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Inhibition of Inflammatory Vasculopathic Processes by Interleukin-1 Receptor Antagonist - transduced Endothelial Progenitor Cells

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Vorwort

Die Regeneration des geschädigten Blutgefäßsystems durch die Transplantation körpereigener Stammzellen ist eine Zielvorstellung in der Therapie entzündlicher, atherosklerotisch bedingter und diabetischer Gefäßkrankheiten. Durch genetische Veränderung sollen geeignete Stammzellen den individuellen durch die jeweilige Erkrankung hervorgerufenen Bedürfnissen des Organismus funktionell angepasst und somit deren therapeutischer Effekt optimiert werden.

In Zukunft könnte eine Therapie dieser Art Wirklichkeit werden. Die vorliegende Arbeit soll dazu beitragen, diesem Ziel näher zu kommen.

Abstract

Human umbilical cord blood constitutes a rich source of endothelial progenitor cells (EPC) capable of differentiation into functional mature endothelial cells. The endothelium is a key modulator in the pathogenesis of inflammatory vasculopathies such as atherosclerosis. Specifically, Interleukin-1 (IL-1) plays a major role in the proinflammatory activation of the endothelium and the propagation of the vascular inflammatory process. Aim of this study was to examine the potential of Interleukin-1 receptor antagonist (IL-1ra) - transduced cord blood-derived endothelial cells (CBEC) to inhibit the vascular inflammatory aspects involved in the pathogenesis of atherosclerosis.

For this purpose, cord blood-derived $CD34^+$ EPC were differentiated into CBEC and retrovirally transduced with the constructs pLXSN and pLXSN-icIL-1ra, respectively, and expanded up to 10^{20} cells during in vitro culture. Transgene expression of the intracellular isoform of IL-1ra was confirmed by RT-PCR and Western Blot and quantified by ELISA throughout the expansion period, demonstrating that transduced cells showed a stable expression of IL-1ra over at least 4 months of culture.

In the following, the effect of transgenic icIL-1ra expression on distinct processes involved in inflammatory vasculopathies was investigated. CBEC gene expression analysis (real time RT-PCR) revealed that steady state levels of the adhesion receptor ICAM-1 and the proinflammatory mediators MCP-1 and thrombin receptor were significantly reduced in transgenic CBEC. Endothelial cell activation induced by monocytes, IL-1ß and TNFα stimulation was assessed by flow cytometry and real time RT-PCR. We observed that monocyte-induced upregulation of ICAM-1, VCAM-1, and tissue factor was significantly inhibited in icIL-1ra-transduced CBEC. Furthermore, transgenic CBEC showed a decreased proinflammatory activation profile upon cytokine stimulation. Interestingly, the ectopically expressed icIL-1ra did not only reduce IL-1ß-induced CBEC activation, but also influenced TNF-induced stimulation. In particular, TNF-induced ICAM-1 expression was reduced by icIL-1ra. Functionally, as assessed by flow chamber measurements and adhesion assays, expression of the icIL-1ra transgene reduced leukocyte rolling and adhesion to resting CBEC as well as IL-1ß stimulated CBEC. Furthermore, transendothelial migration of monocytes through transgenic CBEC was markedly diminished as determined in an in vitro transmigration chamber.

Our findings demonstrate that CBEC can reliably be isolated, transduced, and expanded into clinically relevant numbers. CBEC stably expressing icIL-1ra show an enforced protection against activation by proinflammatory stimuli and display a significantly reduced response in the decisive pathomechanisms of inflammatory vasculopathies - rolling, adhesion, and transmigration of leukocytes. Therefore, icIL-ra transgenic CBEC show potential to be used in the treatment of IL-1ß- as well as TNFα-mediated inflammatory vasculopathies.

Keywords: Cord Blood - Endothelial Progenitor Cells - IL-1ra - Anti-inflammatory Therapy

Zusammenfassung

Menschliches Nabelschnurblut enthält eine große Anzahl Endothelvorläuferzellen (EPC), die in reife, funktionstüchtige Endothelzellen differenzieren können. Das Endothel nimmt eine Schlüsselposition in der Pathogenese entzündlicher Gefäßkrankheiten wie der Atherosklerose ein. Insbesondere das Zytokin Interleukin-1 (IL-1) spielt eine wichtige Rolle in der Aktivierung des Endothels und der Vermittlung entzündlicher Prozesse in der Gefäßwand. Ziel der vorliegenden Untersuchung war es, aus Stammzellen des Nabelschnurbluts Endothelzellen (CBEC) zu differenzieren, sie mit dem Transgen Interleukin-1 Rezeptorantagonist (IL-1ra) genetisch zu modifizieren und ihr Potential zu ermitteln, gefäßentzündliche Aspekte in der Pathogenese der Atherosklerose zu hemmen.

Dafür wurden $CD34^+$ EPC aus Nabelschnurblut in CBEC differenziert, mit den retroviralen Konstrukten pLXSN und pLXSN-IL-1ra transduziert und auf über 10^{20} Zellen expandiert. Die transgene Expression der intrazellulären Isoform des IL-1ra wurde mittels RT-PCR und Western Blot nachgewiesen und durch ELISA während des gesamten Expansionszeitraums quantifiziert. Dabei konnte eine stabile Expression von IL-1ra über mindestens 4 Monate der in vitro Kultur nachgewiesen werden. Nachfolgend wurde der Effekt der transgenen Expression von icIL-1ra in CBEC auf für entzündliche Gefäßkrankheiten charakteristische Prozesse untersucht. Genexpressionsanalysen (real time RT-PCR) ergaben, dass transgene CBEC signifikant geringere basale Level des Adhäsionsrezeptors ICAM-1 und der proinflammatorischen Mediatoren MCP-1 und Thrombinrezeptor aufwiesen. Durch Monozyten, IL-1ß und TNFα (tumor necrosis factor alpha) induzierte endotheliale Aktivierung wurde mittels Flusszytometrie und real time PCR untersucht. Dabei stellte sich eine signifikante Reduktion der monozyteninduzierten Expression von ICAM-1, VCAM-1 und Tissue Factor in icIL-1ra-transduzierten CBEC heraus. Desweiteren ergab die Analyse ein vermindertes proinflammatorisches Aktivierungsmuster transgener CBEC nach Zytokinstimulation. Der ektopisch exprimierte icIL-1ra hemmte dabei nicht nur IL-1ß-induzierte, sondern auch TNFα-mediierte endotheliale Stimulation. So wurde die TNFα-induzierte ICAM-1 Expression durch icIL-1ra vermindert. Funktionelle Untersuchungen in Fließkammermessungen und Adhäsionsexperimenten ergaben, dass die Produktion von icIL-1ra sowohl das Rolling als auch die feste Adhäsion von Leukozyten auf nicht aktivierten und IL-1ß-stimulierten CBEC signifikant abschwächte. Weiterhin war die transendotheliale Migration von Monozyten durch transgene CBEC stark vermindert, wie Messungen in Transmigrationskammern zeigten.

