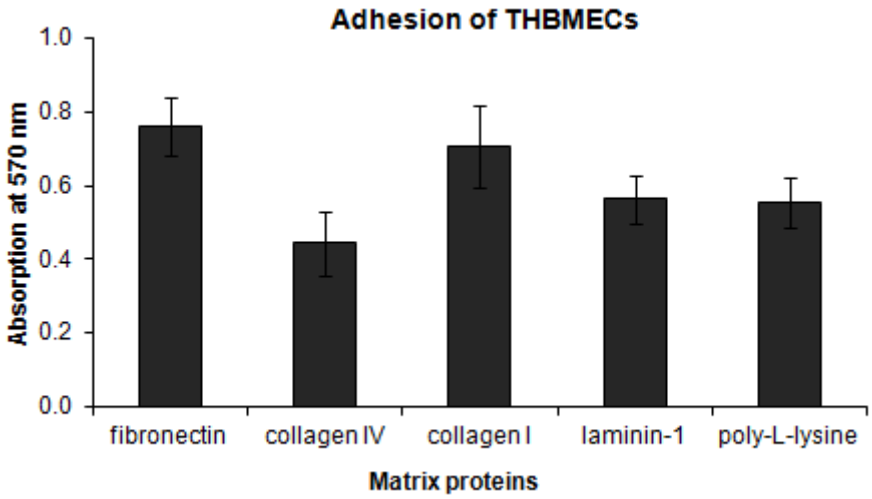


**Figure 14: Adhesion of THBMECs on different matrix proteins**

Confluent THBMECs were serum-starved for 1 h and then plated onto matrix proteins for 1 h. Cells were washed, fixed and stained with crystal violet. Following Triton X-100 dye solubilisation, absorption at 570 nm was measured. Absorption of cells, attached to wells blocked with 1% BSA, were subtracted from absorption of cells adhered to matrix. Approaches were performed in quadruplicate. Four independent experiments were summarised.

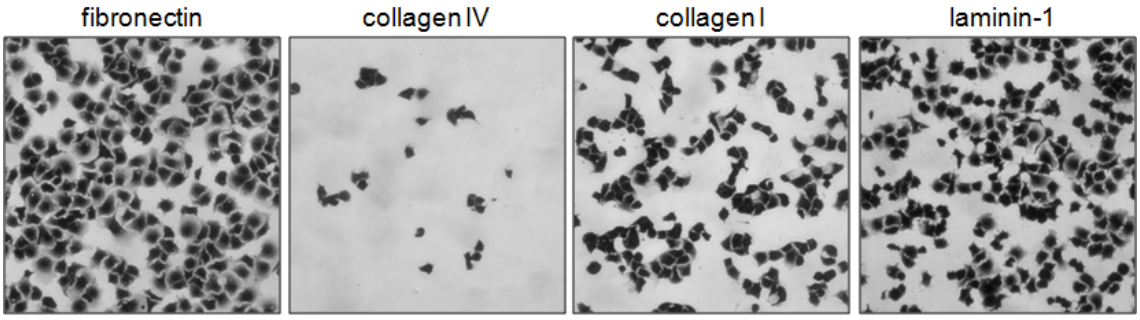


**Figure 15: Morphology of THBMECs on different matrix proteins**

Cells were serum-starved for 1 h and then plated onto matrix proteins for 1 h. Cells were washed, fixed and stained with crystal violet. Images were taken using a Nikon TMS-F microscope at a magnification of 10x.

In order to ascertain the impact of different integrin subunits on adhesion behaviour of THBMECs, adhesion assays were performed in the presence of function-blocking antibodies raised against the integrin subunits β1, α5 and α6. Cells, incubated in the presence of an anti-IgG antibody, served as a negative control (figure 16). Inhibition of the β1 integrin subunit decreased significantly the adhesion to all matrix proteins tested. Adhesion to fibronectin and collagen IV was diminished by about 40%. However, the most pronounced effect was observed for collagen I and laminin-1, to which adhesion was reduced by 70% relative to control cells. Blocking of the α5 integrin subunit resulted in significant reduction of adhesion to fibronectin by about 30% (figure 16). Adhesion to collagen IV, collagen I and laminin-1 was not affected by this antibody. Incubation of THBMECs with an α6 integrin function-blocking antibody decreased adhesion to laminin-1 by about 30% compared to control cells. Adhesion to fibronectin, collagen IV and collagen I was unaffected. Attachment of THBMECs to poly-L-lysine was not altered by inhibition of any integrin subunit (figure 16).

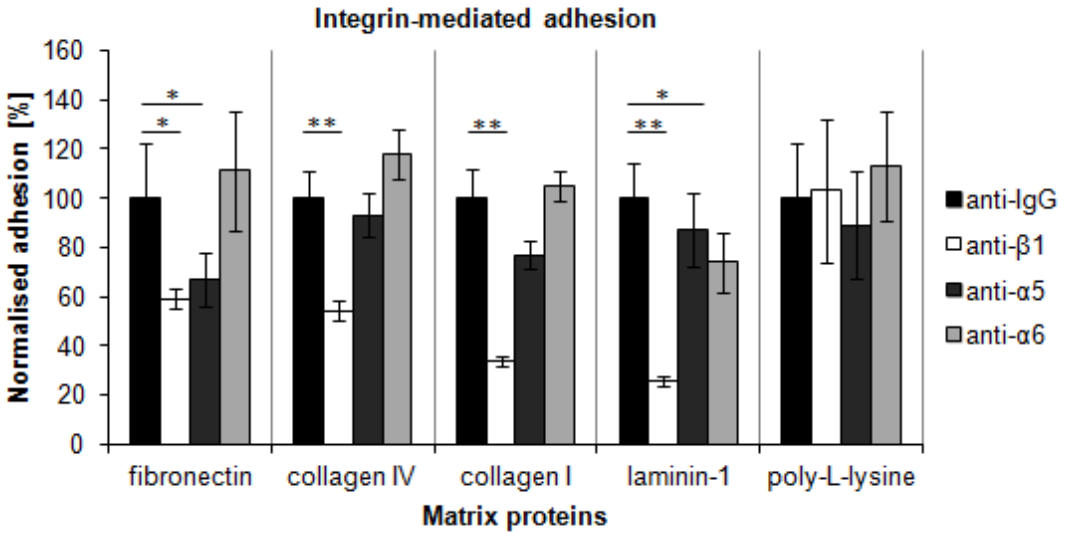


**Figure 16: Integrin-mediated adhesion of THBMECs in the presence of function-blocking antibodies**

THBMECs were serum-starved for 1 h, incubated with function-blocking antibodies (10 µg/ml each) for 30 min at 37°C and then plated onto matrix proteins as indicated for 1 h. Cells were washed, fixed and stained with crystal violet. Following Triton X-100 dye solubilisation, absorption at 570 nm was measured. Absorption of cells attached to wells blocked with 1% BSA were subtracted from absorption of cells adhered to matrix. Each approach was performed in quadruplicate. Absorption of cells, incubated in the presence of an anti-IgG antibody, was set to 100%. Three independent experiments were summarised. Asterisks indicate significant difference to control (* $p<0.05$; ** $p<0.001$).

These results confirmed the expression of the fibronectin receptor α5β1 integrin and the laminin receptor α6β1 integrin. Furthermore, the data demonstrated that adhesion to fibronectin was mediated not only by α5β1 integrin but probably also by other fibronectin receptors, such as the αvβ3 integrin.

## 3.2 Impact of pro-inflammatory cytokines on the *in vitro* BBB model

To investigate the role of β1 integrins during inflammatory conditions in the *in vitro* BBB model, an inflammation was induced using the classical pro-inflammatory cytokines TNFα and IL-1β. Moreover, the effects of recombinant IFN β (IFNβ 1a and IFNβ 1b), which are applied in the treatment of multiple sclerosis and have been demonstrated to have a stabilizing effect on the BBB integrity *in vivo* (Kraus and Oschmann, 2006), were analysed. In the following section, the influence of these four cytokines on the *in vitro* BBB model was evaluated.

### 3.2.1 Expression of adhesion receptors

During inflammation, the expression of adhesion proteins such as ICAM-1 and VCAM-1 is increased due to the secretion of pro-inflammatory cytokines. To determine whether the cytokines TNFα, IL-1β, IFNβ 1a and IFNβ 1b have an impact on the expression of these adhesion molecules, THBMECs were stimulated for 24 h, 48 h as well as 72 h, and expression of ICAM-1 and VCAM-1 was determined on mRNA and protein level.

To investigate gene transcription, cellular mRNA was extracted, transcribed into cDNA and the latter was subjected to qRT-PCR. Whereas incubation with TNFα had no impact on the mRNA expression of ICAM-1 after 24 h, 48 h or 72 h, IL-1β led to an increase of ICAM-1 mRNA expression by 100 - 300% (figure 17). The most pronounced effect was detected after 24 h, and this decreased over time. Treatment with both IFNβ 1a and IFNβ 1b for 24 h as well as 48 h enhanced ICAM-1 mRNA level up to 150% relative to untreated control cells. Incubation with IFNβ 1a and IFNβ 1b for 72 h had no significant effect on ICAM-1 mRNA expression (figure 17).

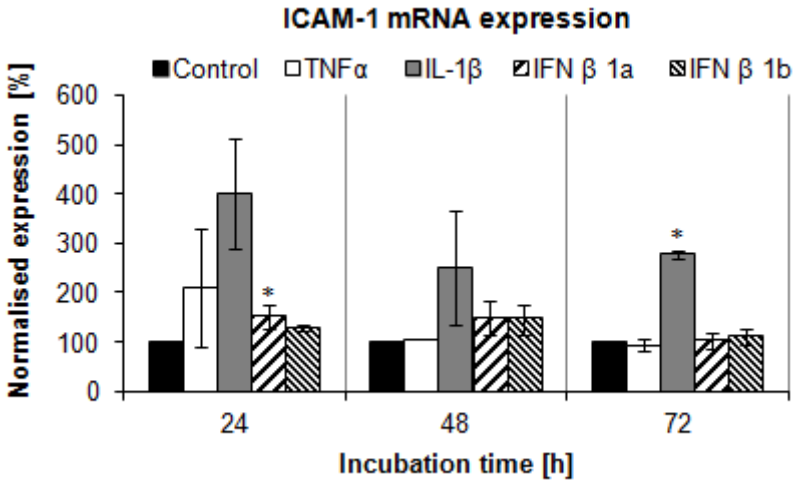


**Figure 17: Impact of cytokines on ICAM-1 mRNA expression**

Confluent THBMECs were stimulated with 10 ng/ml of the respective cytokine in serum-free medium or left untreated (negative control) for 24 h, 48 h and 72 h. Total RNA was extracted, cDNA was synthesised, and qRT-PCR was performed with primers specific for ICAM-1. Each approach was performed in quadruplicate. Untreated control cells were set to 100%. Three independent experiments were summarised. Asterisks indicate significant difference to control (* $p<0.05$).

To evaluate whether the functional ICAM-1 is also expressed at the cell surface, stimulated cells were analysed by flow cytometry after 24 h, 48 h and 72 h with an ICAM-1 specific antibody. Consistent with the data obtained by qRT-PCR, treatment with TNFα did not influence the expression of ICAM-1 at the cell surface (figure 18). However, expression of ICAM-1 was increased threefold after stimulation with IL-1β for 24 h. This increase in ICAM-1 protein expression persisted after 48 h and 72 h. Incubation of endothelial cells with IFNβ 1a and IFNβ 1b revealed no influence on ICAM-1 expression after 24 h but showed an enhancement in surface protein expression by 40-60% after 48 h and 72 h (figure 18).

Moreover, surface expression of the adhesion molecule VCAM-1 was analysed by flow cytometry after 24 h, 48 h and 72 h stimulation. In contrast to ICAM-1, which was constitutively expressed in endothelial cells, VCAM-1 was expressed at a very low level in resting THBMECs (Ubogu *et al*., 2006b). As already observed for ICAM-1 expression, stimulation of THBMECs with TNFα had no significant effect on the expression of VCAM-1 (figure 19). However, VCAM-1 was induced by stimulation with IL-1β. The most pronounced effect was seen after 72 h, when expression of VCAM-1 was doubled (figure 19B). Treatment of THBMECs with IFNβ 1a and IFNβ 1b failed to induce VCAM-1.

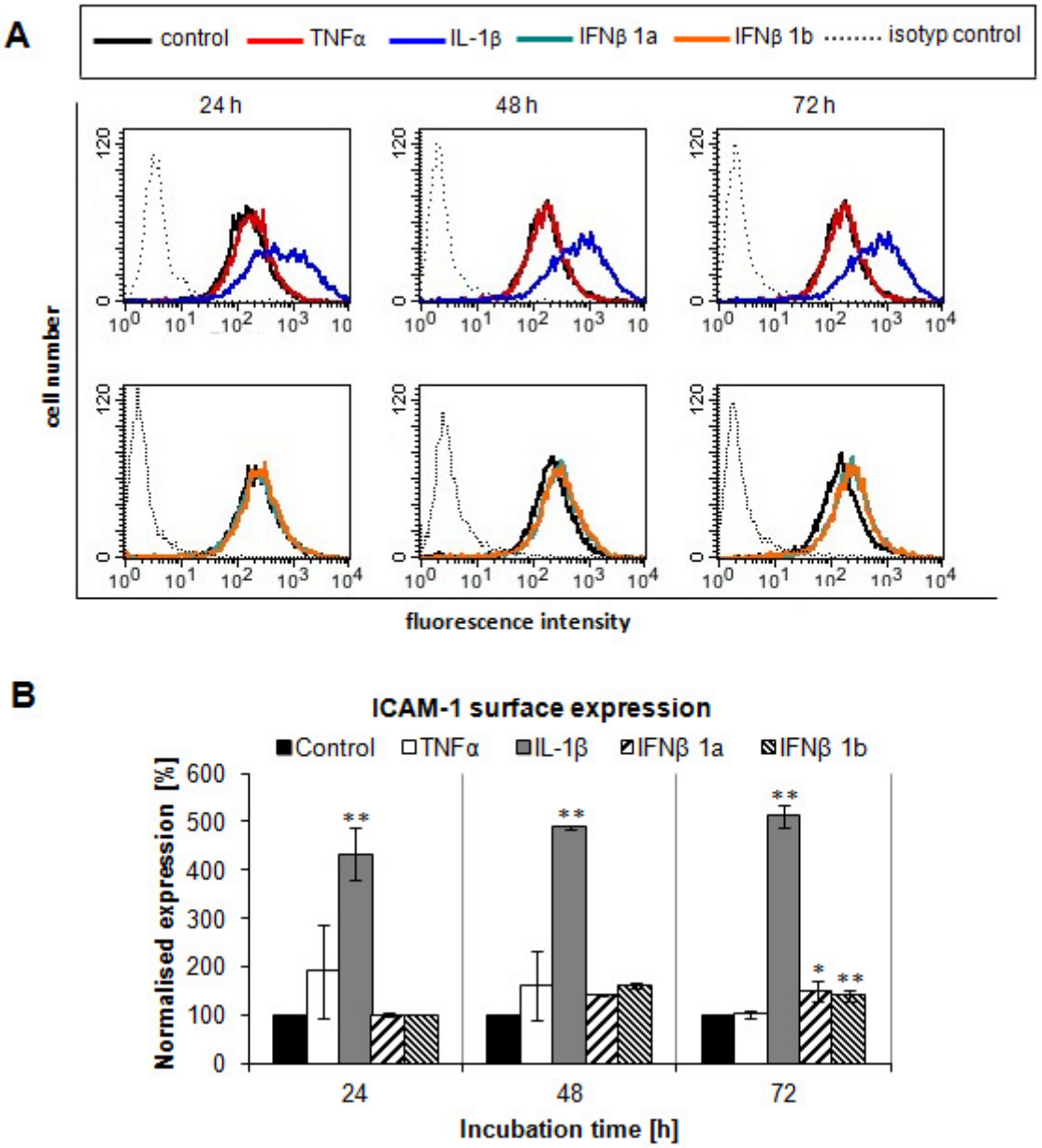


**Figure 18: Impact of cytokines on ICAM-1 protein expression**

Confluent THBMECs were stimulated with 10 ng/ml of the respective cytokine in serum-free medium or left untreated (negative control) for 24 h, 48 h and 72 h. Non-permeabilised cells were analysed by flow cytometry using an antibody raised against ICAM-1. One representative experiment out of three independent experiments is depicted. B) Mean fluorescence intensity was determined using CellQuest software (Version 5.2.1). Untreated control cells were set to 100%. Three independent experiments were summarised. Asterisks indicate significant difference to control (* $p<0.05$, ** $p<0.001$).

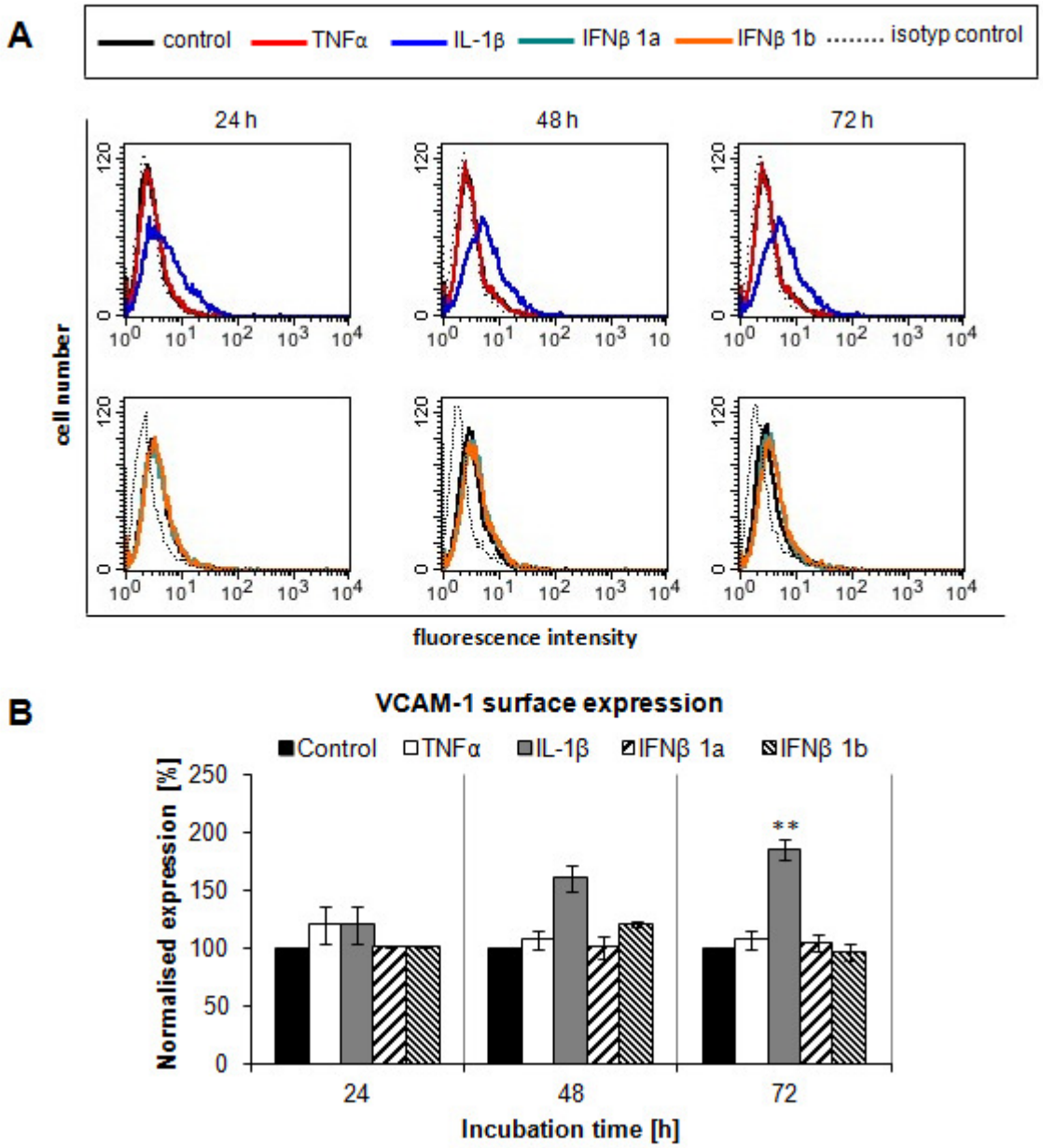


**Figure 19: Impact of cytokines on VCAM-1 protein expression**

Confluent THBMECs were stimulated with 10 ng/ml of the respective cytokine in serum-free medium or left untreated (negative control) for 24 h, 48 h and 72 h. Non-permeabilised ells were analysed by flow cytometry using an antibody raised against VCAM-1. One representative experiment out of three independent experiments is depicted. B) Mean fluorescence intensity was determined using CellQuest software (Version 5.2.1). Untreated control cells were set to 100%. Three independent experiments were summarised. Asterisks indicate significant difference to control (* $p<0.001$).

### 3.2.2 Synthesis and secretion of cytokines

Activation of the endothelium during inflammation is additionally accompanied by the production of cytokines. To analyse whether TNFα, IL-1β, IFNβ 1a and IFNβ 1b influence the cytokine expression profile of THBMECs, qRT-PCR was performed for the detection of the pro-inflammatory cytokines IL-1α, IL-1β, IL-6, IL-8 and TNFα using RNA that was isolated from cells stimulated for 24 h with the respective cytokine. Treatment of THBMECs with TNFα increased the mRNA level of IL-1α and IL-1β by 30% and 86%, respectively, but expression of IL-6, IL-8 and TNFα appeared to be unchanged (figure 20). IL-1β stimulation enhanced the mRNA expression of all analysed cytokines. While IL-1α mRNA level was only slightly increased, expression of IL-1β, IL-6, IL-8 and TNFα was dramatically up-regulated by treatment with IL-1β (1.3 fold and 25 fold, respectively). Treatment with IFNβ 1a augmented the mRNA expression of IL-1α by 20% but had no influence on the mRNA expression of IL1β. Expression of IL-6, IL-8 and TNFα mRNA was enhanced by about 70% after stimulation with IFNβ 1a. Treatment of THBMECs with IFNβ 1b slightly increased the mRNA level of IL-1α and IL-1β but had no impact on the expression of IL-6 mRNA. Nevertheless, IFNβ 1b marginally enhanced the mRNA level of IL-8 and TNFα (10-30%).

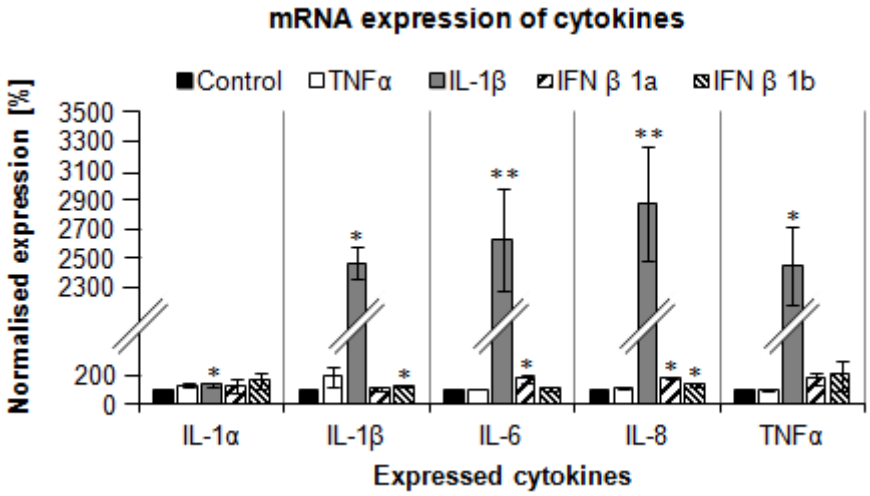


**Figure 20: Impact of cytokines on mRNA expression of different pro-inflammatory cytokines**

THBMECs were stimulated with 10 ng/ml of the respective cytokine in serum-free medium or left untreated (negative control) for 24 h. Total RNA was extracted, cDNA was synthesised, and qRT-PCR was performed with primers specific for different cytokines. Each approach was performed in quadruplicate. Untreated control cells were set to 100%. Three independent experiments were summarised. Asterisks indicate significant difference to control (* $p<0.05$; ** $p<0.001$).