Zusammengefasst zeigen die Ergebnisse dieser Studie, dass CBEC zuverlässig isoliert, transduziert und in klinisch relevante Mengen expandiert werden können. CBEC mit stabiler Expression des icIL-1ra Transgens haben einen erhöhten Schutz gegen die Aktivierung durch proinflammatorische Stimuli und weisen eine verminderte Reaktion in den für entzündliche Gefäßerkrankungen entscheidenden Pathomechanismen - Rolling, Adhäsion und Transmigration von Leukozyten - auf. Somit zeigen icIL-1ra-transduzierte CBEC das Potential, in der Therapie entzündlicher Gefäßkrankheiten eingesetzt zu werden.

Schlagwörter: Nabelschnurblut - Endothelvorläuferzellen - IL-1ra - Entzündungshemmende Therapie

Content

Abbreviations

(m)RNA	(messenger) Ribonucleic acid
ApoE	Apoenzyme E
BM	Bone Marrow
BSA	Bovine Serum Albumine
CBEC	Cord blood derived Endothelial Cell
CD	Cluster of Differentiation
CFU-EC	Endothelial Cell - Colony Forming Units
COX-2	Cyclooxygenase type 2
DMEM	Dulbecco`s modified Eagle Medium
DNA	Desoxyribonucleic acid
EBM-2	Endothelial Cell Basal Medium 2
EGF	Epidermal Growth Factor
ELAM-1	Endothelial-Leukocyte Adhesion Molecule 1
ELISA	Enzyme Linked ImmunoSorbent Assay
EMAP-2	Endothelial-Monocyte Activating Protein 2
EPC	Endothelial Progenitor Cell
FACS	Fluorescence Activated Cell Sorting
FCS	Fetal Calf Serum
FGF	Fibroblast Growth Factor
FITC	Fluoresceinisothiocyanate
GAPDH	Glyceraldehydphosphate Dehydrogenase
GM-CSF	Granulocyte Macrophage - Colony Stimulating Factor
HBSS	Hank's Balanced Salt Solution
HCAEC	Human Coronary Artery Endothelial Cells
HUVEC	Human Umbilical Vein Endothelial Cells
ICAM-1	Intracellular Adhesion Molecule 1
ICE	Interleukin-1 Converting Enzyme
IGF-1	Insulin-like Growth Factor 1
IL-1	Interleukin-1
IL-1ra	Interleukin-1 receptor antagonist
IL1R-AP	Interleukin-1 receptor accessory protein
kDa	kilo Dalton
LDL	Low density Lipoprotein
LPS	Lipopolysaccharides
MAPK	Mitogen Activated Protein Kinase
MCP-1	Monocyte Chemoattractant Protein 1
MIP-1α	MacrophageInflammatory Protein 1α

NF-κB	Nuclear Factor κB
NO	Nitric Oxide
NOS-3	Nitric Oxide Synthase 3
PAF	Platelet Activating Factor
PAI	Plasminogen Activator Inhibitor
PBGD	Porphobilinogendesaminase
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate-buffered Saline
PDGF	Platelet-derived GrowthFactor
PE	Phycoerythrin
PECAM-1	Platelet-Endothelial Cell Adhesion Molecule
PGI_2	Prostaglandin I_2
PI	Propidiumiodide
PSGL-1	P-Selectin Glycoprotein Ligand 1
RT	Room temperature
RT-PCR	Real-time – Polymerase Chain Reaction
SCF	Stem Cell Factor
SCGF-ß	Stem Cell Growth Factor β
SMC	Smooth muscle cell
TF	Tissue Factor
TFPI	Tissue Factor Pathway Inhibitor
TGF-ß	Tumour Growth Factor β
TNF-α	Tumour Necrosis Factor α
tPA	Tissue Plasminogen Activator
UEA-1	Ulex Europeus Agglutinin 1
VCAM-1	Vascular Cell Adhesion Molecule 1
VE-Cadherin	Vascular Endothelial Cadherin
VEGF	Vascular Endothelial Growth Factor
vWF	Von Willebrand Factor

1 Introduction

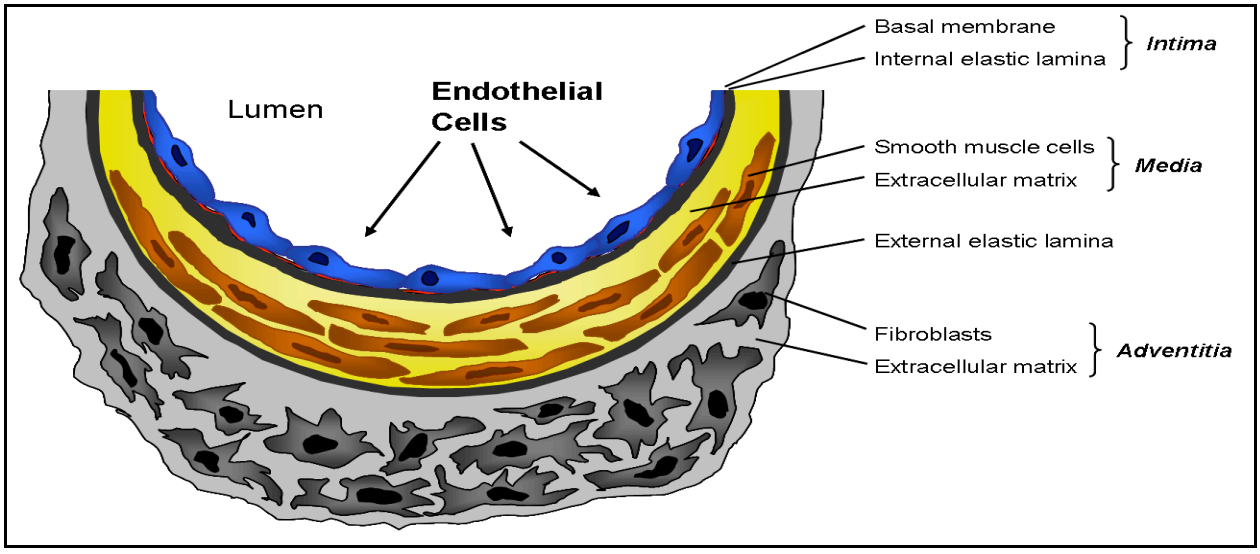
1.1 General introduction

Physiologic vascular homeostasis - the interaction between the endothelium, blood cells and blood plasma - is a prerequisite for undisturbed functioning of the circulatory system. It regulates blood flow and -distribution and thereby ascertains organ and tissue oxygenation adjusted to the requirements. Furthermore, these vascular interactions are involved in local hemostasis, wound healing and repair of vascular injuries.

The endothelium constitutes the interface of the vasculature to the blood stream **(scheme 1)**. As such, it is a main regulator of vascular inflammation, coagulation, fibrinolysis and vascular tone and thereby holds an outstanding position in vascular homeostasis. Vascular repair at sites of acute or chronic injury and renewal of senescent malfunctioning endothelial cells is an indispensable precondition for this physiologic function of the endothelium in the circulatory system.

Endothelial dysfunction and insufficient self-renewal and repair of the endothelium in response to pathologic influences are central to the pathogenesis of inflammatory vasculopathies such as atherosclerosis, neointimahyperplasia, and tissue ischemia. Interleukin-1 - a strong proinflammatory cytokine locally released at sites of vascular inflammation - is a main initiator of endothelial dysfunction and strongly promotes the inflammatory process at the vessel wall.

This introduction aims to characterize endothelial function and the role of endothelial progenitor cells in endothelial regeneration. Furthermore, endothelial dysfunction and the ensuing vascular inflammatory process - hallmarks of inflammatory vasculopathies - are described. Lastly, the predominant role of Interleukin-1 in vascular inflammation and the ability of Interleukin-1 receptor antagonist to inhibit the IL-1-induced processes are elucidated.



Scheme 1 Cross section of a normal blood vessel

1.2 Endothelium

The endothelium is an essential component of the vascular wall. This single-cell-thick lining of the cardiovascular system with a thickness of 1µm comprises about 10^{13} endothelial cells and covers approximately 7 m^2 in an average adult (Cines et al., 1998).

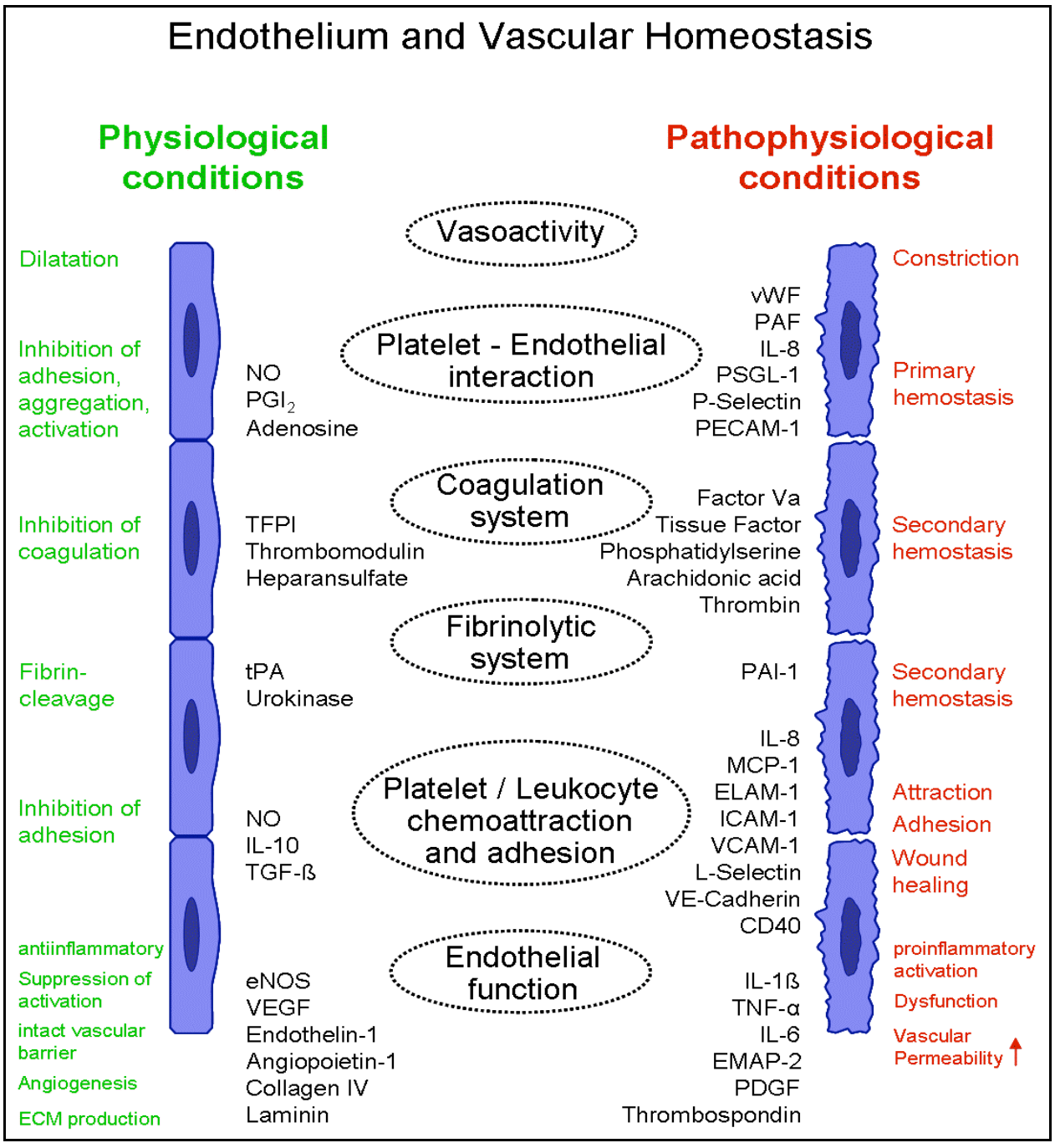
As innermost stratum of all blood vessels endothelial cells provide the physical interface between the blood stream and surrounding tissue **(scheme 1)**. Situated like this, the endothelial layer acts as a selective semipermeable membrane and actively regulates nutrient and blood component traffic. Equipped with various receptors for cytokines (e.g. IL-1) and growth factors (e.g. FGF, VEGF, Insulin) the endothelium receives signals from the blood stream and converts them into specific reactions (Roesen, 2002). Furthermore, it reacts to mechanic stimuli such as shear stress and wall tension caused by the pulsatile blood stream in order to adapt the vascular tone to physiologic requirements. As a regulator of coagulation, fibrinolysis and adhesivity of the vessel wall it plays a major role in many physiologic and pathologic processes such as hemostasis, angiogenesis and inflammation (Lin et al., 2000; Rodgers, 1988).