To evaluate whether the cells synthesise and secrete functional cytokines, ELISAs specific for IL-1β, IL-6, IL-8 and TNFα were performed. Cell culture supernatants of cells, stimulated with TNFα, IL-1β, IFNβ 1a and IFNβ 1b for 24 h, were applied. Untreated control cells did not secrete detectable amounts of IL-1β and IL-6 into cell culture medium under these conditions (figure 21). However, 200 pg/ml of IL-8 and 50 pg/ml of TNFα were detected in the conditioned medium of unstimulated THBMECs. Stimulation with TNFα did not change the expression of IL-1β. However, the production of IL-6 and IL-8 was slightly elevated. Secretion of TNFα remained unchanged after stimulation of endothelial cells with TNFα. Treatment of THBMECs with IL-1β dramatically increased the expression of IL-1β (to 170 pg/ml), IL-6 (to 1200 pg/ml), IL-8 (to 880 pg/ml) and TNFα (to 215 pg/ml) (figure 21). After stimulation with both IFNβ 1a and IFNβ 1a, expression of IL-1β remained unchanged and secretion of IL-6, IL-8 and TNFα was only marginally elevated.

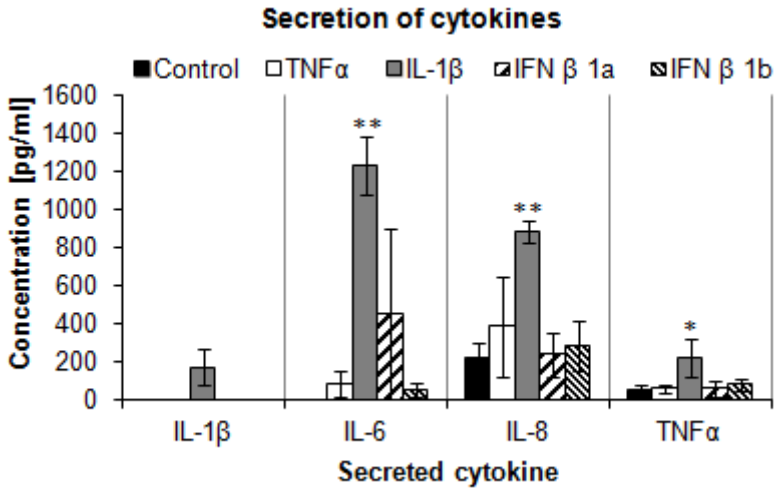


**Figure 21: Concentration of different cytokines in cell culture supernatant of stimulated THBMECs**

THBMECs were stimulated with 10 ng/ml of the respective cytokine in serum-free medium or left untreated (negative control) for 24 h. Cell culture supernatants were applied to ELISA specific for different cytokines. Each approach was performed in triplicate. Three independent experiments were summarised. Asterisks indicate significant difference to control (* $p<0.05$; ** $p<0.001$).

### 3.2.3 Expression of tight junction proteins

Inflammation at the BBB results in changes in the expression of endothelial tight junction proteins (Coisne and Engelhardt, 2011). To investigate the influence of TNFα, IL-1β, IFNβ 1a and IFNβ 1b on the expression of tight junction proteins, THBMECs were stimulated for 24 h, 48 h and 72 h. Subsequently, mRNA expression of the tight junction proteins ZO-1 and occludin was determined by qRT-PCR.

Expression of ZO-1 mRNA remained unchanged after treatment of THBMECs with TNFα for 24 h (figure 22). However, longer stimulation with TNFα resulted in a significant reduction of the ZO-1 mRNA level by 30% relative to untreated control cells. Stimulation with IL-1β revealed similar results (figure 22). Treatment with IFNβ 1a had an opposite effect. It increased the mRNA level of ZO-1 by 60% after 24 h, by 10% after 48 h and by 40% after 72 h (figure 22). Results for the treatment with IFNβ 1b were comparable.

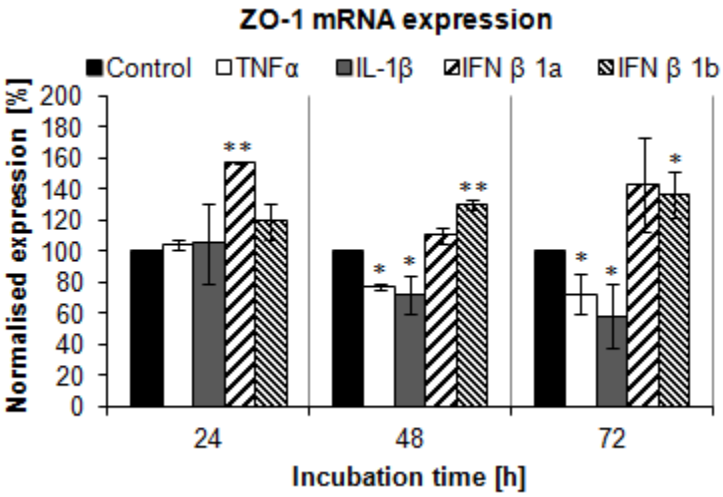


**Figure 22: Impact of cytokines on mRNA expression of ZO-1**

THBMECs were stimulated with 10 ng/ml of the respective cytokine in serum-free medium or left untreated (negative control) for 24 h, 48 h and 72 h. Total RNA was extracted, cDNA was synthesised, and qRT-PCR was performed with primers specific for ZO-1. Three independent sets of qRT-PCR were summarised. Untreated control cells were set to 100%. Asterisks indicate significant difference to control (* $p<0.05$; ** $p<0.001$).

Treatment of THBMECs with TNFα for 24 h had no influence on mRNA expression of occludin (figure 23). However, stimulation with TNFα for 48 h and 72 h significantly reduced the mRNA level of occludin by 10-40% relative to untreated control cells. These effects were comparable with those of IL-1β. The most pronounced effects of IL-1β on mRNA expression of occludin were observed after 72 h (figure 23). Whereas treatment of THBMECs with IFNβ 1a for 24 h resulted in a reduction of occludin mRNA expression in comparison to control cells, stimulation for 48 h increased the occludin mRNA level by 40% (figure 23). After treatment of THBMECs with IFNβ 1a for 72 h the occludin mRNA level decreased to control level. Stimulation with IFNβ 1b revealed comparable results.

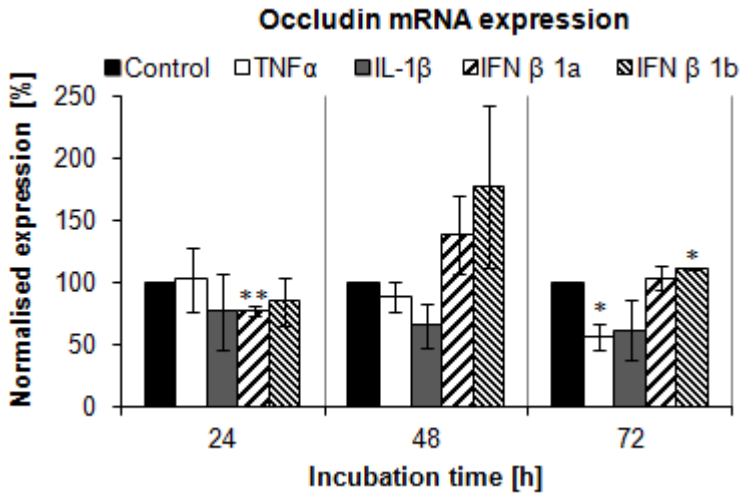


**Figure 23: Impact of cytokines on mRNA expression of occludin**
THBMECs were stimulated with 10 ng/ml of the respective cytokine in serum-free medium or left untreated (negative control) for 24 h, 48 h and 72 h. Total RNA was extracted, cDNA was synthesised, and qRT-PCR was performed with primers specific for occludin. Three independent sets of qRT-PCR were summarised. Untreated control cells were set to 100%. Asterisks indicate significant difference to control (* $p<0.05$; ** $p<0.001$).

In order to ascertain whether cytokine stimulation of THBMECs also effects protein expression of tight junction proteins, cells were stimulated with TNFα, IL-1β, IFNβ 1a and IFNβ 1b or left untreated for 24 h, 48 h and 72 h. Cells were lysed and analysed by western blotting for the expression of ZO-1 and occludin. Figure 24 depicts the results of one representative experiment. Treatment of THBMECs with TNFα did not influence the expression of ZO-1 and occludin after 24 h, 48 h or 72 h. Whereas stimulation with IL-1β for 24 h and 48 h had no impact on the expression of ZO-1, treatment with IL-1β for 72 h led to an obvious reduction in ZO-1 expression. However, the expression of occludin remained unaffected after stimulation of THBMECs with IL-1β for 24 h, 48 h and 72 h. Treatment of THBMECs with IFNβ 1a had no effect on the expression of ZO-1 at any time. However, the protein level of occludin was dramatically increased after 72 h treatment with IFNβ 1a. Similar results were observed for IFNβ 1b.

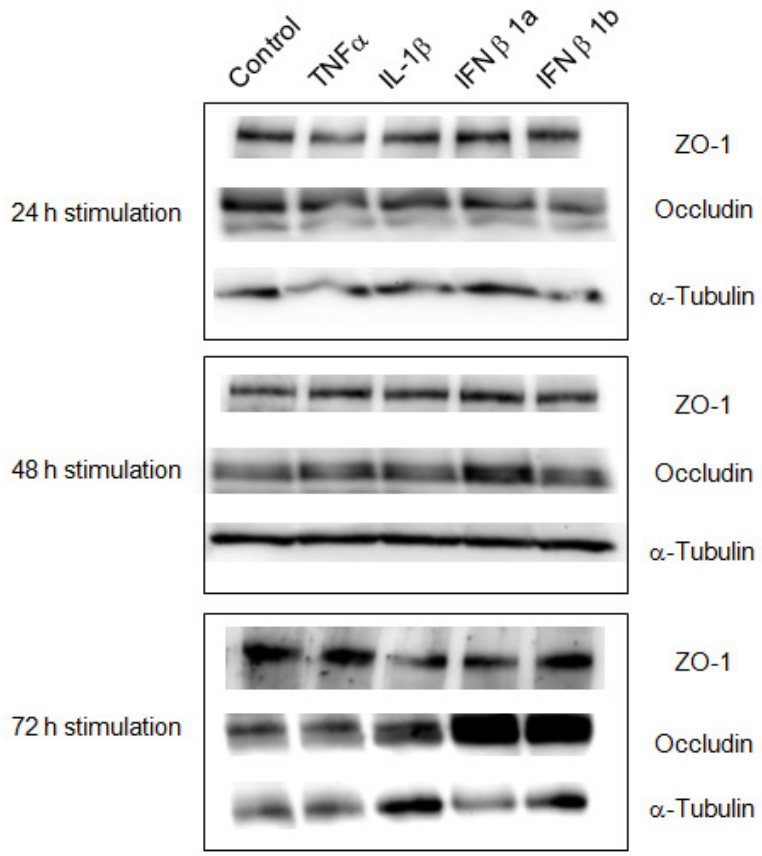


**Figure 24: Impact of cytokines on protein expression of ZO-1 and occludin**

Confluent THBMECs were stimulated with 10 ng/ml of the respective cytokine in serum-free medium or left untreated (negative control) for 24 h, 48 h and 72 h. Cells were lysed and analysed by western blotting using antibodies raised against ZO-1, occludin and α-tubulin. One representative experiment out of three independent experiments is depicted.

### 3.2.4 Transendothelial electrical resistance

Changes in expression levels of tight junction proteins might result in altered barrier function of the *in vitro* BBB model. To investigate whether the cytokines TNFα, IL-1β, IFNβ 1a and IFNβ 1b modify transendothelial resistance of the THBMEC monolayer, the *in vitro* BBB model was incubated with the respective cytokine, and the TEER was determined after 0 h, 24 h, 48 h and 72 h. As depicted in figure 25, treatment with TNFα had no influence on the TEER for the indicated times. In contrast, IL-1β stimulation led to significant reduction in TEER. Electrical resistance of the monolayer was decreased by 20% after 24 h, by 35% after 48 h and by 30% after 72 h stimulation in the presence of IL-1β relative to untreated control cells. Whereas stimulation of the *in vitro* BBB model with IFNβ 1a as well as IFNβ 1b for 24 h had no effect on the resistance, both cytokines induced a weak but significant increase in TEER by about 15% after 48 h and 72 h.

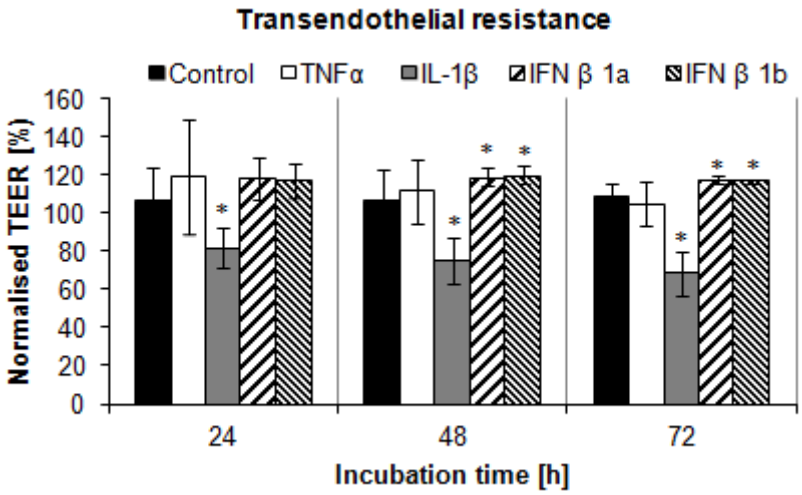


**Figure 25: Impact of cytokines on transendothelial resistance of the *in vitro* BBB model**

Confluent THBMECs with a TEER of at least 150 Ω x cm$^2$ and higher were stimulated with 10 ng/ml of the respective cytokine in growth medium or left untreated (negative control). TEER was measured by means of an Endohm-12 chamber (WPI, USA) and the volt-ohm-meter Millicell® ERS-2 (Millipore, USA) after 0 h, 24 h, 48 h and 72 h. TEER at time point 0 h was set to 100% for each condition. Approaches were performed in duplicate. Four independent experiments were summarised. Asterisks indicate significant difference to control (* $p<0.05$).

### 3.2.5 Endothelial permeability

Reduction in transendothelial electrical resistance of an endothelial monolayer is often accompanied by an increase in permeability. To evaluate the influence of the cytokines TNFα, IL-1β, IFNβ 1a and IFNβ 1b on the paracellular permeability coefficient of the *in vitro* BBB model, THBMEC monolayers with a TEER higher than 150 Ω x cm$^2$ were stimulated for 24 h, 48 h and 72 h. Subsequently, the permeability assay with sodium fluorescein was performed. Consistent with the transendothelial electrical resistance, stimulation of TNFα did not influence the permeability coefficient of the BBB model, while IL-1β caused a significant increase in paracellular permeability (figure 26). The permeability coefficient of sodium fluorescein was augmented by 50% after 24 h, 60% after 48 h and 90% after 72 h stimulation with IL-1β relative to permeability coefficient of control cells. Treatment of the cell layer with IFNβ 1a or IFNβ 1b did not affect its paracellular permeability coefficient after the indicated times.

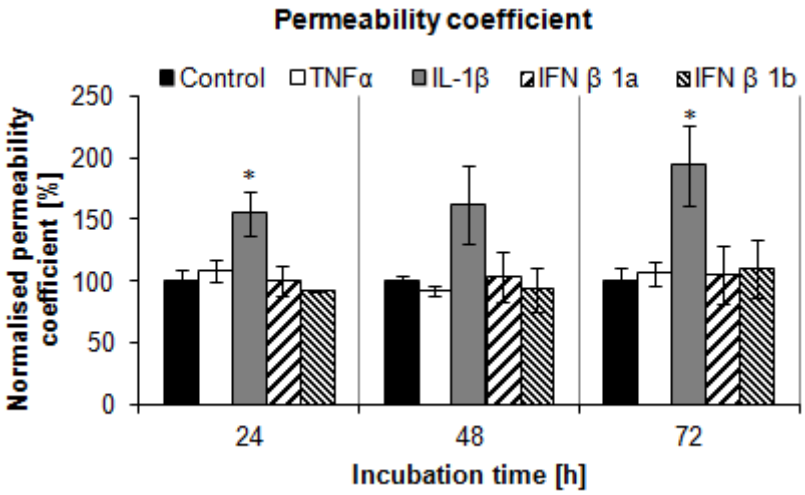


**Figure 26: Impact of cytokines on the paracellular permeability coefficient for sodium fluorescein of the *in vitro* BBB model**

Confluent THBMECs with a TEER of at least 150 Ω x $cm^2$ and higher were stimulated with 10 ng/ml of the respective cytokine in growth media or left untreated (negative control). Permeability coefficient of sodium fluorescein was determined after 24 h, 48 h and 72 h. Duplicates for each value were performed. Three independent experiments were summarised. Untreated control cells were set to 100%. Asterisks indicate significant difference to control (* $p<0.05$).

### 3.2.6 Expression of matrix metalloproteinases

MMPs are reported to cause an increase in permeability of the BBB by attacking the basal lamina surrounding the capillaries as well as the tight junctions that physiologically maintain the integrity of the endothelium (Rosenberg, 2009). The most studied MMPs in the brain are the gelatinases MMP-2 and MMP-9, the expression of which can be induced by pro-inflammatory cytokines (Sorokin, 2010). To investigate the influence of the cytokines TNFα, IL-1β, IFNβ 1a and IFNβ 1b on the expression and secretion of MMP-2 and MMP-9, THBMECs were stimulated for 24 h and 48 h. Subsequently, a gelatine zymography was performed with the cell culture supernatants to analyse secreted gelatinases and their activity. Human recombinant MMP-2 and MMP-9 served as respective positive controls. Since MMPs are activated by proteolytic cleavage, two bands were detected by zymography: one band presents the inactive pro-form of the MMP and a lower band the active form of the respective MMP.

Untreated control cells expressed only little amounts of the inactive pro-form of MMP-2, and the active MMP-2 was not detected under this condition (figure 27). Treatment of THBMECs with TNFα for 24 h dramatically increased the expression and secretion of the inactive proMMP-2 as well as the active MMP-2. Stimulation of endothelial cells with IL-1β, IFNβ 1a and IFNβ 1b for 24 h had similar effects. However, the influence of those cytokines on the

expression and secretion of MMP-2 was not as pronounced as that of TNFα. Similar observations were made after the treatment of THBMECs with TNFα, IL-1β, IFNβ 1a and IFNβ 1b for 48 h (figure 27).

MMP-9 was not expressed in detectable amounts by untreated THBMECs (data not shown). Nevertheless, treatment of THBMECs with TNFα, IL-1β, IFNβ 1a and IFNβ 1b for 24 h resulted in a slight increase in the expression and secretion of the inactive proMMP-9. Similar results were obtained for the stimulation of endothelial cells with TNFα, IL-1β, IFNβ 1a and IFNβ 1b for 48 h. However, these results were not able to be visualised with the technical means available.

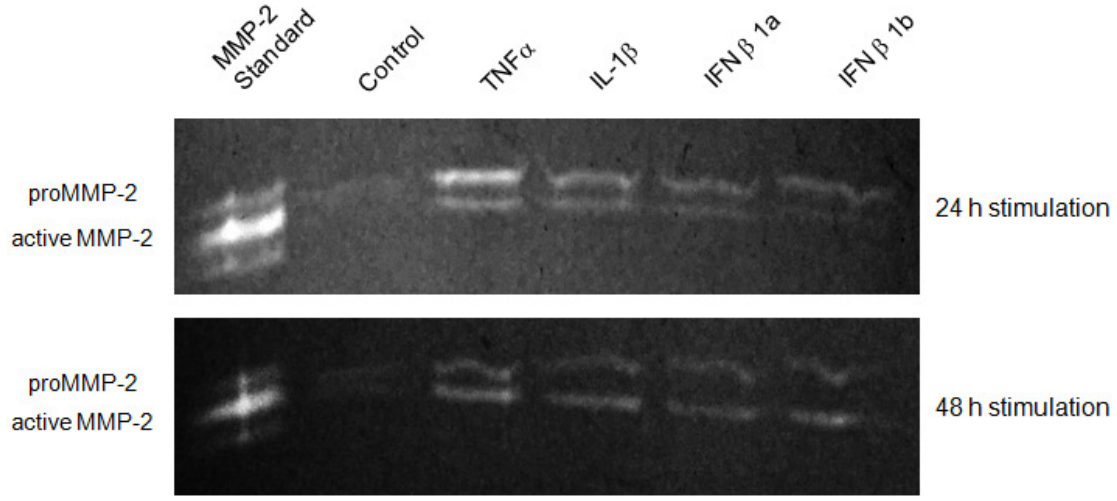


**Figure 27: Impact of cytokines on the secretion of matrix metalloproteinase-2**

5 x $10^5$ THBMECs were grown for 3 days in growth medium. Confluent cells were stimulated with 10 ng/ml of the respective cytokine in serum-free medium or left untreated (negative control). Zymography was performed with cell culture supernatants collected after 24 h and 48 h stimulation. Human recombinant MMP-2 served as positive control. Inactive proMMP-2 (72 kDa) and active MMP-2 (66 kDa) were detected. One representative experiment out of four independent experiments is depicted.

### 3.2.7 Transendothelial migration

The data presented indicate that the applied cytokines influence the physical properties of the endothelial cell layer. Alteration in transendothelial resistance and permeability of the BBB as well as increased expression of MMPs are often accompanied by changes in the barrier function. To evaluate whether the cytokines TNFα, IL-1β, IFNβ 1a and IFNβ 1b also affect the functionality of the *in vitro* BBB model, transmigration of different subpopulations of immune cells across the THBMEC layer was determined after stimulation for 72 h with different cytokines. Transmigration assays with PBMCs as well as pure T lymphocytes and pure monocytes were performed.

Treatment of the endothelial cells with TNFα did not influence the amount of transmigrated PBMCs in comparison to the untreated THBMEC layer (figure 28). In contrast, stimulation of the *in vitro* BBB model with IL-1β significantly increased the number of transmigrated PBMCs by about 50%. The treatment of THBMECs with both IFNβ 1a and IFNβ 1b had no effect on the transmigration of PBMCs across the *in vitro* model. Whereas treatment of the THBMEC layer with TNFα marginally decreased the amount of transmigrated T lymphocytes, stimulation of the endothelial cells with IL-1β resulted in a significant increase of transmigrated T lymphocytes of about 20% in comparison to untreated control cells (figure 28). Stimulation with IFNβ 1a as well as IFNβ 1b did not influence the transmigration of T lymphocytes. The number of transmigrated monocytes was significantly enhanced by about 10% after the treatment of the THBMEC layer with TNFα (figure 28). While stimulation of the endothelial cells with IL-1β increased monocyte transmigration by 50% relative to untreated control cells, treatment with IFNβ 1a as well as IFNβ 1b had no impact on the amount of monocytes transmigrated across the *in vitro* BBB model.

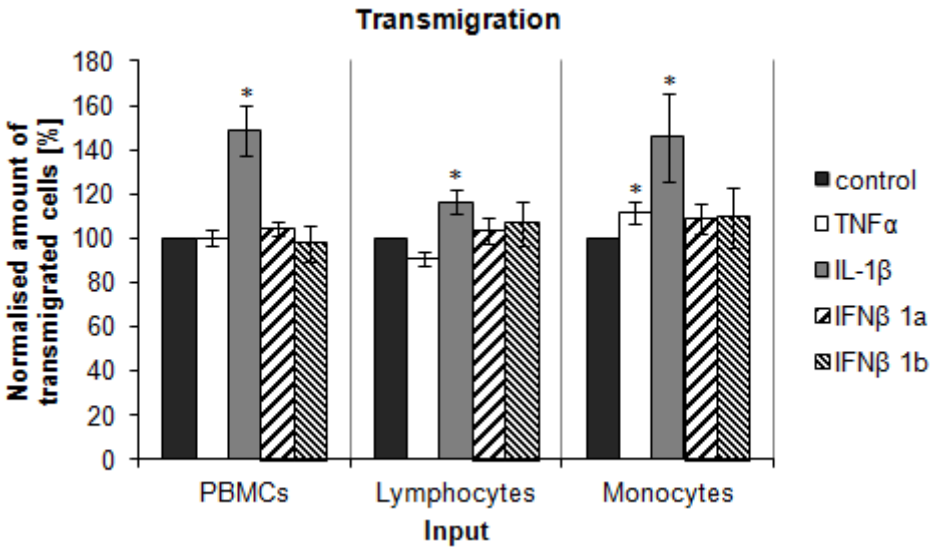


**Figure 28: Transmigration of PBMCs, T lymphocytes and monocytes across the *in vitro* BBB model after cytokine stimulation**

Confluent THBMECs with a TEER of at least 150 Ω x $cm^2$ and higher were stimulated with 10 ng/ml of the respective cytokine in growth medium for 72 h or left untreated (negative control). Calcein AM labelled immune cells were allowed to transmigrate across the THBMEC layer for 18 h. Amounts of transmigrated cells were calculated by means of a standard curve. Each approach was performed in duplicate. Three independent experiments were summarised. Untreated control cells were set to 100% for each condition. Asterisks indicate significant difference to control (* $p<0.05$).