Physiologically, the endothelium holds an antiinflammatory and anticoagulatory function and presents a non-thrombogenic surface to the blood stream which prevents adhesion and aggregation of circulating blood cells at the vessel wall **(scheme 2).** This is the result of the preclusion of intravascular thrombin formation by expression of tissue factor pathway inhibitor (TFPI), activation of antithrombin III by heparansulfate-proteoglycanes of the endothelial surface and activation of protein C via thrombomodulin (Bertina, 1997; Boehme et al., 1996; Boehme et al., 2000; Bombeli et al., 1997; Esmon, 1995; Esmon and Fukudome, 1995). Thrombomodulin additionally inhibits fibrinogen clotting, platelet and endothelial cell activation and factor V activation and thereby holds important anticoagulative capacity in intact endothelium (Esmon, 2001). Endothelial cells additionally exert fibrinolytic effects by preparation of tissue plasminogen activator (tPA) and urokinase **(scheme 2)**. Furthermore, endothelium mediates vascular dilatation and prevents platelet adhesion by production of nitric oxide (NO), Prostacyclin (PGI_2) and adenosine (Busse and Fleming, 1998; Gerlach, 1987). Endothelial cells prevent vascular inflammation by releasing NO (nitric oxide, endothelium derived relaxing factor), IL-10 and TGF-β which attenuate adhesion and subsequent transmigration of leukocytes and monocytes and prevent local proliferation of these cells (Kupatt et al., 1996).

These multifarious endothelial functions are of great importance for the maintenance of a physiologic blood stream, inhibition of inflammatory processes and both regulation and limitation of local as well as systemic coagulation. In this framework, the endothelium influences the interacting components of primary and secondary hemostasis: the vessel wall (smooth muscle cells and extracellular matrix), the blood cells (platelets granulocytes, monocytes and leukocytes) and the clotting and fibrinolytic system of the plasma **(scheme 2)**.

Indispensable prerequisite for physiologic vascular homeostasis - which eventually guarantees appropriate organ/tissue blood supply - is the structural and functional integrity of the endothelium. Chronic or disproportionate damage either functionally or structurally disrupts endothelial integrity in such a way that the antiinflammatory and anticoagulatory versus proinflammatory and procoagulatory balance is dysregulated (Becker et al., 2000).

Furthermore, renewal of the endothelial monolayer with replacement of senescent or damaged endothelial cells at sites of vascular injury (mechanic, ischemic or toxic) plays another integral part in the maintenance of physiologic endothelial function in vascular homeostasis as well as for ensuring structural integrity of the vessel wall.



Scheme 2 The endothelium is a key regulator of vascular homeostasis

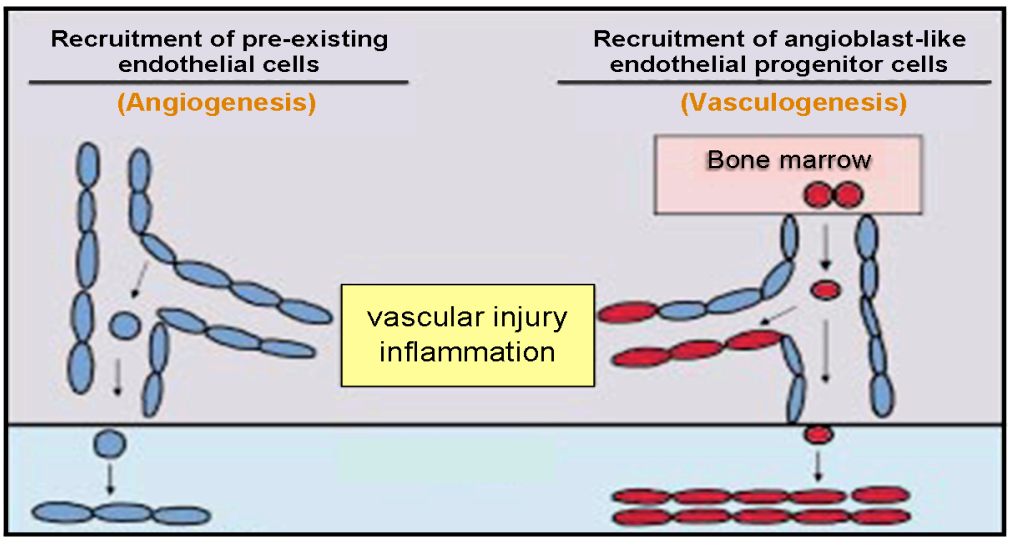
Two states of the endothelium can be differentiated in vascular homeostasis: Antiinflammatory, antithrombotic endothelium versus endothelium with a proinflammatory, prothrombotic nature. Physiologically, the endothelium supports vasodilation, suppresses adhesion and activation of circulating blood cells, inhibits the coagulation cascade, promotes fibrinolysis, mediates angiogenesis and modulates ECM composition. Dysfunctional endothelium enhances vasoconstriction, promotes platelet/leukocyte adhesion, mediates thrombin formation and fibrin deposition. Additionally, it releases proinflammatory mediators into the blood stream and increases vascular permeability. For abbreviations see page 8 / 9.

1.3 Endothelial Progenitor Cells

1.3.1 Characterization and Isolation

Physiological and pathophysiological vascular processes such as vascular injury, wound healing and ischemic tissue damage necessitate repair and development of blood vessels in order to maintain tissue perfusion required for physiologic organ function. Migration, proliferation and stabilisation of intact endothelial cells on the sites of vascular injury are prerequisites for vascular regeneration.