Table 4 summarises all effects of the cytokines determined in the *in vitro* BBB model.

**Table 4: Summary of the effects of cytokines on the *in vitro* BBB model**

→: no effect, ↑: slight up regulation; ↑↑: strong up regulation; ↓: down regulation

| | **Effects of** | | | |
|---|---|---|---|---|
| **Effects on** | **TNFα** | **IL-1β** | **IFNβ 1a** | **IFNβ 1b** |
| **Adhesion receptor expression** | | | | |
| ICAM-1 | → | ↑↑ | ↑ | ↑ |
| VCAM-1 | → | ↑↑ | → | → |
| **Cytokine expression** | | | | |
| IL-1α | → | ↑ | → | → |
| IL-1β | → | ↑↑ | → | → |
| IL-6 | → | ↑↑ | ↑ | ↑ |
| IL-8 | → | ↑↑ | ↑ | ↑ |
| TNFα | → | ↑↑ | → | → |
| **Tight junction protein expression** | | | | |
| ZO-1 | → | ↓ | → | → |
| Occludin | → | → | ↑↑ | ↑↑ |
| **Transendothelial resistance** | → | ↓ | → | → |
| **Endothelial permeability** | → | ↑ | → | → |
| **Matrix metalloproteinases expression** | | | | |
| MMP-2 | ↑ | ↑ | ↑ | ↑ |
| MMP-9 | ↑ | ↑ | ↑ | ↑ |
| **Transendothelial migration** | | | | |
| PBMCs | → | ↑↑ | → | → |
| T lymphocytes | → | ↑ | → | → |
| monocytes | → | ↑↑ | → | → |

The data presented in chapter 3.2 revealed that the physical and functional properties of the generated *in vitro* BBB model were modified by stimulation with different cytokines. Moreover, IL-1β induced an inflammation at the THBMEC layer. For this reason, this system was appropriate to investigate the role of β1 integrins at the BBB during inflammatory processes.

## 3.3 Impact of pro-inflammatory cytokines on endothelial integrin function

As mentioned in chapter 1.4.5 integrins are involved in the transmigration process of immune cells. While the role of β2 integrins as well as β1 integrins - especially the α4β1 integrin - expressed by leukocytes in transendothelial migration is well studied, less is known about the importance of β1 integrins expressed by endothelial cells under inflammatory conditions. In order to clarify the role of endothelial β1 integrins during inflammation, the impact of the pro-inflammatory cytokines on the function, expression, activity as well as on localisation of β1 integrins was evaluated.

### 3.3.1 Cell matrix adhesion

B1 integrins function as receptors for proteins of the ECM and thereby mediate cell matrix adhesion. To investigate whether cytokines influence the adhesion of endothelial cells to proteins of the ECM, THBMECs were stimulated with TNFα, IL-1β, IFNβ 1a and IFNβ 1b for 24 h and 72 h, and adhesion assays on fibronectin, collagen IV, collagen I, laminin-1 and poly-L-lysine were performed.

After 24 h, none of the cytokines tested significantly altered the adhesion to fibronectin, collagen IV, collagen I and laminin-1 (data not shown). However, adhesion to fibronectin was enhanced by prolonged treatment of THBMECs with TNFα, IL-1β, IFNβ 1a and IFNβ 1b for 72 h (figure 29). The most pronounced effect was observed for IL-1β. This cytokine almost doubled the adhesion of THBMECs to fibronectin relative to untreated cells. While treatment with TNFα had no effect on the adhesion to collagen IV, stimulation of THBMECs with IL-1β, IFNβ 1a and IFNβ 1b was found to augment cell adhesion to this protein. Again, the most striking effect was observed after treatment with IL-1β. Adhesion to collagen I was not affected by treatment of THBMECs with TNFα for 72 h. Nevertheless, stimulation with IL-1β led to a significant twofold increase in adhesion to collagen I. Treatment of endothelial cells with both IFNβ 1a and IFNβ 1b caused a slight rise of adhesion to collagen I. Adhesion of THBMECs to laminin-1 was unaffected by treatment of endothelial cells with TNFα. However, stimulation with IL-1β, IFNβ 1a and IFNβ 1b for 72 h resulted in an increase of adhesion to laminin-1 by 50% relative to control cells. Integrin-independent adhesion to poly-L-lysine remained nearly unchanged under all conditions tested (figure 29).

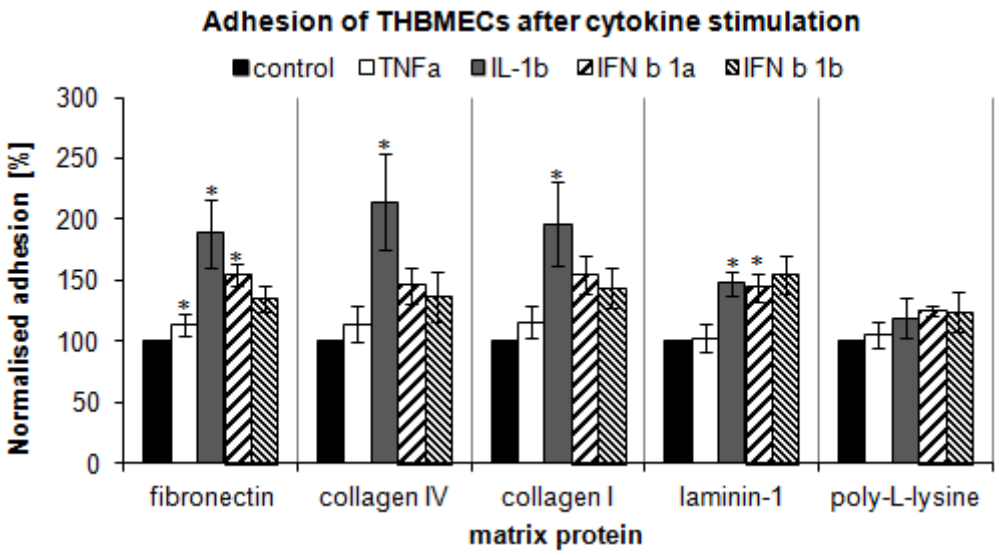


**Figure 29: Impact of cytokines on adhesion of THBMECs**

Confluent THBMECs were stimulated with 10 ng/ml of the respective cytokine in serum-free medium or left untreated (negative control) for 72 h. Adhesion assays were performed on fibronectin, collagen IV, collagen I and laminin-1. Poly-L-lysine served as control for integrin-independent attachment. Adhesion of untreated control cells were set to 100%. At least three independent experiments were summarised. Asterisks indicate significant difference to control (* $p<0.05$).

### 3.3.2 Integrin expression

To evaluate whether increased matrix adhesion resulted from altered integrin expression levels in cytokine-stimulated endothelial cells, THBMECs were treated with TNFα, IL-1β, IFNβ 1a and IFNβ 1b for 24 h, 48 h and 72 h. Subsequently, cell surface expression of different α and β integrin subunits was determined by flow cytometry. None of the cytokines tested affected the expression of α1, α2, α3, α5, α6 and αv as well as β1 and β3 integrin subunit after 24 h and 48 h stimulation (data not shown).

After 72 h, TNFα did not to induce the expression of the α1 integrin subunit either (figure 30A), and the expression of the α2, α3, α5, α6 and β1 integrin subunits remained unchanged (figure 30). The surface expression of the αv and β3 integrin subunit was only marginally increased after TNFα stimulation. IL-1β also failed to induce the expression of the α1 integrin subunit (figure 30A) and did not alter the expression of α2 integrin subunit relative to control cells after 72 h. However, treatment with IL-1β resulted in a slight but significant increase in the expression of the subunits α3, α5, α6 and αv (figure 30B). While expression of the β1 integrin subunit was unaffected after IL-1β treatment, the level of β3 subunit was increased by 70% (figure 30B). After 72 h, IFNβ 1a did not induce the expression of the α1 integrin subunit (figure 31A) and had no influence on the surface expression of integrin subunits α2, α3, α6 and αv (figure 31A). However, the expression level of α5 integrin subunit was

significantly enhanced by about 40% (figure 31B). The levels of β1 and β3 integrin subunit remained unaffected after stimulation with IFNβ 1a. Similar results were obtained for the treatment of THBMECs with IFNβ 1b for 72 h (figure 31).

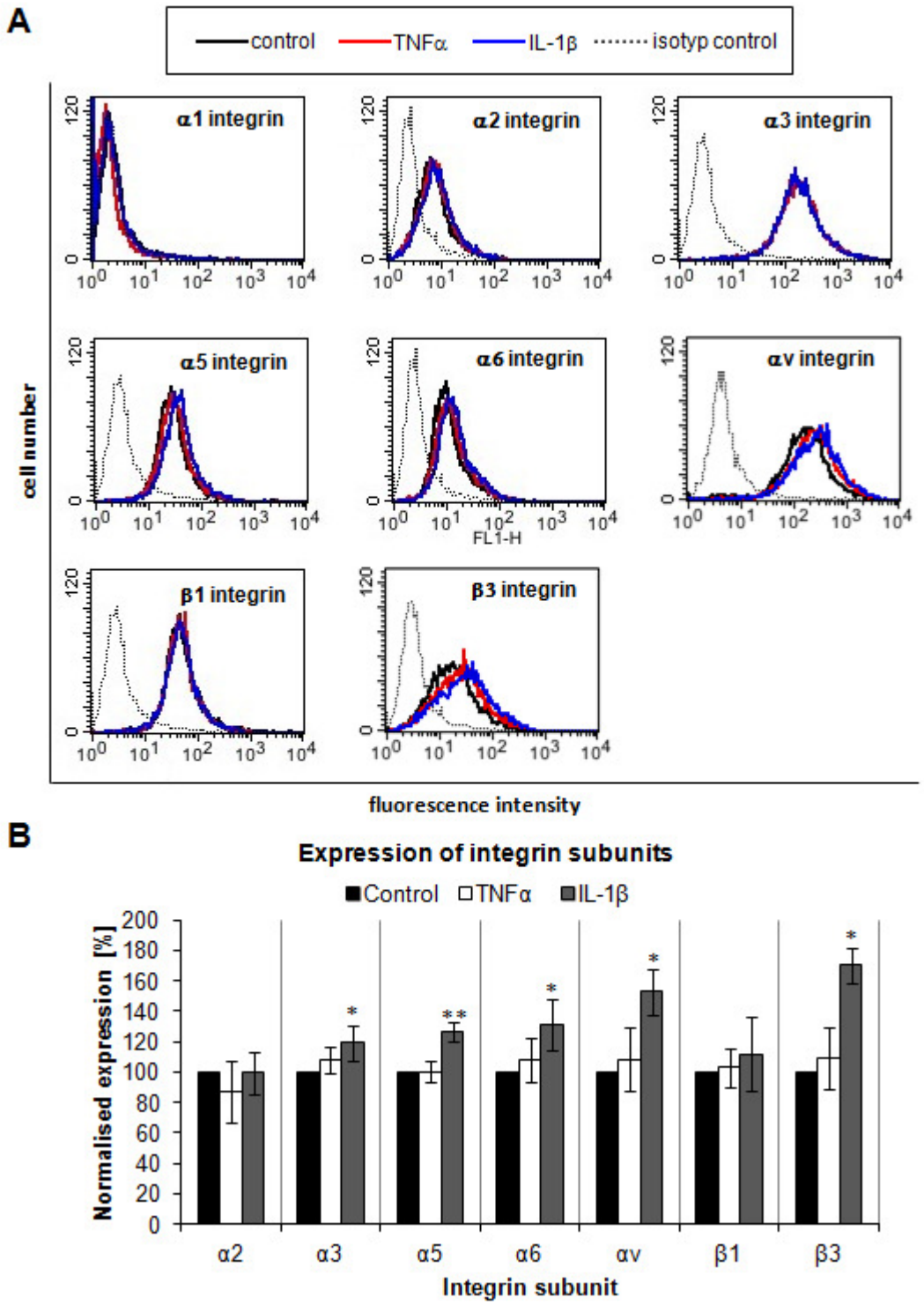


**Figure 30: Impact of TNFα and IL-1β on the expression of integrin subunits**

Confluent THBMECs were stimulated with 10 ng/ml TNFα or IL-1β in serum-free medium or left untreated (negative control) for 72 h. Cells were analysed by flow cytometry using different integrin-specific antibodies. One experiment out of four independent experiments is depicted. B) Mean fluorescence intensity was determined using CellQuest Pro software (Version 5.2.1). Untreated control cells were set to 100%. Four independent experiments were summarised. Asterisks indicate significant difference to control (* $p<0.05$, ** $p<0.001$).

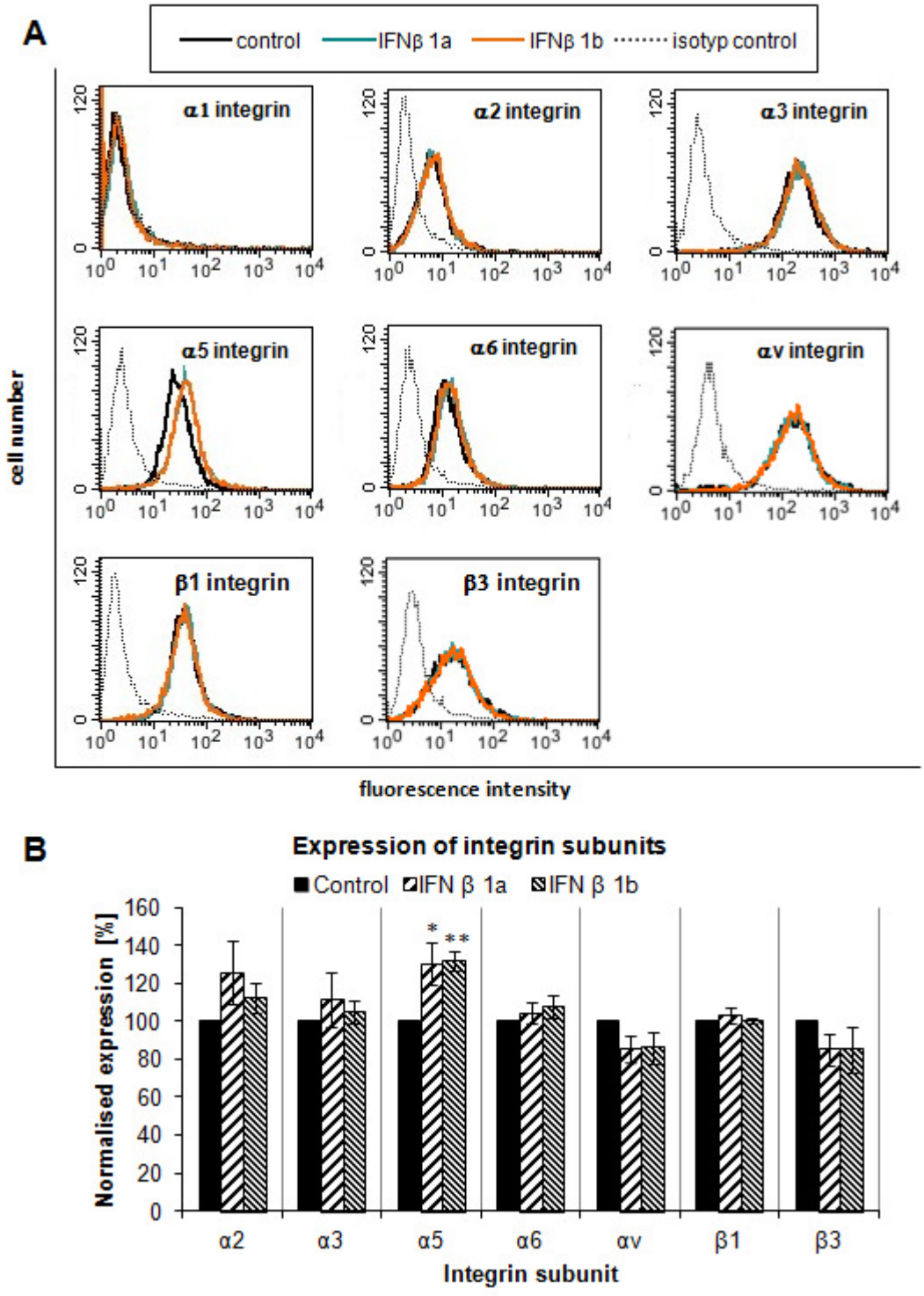


**Figure 31: Impact of IFNβ 1a and IFNβ 1b on the expression of integrin subunits**

Confluent THBMECs were stimulated with 10 ng/ml IFNβ 1a or IFNβ 1b in serum-free medium or left untreated (negative control) for 72 h. Cells were analysed by flow cytometry using different integrin-specific antibodies. One experiment out of four independent experiments is depicted. B) Mean fluorescence intensity was determined using CellQuest Pro software (Version 5.2.1). Untreated control cells were set to 100%. Four independent experiments were summarised. Asterisks indicate significant difference to control (* $p<0.05$, ** $p<0.001$).

### 3.3.3 Integrin affinity and avidity

To examine whether the increase in adhesion to fibronectin and collagens after cytokine treatment is only regulated by the amount of integrin subunits at the cell surface or caused by alterations in receptor affinity or avidity, experiments were performed using the integrin-specific antibody 12G10, which recognizes an epitope only present in the high-affinity state of β1 integrins (Mould *et al.*, 1995). To analyse integrin affinity, THBMECs were subjected to flow cytometry. By indirect immunofluorescence microscopy analysis, the avidity of β1 integrins was evaluated.

For flow cytometry, THBMECs were incubated with TNFα, IL-1β, IFNβ 1a and IFNβ 1b for 24 h, 48 h, and 72 h. Subsequently, cells were analysed as described above. Cells, which were incubated in a buffer containing 5 mM $Mn^{2+}$ a known inducer of integrin affinity, served as positive control (Mould *et al.*, 2002; figure 32, upper panel, left). To control integrin expression under the applied conditions, an integrin antibody that recognises the β1 integrin subunit independent of its activation state was applied in parallel (figure 32, upper panel, right). Analysis of cytokine-stimulated THBMECs revealed that none of the tested cytokines had an impact on the affinity state of β1 integrins (figure 32, lower panels).

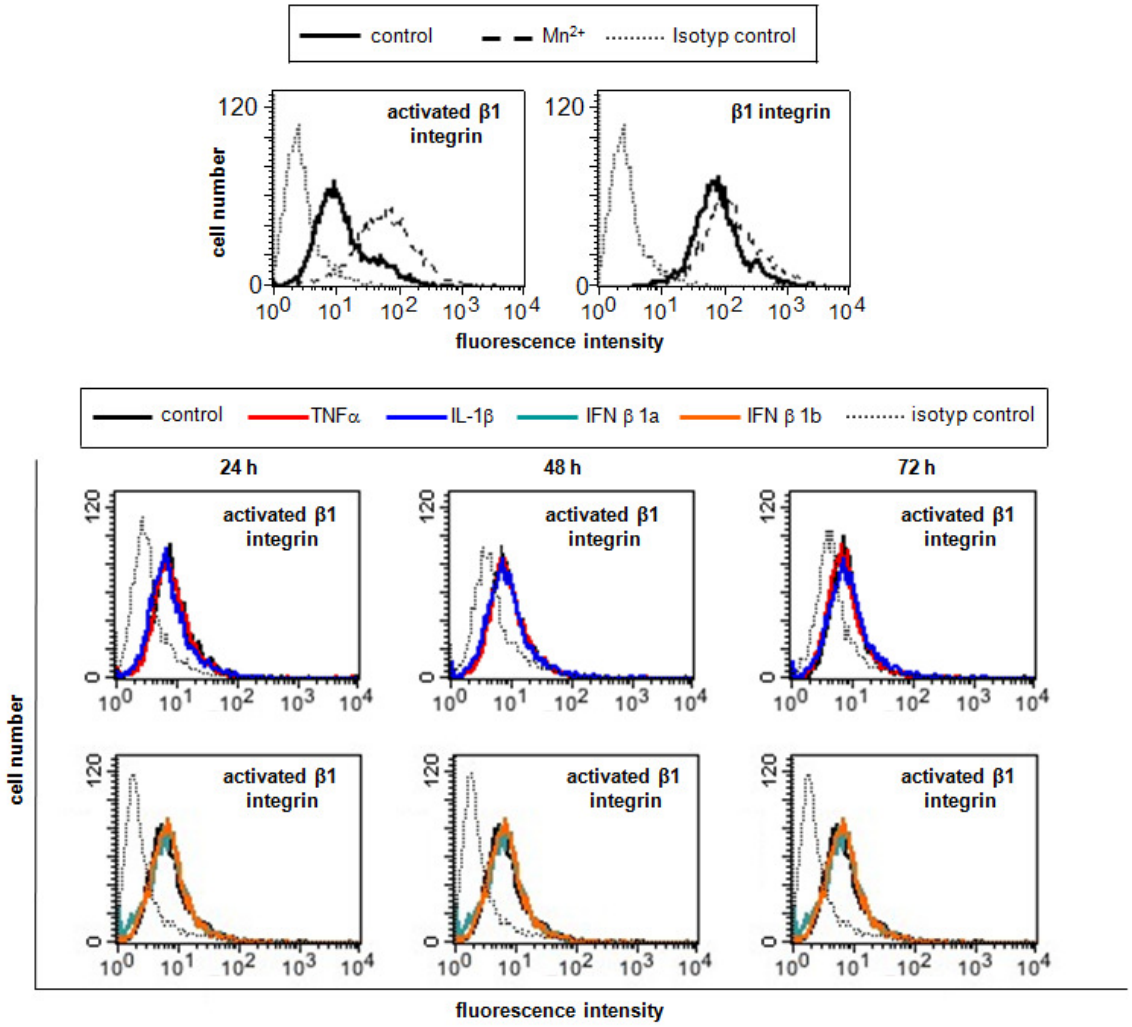


**Figure 32: Impact of cytokines on the affinity of endothelial β1 integrins**

Confluent THBMECs were stimulated with 10 ng/ml of the respective cytokine in serum-free medium or left untreated (negative control) for 24 h, 48 h and 72 h. Cells were analysed by flow cytometry using the antibody 12G10 which recognizes β1 integrins only in their high-affinity state. One experiment out of four independent experiments is depicted.

Since activation of integrins is not only achieved by changes in ligand affinity but also by alteration in integrin avidity, clustering of β1 integrin molecules in subconfluent and confluent THBMECs was analysed by indirect immunofluorescence microscopy using the 12G10 antibody. In subconfluent control cells, activated β1 integrins are localised in focal adhesions (figure 33A, upper panel, left, arrows). In cells treated with IL-1β, the amount of β1 integrins containing focal adhesions was enhanced, and focal adhesions were often increased in size (figure 33A, upper panel, right, arrows). In cells stimulated with IFNβ 1a and IFNβ 1b, clustering of activated β1 integrins was similar to control cells (figure 33A, lower panels). In confluent untreated THBMECs, active β1 integrins were distributed diffusely over the cell. However, active β1 integrins also accumulated in vesicles (figure 33B, upper panel, left, arrows). Treatment with IL-1β resulted in increased clustering of active β1 integrins, which

seems to be located at the borders of adjacent cells (figure 33B, upper panel, right, arrows). Treatment of confluent THBMECs with IFNβ 1a and IFNβ 1b caused enlarged clusters of active β1 integrins compared to control cells. The distribution seemed to be similar to the distribution in subconfluent cells (figure 33B, lower panels).

To compare the localisation of active β1 integrins with the distribution of total β1 integrin subunits, immunofluorescence microscopy using an antibody, which recognises both the active and the inactive form of this subunit, was performed. In contrast to the analysis with the 12G10 antibody, the β1 integrin subunits was found all over the cell, and their expression was lightly enriched at the lamellipodium and cell contact regions in untreated subconfluent THBMECs (figure 13A, lower panel, left and figure 34A, upper panel, left). None of the cytokines applied had an impact on the localisation of the β1 integrin subunit in subconfluent cells (figure 34A). Localisation of all β1 integrin subunits in confluent THBMECs was mainly restricted to cell-cell contacts (figure 34, upper panel, left). Localisation of β1 integrin subunit after treatment with IL-1β, IFNβ 1a and IFNβ 1b was found to be comparable with that in untreated THBMECs (figure 34B).

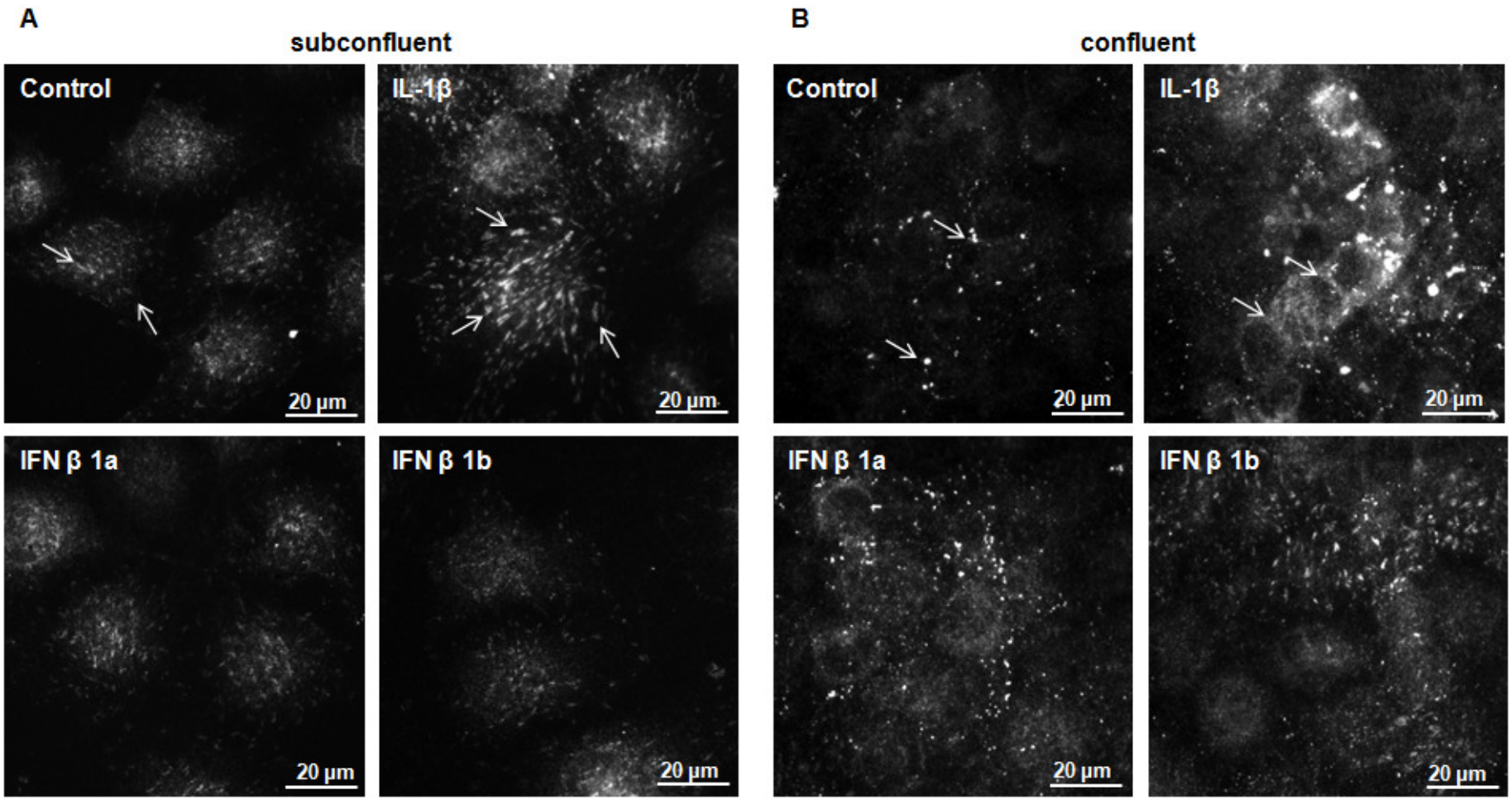


**Figure 33: Impact of cytokines on the localisation of active β1 integrin subunits**

Subconfluent (A) and confluent (B) THBMECs, grown onto a matrix of fibronectin and collagen IV, were treated with the respective cytokine for 72 h. Subsequently, cells were washed in PBS, fixed, permeabilised and incubated with the 12G10 antibody as well as the respective secondary antibody. Images were taken on a Zeiss Axiovert 200 microscope with a magnification of 64x.

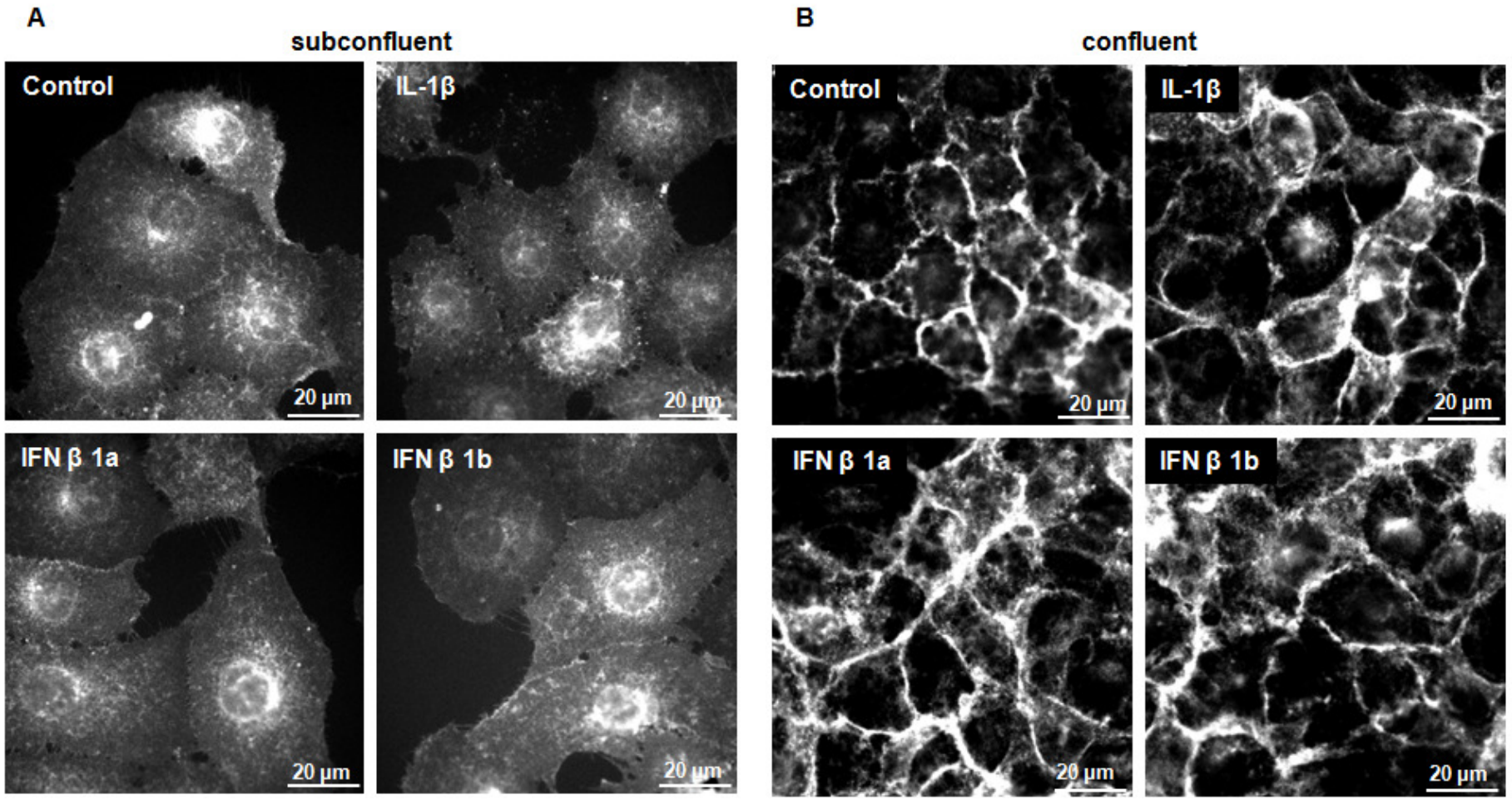


**Figure 34: Impact of cytokines on the localisation of total β1 integrin subunits**

Subconfluent (A) and confluent (B) THBMECs, grown onto a matrix of fibronectin and collagen IV, were treated with the respective cytokine for 72 h. Subsequently, cells were washed in PBS, fixed, permeabilised and incubated with an antibody raised against β1 integrin subunit as well as the respective secondary antibody. Images were taken on a Zeiss Axiovert 200 microscope with a magnification of 64x.

### 3.3.4 Integrin localisation

To explore whether cytokines influence localisation of endothelial integrins, subconfluent and confluent THBMECs were treated with IL-1β, IFNβ 1a and IFNβ 1b for 72 h and analysed by indirect immunofluorescence microscopy for the expression of different integrin subunits.

In subconfluent untreated THBMECs, the α3 integrin subunit was evenly distributed at the plasma membrane. Furthermore, this subunit was lightly enriched in cell protrusions as well as in formed cell-cell contacts (figure 13A). No differences in the localisation of α3 integrin subunit in subconfluent cells were observed after treatment with IL-1β, IFNβ 1a or IFNβ 1b (data not shown). In confluent THBMECs, α3 integrin was mainly localised at cell-cell contacts of adjacent cells (figure 35A, upper panel, right). Treatment with IL-1β led to less diffuse staining pattern of this integrin at the cell-cell contacts (figure 35A, upper panel, right, arrows). In THBMECs treated with IFNβ 1a or IFNβ 1b, localisation of α3 integrin subunit was comparable to that of control cells (figure 35A, lower panels).

To investigate the localisation of the α3 integrin subunit in more detail, confocal laser scanning microscopy was performed in cooperation with Dr. Annett Koch (AG Krüger, Institut für Biochemie, Charité Universitätsmedizin, Berlin). To this end, an x-z sectional scan of the monolayer was performed. The α3 integrin subunit was expressed at the abluminal and the luminal surface in untreated as well as in IL-1β treated cells (figure 35B, left panels, arrows). Furthermore, accumulation of integrin α3 subunit at cell-cell contacts was detected (figure 35B, upper panel, left, arrowhead). After stimulation of THBMECs with IL-1β, localisation of α3 integrin subunit at cell-cell contacts was enhanced (figure 35B, lower panel, left, arrowheads).

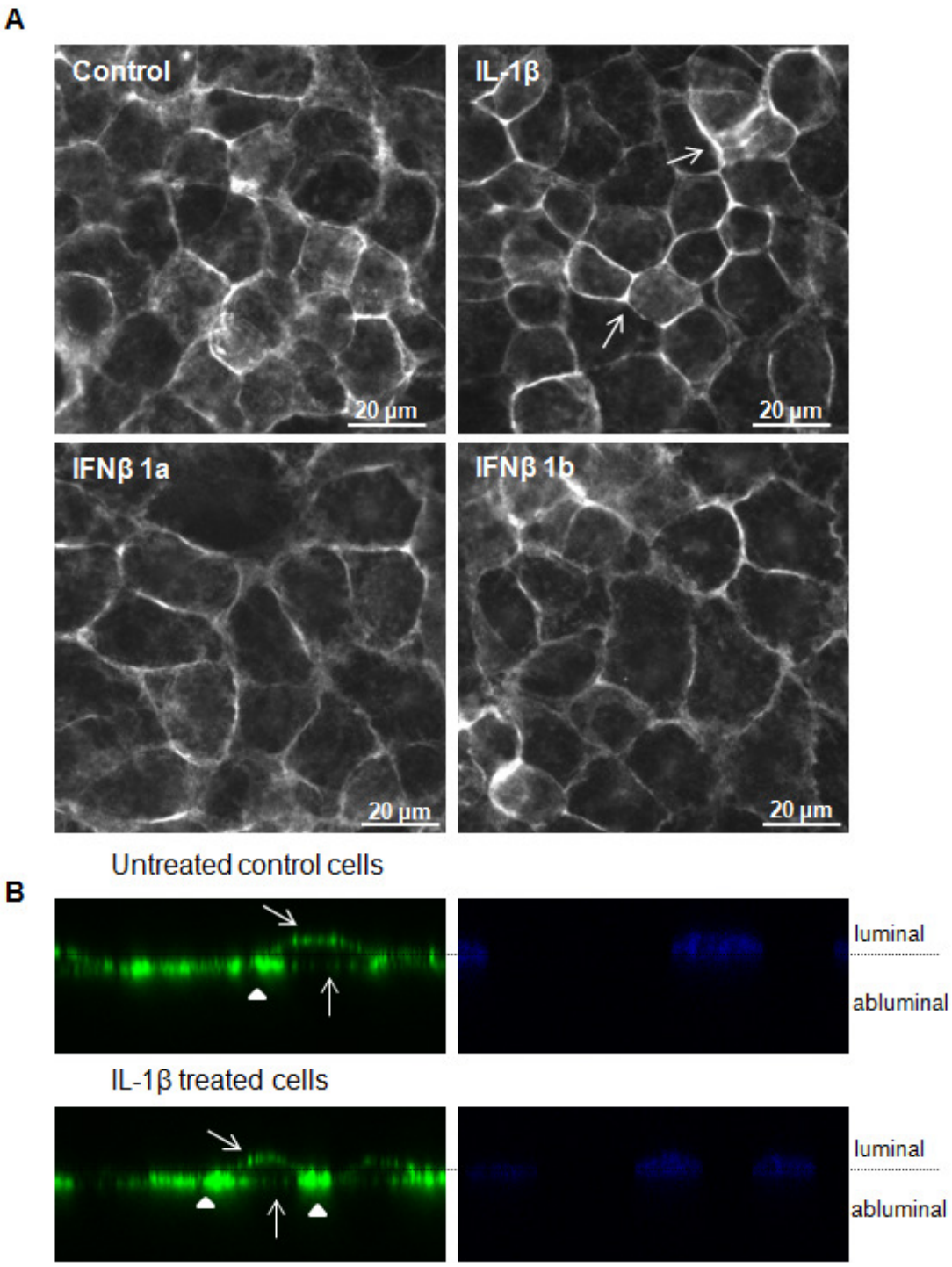


**Figure 35: Impact of cytokines on the localisation of α3 integrin subunit**

Confluent THBMECs were treated with the 10 ng/ml of the respective cytokine or left untreated (negative control) for 72 h. Subsequently, cells were washed in PBS, fixed, permeabilised and incubated with Hoechst to stain the nucleus or an antibody raised against α3 integrin subunit as well as the corresponding secondary antibody. A) Images were taken on a Zeiss Axiovert 200 microscope with a magnification of 64x. B) Confocal images of a confluent cell monolayer were obtained with a Leica TCS SP2 microscope with a magnification of 63x. The α3 integrin subunit appears in green. The nucleus appears in blue. An auxiliary line indicates the luminal and abluminal side of the THBMEC layer.

In untreated subconfluent THBMECs, staining of the α5 integrin subunit revealed a perinuclear localisation. Furthermore, it was detected in focal contacts at the plasma membrane (figure 13A). None of the tested cytokines altered the localisation of this integrin subunit in subconfluent endothelial cells (data not shown). In confluent cells, the α5 integrin was diffusely distributed all over the cell and accumulated in regions of cell-cell contacts (figure 36A, upper panel, left, arrows). Treatment with IL-1β, IFNβ 1a as well as IFNβ 1b resulted in a more intensive staining for this subunit, which indicated an increase in expression (figure 36A). By confocal laser scanning microscopy it was shown that the α5 integrin subunit was expressed at both the luminal and abluminal side of endothelial cells (figure 36B, upper panel, left, arrows). In the presence of IL-1β, α5 integrin subunit accumulated in cell-cell contacts in favour of the luminal signal (figure 36B, lower panel, left, arrowheads).

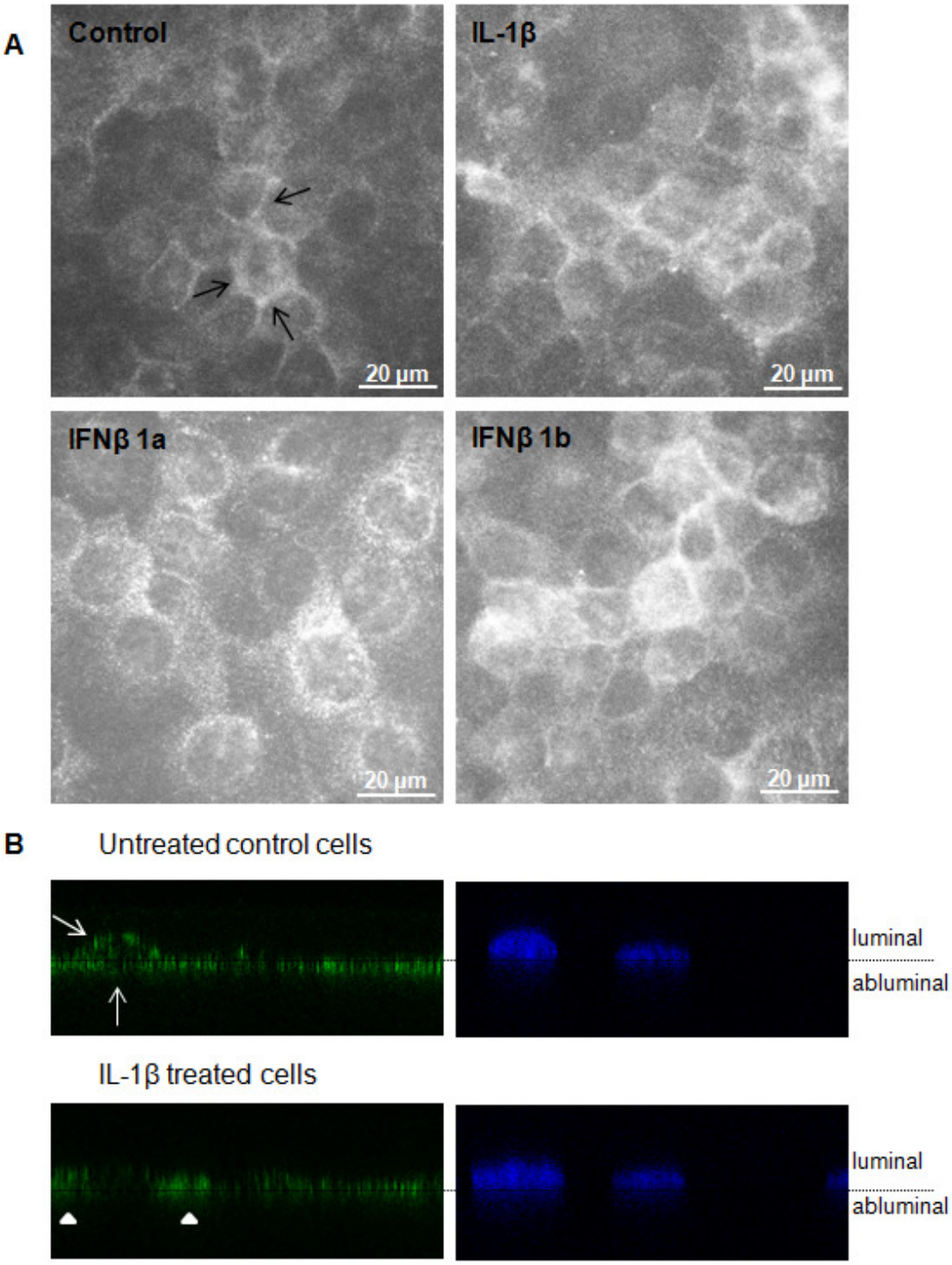


**Figure 36: Impact of cytokines on the localisation of α5 integrin subunit**

Confluent THBMECs were treated with the 10 ng/ml of the respective cytokine or left untreated (negative control) for 72 h. Subsequently, cells were washed in PBS, fixed, permeabilised and incubated with Hoechst to stain the nucleus or an antibody raised against the α5 integrin subunit as well as the corresponding secondary antibody. A) Images were taken on a Zeiss Axiovert 200 microscope with a magnification of 64x. B) Confocal images of a confluent cell monolayer were obtained with a Leica TCS SP2 microscope with a magnification of 63x. The α5 integrin subunit appears in green. The nucleus appears in blue. An auxiliary line indicates the luminal and abluminal side of the THBMEC layer.

In subconfluent THBMECs, staining of the integrin subunit α6 revealed a perinuclear distribution as well as localisation at the plasma membrane ruffles (figure 13A). Stimulation with the cytokines IL-1β, IFNβ 1a and IFNβ 1b did not influence the localisation of α6 integrin subunits in subconfluent THBMECs (data not shown). Under confluent conditions, this integrin subunit was distributed diffusely over the cell body. Moreover, in some regions of the THBMEC monolayer the α6 integrin subunit was concentrated in cell-cell contacts (figure 37, upper panel, left, arrows). After stimulation with IL-1β, the α6 integrin subunit seemed to accumulate in cell-cell contacts (figure 37, upper panel, right, arrows). This effect was also observed after treatment with IFNβ 1a and IFNβ 1b (figure 37, lower panels, arrows).

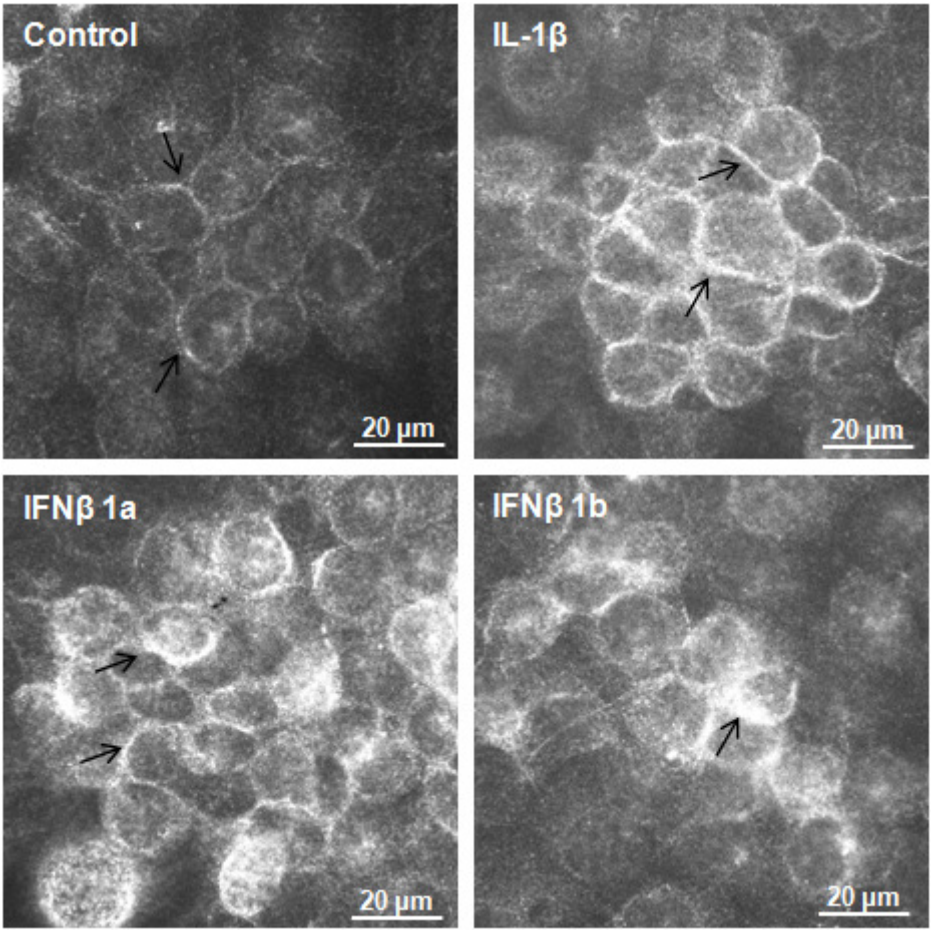


**Figure 37: Impact of cytokines on the localisation of α6 integrin subunit**

Confluent THBMECs were treated with the 10 ng/ml of the respective cytokine or left untreated (negative control) for 72 h. Subsequently, cells were washed in PBS, fixed, permeabilised and incubated with an antibody raised against the α6 integrin subunit as well as the corresponding secondary antibody. Images were taken on a Zeiss Axiovert 200 microscope with a magnification of 64x.