Currently, two different mechanisms of (re-) endothelialisation and neoangiogenesis have been described: The first one operates by in situ proliferation, migration and sprouting of resident adjacent mature vascular wall endothelial cells and is named angiogenesis (Carmeliet, 2000; Carmeliet, 2003; Isner and Asahara, 1999). It occurs predominantly in minor scale vascular regeneration due to the limited life span of mature endothelial cells and their inability to migrate into remote target sites. The second mechanism consists in recruitment of bone marrow-derived endothelial progenitor cells (EPC) from the circulation, their maturation and incorporation into the vessel wall **(scheme 3)** (Caprioli et al., 1998). The latter process is referred to as vasculogenesis (Risau and Flamme, 1995) and was thought to be restricted to early embryogenesis for a long time (Rafii, 2000). In the last years evidence has accumulated that EPC circulate in adult peripheral blood and contribute to a large part to postnatal neovascularization at sites of vascular damage/repair (Masuda and Asahara, 2003). This process takes place in more extensive vascular regenerative processes, activated by cytokines and growth factors derived from the site of neovascularization. Hence, both angiogenesis and vasculogenesis are involved in maintenance and repair of the adult vascular endothelium (Crosby et al., 2000; Murayama et al., 2002; Shi et al., 1998).



Scheme 3 Models of postnatal angiogenesis and vasculogenesis

Recruitment of endothelial cells from pre-existing vessel wall or circulating endothelial progenitor cells (CEPs) plays a critical role in the regulation of postnatal angiogenesis and vasculogenesis. Mobilized BM-derived CEPs with high proliferative capacity may have the potential to home and incorporate into the injured tissue or inflamed vascular bed (modified from Rafii, 2000).

Endothelial progenitor cells (EPC) can be isolated from peripheral blood (Asahara et al., 1997; Lin et al., 2000), cord blood (Murohara, 2001; Nieda et al., 1997), and bone marrow (Lin et al., 2000; Shi et al., 1998). EPC and mature vascular endothelial cells are characterized by the expression of markers including VEGF-R2 (KDR, Flk-1) (Eichmann et al., 1997), stem cell factor (SCF) receptor c-kit, Tie-1, Tie-2 (Sato et al., 1993; Sato et al., 1995), VE-cadherin (Vittet et al., 1996), CD31, CD34, CD146 and vWF (Ziegler et al., 1999). In addition, they are characterized by their ability to take up acetylated LDL and to bind the Ulex europeus agglutinin (Rafii and Lyden, 2003). This similarity of surface marker pattern causes some difficulties in the delimitation of endothelial cells from their progenitors. Isolation of EPC is additionally complicated since many of these markers are not only expressed by EPC and mature endothelial cells but also by subsets of haematopoietic cells. Indeed, recent studies uncovered that the majority of cells defined by uptake of acetylated LDL and the ability to bind UEA-1 are, apparently, of myelocytoid origin (Gulati et al., 2003; Gulati et al., 2004; Rehman et al., 2003) and that $CD14^{+}$ $CD34^{low}$ cells represent the major source of circulating endothelial progenitors (Romagnani et al., 2005). Moreover, CD14+ monocytes can be induced to an EC-like phenotype in culture (Fernandez Pujol et al., 2000; Schmeisser et al., 2003). The reason for this phenotypic overlap between haematopoietic and endothelial cells is their origin from the same precursor, the hemangioblast (Choi et al., 1998; Schmeisser and Strasser, 2002).

Only AC133, CD34, and KDR have, so far, been identified to distinguish between mature endothelial cells and their progenitors. As AC133+CD34+VEGF-R2+ cells account for less than 0.01% of peripheral blood (PB), bone marrow (BM) or cord blood (CB) mononuclear cells (Peichev et al., 2000) optimized in vitro expansion of endothelial progenitor cells is an indispensable prerequisite prior to research and clinical application for neovascularization.

Among the named sources, cord blood contains the largest number of progenitor cells (10-fold more than peripheral blood) with a more rapid outgrowth of EPC (Murohara et al., 2000). Moreover, cord blood-derived endothelial progenitor cells have distinctive proliferative advantages such as longer telomeres, a higher cell cycle rate and self-renewal potential compared to progenitors obtained from the other sources (Broxmeyer et al., 1989; Mayani and Lansdorp, 1994; Vaziri et al., 1994). That is why cord blood represents a reliable and high-yield clinically relevant source of EPC.

1.3.2 Mobilization of Endothelial Progenitor Cells

To achieve neovasculogenesis, mobilization of EPC from distant sources has to be initiated. Endogenous mobilization of endothelial progenitor cells from the bone marrow into circulation is induced after vascular injury (Gill et al., 2001) and states of peripheral ischemia (Takahashi et al., 1999).

Moreover, elevated levels of EPC have been detected in response to ischemic events such as myocardial infarction (Massa et al., 2005; Shintani et al., 2001), and acute vascular trauma secondary to burns (Gill et al., 2001). Recruitment of EPC from the bone marrow to sites of neovascularization is associated with increased concentrations of soluble mediators such as VEGF (Shintani et al., 2001), GM-CSF (Takahashi et al., 1999), SDF-1 (Hattori et al., 2001; Kim and Broxmeyer, 1998; Lapidot and Kollet, 2002), angiopoietin (Moore et al., 2001), SCF (Donahue et al., 1996), erythropoietin (Heeschen et al., 2003) and estrogen (Strehlow et al., 2003). In addition, activation of metalloproteinases (Heissig et al.,

2002; Heissig et al., 2003) and adhesion molecules (Hattori et al., 2002; Lapidot and Petit, 2002) are involved in the release of bone marrow-derived EPC. Furthermore, administration of HMG-CoA reductase inhibitors (statins) was found to increase circulating EPC (Dimmeler et al., 2001; Vasa et al., 2001a; Walter et al., 2002).

The exact kinetics of endothelial progenitor mobilization and homing to sites of neovascularization remain to be pinpointed. But, it emerged that immigration of EC-like cells into damaged/ischemic stromal tissues follows closely behind the extravasation of monocytes/macrophages within the inflammatory vascular response (Dvorak, 1986) suggesting an intimate communication between hematopoietic proangiogenic cells and EPC. This is consistent with the highest levels of circulating progenitors and VEGF in peripheral blood occurring around day 7 after an acute ischemic event (Rivard et al., 1999; Shintani et al., 2001).

1.3.3 Endothelial Progenitor Cells in inflammatory vascular disorders

Decreased levels of circulating EPC and dysfunction of mature endothelium have been observed in several pathologic conditions in humans. In inflammatory vascular disorders such as atherosclerosis (Shimokawa, 1999), normal endothelial cell function and regeneration is maintained not only through angiogenesis but also by vasculogenesis involving EPC recruited from the bone marrow (Rauscher et al., 2003; Ross, 1993). Physiologically, there exists an age-related failure of mature endothelial cell function (e.g. proliferation, migration, NO release and vasodilatation) and a progressive phenotype switch of endothelial cells from anti- towards proinflammatory and proatherosclerotic properties in the course of human lifetime (Gerhard et al., 1996; Hadziselimovic, 1981; Minamino et al., 2002; Rivard et al., 1999). These changes in endothelial cell phenotype are central to the pathogenesis of atherosclerotic lesions in the vasculature.