The effects of the cytokines TNFα, IL-1β, IFNβ 1a and IFNβ 1b on matrix adhesion and β1 integrin function are summarised in table 5.

**Table 5: Summary of the effects of cytokines on integrin function**

→: no effect, ↑: slight up regulation; ↑↑: strong up regulation

| | **Effects of** | | | |
|---|---|---|---|---|
| **Effects on** | **TNFα** | **IL-1β** | **IFNβ 1a** | **IFNβ 1b** |
| **matrix adhesion** | | | | |
| fibronectin | ↑ | ↑↑ | ↑ | ↑ |
| collagens | → | ↑↑ | ↑ | ↑ |
| laminin-1 | → | ↑ | ↑ | ↑ |
| **integrin expression** | | | | |
| α1 integrin subunit | not expressed | not expressed | not expressed | not expressed |
| α2 integrin subunit | → | → | → | → |
| α3 integrin subunit | → | ↑ | → | → |
| α5 integrin subunit | → | ↑ | ↑ | ↑ |
| α6 integrin subunit | → | ↑ | → | → |
| αv integrin subunit | → | ↑↑ | → | → |
| β1 integrin subunit | → | → | → | → |
| β3 integrin subunit | → | ↑↑ | → | → |
| **β1 integrin affinity** | → | → | → | → |
| **β1 integrin avidity** | | | | |
| subconfluent | not investigated | more focal adhesions and increased in size | → | → |
| confluent | not investigated | increased clustering, especially at cell borders | enlarged clusters | enlarged clusters |
| **β1 integrin localisation** | | | | |
| subconfluent | not investigated | → | → | → |
| confluent | not investigated | increased expression and localisation at cell-cell contacts of α3, α5 and α6 integrin subunit | increased expression and localisation at cell-cell contacts of α5 integrin subunit | increased expression and localisation at cell-cell contacts of α5 integrin subunit |

## 3.4 Impact of pro-inflammatory cytokines on the expression of proteins of the extracellular matrix

The data presented so far revealed that cytokines affect the expression levels, activation state and localisation of endothelial β1 integrins. Hence, expression and localisation of extracellular matrix proteins, which are ligands for integrins and synthesised and secreted by endothelial cells, might be influenced by pro-inflammatory cytokines, too. For that reason, extracts of THBMECs that had been stimulated with TNFα, IL-1β, IFNβ 1a and IFNβ 1b for 72 h were prepared and analysed for expression of the matrix components fibronectin, collagen IV and laminin on mRNA and protein level.

By qRT-PCR, it was shown that fibronectin mRNA expression was unchanged after treatment of THBMECs with TNFα (figure 38). However, stimulation with IL-1β resulted in a significant increase in fibronectin mRNA expression of about 40%. Treatment of endothelial cells with IFNβ 1a and IFNβ 1b revealed an opposite effect on fibronectin mRNA expression. Both IFNβ 1a and IFNβ 1b significantly reduced the mRNA level of this matrix protein. To investigate the expression of collagen IV mRNA, primers that specifically anneal to the coding sequence of the α1 chain of collagen IV were used. Analysis of collagen IV mRNA levels exhibited no alterations after treatment of THBMECs with TNFα relative to control cells (figure 38). In contrast, stimulation with IL-1β elevated expression of collagen IV mRNA by 20%, but this increase was not significant. IFNβ 1a as well as IFNβ 1b had no influence on the expression of collagen IV mRNA in THBMECs. To analyse the expression of laminins, qRT-PCR was performed with primers that specifically anneal to the coding sequence of the β1 chain of laminin. The laminin β1 chain is a component of laminin-8 and laminin-10, which are found in the endothelial basement membrane (Korpos *et al.*, 2010). Additionally, it is part of the laminin-1, laminin-2, laminin-6 and laminin-12 (Aumailley *et al.*, 2005). As illustrated in figure 38, treatment with TNFα did not affect the mRNA expression of laminin. Nonetheless, after stimulation of THBMECs with IL-1β, a significant reduction of laminin mRNA level was observed. IFNβ 1a and IFNβ 1b had no effect on mRNA levels of the laminin β1 chain relative to untreated cells.

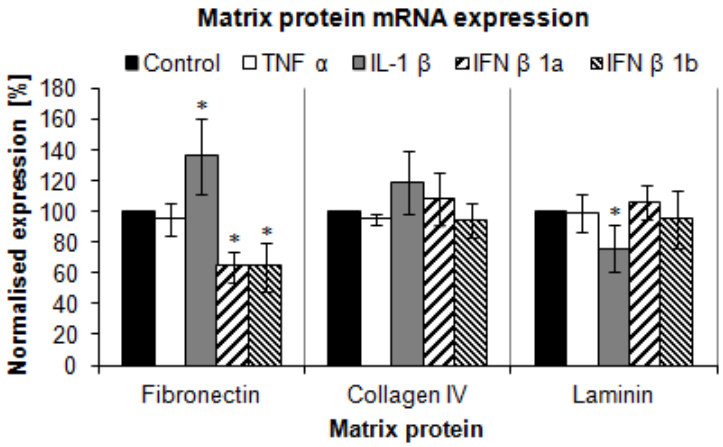


**Figure 38: Impact of cytokines on mRNA expression of matrix proteins**

Confluent THBMECs were stimulated with 10 ng/ml of the respective cytokine in serum-free medium or left untreated (negative control) for 72 h. Total RNA was isolated and applied for cDNA synthesis. qRT-PCR was performed with primers chosen for fibronectin, the α1 chain of collagen IV and the β1 chain of laminin. Three independent sets of qRT-PCR were summarised. Untreated control cells were set to 100%. Asterisks indicate significant difference to control (* $p<0.05$).

In order to investigate whether protein expression of the matrix components was also influenced by stimulation with cytokines, THBMECs were grown on plastic and treated for 72 h with TNFα, IL-1β, IFNβ 1a or IFNβ 1b. Matrix extracts were prepared and applied to western blotting using antibodies directed against fibronectin, the α1 chain of collagen IV as well as the β1 chain of laminin. Plasma fibronectin, collagen IV produced by human fibroblasts and epithelial cells as well as laminin-1 from Engelbreth-Holm-Swarm murine sarcoma served as respective positive controls. A-tubulin was used as a loading control.

Untreated control cells expressed a small amount of fibronectin, which had an apparent molecular weight of about 250 kDa (figure 39). A smaller 220 kDa soluble form of fibronectin isolated from bovine plasma was used as a positive control. Consistent with the mRNA analysis, treatment with IL-1β increased the amount of fibronectin on the protein level relative to control cells while stimulation with IFNβ 1a resulted in a slight decrease in the expression of fibronectin. This effect was more pronounced in cells stimulated with IFNβ 1b. TNFα had no influence on the fibronectin expression (figure 39, upper panel). Collagen IV detected by an antibody raised against the α1 chain was slightly elevated by IL-1β relative to untreated cells. However, collagen IV protein levels remained unaffected by treatment with TNFα, IFNβ 1a and IFNβ 1a (figure 39, lower panel). B1 chain containing laminins were expressed at a very low level in THBMECs (figure 39, lower panel). The expression was not altered by the treatment of the respective cytokines.

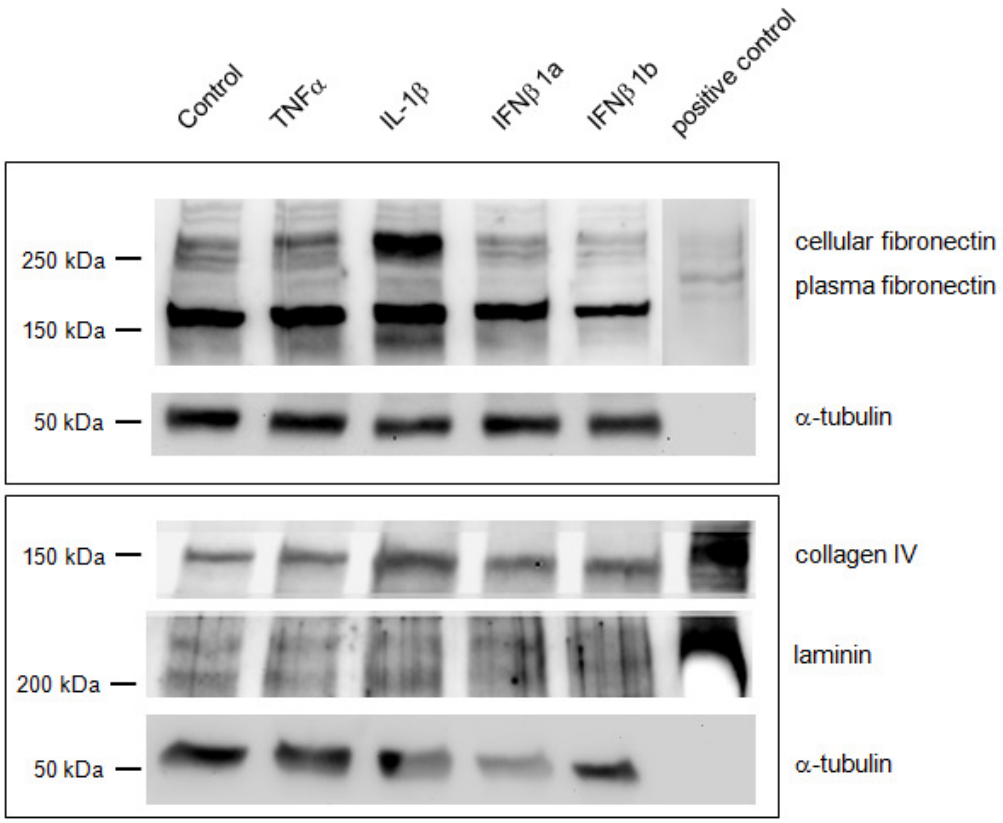


**Figure 39: Impact of cytokines on the expression of extracellular matrix proteins**

Confluent THBMECs were stimulated with 10 ng/ml of the respective cytokine in serum-free medium or left untreated (negative control) for 72 h. Matrix extracts were prepared and applied to western blotting using antibodies raised against fibronectin, collagen IV α1 chain and laminin β1 chain. 10 µg/ml of plasma fibronectin (220 kDa), collagen IV (160 kDa) and laminin-1 (210 kDa) served as respective positive controls. A-tubulin was used as a loading control. One representative experiment out of four independent experiments is depicted.

The influence of IL-1β on the fibronectin expression was investigated in more detail. Localisation of this protein was analysed in confluent THBMECs by indirect immunofluorescence microscopy. In untreated endothelial cells, some dot-like fibronectin-positive structures that resemble focal adhesions were observed (figure 40A, left, arrows). In IL-1β-treated THBMECs, the assembly of fibronectin into a fibrillary matrix was detected (figure 40A, right, arrows). Furthermore, the fluorescence intensity was much stronger than in control cells indicating an increase in expression. Images obtained by confocal microscopy confirmed this observation (figure 40B). The fibronectin signal was enriched at the abluminal side of the cells.

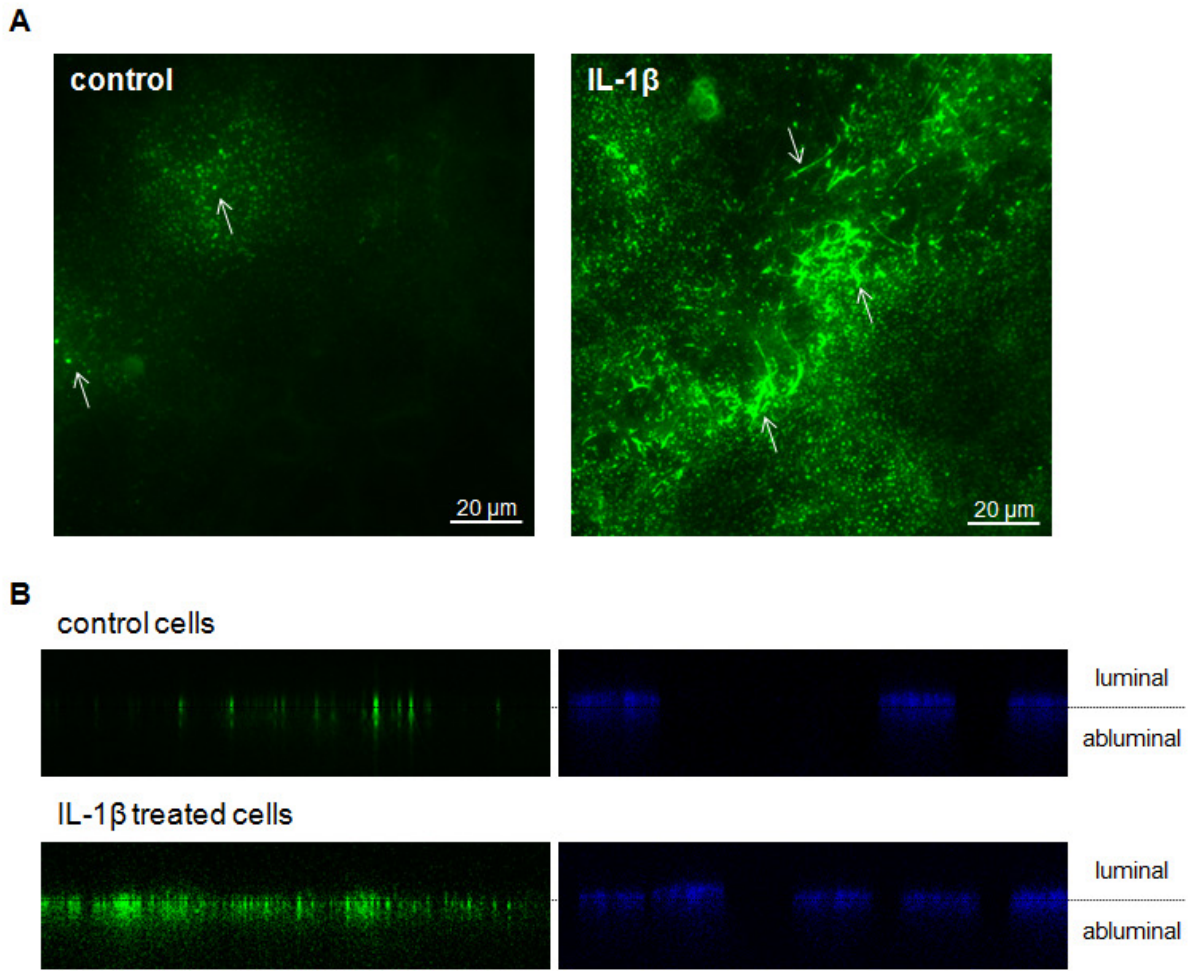


**Figure 40: Impact of IL-1β on localisation of fibronectin**

8 x $10^4$ THBMECs were seeded onto plastic and grown for 72 h. Afterwards, cells were treated with IL-1β for 72 h. Cells were washed in PBS, fixed and permeabilised and incubated with Hoechst to stain the nucleus or an antibody raised against fibronectin and in the respective secondary antibody. A) Images were taken on a Zeiss Axiovert 200 microscope with a magnification of 64x. Arrows indicate accumulation of fibronectin. B) Confocal pictures were obtained with a Leica TCS SP2 microscope with a magnification of 63x. Fibronectin appears in green. Nucleus appears in blue. An auxiliary line indicates luminal and abluminal side of THBMECs.

## 3.5 Impact of endothelial β1 integrins on the blood-brain barrier integrity and transendothelial migration

The data presented revealed that the expression of the α5β1 integrin and its ligand fibronectin was increased during inflammation. Additionally, up-regulation of α3β1 and α6β1 integrin during inflammatory conditions indicated that β1 integrins are involved in inflammatory processes at the BBB. To ascertain whether endothelial β1 integrins also play a role in maintaining the BBB integrity, the TEER of the *in vitro* BBB model was monitored in the presence of function-blocking antibodies raised against the integrin subunits β1, α5 and α6 using the automatic cell monitoring system cellZscope® (nanoAnalytics GmbH, Münster).

Cells treated with anti-IgG served as a negative control. Blocking of the β1 integrin subunit for 30 min resulted in an only marginal reduction of the TEER (figure 41). However, inhibition of the α5 integrin subunit of THBMECs led to a slight but significant decrease in TEER. After incubation of endothelial cells with an α6 integrin function-blocking antibody a faintly reduced TEER relative to cells, treated with anti-IgG, were also observed. However, this decrease was not significant (figure 41). With elongated incubation the reducing effect of β1 integrin function-blocking antibodies on TEER was abolished, probably due to endocytosis of the antigen-antibody complex (appendix; figure 45).

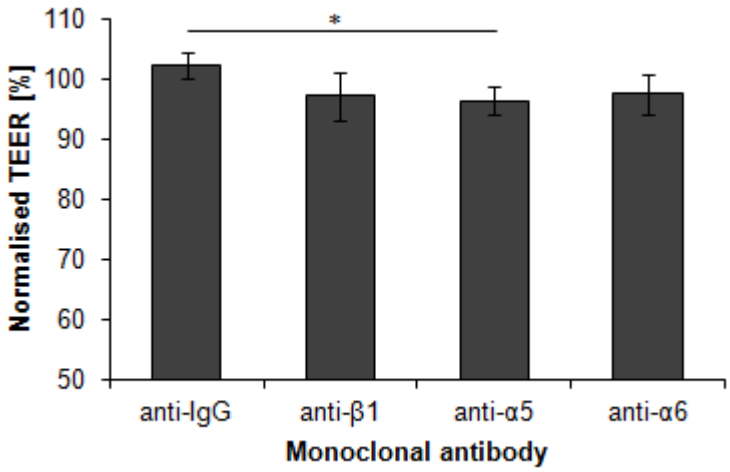


**Figure 41: Impact of β1 integrin function-blocking antibodies on TEER of the *in vitro* BBB model**

Confluent THBMECs with a TEER of at least 150 Ω x $cm^2$ and higher were incubated with 10 ng/ml of the respective function-blocking antibody in growth medium for 30 min. THBMECs incubated with anti-IgG served as negative control. TEER was monitored using the automatic cell monitoring system cellZscope® (nanoAnalytics GmbH, Münster). TEER at time point 0 h was set to 100% for each condition. Approaches were performed in duplicate. Three independent experiments were summarised. Asterisks indicate significant difference to control (* $p<0.05$).

To analyse whether endothelial β1 integrins are involved in the transmigration of immune cells across the *in vitro* BBB, THBMECs were incubated in the presence of function-blocking antibodies raised against the integrin subunits β1, α5 and α6 for 30 min. Subsequently, transmigration assays were performed with PBMCs, isolated T lymphocytes and monocytes. Blockade of the β1 integrin subunit had no impact on transmigration of PBMCs (figure 42). Nevertheless, inhibition of endothelial α5 integrin subunit reduced transmigration of PBMCs by about 10%. However, this decrease was statistically not significant. Incubation of THBMECs with an α6 function-blocking antibody had no effect on transmigration of PBMC at all.

Transmigration of isolated T lymphocytes was not affected by inhibition of any of the mentioned integrin subunits (figure 42). However, transmigration of isolated monocytes was reduced by blockade of the α5 or α6 integrin subunit on endothelial cells by about 15%. Inhibition of the endothelial β1 integrin subunit had no impact on monocytes migration (figure 42).

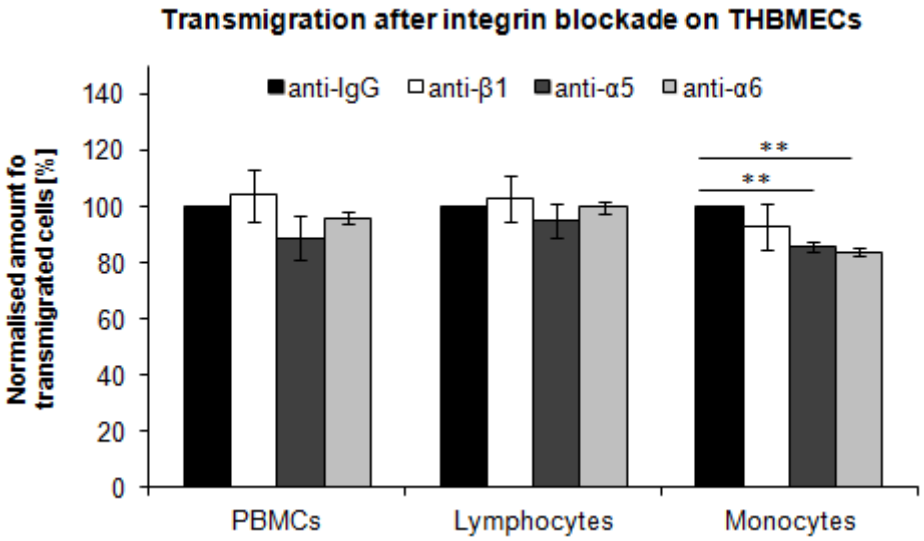


**Figure 42: Impact of β1 integrin function-blocking antibodies on transmigration of PBMCs, isolated T lymphocytes and isolated monocytes**

Confluent THBMECs with a TEER of at least 150 Ω x $cm^2$ and higher were incubated with 10 ng/ml of the respective function-blocking antibody in growth medium for 30 min. Subsequently, cells were washed and calcein AM labelled immune cells were allowed to transmigrate across the THBMEC layer for 18 h. Amounts of transmigrated cells were calculated by means of a standard curve. Each approach was performed in duplicate. Cells treated with anti-IgG were set to 100% for each PBMC population. At least three independent experiments were summarised. Asterisks indicate significant difference to control (** $p<0.001$).

To investigate whether the effects on the transmigration of immune cells by inhibition of endothelial β1 integrins PBMCs was more pronounced under inflammatory conditions, THBMECs were stimulated with IL-1β for 72 h and subsequently incubated with function-blocking antibodies raised against the integrin subunit β1, α5 and α6 for 30 min. PBMCs were allowed to transmigrate for 18 h. In contrast to unstimulated endothelial cells, blocking of the β1 integrin subunit on IL-β-stimulated THBMECs reduced the transmigration of PBMCs by about 10% (figure 43). Additionally, the inhibition of the α5 integrin subunit on IL-1β-treated THBMECs significantly reduced the transmigration of PBMCs by about 15%. Incubation of endothelial cells stimulated with IL-1β, with an α6 function-blocking antibody only marginally reduced the transmigration of PBMC relative to cells which were incubated in anti-IgG (figure 43).

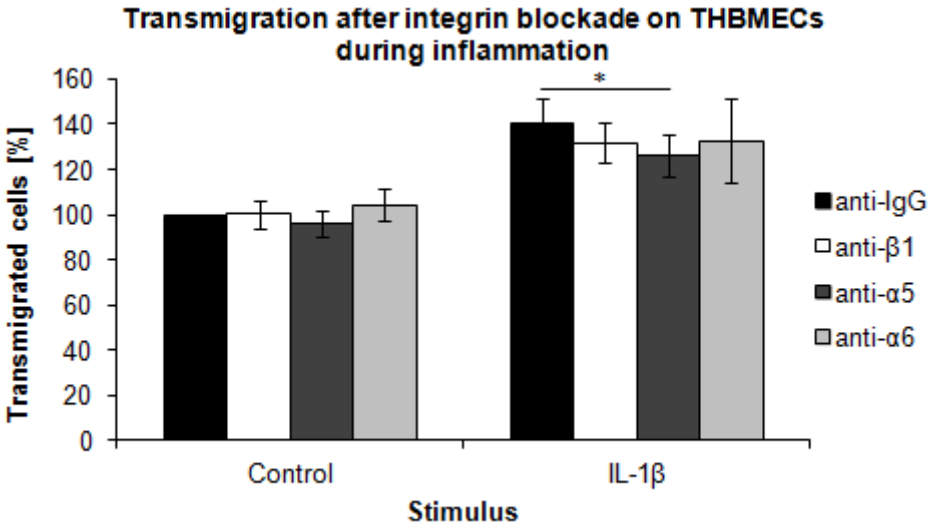


**Figure 43: Impact of β1 integrin function-blocking antibodies on transmigration of PBMCs during inflammation**

Confluent THBMECs with a TEER of at least 150 Ω x $cm^2$ and higher were stimulated with 10 ng/ml of IL-1β for 72 h. Afterwards, cells were pre-incubated with the respective function-blocking antibody in growth medium for 30 min. Subsequently, cells were washed and calcein AM labelled PBMCs were allowed to transmigrate across the THBMEC layer for 18 h. Amounts of transmigrated cells were calculated by means of a standard curve. Each approach was performed in duplicate. Untreated cells, which ware incubated with anti-IgG, were set to 100%. Three independent experiments were summarised. Asterisks indicate significant difference to control (* $p<0.05$).

# 4 Discussion

## 4.1 Establishment of an *in vitro* blood-brain barrier model

The main role of the current study addressed the question whether endothelial β1 integrins play a certain role for the BBB function in general or especially during inflammation.

Therefore, an *in vitro* BBB model had to be established using the endothelial cell line THBMEC. This model met some of the important physical and morphological features required for the maintenance of the BBB function. In the THBMEC-based model presented here, the endothelial cells showed the characteristic morphology which was also observed for primary endothelial cells (Steiner *et al.*, 2011). Furthermore, expression of the tight junction proteins claudin-5, occludin and ZO-1 was confirmed. Localisation of these proteins was restricted mainly to cell-cell junctions as typical for brain endothelial cells and had been described for different *in vitro* BBB models (Brown *et al.*, 2007; Man *et al.*, 2008; Cohen-Kashi Malina *et al.*, 2009). Moreover, the F-actin cytoskeleton was organised in cortical rings and only a few F-actin stress fibres were detected. In contrast to other endothelial cell lines such as bEND5, this resembles the F-actin organisation detected in primary cells (Steiner *et al.*, 2011).

Furthermore, the *in vitro* BBB model established here was characterised by a maximum TEER of about 250 Ω x $cm^2$. Thus, higher resistances were achieved than have been published for this cell line so far. Other research groups that worked with this cell line reached TEER values of 30 to 120 Ω x $cm^2$ (Stins *et al.*, 2001, Callahan *et al.*, 2004; Ubogu *et al.*, 2006b; Man *et al.*, 2008). *In vitro* models based on the human endothelial cell line hCMEC/D3 either achieved lower resistances of 40 to 140 Ω x $cm^2$ (Hatherell *et al.*, 2011; Daniels *et al.*, 2013; Griep *et al.*, 2013). However, *in vitro* BBB models comprised of primary cells exhibited higher resistances ranging from 350 Ω x $cm^2$ for human endothelial cells to 1,800 Ω x $cm^2$ for porcine endothelial cells (Joó, 1992; Zozulya *et al.*, 2008; Cohen-Kashi Malina *et al.*, 2009). These values closely resemble the physiological resistance of more than 1,000 Ω x $cm^2$ (Deli *et al.*, 2005; Santaguida *et al.*, 2006). Besides high transendothelial resistance the barrier function of an appropriate *in vitro* model needed to be additionally characterised by low paracellular permeability. Using the paracellular transport marker sodium fluorescein a minimal paracellular permeability coefficient of about 1 x $10^{-6}$ cm/s was determined for the model established here. Paracellular permeability coefficients published for other *in vitro* models using rat brain endothelial cell lines were one order of magnitude

higher and ranged from 1 to 2 x $10^{-5}$ cm/s (Blasig *et al.*, 2001). However, for *in vitro* BBB models using primary bovine and rat brain endothelial cells a permeability coefficient for sodium fluorescein of about 2 - 3 x $10^{-6}$ cm/s was published, which is in a similar range as for the model presented here (Deli *et al.*, 2005).

Moreover, it was shown that this *in vitro* BBB model represented a barrier for PBMCs under chemokine-free conditions. The amount of transmigrated cells rose in a linear fashion over time. This is consistent with data obtained by chemokine-free transmigration assays performed in other THBMEC-based *in vitro* BBB models (Callahan *et al.*, 2004; Man *et al.*, 2008). Nevertheless, the amounts of transmigrated PBMCs were higher. Whereas in the model presented here only 5% of the PBMCs transmigrated after 3 h, 7% to 10% of the PBMCs crossed the barrier in the other THBMEC-based *in vitro* BBB models (Callahan *et al.*, 2004; Man *et al.*, 2008). These findings indicate that the barrier function of these published systems was less well developed compared to the model presented in this study. Analysis of the fraction of migrated cells gave insight, in which leukocyte subpopulation actually transmigrated. The input consisted mainly of T lymphocytes, to a lesser extent of monocytes, macrophages, NK cells and neutrophils, and of only a few B lymphocytes. Analysis of the migrated cells revealed that mainly monocytes migrated after 3 h, whereas T lymphocytes, NK cells and B lymphocytes persisted in the upper compartment. This finding is in line with published data that demonstrated higher migration efficiency for monocytes (Roth *et al.*, 1995; Ubogu *et al.*, 2006; Man *et al.*, 2008). Surprisingly, many cells, which migrated within 3 h, were tested negative for all of the investigated CD proteins. A possible explanation for this phenomenon might be that also cell debris and endothelial cells were detached from the membrane through the rough washing procedure. Since endothelial cells did not express CD3, CD14, CD16 and CD19, they might enhance the percentage of cells that were tested negative for these proteins. Surprisingly, after 18 h of transmigration these negative cells were not observed anymore. This might be due to the enhanced percentage of PBMCs which migrated to the lower compartment. Under these conditions, the amount of endothelial cells in the fraction of migrated cells was negligible. After 18 h, higher amounts of T lymphocytes, NK cells and neutrophils were detected in the fraction of migrated cells than after 3 h. These results indicate that monocytes are the first subpopulation of PBMCs, which transmigrate across the endothelial cell layer, followed by T lymphocytes, NK cells and neutrophils. The small amount of B lymphocytes in the fraction of migrated cells even after 18 h incubation indicates that B lymphocytes were unable to overcome the cell layer. This is in line with other studies which show a low migratory efficiency for this PBMC subpopulation (Roth *et al.*, 1995; Man *et al.*, 2008).

In summary, the in *vitro* BBB model established in this study presents a more restrictive barrier than the models that have been published so far for THBMEC and other endothelial cell lines. However, models using primary endothelial cells in co-culture with astrocytes and/or pericytes resemble more closely the physiological conditions of the BBB. Nevertheless, these *in vitro* models have several disadvantages. The availability of human brain material is restricted. The isolation of primary endothelial cells is expensive and necessitates special skills. Furthermore, primary cells lose their specific characteristics in culture within limited passages.

Since the *in vitro* BBB model presented here nearly approaches the physical and functional characteristics of models generated by primary endothelial cells, it can be regarded as an appropriate system to provide first indications about the role of β1 integrins at the BBB.

## 4.2 Impact of pro-inflammatory cytokines on the *in vitro* blood-brain barrier model

Brain inflammation is associated with activation of the local innate immune system (Griffiths *et al.*, 2007). Activation of microglia and astrocytes results in expression of key inflammatory mediators such as cytokines, chemokines and prostaglandins, which in turn up-regulate adhesion molecules and increase the permeability of the BBB (Simi *et al.*, 2007). This facilitates the invasion of innate immune cells like NK cells, neutrophils, dentritic cells and macrophages which are involved in the selective recognition and clearance of pathogens and toxic cell debris (Griffiths *et al.*, 2007).

The *in vitro* BBB model was further extended to a model of inflammation in order to study the role of β1 integrins at the BBB during inflammatory processes. Since cytokines can be considered as the primary mediators of the inflammatory response, the pro-inflammatory cytokines TNFα and IL-1β were chosen to be applied in this model. Their effects on the BBB during inflammation were well investigated and include expression of adhesion proteins, secretion of cytokines, alterations in tight junction structure as well as increased expression of matrix metalloproteinases and result in leakage of the BBB as well as enhanced transmigration of leukocytes (Szmitko *et al.*, 2003; Stolp and Dziegielewska, 2009; Pate *et al.*, 2010).

Besides the pro-inflammatory cytokines TNFα and IL-1β, the influence of IFNβ 1a and IFNβ 1b on the *in vitro* BBB model was examined. These two kinds of recombinant IFNβ are used in the treatment of the relapsing-remitting course of multiple sclerosis. IFNβ 1a, which is applied as Rebif™ (Ares-Serono, Switzerland) or Avonex™ (Biogen, USA), is expressed in

CHO cells. Its molecular structure as well its glycosilation pattern is identical to human IFNβ. IFNβ 1b, which is applied as Betaseron™ (Berlex, USA) or Betaferon™ (Schering, Berlin), is expressed in *Escherichia coli* and is unglycosilated (Kraus and Oschmann, 2006). IFNβ 1b has been additionally approved in the secondary progressive form of multiple sclerosis. Physiologically, IFNβ is known to have anti-inflammatory effects and stabilise the BBB integrity (Kraus *et al.*, 2004; Kraus and Oschmann, 2006).

### 4.2.1 TNFα

In healthy brain, TNFα is involved in CNS development, brain homeostasis and synaptic plasticity. However, under pathological conditions it drives acute and chronic inflammatory responses resulting in BBB impairment and cell death (Montgomery and Bowers, 2012).

In this *in vitro* BBB model, TNFα had no significant effect on the expression of adhesion receptors in THBMECs. It failed to induce the expression of ICAM-1 as well as VCAM-1. Furthermore, TNFα did not change the protein expression levels of tight junction proteins. TNFα had no significant effect on the physical and functional properties of the BBB model presented here, indicating that there were no alterations in the BBB integrity. Solely, slightly elevated levels of the cytokines IL-6 and IL-8 as well as the matrix metalloproteinases MMP-2 and MMP-9 were detected in the culture media of TNFα treated cells. Although THBMECs express TNFα receptor I (data not shown), this cytokine failed to induce an inflammation in the present *in vitro* BBB model. However, in other systems TNFα induced inflammatory processes. Treatment of *in vitro* models, generated by primary bovine brain microvascular endothelial cells, with TNFα decreased significantly the permeability, indicating its ability to induce an inflammation in cultured endothelial cells (Anda *et al.*, 1997; Descamps *et al.*, 1997). Moreover, in human umbilical vein endothelial cells (HUVEC), TNFα have been reported to increase the expression of ICAM-1, VCAM-1 and E-selectin in a concentration-dependent manner (Pober *et al.*, 1986; Sun *et al.*, 2010). In other in non-cerebral endothelial cells, TNFα induced redistribution of ZO-1, enhanced endothelial permeability as well as decreased TEER (Shivanna and Srinivas *et al.*, 2010).

Ubogu and colleagues established an inflammatory *in vitro* BBB model by stimulating THBMECs with a combination of TNFα and INFγ (Ubogu *et al.*, 2006a). This mixture of cytokines increased the expression of ICAM-1 as well as the secretion of the chemokines CXCL1, CXCL2 and CXCL3 (Ubogu *et al.*, 2006a; Ubogu *et al.*, 2006b; Man *et al.*, 2009). These findings suggest that TNFα, at least in combination with IFNγ, can induce activation of THBMECs. However, no effects on the TEER were detected (Ubogu *et al.*, 2006b). So it

remains unclear whether TNFα can induce leakiness of an *in vitro* BBB model generated by THBMECs.

### 4.2.2 IL-1β

Members of the IL-1 family are expressed at low levels in the healthy brain and regulate many important physiological functions within the brain including metabolism, temperature regulation, sleep, food and water intake as well as memory (Basu *et al.*, 2004; Simi *et al.*, 2007) However, under pathological conditions the expression of IL-1 and its receptor is rapidly up-regulated (Simi *et al.*, 2007).

In the THBMEC-based model, IL-1β had the strongest impact on the endothelial cell layer including expression of adhesion proteins, secretion of cytokines as well as increased expression of matrix metalloproteinases, impairment of the BBB integrity as well as transmigration of leukocytes. Whereas resting THBMECs constitutively expressed ICAM-1, VCAM-1 was not detected on resting cells. This has also been described for primary human brain endothelial cells as well as in non-cerebral HUVEC (Pober *et al.*, 1986; Carlos *et al.*, 1990; Wong and Dorovini-Zis, 1992; Wong and Dorovini-Zis, 1995). Stimulation of THBMECs with IL-1β resulted in up-regulation of ICAM-1 mRNA and protein after 24 h, which persisted over 72 h. These findings are in line with other studies showing an increase in ICAM-1 expression in a time and concentration-dependent manner (Pober *et al.*, 1986; Carlos *et al.*, 1990; Wong and Dorovini-Zis, 1992). Treatment with IL-1β induced additionally the expression of VCAM-1 on the endothelial cells, but the increase in VCAM-1 expression was delayed compared to the expression of ICAM-1 and reached its peak after 72 h. This is in contrast to published data achieved with HUVEC and primary human brain endothelial cells, in which a maximum increase in VCAM-1 expression was observed in the presence of IL-1β after just 4 h and 12 h, respectively (Pober *et al.*, 1986; Wong and Dorovini-Zis, 1995). Besides the increase in expression of these inflammation-related adhesion molecules, enhanced expression and secretion of the pro-inflammatory cytokines IL-1α, IL-1β, IL-6, IL-8 and TNFα was detected after stimulation of THBMECs with IL-1β. Similar findings have been reported for primary HUVEC and human intestine microvascular endothelial cells after treatment with IL-1β (Nilsen *et al.*, 1998). These pro-inflammatory cytokines trigger signal transduction pathways, which lead to the activation of the transcription factor nuclear factor-κB (NF-κB). NF-κB induces different target genes, which are involved in inflammation, immunity, cell survival and cell death (Kataoka, 2009). Furthermore, pro-inflammatory cytokines such as IL-1, IL-6 and TNFα induce the release of nitric oxide, which in turn

increases the expression and secretion of these pro-inflammatory cytokines (Merrill and Murphy, 1997). This positive feedback-loop further promotes the inflammation.

During inflammation the molecular composition of tight junctions or the functional state of tight junction proteins are often altered (see chapter 1.3.1). In this work it was demonstrated that IL-1β decreased expression of the tight junction proteins ZO-1 and occludin. Furthermore, redistribution of the tight junctions proteins claudin-5, ZO-1, occludin and JAM-A was shown in primary endothelial cells after inflammatory stimuli (Stamatovic *et al.*, 2012). Stamatovic and colleagues revealed that the inflammation-induced remodelling of the tight junction complex is associated with a caveolae-dependent internalisation of occludin and claudin-5 (Stamatovic *et al.*, 2009). Recently, it has been reported that IL-1β also influenced adherence junctions. In primary human coronary artery endothelial cells, it decreased the expression of VE-cadherin, α-catenin and β-catenin (Ferreira *et al.*, 2005). Alterations in the structure of endothelial junctions led to impaired integrity of endothelial cells, which was represented by reduced transendothelial resistance and increased paracellular permeability in the THBMEC-based model. IL-1β additionally decreased the TEER in primary human endothelial cells (Ferreira *et al.*, 2005; Rigor *et al.*, 2012). Leakage of the endothelial cell monolayer as well as up-regulation of adhesion molecules in turn facilitated the transmigration of leukocytes. Moreover, IL-1β induced the expression of MMP-2 and MMP-9 in THBMECs, which additionally contributed to an increase in transmigration of immune cells. In this model, IL-1β enhanced the amount of transmigrated PBMCs by 50%. Furthermore, it was demonstrated that IL-1β had a stronger impact on the transmigration of monocytes than on T lymphocytes. Ferreira and colleagues analysed transmigration of monocytes across the IL-1β-treated endothelium. It turned out that IL-1β reduced the transcellular migration and promote paracellular migration by altering the distribution of adherens junction components and F-actin organisation (Ferreira *et al.*, 2005). These observations indicated that IL-1β not only influences the amount of transmigrating leukocytes, but also the pathways they use.

In summary, all these findings demonstrate that IL-1β leads to THBMEC activation represented by enhanced expression of inflammation-related adhesion molecules, cytokines and matrix metalloproteinases. Furthermore, IL-1β induces leakage and increases leukocyte transmigration in this THBMEC-based *in vitro* BBB model.

### 4.2.3 IFNβ 1a and IFNβ 1b

IFNβ is known to be an anti-inflammatory, anti-proliferating and anti-viral cytokine (Kraus *et al.*, 2004; Kraus and Oschmann, 2006). Furthermore, IFNβ is used in multiple sclerosis therapy and has been demonstrated to have a stabilizing effect on the BBB integrity *in vivo* (Kraus *et al.*, 2004). However, the mechanism of action of IFNβ is still poorly understood, but changes in T lymphocyte activation, cytokine production and migration have been reported in multiple sclerosis patients treated with IFNβ (Jensen *et al.*, 2005). Moreover, the stabilising effect of IFNβ on the BBB might be achieved by changes in the molecular structure of endothelial junctions (Kraus and Oschmann, 2006).

The effects on the THBMEC-based *in vitro* BBB model caused by IFNβ 1a and IFNβ 1b were very similar and are discussed together in the following. Treatment of THBMECs with IFNβ slightly increased the expression of ICAM-1 but did not influence the expression of VCAM-1. This is consistent with published data, demonstrating that stimulation of HUVEC with IFNβ enhanced expression of ICAM-1 but not VCAM-1 (Jiang *et al.*, 1997). However, the IFNβ-induced increase in the expression of ICAM-1 in this system was not comparable with those of IL-1β, which induced a much stronger up-regulation of ICAM-1. Jiang and colleagues demonstrated that IFNβ was not able to reduce the rise in VCAM-1 and ICAM-1 expression induced by IL-1β and TNFα in HUVEC (Jiang *et al.*, 1997). First investigations in the *in vitro* model established here provide similar results (data not shown). Furthermore, elevated levels of soluble ICAM-1 and VCAM-1, generated by proteolytic cleavage of the membrane-bound form, was detected in dependence of IFNβ in serum of multiple sclerosis patients (Kilinc *et al.*, 2003; Jensen *et al.*, 2005; Graber *et al.*, 2005). Soluble forms of the adhesion proteins ICAM-1 and VCAM-1 are suggested to catch circulating lymphocytes by binding to leukocyte integrins and thereby prevent the extravasation of lymphocytes into the brain (Graber *et al.*, 2005).

In this study, it was shown that IFNβ 1a and IFNβ 1b only marginally elevated the expression and secretion of the pro-inflammatory cytokines IL-1β, IL-6 and IL-8 by THBMECs. These findings indicate that the endothelial cells were not strongly activated by IFNβ, and an inflammatory response seems not to be induced. Whereas no data is available for the expression of cytokines by other endothelial cells after stimulation with IFNβ, several studies reported an increase in the expression of the anti-inflammatory cytokines IL-4, IL-5 and IL-10 by lymphocytes (Corsini *et al.*, 1997; Jensen *et al.*, 2005). These data support the anti-inflammatory role of IFNβ.

Although IFNβ had no significant effect on the expression of the tight junction protein ZO-1 in THBMECs, it increased the expression of occludin mRNA and protein. To date, not much

data is published about a possible influence of IFNβ on the expression levels of tight junction proteins. However, Kraus and colleagues presented data indicating a modulatory effect of IFNβ on the distribution of claudin-3, claudin-5, occludin, ZO-1 and ZO-2 in primary bovine brain endothelial cells (Kraus *et al.*, 2004). Using immunofluorescence they demonstrated a continuous and more homogeneous expression for these tight junction proteins after treatment with IFNβ. Moreover, in the murine endothelial cell line bEND5 it was shown that IFNβ influenced microfilament-associated cell-cell contacts by increasing the expression of N-cadherin and vinculin (Harzheim *et al.*, 2004). These findings indicate that IFNβ modulate the structure of endothelial tight and adherens junctions and thereby contribute to stabilise the BBB. In fact, IFNβ increased the TEER of the *in vitro* BBB model, confirming a stabilising role of IFNβ in this system. However, no changes in the permeability coefficient of sodium fluorescein were determined. In other *in vitro* BBB models based on the endothelial cell line THBMEC, stimulation with IFNβ and serum of IFNβ-treated patients led to a significant decrease in the permeability for sucrose and insulin (Kraus *et al.*, 2008; Müller *et al.*, 2012). Additionally, an IFNβ-induced increase in TEER as well as a decrease in permeability was detected in other systems using primary bovine and porcine brain endothelial cells as well as the murine endothelial cell line bEND5 (Kraus *et al.*, 2004; Kraus *et al.*, 2008).

The present work revealed that IFNβ lightly enhanced the expression of the matrix metalloproteinases MMP-2 and MMP-9 in the endothelial cell line THBMEC. To date, no data, dealing with the influence of IFNβ on MMPs expressed by endothelial cells, have been published. However, several studies showed a reduced level of MMP-9 expressed by leukocytes *in vitro* as well as reduced amounts of MMP-8 and MMP-9 in serum of patients treated with IFNβ (Lou *et al.*, 1999; Boz *et al.*, 2006; Comabella *et al.*, 2009; Alexander *et al.*, 2010). These data support an anti-inflammatory role of IFNβ. Despite its positive effect on the TEER, IFNβ did not influence the transmigration of PBMCs, T lymphocytes and monocytes in the model presented here. These results differ from other studies. Using human *in vitro* BBB models, it was shown that IFNβ significantly reduced the number of transmigrated PBMCs and T lymphocytes (Lou *et al.*, 1999; Prat *et al.*, 2005; Kraus and Oschmann, 2006). Furthermore, transmigration of monocytes across primary rat brain endothelial cells was significantly reduced after treatment with IFNβ *in vitro* and *in vivo* (Floris *et al.*, 2002). Differences might result from the usage of primary cells or different incubation time and concentrations of IFNβ. Furthermore, Floris and colleagues analysed transmigration with a time-lapse videomicroscopy migration assay, and not with the classical transmigration assay used in this work.

In summary, the presented data suggest that IFNβ has an anti-inflammatory and stabilising effect of the THBMEC-based *in vitro* BBB model.

## 4.3 Characterisation of THBMECs concerning its integrin expression and adhesion behaviour

Integrins are of vital importance for the physiological function of endothelial cells. They promote cell adhesion to matrix proteins and participate in cell migration, proliferation as well as remodelling during angiogenesis and wound healing (Dejana *et al.*, 1993; Luscinskas and Lawler, 1994). However, endothelial integrins are also involved in pathological events, such as inflammation and multiple sclerosis (Sobel *et al.*, 1998; Bank *et al.*, 2002). Endothelial cells express several integrins of the β1 and αv classes (Conforti *et al.*, 1992). They mediate the adhesion to extracellular matrix proteins, such as laminin, collagen, fibronectin and vitronectin, as well as to soluble proteins, such as von Willebrand factor, fibrinogen and thrombin (Conforti *et al.*, 1992; Dejana *et al.*, 1993; Luscinskas and Lawler, 1994).

In agreement with a previous study, analysis of the immortalised endothelial cell line THBMEC revealed expression of β1 integrins including α2β1, α3β1, α5β1, α6β1 as well as the αvβ3 integrin (Pilorget *et al.*, 2007). For the integrins α3β1 and α5β1, a luminal and abluminal localisation was shown, which is consistent with other studies (Conforti *et al.*, 1992). Furthermore, the integrin subunits α3 and β1 are located mainly at cell-cell contacts under confluent conditions, whereas the α5 and α6 integrins subunit were distributed diffusely over the cell body with accumulation at the cell borders of adjacent cells. These findings differ from another study which demonstrated that only the α2β1 and α5β1integrins and not the α3β1 and α6β1integrins were located at cell-cell borders in endothelial cells isolated from umbilical vein (Lampugnani *et al.*, 1991). Differences might result from the affinity of the integrin-specific antibodies, the different endothelial cell types or the culture conditions. Additionally, the authors demonstrated that the intercellular localisation of the α2β1 and α5β1 integrins did not depend on the substrate, where the endothelial cells were initially grown. They suggested that - if a matrix ligand was indeed required for the localisation of these integrins at the cell-cell contacts - it might be produced and organised by the endothelial cells themselves (Lampugnani *et al.*, 1991).