However, it is not exclusively the mature endothelium of the vascular intima that undergoes age-related reduction of its physiological functions. As recently reported, aging equally causes a decrease in survival, proliferation and migration properties of endothelial progenitor cells with an impairment of endothelial repair and vasculogenesis (Heiss et al., 2005). Indeed, atherosclerosis appears to be partly a result of age-related declines in the production of endothelial progenitors, insufficient recruitment to sites of vascular inflammation and their reduced capabilities to replace dysfunctional senescent and apoptotic endothelium and repair vascular damages (Choy et al., 2001; Dimmeler et al., 1998; Dimmeler and Vasa-Nicotera, 2003; Guevara et al., 2001; Kravchenko et al., 2005; Murayama et al., 2001; Rauscher et al., 2003). Constituting a disequilibrium between vascular injury and vascular repair this pathogenetic process likewise seems to form the basis of the impact of additional risk factors for accelerated progression of atherosclerosis and further inflammatory vascular diseases (Goldschmidt-Clermont, 2003; Goldschmidt-Clermont et al., 2005).

Indeed, EPC levels are also reduced in the presence of several risk factors for coronary artery disease (CAD) (Vasa et al., 2001b), endothelial dysfunction (Hill et al., 2003), hypercholesterolemia (Chen et al., 2004), smoking (Kondo et al., 2004; Wang et al., 2004) and chronic renal failure (Choi et al., 2004). Moreover, an accelerated senescence and reduced telomerase activity of endothelial progenitor cells could also be proven in subjects with chronic essential hypertension (Imanishi et al., 2005). Furthermore, number of circulating EPC was found to be reduced by 40% in type II diabetes mellitus patients and severity of peripheral vascular disease (caused by impaired

collateralization of vascular ischemic beds) was shown to be negatively correlated with peripheral levels of progenitor cells (Fadini et al., 2005).

Obviously, inflammatory and thrombotic processes in atherosclerotic lesions are equally in part sustained by an insufficient substitution of senescent, malfunctioning endothelium of the vessel wall by bone marrow-derived EPC. These assumptions were confirmed by the finding that in mouse models of atherosclerosis the content of EPC in bone marrow was significantly decreased in aging animals with an advanced state of atherosclerosis. Most strikingly, transplantation of bone marrow cells of young healthy animals into older, atherosclerosis-prone ApoE-deficient mice engrafted in areas at risk for atherosclerotic injury, differentiated into endothelial cells and in this way significantly diminished lipid deposition in those regions. This replacement of senescent arterial intimal cells by younger endothelial cells had an atheroprotective effect in prediseased animals (Rauscher et al., 2003). These observations are supported by two further studies, in which systemic administration and cytokine-induced mobilisation of endothelial progenitor cells resulted in diminished neointima formation after mechanical vascular injury in mice (Griese et al., 2003b; Werner et al., 2003). Remarkably, reendothelialization of the denuded vessels by mobilized endothelial progenitors was accompanied by a decrease of inflammation in the vessel wall (Kong et al., 2004a). Vice versa, it seems conceivable that inadequate endothelial repair due to exhaustion of endothelial progenitor supply or functional impairment of EPC might lead to progressive intensification of the inflammatory signals in the vasculature (Dong et al., 2005).

Hence, the finding that endothelial progenitor cells home to foci of angiogenesis and successfully promote neovascularization suggests potential utility for cell-mediated regeneration of endothelial dysfunction, vascular inflammation, and ischemic tissues.

1.4 Endothelial dysfunction in inflammatory vasculopathies

1.4.1 General information

Disturbances of vascular homeostasis are the main factor in the pathogenesis of inflammatory vasculopathies. Endothelial cells of the vascular intima play the central role in the development of atherosclerosis as this single-cell-thick lining of the cardiovascular system maintains the interface between blood components and paravascular tissue and actively interacts with all the cells and structures participating in the inflammatory process (Fischer et al., 2005; Gimbrone, 1995; Gimbrone et al., 2000; Landmesser and Drexler, 2005). By the release of a variety of cytokines and growth factors the endothelium activates and influences the vessel wall at the site of injury as well as distant areas of the vasculature, remote tissues and organs, for example the bone marrow (Mantovani et al., 1998). The central role of the endothelium as a modulator of vascular inflammation is highlighted in experiments studying gene expression changes in response to proinflammatory stimuli e.g. IL-1 and TNF-α: While in vascular smooth muscle cells only 40 genes are regulated, endothelial cells show transcriptional regulation of over 200 genes involved in inflammatory processes (Bandman et al., 2002). The magnitude and endothelial

selectivity of functional alterations shows its importance in maintenance of hemostasis, coagulation, inflammation and leukocyte-vessel wall interactions **(scheme 2)**. These studies further indicate that controlled and limited release of vasoconstrictive, prothrombotic and proinflammatory factors represents an adaptive physiologic response of endothelial cells to local vascular trauma and initiates repair processes (Boyle et al., 1996) whereas prolonged or excessive endothelial irritation by proinflammatory stimuli, infectious agents, lipoproteins, ischemia and oxidative stress will result in adverse changes of the endothelial function (Dignat-George and Sampol, 2000; Dignat-George et al., 2003).