The α1β1 integrin is involved in the progression of many chronic-inflammatory diseases, such as rheumatoid arthritis, multiple sclerosis, psoriasis and arthrosclerosis (Ben-Horin and Bank, 2004). Furthermore, endothelial cells up-regulate the α1 integrin subunit both in active

and chronic-inactive lesions of multiple sclerosis (Sobel *et al.*, 1998). By PCR, the mRNA expression of the α1 integrin subunit, which is part of the collagen and laminin receptor α1β1, was detected. However, protein expression of this subunit was not observed using flow cytometry and western blotting. Several studies reported that expression of the α1 integrin subunit was restricted to microvascular endothelial cells and that large vessels lacked this subunit (Defilippi *et al.*, 1991; Dejana *et al.*, 1993; Luscinskas and Lawler, 1994). Since the endothelial cell line THBMEC is derived from the microvasculature of human brain, it shall express this integrin subunit. However, Pilorget and colleagues were likewise not able to detect the α1 integrin subunit in this cell line by flow cytometry, and no data concerning the α1 integrin mRNA expression was published (Pilorget *et al.*, 2007). Furthermore, surface expression of the α1 integrin subunit was neither induced by TNFα nor by TGFβ (data not shown), which are known regulators of the α1 integrin subunit expression (Heino *et al.*, 1989; Defilippi *et al.*, 1991). All attempts to transfect THBMECs stably with a plasmid, encoding for the α1 integrin subunit, failed. After several passages, surface expression of the α1 integrin subunit was abolished although the incorporated plasmid was detected by PCR (data not shown).

THBMECs adhered strongly to fibronectin as well as collagen I and attached to laminin-1 and collagen IV to a lower extent. These findings are in line with published data, which revealed that this cell line adhered to fibronectin, laminin-1, vitronectin and collagen I (Pilorget *et al.*, 2007). A more detailed analysis of matrix adhesion of THBMECs with integrin-specific, function-blocking antibodies turned out that adhesion to fibronectin, collagen IV, collagen I and laminin was mediated primarily by β1 integrins. It was demonstrated that adhesion to fibronectin was mainly mediated by α5β1 integrin. However, since blocking of this receptor not fully reduced adhesion to fibronectin, also other integrins might be involved in adhesion of THBMECs to this matrix protein. In fact, Cheng and colleagues demonstrated that primary endothelial cells use several integrins, including α5β1, αvβ3 and αvβ5, to adhere to fibronectin (Cheng *et al.*, 1991). By inhibition of the α6 integrin subunit it turned out that adhesion to laminin-1 was mainly mediated by the α6β1 integrin. However, also other integrins, such as α2β1, α3β1 and αvβ3 integrin, have been reported to participate in the adhesion of endothelial cells to laminins (Languino *et al.*, 1989; Kramer *et al.*, 1990; Dejana *et al.*, 1993).

## 4.4 Impact of pro-inflammatory cytokines on endothelial integrin function

One aim of this work was to investigate whether β1 integrins at the *in vitro* BBB model are influenced by cytokines and might contribute to pathological events at the BBB.

It was shown that under inflammatory conditions, induced by IL-1β, adhesion of THBMECs to the matrix proteins fibronectin, collagen IV, collagen I and laminin-1 was significantly enhanced. This was accompanied by elevated expression of the α3β1, α5β1, α6β1 and αvβ3 integrin and activation of β1 integrins achieved by clustering under inflammatory conditions. Surprisingly, IFNβ, which has a more stabilising role in this *in vitro* BBB model, also increased the expression of the α5β1 integrin as well as the adhesion to fibronectin and laminin-1.

Altered integrin expression patterns on endothelial cells during inflammation have also been reported in previous studies. In agreement with this work, Sun and co-workers reported higher expression levels for the α5β1 integrin as well as activation of β1 integrins in the non-cerebral endothelial cell line HUVEC during TNFα-induced inflammation (Sun *et al.*, 2010). Furthermore, the endothelial α5 integrin subunit was elevated during brain inflammation after neuronal injury (Kloss *et al.*, 1999). Additionally, α5β1 integrin was up-regulated on brain endothelial cells *in vitro* and *in vivo* during cerebral hypoxia, a phenomenon which often accompanies inflammation (Milner *et al.*, 2008a). Moreover, microvessels strongly up-regulated the fibronectin receptors α5β1 integrin and αvβ3 integrin after focal cerebral ischemia, an event which induces an inflammatory phenotype on microvascular endothelial cells (Okada *et al.*, 1996; Milner *et al.*, 2008b; Maddahi and Edvinsson, 2010; Li *et al.*, 2012). Consistent with the presented data, IL-1β enhanced cell adhesion of fibroblasts to complete as well as to degraded fibronectin by activation of β1 integrins (Rajshankar *et al.*, 2012). Beside the inflammation at the BBB, increased expression levels of α5β1 integrin are thought to contribute to the progression of other chronic-inflammatory diseases, such as arthritis and atherosclerosis (Eichinger *et al.*, 2005; Barillari *et al.*, 2001).

In contrast to this work, *in vitro* studies using primary microvascular endothelial cells revealed decreased levels of αvβ3 and α6β1 under inflammatory stimuli (Delfilippi *et al.*, 1991; Delfilippi *et al.*, 1992). Immunohistochemical analysis of active lesions in patients, suffering from multiple sclerosis, showed also decreased expression levels of the β1 and α6 integrin subunit. However, it returned to normal levels in chronic-inactive plaques (Sobel *et al.*, 1998).

Besides endothelial integrins, integrin expression on astrocytes and pericytes was altered during inflammation, too (Milner and Campbell, 2006; Tigges *et al.*, 2013).

Taken together, these data present evidence that endothelial integrins are involved in inflammatory processes at the BBB. Their up-regulation under inflammatory conditions might facilitate the inflammation. This idea is supported by Sun and colleagues, who showed that blockade of the endothelial α5β1 integrins abolished the adhesion of leukocytes to the activated endothelium (Sun *et al.*, 2010). Furthermore, angiopoietin-2, another ligand for α5β1 integrin, is up-regulated on endothelial cells. Accordingly, they suggest a α5β1:α5β1 integrin-dependent mechanism for neutrophil adhesion to activated endothelium, in which angiopoietin-2 acts as a bridge between the two integrins. Moreover, adhesion of endothelial cells to fibronectin, mediated by α5β1 integrin, induced the transcription of genes, known to be controlled by the NF-κB transcription factor or to be induced by inflammatory cytokines (Klein *et al.*, 2002). Up-regulation of cytokines and adhesion molecules, such as VCAM-1, ICAM-1 and E-selectin, was detected after adhesion to fibronectin and might promote inflammatory response in these cells. Furthermore, integrin expression and activation is thought to negatively regulate the cadherin-mediated cell-cell adhesion. Wang and colleagues demonstrated that integrin binding to fibronectin-coated beads resulted in disruption of adherens junctions in endothelial cells (Wang *et al.*, 2006b). Engagement of integrins led to dissociation of γ-catenin and α-catenin form VE-cadherin as well as phosphorylation of β-catenin, γ-catenin and catenin p120. Additionally, alterations of the cortical actin structure and increased formation of F-actin stress fibres were detected by the authors. Furthermore, binding of fibronectin by α5β1 integrin induced the dissociation of tensin and actin form the N-cadherin-β-catenin complex and stimulated the reorganisation of N-cadherin in fibroblasts (Lefort *et al.*, 2011). These findings suggest that up-regulation of endothelial integrins promote inflammation by altering the morphological features of endothelial cells resulting in impaired barrier function.

However, enhanced expression of endothelial integrins during inflammatory conditions might also have a compensatory effect. Following the model of del Zoppo *et al.*, in which the structure of the BBB is influenced by horizontal and vertical components, loosening of the tight and adherens junctions might result in strengthening the adhesion of endothelial cells to the underlying basement membrane (del Zoppo *et al.*, 2006). This theory is supported by Milner and colleagues who proposed that during chronic inflammation vascular cells reinforce their adhesive mechanisms in an attempt to increase the stability of the blood vessels (Milner and Campbell, 2006). Ligation of the α5β1 integrin promotes cell survival and proliferation of endothelial cells and other cell types (Rich *et al.*, 1996; Kitagawa *et al.*, 2006; Milner *et al.*,

2008; Li *et al.*, 2012). Accordingly, up-regulation of this integrin might have a stabilising effect on the BBB during neuroinflammation. Support for this suggestion comes from the observation that IFNβ only increased the expression of α5β1 integrin.

Besides enhanced expression levels and activation of endothelial β1 integrins under inflammatory conditions, the integrin subunits α3, α5 and α6 accumulated in cell-cell contacts. In the x-z-sectional analysis of the endothelial monolayer, it turned out that the integrins α3β1 and α5β1 were mainly located at cell-matrix contacts. However, these integrins were additionally expressed at the lateral plasma membrane and the luminal region of the cell-cell contact. A similar distribution of the αvβ3 integrin was shown in epithelial cells (Sakamoto *et al.*, 2006). Furthermore, Sakamoto *et al.* demonstrated that the αvβ3 integrin co-localised with the adherens junction components E-cadherin and nectin but not with the tight junction protein claudin-1. Additionally, localisation of the integrins α3β1 and α5β1 in adherens junctions was described for epithelial cells and fibroblasts (Nakamura *et al.*, 1995; Lefort *et al.*, 2011). At the cell-cell contacts these integrins were found in a low affinity state (Sakamoto et al., 2006; Lefort *et al.*, 2011). This is in agreement with the present study, since it was shown by immunofluorescence analysis that the β1 integrins, which localised in the cell-cell contacts, are mainly inactive. According to their localisation and their activation state, integrins might have different functions in inflammation. The localisation of integrins at cell-cell contacts in an inactive state might stabilise the integrity of endothelial cells, whereas enhanced engagement of integrins to proteins of the extracellular matrix might result in disruption of cell-cell contact and destabilise the monolayer.

In further experiments, it should be clarified the precise localisation of active integrins in confluent THBMECs. Therefore, co-localisation of active β1 integrins and marker proteins for the luminal (such as P-glycoprotein) and abluminal site (such as glucose transporter 1) of endothelial cells should be analysed using confocal microscopy. Additionally, it should be analysed whether β1 integrins and tight junctions proteins or adherens junctions co-localises in the cell-cell contacts.

## 4.5 Impact of pro-inflammatory cytokines on proteins of the extracellular matrix

The endothelial basement membrane is important for the integrity of blood vessels and maintaining the function of the BBB. Changes in the composition of the ECM have been associated with different pathological conditions, e.g. multiple sclerosis and peritonitis (Sobel and Mitchell, 1989; van Horssen *et al.*, 2005; Sampaio *et al.*, 2010). Since alterations of

integrin expression and function after application of cytokines was observed in this study, it was necessary to investigate the influence of cytokines on the expression of ECM proteins. In the present work, it was shown that expression of fibronectin was dramatically increased under inflammatory conditions, induced by IL-1β. Additionally, slightly elevated levels of collagen IV were observed under these conditions. INFβ caused a reduction in fibronectin levels, whereas expression of collagen IV and laminin remained unaffected.

The observation that inflammation resulted in elevated levels of ECM proteins is consistent with previous reports which described increased fibronectin expression in active lesion of patients with multiple sclerosis (Sobel and Mitchell, 1989). In addition, this increase in fibronectin expression correlated with the degree of inflammation. Moreover, many inflammatory diseases, including multiple sclerosis, meningitis, intraperitoneal inflammation, are also associated with an increase in soluble plasma fibronectin in blood or cerebrospinal fluid (Richards and Saba, 1983; Merino Garcia *et al.*, 2000; Goos *et al.*, 2007). *In vitro* studies demonstrated that IL-1β increased the expression of fibronectin as well as collagen IV in endothelial cells (Boyle *et al.*, 2000; Anderson and Hinds, 2012). Neutralising antibodies against IL-1β reduced fibronectin mRNA levels as well as protein synthesis (Clausell and Rabinovitch, 1993). Furthermore, enhanced synthesis of laminin and collagen IV by glomerular capillary endothelial cells as well as thickening of the glomerular basement membrane following intravenous administration of IFNα was observed *in vivo* (Moss *et al.*, 1988). In addition, the expression of laminin-8 and laminin-10 in the endothelial basement membrane was increased by inflammatory stimuli (Korpos *et al.*, 2010).

Increased expression of ECM proteins by endothelial cells might be a mechanism that promotes leukocyte capturing and migration. Evidence for this suggestion comes from the observation that TNFα up-regulated the integrin receptors α4β1 and α5β1 in PBMCs (appendix; figure 46). In keeping with this, other studies described enhanced expression of the fibronectin receptor α5β1 integrin on monocytes and T lymphocytes as well as increased levels of α6β1 integrin on neutrophils during inflammatory conditions (Dangerfield *et al.*, 2002; Dangerfield *et al.*, 2005; Sampaio *et al.*, 2010; Li *et al.*, 2011). Additionally, blockage of the α5β1 integrin on leukocytes resulted in reduced adhesion and transmigration of lymphocytes across the endothelium (Roth *et al.*, 1995; Szekanecz *et al.*, 1992; Molossi *et al.*, 1995; Sampaio *et al.*, 2010). Further evidence comes from two previous studies, which demonstrated that antibodies, directed against cellular fibronectin and the fibronectin CS-I fragment, decreased transmigration and adhesion of T lymphocytes (Molossi *et al.*, 1995; Man *et al.*, 2009). These data suggest that leukocyte transmigration is mediated by interactions of fibronectin and α5β1 as well as α4β1 integrin. Therefore, the observed

reduction in fibronectin expression during treatment with IFNβ might negatively regulate this process. Wu and colleagues demonstrated that also laminin basement membrane composition selectively influenced the extravasation of T lymphocytes (Wu *et al.*, 2009). Accordingly, enhanced expression of fibronectin and other ECM proteins might have pro-inflammatory effects. This assumption is consistent with reports, which described that binding of fibronectin by leukocytes induced the secretion of IL-1β as well as the expression of receptors for IL-1β and TNFα (Graves *et al.*, 1996; Simms *et al.*, 1997). Furthermore, interaction of fibronectin with α5β1 integrin resulted in increased secretion of MMP-9 by different cell types, which additionally promoted inflammation (Moore *et al.*, 2007; Jin *et al.*, 2011).

However, increased expression of ECM molecules have also been suggested to be important for maintaining a healthy endothelium and stabilising the BBB (Milner and Campbell, 2006; Anderson and Hinds, 2012). Support for a more anti-inflammatory role of fibronectin comes from the observation that fibronectin inhibits the activity of the endothelial nitric oxide synthase, a protein which releases nitric oxide from arginine and thereby promotes inflammation (Viji *et al.*, 2009).

In untreated THBMECs, fibronectin accumulated in vesicle-like structures whereas treatment with IL-1β promoted formation of fibrils. Analysis of images taken by confocal microscopy pointed to an increase of fibronectin at the abluminal site, which might strengthen the adhesion of endothelial cells to the basement membrane. This is in accordance with published data which demonstrated that bovine aortic endothelial cells secreted fibronectin mainly to their abluminal site (Cseh *et al.*, 2010). The authors also showed that this cellular fibronectin was crucial for fibronectin fibrillogenesis and helped to establish and maintain the luminal-abluminal polarity of endothelial cells. Fibronectin fibrillogenesis is a α5β1 integrin-mediated process (Sechler *et al.*, 1997; Cseh *et al.*, 2010). Accordingly, enhanced formation of fibronectin fibrils during IL-1β-induced inflammation is due to enhanced expression of α5β1 integrin and increased abluminal fibronectin.

However, further research has to be done to elucidate the precise localisation of fibronectin in inflamed and non-inflamed cells. More information about the localisation of fibronectin might give insight in its precise function during this process. Using confocal microscopy the co-localisation of fibronectin with proteins expressed on the luminal or abluminal site of endothelial cells should be performed.

## 4.6 Impact of endothelial β1 integrins on the blood-brain barrier's integrity

To evaluate whether β1 integrins are involved in the maintenance of the BBB the transendothelial resistance was monitored in the presence of integrin-specific function-blocking antibodies. These experiments pointed to a possible role for these integrins in supporting the BBB integrity. Again, the most pronounced effect was obtained by blocking the α5 integrin subunit. However, with longer incubation times the reducing effect of β1 integrin antibodies on the TEER was abolished. This might be due to the internalisation of the integrin-antibody complex during the incubation time.

In keeping with this work, a previous study showed that inhibition of β1 integrins reduced TEER of an *in vitro* BBB model generated by primary murine endothelial cells (Osada *et al.*, 2011). However, TEER was measured only after 0 h, 18 h, 24 h, 42 h and 48 h. Thus, short-term effects of the function-blocking antibody were not analysed. In addition, TEER was not monitored in real time as shown in this study. Hence, alterations in transendothelial resistance between two time points were not noticed. Another big difference of the study from Osada *et al.* is that the antibody was applied only one day after seeding the cells on permeable Transwell® filters. At this time, the cell layers had a TEER of only 20 to 25 $\Omega \times cm^2$, which do not represent a restrictive barrier. In contrast, endothelial monolayer in this work exhibited a transendothelial resistance of at least 150 $\Omega \times cm^2$ and antibodies were applied at day 10 after seeding, when the *in vitro* BBB was fully developed. Furthermore, the authors demonstrated that β1 integrin blockade also reduced the permeability of the BBB *in vitro* as well as *in vivo* and decreased the expression of claudin-5. Further evidence for the involvement of β1 integrins in endothelial permeability comes from a study that showed a dose- and time-dependent increase in protein permeability after application of RGD peptides and antibodies to α5β1 integrin (Curtis *et al.*, 1995). Additionally, Lampugnani and colleagues demonstrated a dose-dependent increase in the permeability of a monolayer of primary HUVEC after application of a goat serum to α5β1 integrin (Lampugnani *et al.*, 1991).

Accordingly, the β1 integrin-mediated endothelial cell adhesion is suggested to regulate intracellular signals maintaining the BBB integrity (Engelhardt, 2011). However, it is not known whether the antibodies, applied to the *in vitro* model, only bind to integrins on the luminal site of the endothelial cells or also influence the interactions between integrins on the abluminal site and the underlying matrix.

Taken together, this work and other studies present first evidence for the importance of β1 integrins, especially the α5β1 integrin, in maintaining the BBB integrity. In further investigations, permeability studies in the presence of function-blocking antibodies should be performed. Using immunofluorescence microscopy the influence of function-blocking antibodies directed against the β1, α5 and α6 integrin subunit should give insight whether these integrin subunits influence the morphology of endothelial cell monolayer as well as expression and localisation of tight junction proteins. Furthermore, knock-down of the β1, α5 and α6 integrin subunit using siRNA should clarify whether these integrins have an impact on the physical and morphological properties of the *in vitro* BBB.

## 4.7 Impact of endothelial β1 integrins on the transendothelial migration

Since the data obtained in this work indicate that β1 integrins influence the physical properties of the *in vitro* BBB model, it was evaluated whether they also play a role in endothelial transmigration. Blockade of endothelial β1 integrin subunit did not affect the transmigration of PBMCs, T lymphocytes and monocytes. However, function-blocking antibodies directed against the α5 integrin subunit revealed also a role in transmigration of PBMCs across endothelial cells. The reducing effect on transmigration was much stronger for monocytes than for T lymphocytes. Additionally, inhibition of the α6 subunit had a similar effect. Blockade of all investigated integrin subunits on endothelial cells, treated with IL-1β, reduced transmigration of PBMCs indicating that endothelial α5β1 integrin and α6β1 integrin participate in transendothelial migration during inflammation. These findings present a new view on the transendothelial migration of leukocytes because, to date, solely the interactions of leukocytic integrins with their endothelial ligands, such as E-selectin, ICAM-1, ICAM-2 and VCAM-1, are thought to mediate transendothelial migration.

However, further investigations have to be done. Transmigration of monocytes and T lymphocytes across inflamed endothelium, treated with function-blocking antibodies directed against the β1, α5 and α6 integrin, should be performed. Since the impact of β1 integrin blockade on resting endothelial cells was much stronger on monocytes than on T lymphocytes, it can be expected that neutralising antibodies will reduce the transmigration of monocytes to a greater extent. To clarify how β1 integrins facilitates the transendothelial migration, adhesion studies with PBMCs and PBMC subpopulations in the presence of function-blocking antibodies for β1 integrins should be performed under physiological and inflammatory conditions. As mentioned above a previous study showed that blockade of the

α5β1 integrin on endothelial cells abolished the adhesion of leukocytes to HUVECs treated with TNFα (Sun *et al.*, 2010). The authors suggested an α5β1:α5β1 integrin-dependent mechanism for adhesion of immune cells to activated endothelium. Accordingly, blockade of β1 integrins on leukocytes might have a similar effect on transmigration as observed for the blockade of endothelial β1 integrins. In addition, blocking of β1 integrins on both cell types, endothelial and immune cells, is expected to have a synergistically effect and abolish adhesion as well as transmigration almost completely. Further adhesion studies and transmigration assays should be done to confirm these suggestions.

In summary, the present work presents a novel THBMEC-based *in vitro* BBB model which fulfils the morphological, physical and functional requirements of brain endothelial cells. It was demonstrated that this system is a useful tool to investigate the role of β1 integrins during the inflammatory response at the BBB. The data presented indicate the involvement of endothelial β1 integrins in inflammatory processes at the BBB including integrin-mediated adhesion and matrix production. Furthermore, the proper function of β1 integrins on endothelial cells is important for maintaining the BBB integrity and transendothelial migration of immune cells.

## 4.8 Hypothesis for the role of the α5β1 integrin function during inflammation

The data obtained in this work led to the following hypothesis for the role of the α5β1 integrin during inflammation (figure 44).

Under physiological conditions, the barrier function of the BBB resulted from the formation of tight and adherens junctions. These junctional complexes mediate the cell-cell adhesion of adjacent endothelial cells and thereby contribute to the low paracellular permeability of the BBB (Hawkins and Davis, 2005). The components of the interendothelial junctions are connected to the cytoskeleton via adaptor proteins (figure 44A). They are involved in the organisation of F-actin cytoskeleton in cortical rings and influence the cell morphology (Wang *et al.*, 2006b; Fanning *et al.*, 2012). Furthermore, inactive α5β1 integrin co-localise with adherens junction complexes in cell-cell contacts (Sakamoto *et al.*, 2006; Lefort *et al.*, 2011). Moreover, it might be possible that inactive α5β1 integrin also co-localise with components of the tight junctions. Under these conditions, the inactive α5β1 integrin contribute to stabilise the BBB. Furthermore, α5β1 integrin in a low affinity state is found on the luminal site of

endothelial cells. Cell adhesion of endothelial cells to the surrounding ECM is mediated by active α5β1 and other integrins on the abluminal site of the cells (figure 44A).

During inflammation, pro-inflammatory cytokines induce increased expression of α5β1 and other integrins on endothelial cells. Furthermore, the level of fibronectin is enhanced under inflammatory conditions (Richards and Saba, 1983; Sobel and Mitchell, 1989). Binding of soluble fibronectin to α5β1 integrin result in the translocation of active α5β1 integrin to the abluminal site and the formation of cell-matrix contacts (figure 44B; Lefort *et al.*, 2011). Increased adhesion of endothelial cells to the matrix components lead to altered integrin signalling, which in turn cause dissociation of γ-catenin and α-catenin form VE-cadherin and result in adherens junctions disruption (Wang *et al.*, 2006b). Furthermore, inflammation is accompanied by decreased expression as well as internalisation of tight junction proteins, e.g. occludin and ZO-1 (figure 44B; Stamatovic *et al.*, 2009; Stamatovic *et al.*, 2012). Furthermore, disruption of the junctional complexes lead to reorganisation of the F-actin cytoskeleton and increased formation of F-actin stress fibres (Ferreira *et al.*, 2005; Wang *et al.*, 2006b). Due to these events, the stability of the blood-brain barrier is impaired, and the paracellular permeability is increased. Furthermore, ligation of α5β1 integrins to fibronectin facilitates the fibrillogenesis of fibronectin (figure 44B; Singh *et al.*, 2010; Lefort *et al.*, 2011).