1.4.2 Endothelial dysfunction initiates the vascular inflammation cascade

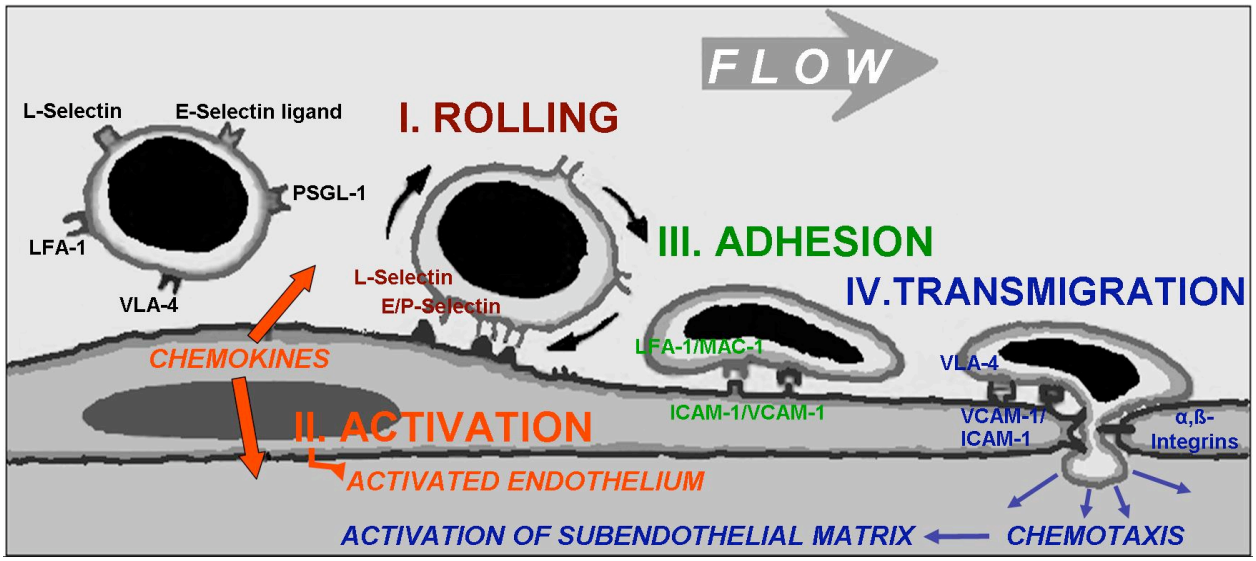
In addition to initiation and promotion of the haemostatic processes, activated endothelium exerts strong proinflammatory effects. Activated endothelium promotes margination and accumulation of monocytes to the vascular wall and recruits localized leukocytes from the circulation into the extravascular space. This aspect of the vascular inflammatory process involves a sequence of four successive events at the vessel wall – rolling, adhesion, transmigration and activation of the subendothelial tissue by mononuclear leukocytes (Butcher, 1991) **(scheme 4)**. This mechanism of vascular inflammation is central to the pathogenesis of atherosclerosis, neointimahyperplasia, ischemic damage and postischemic reperfusion (Becker et al., 2000) and further inflammatory vascular disorders (Luscinskas and Gimbrone, 1996).

The initial interaction between monocytes/leukocytes and activated endothelium is transient and manifests itself as rolling of these cells along the vessel wall. The rolling monocytes/leukocytes then become activated by factors locally generated by the endothelium, resulting in their arrest and firm adhesion to the endothelial layer. Finally, monocytes and leukocytes transmigrate through the endothelium and lead to activation and proliferation of subendothelial tissue (Collins et al., 1995). The initial rolling interactions are mediated by the selectins, whereas firm adhesion and diapedesis are a result of the interaction of leukocytic integrins with immunoglobulin gene superfamily members expressed on the surface of endothelial cells (Armulik, 2002; Collins et al., 1995). Dysfunctional endothelial cells considerably promote these four steps of vascular inflammation as they hold the major role in the interactions between inflammatory cells of the blood stream and the vascular wall (Collins, 1995).

Activation of endothelial cells by circulating inflammatory cytokines (mainly IL-1 and TNF-α), lipoproteins (LDL) and bacterial components (e.g. LPS) or by localized injury of the endothelial surface constitutes the initiation of the inflammatory cascade (Bevilacqua et al., 1985; Schleimer and Rutledge, 1986). The activated endothelial cells mobilize P-selectin from Weibel-Palade bodies onto their surface which leads to platelet and leukocyte rolling on the endothelium mediated by the P-selectin glycoprotein ligand (PSGL-1) **(scheme 2)**. Endothelial-thrombocyte interaction results in further activation and expression of vWF and PECAM-1 by endothelial cells and thereby causes firm adhesion of platelets (Muller et al., 1993). Simultaneously, endothelial expression of E-selectin (ELAM-1) is induced and - binding to glycoproteins (e.g. PSGL-1) of peripheral blood mononuclear cells (PBMC) - gives rise to the rolling of leukocytes, lymphocytes and monocytes on the endothelial surface (Bevilacqua and Nelson, 1993). Besides production and release of the proinflammatory mediators IL-1, TNF-α, PAF and arachidonic acid by stimulated

endothelium (Pober and Cotran, 1990a; Pober and Cotran, 1990b) induction of L-selectin on monocytes additionally contributes to monocyte activation and rolling (Luscinskas et al., 1994) **(scheme 2)**.

Though initial rolling interactions are weak and reversible they generate further activation of the endothelium. In particular, endothelial cells promote adhesion and transmigration by secretion of IL-8, MCP-1, MIP-1α and thrombin (Springer, 1994). These chemoattractants critically enhance mononuclear leukocyte margination to the vessel wall by triggering β_2 (CD11a/CD18 (LFA-1), CD11b/CD18 (MAC-1), CD11c/CD18) - and β_1 (VLA-4) -integrin activation (Elices et al., 1990; Lynam et al., 1998; Meerschaert and Furie, 1994; Rose et al., 2001). Once integrin activation has occurred, monocyte and leukocyte adhesiveness for the endothelium dramatically increases, actually via binding to ligands of the immunoglobulin family such as ICAM-1/2 and VCAM-1 as well as PECAM-1 and VE-cadherin which are strongly upregulated on the endothelial surface (Luscinskas et al., 1995) **(scheme 4)**. Additional monocytes and B-lymphocytes attach to the vessel wall by interaction of CD40 (Mach et al., 1998) with CD40-ligand (CD154) expressed by thrombin-activated platelets (Lutgens et al., 1999). Monocytes and leukocytes then rapidly flatten and extend pseudopods towards the intercellular junctions between adjoining endothelial cells. Mediated by the connection between MAC-1/LFA-1 and ICAM-1/-2 on the one hand (Diamond and Springer, 1993) and VLA-4 and VCAM-1 on the other hand cells eventually change in shape and transmigrate into the subendothelial compartment (Matheny et al., 2000).



Scheme 4 Leukocyte-endothelial interactions during vascular inflammatory processes

Vascular inflammation involves a sequence of four successive events at the vessel wall – rolling, adhesion, transmigration and activation of subendothelial tissue by peripheral blood mononuclear cells (the main adhesion molecules involved in these processes are displayed in the diagram) (modified from Blankenberg et al., 2003).