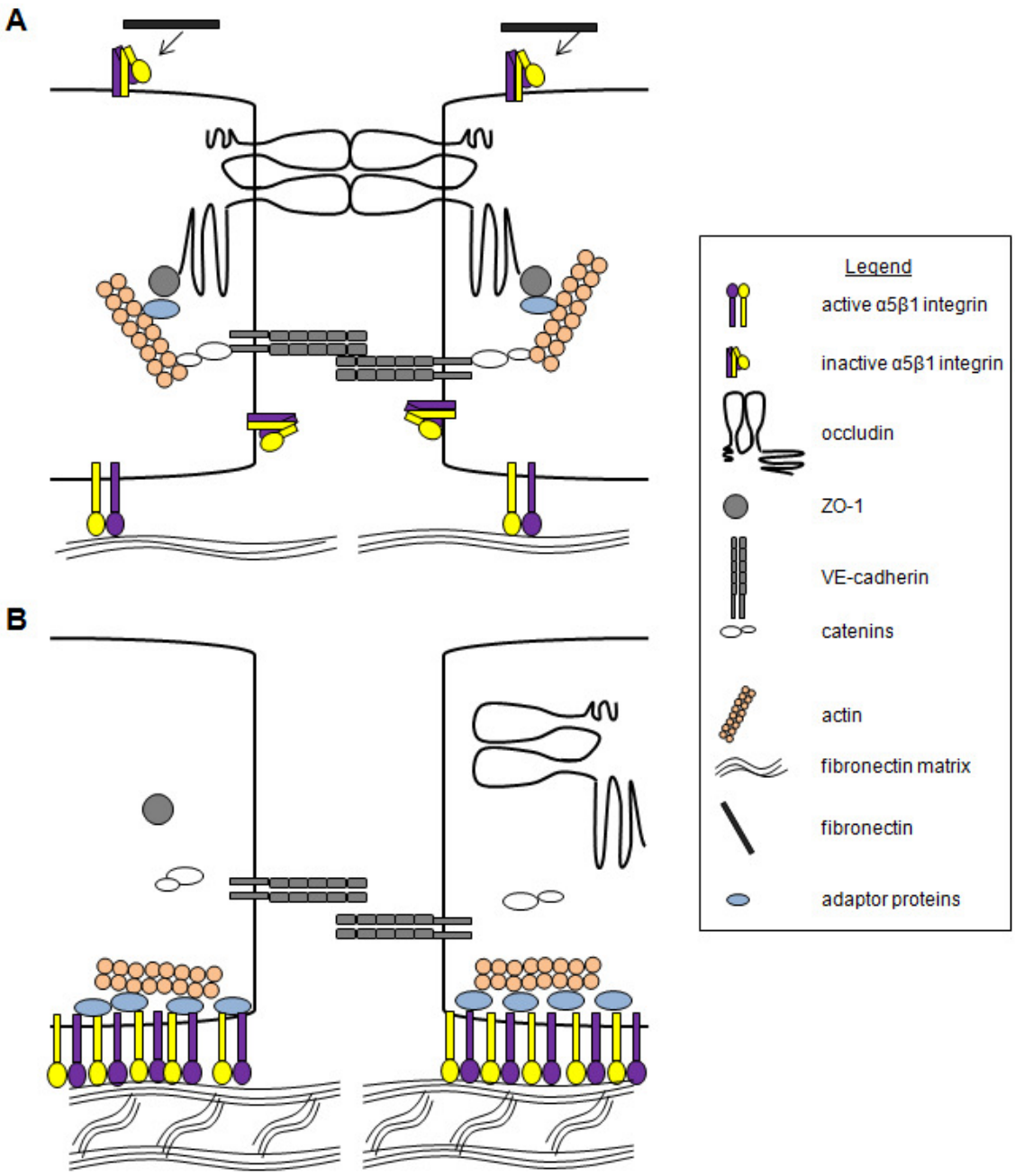


**Figure 44: Schematic representation of the hypothetic model for the role of α5β1 integrin during inflammation**

A) Under physiological conditions, tight and adherens junctions contribute to the stability of the BBB. Furthermore, inactive α5β1 integrin is expressed at cell-cell contacts and at the luminal membrane whereas active α5β1 integrin forms cell-matrix contacts at the abluminal membrane. During inflammation, expression of α5β1 integrin and fibronectin is increased. B) Ligation of inactive α5β1 integrin to fibronectin results in activation and translocation of the α5β1 integrin to the abluminal membrane and the formation of cell-matrix contacts (Lefort *et al.*, 2011). Furthermore, integrin activation is accompanied by disassembly of adherens junctions and the reorganisation of the F-actin cytoskeleton (Wang *et al.*, 2006b). Moreover, inflammation-induced decrease in expression of tight junction proteins additionally contribute to impaired integrity of the BBB. Moreover, ligation of α5β1 integrin to soluble fibronectin facilitates the fibrillogenesis of fibronectin (Singh *et al.*, 2010; Lefort *et al.*, 2011).

# Summary

The blood-brain barrier (BBB) is a specialised layer consisting of endothelial cells, pericytes and astrocytes as well as a basement membrane that restricts the diffusion for small hydrophilic solutes and macromolecules as well as the transmigration of leukocytes into the central nervous system. During inflammation, pro-inflammatory cytokines induce changes in the expression of adhesion proteins and matrix metalloproteinases as well as the composition of tight junctions resulting in BBB leakage and an increase in infiltrating leukocytes. The process of transendothelial migration is characterised by expression and function of leukocyte integrins. But less is known about the impact of integrins expressed on the endothelium during these processes.

The aim of this study was to investigate the role of endothelial β1 integrins at the BBB during inflammation. For this purpose, an appropriate *in vitro* BBB model, characterised by high electrical resistance and low permeability, was established. This model was extended to an inflammatory model by the application of suitable pro-inflammatory cytokines. The functionality of the model as well as the role of endothelial β1 integrins under inflammatory conditions was evaluated.

In the present work, a novel THBMEC-based *in vitro* BBB model was established which fulfils the morphological, physical and functional requirements of the BBB and presents a more restrictive barrier than the models that have been published so far for THBMEC and other endothelial cell lines. It was demonstrated that this system can be modified by pro-inflammatory cytokines. For the first time, it was shown that IL-1β and not TNFα induce the most striking inflammatory effects in these cells. Therefore, this system is a useful tool to investigate the role of β1 integrins during the inflammatory response at the BBB. The data presented indicate the involvement of endothelial β1 integrins in inflammatory processes at the BBB including integrin-mediated adhesion and production of proteins of the extracellular matrix, such as collagen IV and fibronectin. It was shown that β1 integrins are up-regulated and activated on endothelial cells under inflammatory conditions. Furthermore, immunofluorescence studies suggest that β1 integrins accumulate in cell-cell contacts during inflammation. Additionally, the application of function-blocking antibodies directed against β1 integrins revealed that the proper function of the α5β1 integrin on endothelial cells is important for maintaining the integrity of the BBB. Moreover, inhibition of α5β1 integrin and α6β1 integrin on endothelial cells reduce the transmigration of leukocytes - especially monocytes - across the *in vitro* model. Since this effect is enhanced under inflammatory conditions the data indicates that these receptors participate in the transendothelial migration of leukocytes during inflammation.

# Zusammenfassung

Die Blut-Hirn-Schranke ist eine spezialisierte Barriere, die aus Endothelzellen, Astrozyten und Perizyten sowie der Basalmembran besteht. Sie beschränkt die Diffusion kleiner hydrophiler Stoffe und Makromoleküle sowie die Transmigration von Leukozyten in das zentrale Nervensystem. Während einer Entzündung induzieren pro-inflammatorische Zytokine Veränderungen in der Expression von Adhäsionsmolekülen und Matrixmetalloproteinasen sowie in der Zusammensetzung von Tight Junctions. Das bewirkt das Durchlässigwerden der Blut-Hirn-Schranke und ermöglicht so das Eindringen von Leukozyten in das Hirnparenchym. Der Prozess der transendothelialen Migration geht einher mit der vermehrten Expression und Aktivierung von Leukozyten-Integrinen. Wenig ist allerdings darüber bekannt, ob auch Integrine, die auf dem Endothel exprimiert werden, an diesem Prozess beteiligt sind.

Das Ziel der Arbeit war es, die Rolle der endothelialen β1 Integrine an der Blut-Hirn-Schranke während der Entzündung zu untersuchen. Dazu sollte ein geeignetes *in vitro* Blut-Hirn-Schranken-Modell etabliert werden, welches sich durch einen hohen elektrischen Widerstand sowie eine geringe Permeabilität auszeichnet. Dieses Model sollte durch den Einsatz geeigneter pro-inflammatorischer Zytokine zu einem Entzündungsmodell erweitert werden. Die Funktionalität des Modelles als auch die Bedeutung der endothelialen β1 Integrine sollte unter entzündlichen Bedingungen untersucht werden.

In der vorliegenden Arbeit konnte mit Hilfe der Endothelzelllinie THBMEC ein neuartiges *in vitro* Blut-Hirn-Schranken-Modell etabliert werden, welches die morphologischen, physikalischen und funktionellen Anforderungen einer Blut-Hirn-Schranke erfüllt. Desweiteren zeichnet sich dieses Modell dadurch aus, dass es eine stärkere Barriere darstellt, als bisher für diese und andere Endothelzelllinien publiziert worden ist. Weiterhin lässt sich dieses System durch die Zugabe von pro-inflammatorischen Zytokinen beeinflussen. Es konnte zum ersten Mal gezeigt werden, dass die stärksten entzündlichen Effekte in dieser Endothelzelllinie durch IL-1β und nicht durch TNFα ausgelöst werden. Diese Ergebnisse demonstrieren, dass dieses *in vitro* Modell ein nützliches Hilfsmittel für die Untersuchung der Bedeutung der β1 Integrine während der Immunantwort an der Blut-Hirn-Schranke ist. Die in dieser Arbeit vorgestellten Ergebnisse deuten darauf hin, dass β1 Integrine, die auf Endothelzellen exprimiert werden, an entzündlichen Prozessen an der Blut-Hirn-Schranke beteiligt sind. Diese Prozesse beinhalten die Integrin-abhängige Zelladhäsion sowie die Synthese von Proteinen der Extrazellularen Matrix, wie Kollagen IV und Fibronektin. Darüber hinaus geht die Entzündung mit einer vermehrten Expression und Aktivierung von β1 Integrinen auf Endothelzellen einher. Außerdem gaben

Immunfluoreszenz-Studien Hinweise darauf, dass β1 Integrine unter entzündlichen Bedingungen in den Zell-Zell-Kontakten akkumulieren. In funktionelle Studien mit funktions-blockierenden Antikörpern gegen β1 Integrine konnte demonstriert werden, dass die korrekte Funktion des α5β1 Integrins wichtig für die Aufrechterhaltung der Integrität der Blut-Hirn-Schranke ist. Zusätzlich konnte gezeigt werden, dass die Blockade der Integrine α5β1 und α6β1 auf Endothelzellen die Transmigration von Leukozyten, besonders die von Monozyten, über das Blut-Hirn-Schranken-Modell verringert. Da dieser Effekt unter entzündlichen Bedingungen verstärkt ist, lässt sich schlussfolgern, dass diese Rezeptoren an der transendothelialen Migration von Leukozyten während der Entzündung beteiligt sind.

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# Appendix

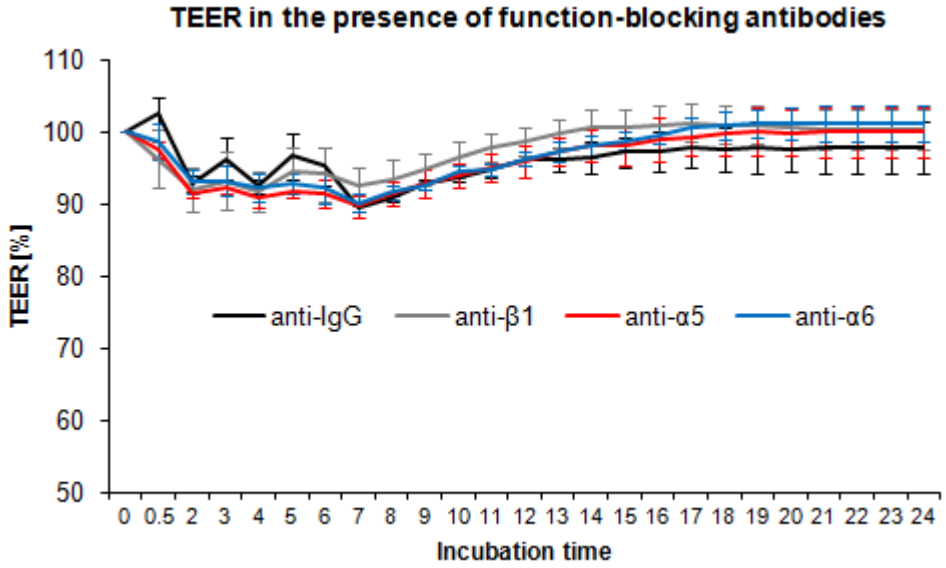


**Figure 45: Monitoring of the transendothelial resistance in the presence of integrin function-blocking antibodies**

Confluent THBMECs with a TEER of at least 150 Ω x $cm^2$ and higher were stimulated with 10 ng/ml of the respective function-blocking antibody in growth medium for 24 h. THBMECs incubated with anti-IgG served as negative control. TEER was monitored using the automatic cell monitoring system cellZscope® (nanoAnalytics GmbH, Münster). TEER at time point 0 h was set to 100% for each condition. Three independent experiments were summarised.

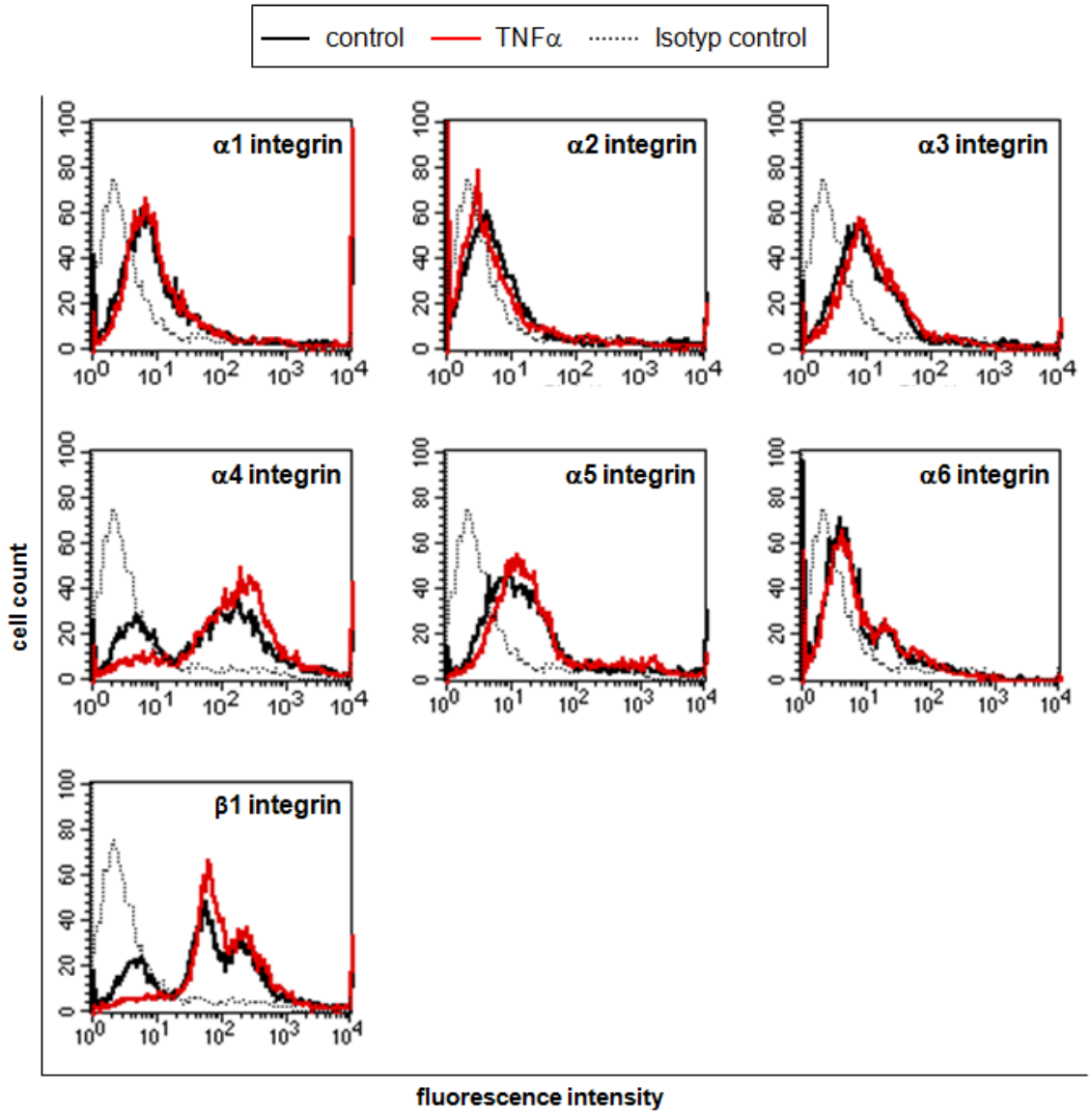


**Figure 46: Influence of TNFα on expression of integrin on leukocytes**

PBMCs were isolated from heparised whole blood and stimulated with 10 ng/ml TNFα in serum-free medium or left untreated (negative control) for 72 h. Cells in suspension and adherent cells were collected and analysed for integrin expression by flow cytometry using appropriate fluorophor-coupled antibodies. Cells incubated with an isotype control antibody served as negative control.

**Table 6: Primer for polymerase chain reaction**

bp: base pairs

| Name | Sequence | Fragment length |
|---|---|---|
| Collagen IV antisense | CATTGCCTTGCACGTAGAGC | 129 bp |
| Collagen IV sense | CAGGCACCCCATCTGTTGAT | |
| Fibronectin antisense | AGCTTCTTGTCCTACATTCGGC | 258 bp |
| Fibronectin sense | ACCCTTCCACACCCCAATCTTC | |
| GAPDH antisense | CATCACCATCTTCCAGGAGC | 756 bp |
| GAPDH sense | ACCACCCTGTTGCTGTAG | |
| ICAM-1 antisense | TGGCGGTTATAGAGGTACGTGC | 78 bp |
| ICAM-1 sense | GATTGTCATCATCACTGTGGTAGCA | |
| Integrin α1 antisense | GGCCCACAAGCCAGAAATCCTC | 288 bp |
| Integrin α1 sense | AATGACTTTCAGCGGCCCGGT | |
| Integrin α2 antisense | TCAAGGGCAGGGCTAGTGCCA | 155 bp |
| Integrin α2 sense | CACAGAGTTGCCCCGAGCACA | |
| Integrin α3 antisense | TGTAATCCTCGATGAAGGTGCTG | 88 bp |
| Integrin α3 sense | CTAGAGTGCCCCATCCCTGAT | |
| Integrin α5 antisense | ACTCCAGGAGCCGAGAGCCTTT | 257 bp |
| Integrin α5 sense | CAGGGTCGGGGGCTTCAACTTAGA | |
| Integrin α6 antisense | TGAACTCTTGAGGATAGCCC | 184 bp |
| Integrin α6 sense | CCTAACAGAATTGACCTCCG | |
| Integrin β1 antisense | TGTCATCTGGAGGGCAACCCTTCT | 254 bp |
| Integrin β1 sense | ACGCCGCGCGGAAAAGATGAAT | |
| Interleukin 1α antisense | CCAGACCTACGCCTGGTTTT | 154 bp |
| Interleukin 1α sense | CTTCTGGGAAACTCACGGCA | |

| Name | Sequence | Fragment length |
|---|---|---|
| Interleukin 1β antisense | GGAGCGAATGACAGAGGGTT | 113 bp |
| Interleukin 1β sense | TCCCCAGCCCTTTTGTTGAG | |
| Interleukin 6 antisense | GCAAGTCTCCTCATTGAATCC | 85 bp |
| Interleukin 6 sense | GGCACTGGCAGAAAACAACC | |
| Interleukin 8 antisense | CTCCACAACCCTCTGCAC | 197 bp |
| Interleukin 8 sense | TGCCAAGGAGTGCTAAAG | |
| Laminin β1 antisense | GAGACACCTCCCAGTCTCCT | 136 bp |
| Laminin β1 sense | CAGATGTGACGACTGTGCCT | |
| Occludin antisense | GTGTCGGCCTCCTCCCTCGGT | 246 bp |
| Occludin sense | TCAGACACCCCAAGGTTCCATCCGA | |
| TNFα antisense | GCTACAGGCTTGTCACTCGG | 81 bp |
| TNFα sense | CCCCAGGGACCTCTCTCTAATCA | |
| vWF antisense | GCAGCACCCCGGCAAATCTGG | 259 bp |
| vWF sense | AAAGGGAGGGTGGTTGGTGGATGTC | |
| ZO-1 antisense | GTGGGCTCCTCCAGTCTGACATT | 335 bp |
| ZO-1 sense | GAAGGCGGGGCCTACACTGAT | |

# List of Abbreviations

| | |
|---|---|
| ADMIDAS | metal ion dependent adhesion site |
| AM | acetomethoxy derivate |
| BBB | blood-brain barrier |
| Block | blocking |
| BMEC | brain microvascular endothelial cell |
| bp | base pairs |
| CD | cluster of differentiation |
| CNS | central nervous system |
| ECM | extracellular matrix |
| FC | flow cytometry |
| GAPDH | glyceraldehyde 3-phosphate dehydrogenase |
| GFAP | glial fibrillary acidic protein |
| hCMEC/D3 | human cerebral microvascular endothelial cell line |
| HUVEC | human umbilical vein endothelial cells |
| ICAM-1 | intracellular cell adhesion molecule-1 |
| IF | immunofluorescence microscopy |
| IFN | interferon |
| Ig | immunoglobulin |
| IL | interleukin |
| JAM | junctional adhesion molecule |
| LIMBS | ligand induced metal binding site |
| MACS | magnetic cell separation |
| MAGUK | membrane associated guanylate kinases |
| MAP kinase | mitogen-activated protein kinase |
| MHC | major histocompatibility complex |
| MIDAS | metal ion dependent adhesion site |
| MMP | matrix metalloproteinase |

| | |
|---|---|
| PBS | phosphate-buffered saline |
| PECAM | platelet endothelial cell adhesion molecule |
| PIC | protease inhibitor cocktail |
| POD | peroxidase |
| Src kinase | Sarcoma kinase |
| SV40-LT | simian virus 40 large T antigen |
| TAE | tris-acetate-EDTA |
| TBS | tris-buffered saline |
| TEER | transendothelial electrical resistance |
| TEM | transendothelial migration buffer |
| THBMECs | transformed human brain endothelial cells |
| TNFα | tumour necrosis factor α |
| VCAM-1 | vascular cell adhesion molecule-1 |
| VE-cadherin | vascular endothelial cadherin |
| VVO | vesiculo-vacuolar organelles |
| vWF | von Willebrand factor |
| WB | western blotting |
| ZO | *Zona occludens* |

# Publications

## Original articles

Labus J, Häckel S, Danker K

IL-1β induces an inflammatory response in an improved human THBMEC-based *in vitro* blood-brain barrier model and breakdown of the endothelial cell layer, submitted to Journal of Neuroscience Methods

Labus J, Woeltje K, Häckel S, Hildmann A, Danker K

The endothelial α5β1 integrin is involved in blood-brain barrier integrity and transendothelial migration, in preparation

Labus J, Tang K, Woeltje K, Krüger U, Hondke S, Semini G, Lucka L, Danker K

Membrane proximal lysines of the α1 Integrin cytoplasmic tail are important for activity regulation of the α1β1 integrin, in preparation

## Posters

Labus J, Häckel S and Danker K

"The role of β1 integrins in a novel *in vitro* blood-brain barrier model", Fifteenth International Symposium on signaltransduction in the blood-brain barriers, Potsdam, 13.09.-16.09.2012

# Curriculum vitae

For reasons of data protection, the curriculum vitae is not included in the online version.

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# Statement of the authorship

I declare that my PhD thesis “The role of β1 integrins I a novel *in vitro* blood-brain barrier model during inflammation” has been written independently and with no other sources and aids then quoted.

Josephine Labus