Once arrived in the interstitial space the inflammatory cells directly impact on the endothelium and the subendothelial tissue, e.g. vascular smooth muscle cells. For example, tissue factor expression by activated monocytes and immunoglobulin production by adhering B-lymphocytes is supposed to trigger endothelial cell apoptosis. The predominant detrimental effects emanating from the damaged endothelium are further release of proinflammatory cytokines

(e.g. IL-1β, TNF-α, COX-2, Caspase-1 (ICE), MCP-1, IL-6 and 8, G-CSF, M-CSF), increased endothelial adhesion receptor expression (ELAM-1, ICAM-1, VCAM-1), production of growth factors (angiopoetin-1, PDGF) and extracellular matrix components (collagen IV, laminin) and various proinflammatory alterations in endothelial regulation of coagulation and vasotone (tissue factor, PAI-1, thrombospondin, thrombomodulin, thrombin receptor, prostacyclin synthase and NOS-3) (Lentsch and Ward, 2000). These processes sustain, prolong and boost the inflammatory actions and involve further circulating cells and the surrounding subendothelial tissue into the pathologic process (Introna et al., 1993; Johnson et al., 1998).

In conclusion, one can say that the endothelium is the nodal point of vascular inflammatory processes. Some of the phenotypic alterations of the endothelium are essential for adequate tissue repair and immune response whereas others intensify and escalate the pathologic effects of the initial destructive event.

1.5 Interleukin-1 - a major player in inflammatory vasculopathies

Interleukin-1 (IL-1) is a potent intercellular mediator of inflammatory processes and immunological responses. Besides its predominant role as a strong proinflammatory cytokine it is also involved in tissue degradation (e.g. apoptosis of endothelial cells) as well as it exerts growth promoting properties (e.g. smooth muscle and fibroblast proliferation) and furthermore acts as a regulator of haematopoiesis (Dinarello, 1997a). IL-1 is part of a large family of cytokines including the interleukins, tumor necrosis factor and colony-stimulating factors (Helmreich, 2001).

1.5.1 General information

Interleukin 1 is the first member of the interleukins that was characterized. In the early 80's, three members of the Interleukin-1 gene family were described: IL-1α, IL-1β and IL-1 receptor antagonist (IL-1ra). The cDNA of the two isoforms of Interleukin-1, IL-1α and IL-1β, was isolated by Auron et al. (Auron et al., 1984) and March et al. (March et al., 1985). Both IL-1α and IL-1β as well as their naturally occurring antagonist IL-1 receptor antagonist are assigned to the long arm of chromosome 2 (2q14) (Nicklin et al., 1994; Patterson et al., 1993; Webb et al., 1985; Webb et al., 1986).

IL-1α and IL-1β are structurally related polypeptides with approximately 25% identity. They bind to the same receptors, and there are no obvious differences in the spectrum of biological activities induced by IL-1α or IL-1β (Dinarello, 1996). IL-1α and IL-1β are regulated at the transcriptional and translational level. They are both produced as 31 kDa precursor proteins (proIL-1α and proIL-1β, respectively) which undergo posttranslational proteolytic cleavage mediated by Interleukin-1 converting enzyme (ICE, Caspase-1) resulting in mature IL-1 proteins of approximately 17.5 kDa (Schindler et al., 1990). Additionally involved in this conversion are unspecific cleavage enzymes such as elastase, trypsin, chymotrypsin and granzyme A found in extracellular fluids at sites of tissue degradation (Dinarello et al., 1986; Fantuzzi et al., 1997; Irmler et al., 1995).

However, processing of IL-1α and IL-1β is different:: While IL-1β is active only as mature product, both IL-1α and its precursor proIL-1α show biological effects (Jobling et al., 1988). Lacking

a leader peptide, IL-1α remains mostly cytosolic or can be found membrane-associated where it has autocrine cell specific effects on growth and differentiation (e.g. promoting senescence in endothelial cells) (Kurt-Jones et al., 1985; Stevenson et al., 1993). ProIL-1α, IL-1α and their complexes with intracellular IL-1RI can translocate into the nucleus, bind to nuclear DNA and thereby act as a transcription regulator (Curtis et al., 1990; Grenfell et al., 1989; Hofmeister et al., 1995; Mizel et al., 1987; Wessendorf et al., 1993).

In contrast, IL-1β is in the main secreted into the extracellular space and can be detected in the circulation. In this manner IL-1β eventually binds to IL-1 receptors both near and distant from the sites of its synthesis (Voronov et al., 2003).

Thus one can say that IL-1β is a systemic, hormone-like mediator released from cells, whereas IL-1α is primarily a regulator of intracellular events and mediator of local inflammation.

Interleukin 1 (IL-1) was first detected as “lymphocyte-activating factor” from macrophages required for initiating lymphocyte proliferation through production of T cell growth factor (IL-2) (Diamantstein et al., 1979; Togawa et al., 1979). Though the main sources of IL-1 are monocytes and activated macrophages, both IL-1α and IL-1β are produced by a wide variety of cells e.g. endothelial cells, thrombocytes, leucocytes and further tissue specific cells (Bevilacqua et al., 1984).

Inducers of IL-1 production are bacterial lipopolysaccharides as well as non-bacterial factors such as hypoxia/ischemia (Clark et al., 1995), adherence of peripheral blood mononuclear cells to endothelium, radiation and thermal injury, complement components C5a/C5b-9 (membrane attack complex), cell matrix components (collagen, fibronectin), clotting factors (e.g. fibrin degradation products, plasmin and thrombin) and platelet activation factor (PAF). Furthermore, IL-1 production is induced by a multitude of cytokines including TNF-α, IL-1β, IL-2, IL-3, IL-12, GM-CSF, SCF and PDGF (reviewed by Dinarello, 1997b; Dinarello, 2000a).

1.5.2 Interleukin-1 signaling

Two receptors are at disposal for binding of Interleukin-1: IL-1R1 and IL-1R2. Analogous to the ligands their genes localize to chromosome 2.

The IL-1 receptor type I (IL-1RI), an 80-kDa glycoprotein (prominently found on endothelial cells, smooth muscle cells, epithelial cells, fibroblasts and various haematopoietic cell types) has a single transmembrane segment and a cytoplasmatic domain and is the primary signal transducing receptor (Sims et al., 1993). This receptor contains a subunit, the IL-1 receptor accessory protein (IL-1R-AP) which is, upon binding of IL-1 to the IL-1RI recruited to the complex. The heterodimerization of IL-1RI and IL-1R-AP stabilizes the binding of IL-1, mediates recruitment of signaling proteins and thereby activates the signal cascade (Dunne and O'Neill, 2003; Greenfeder et al., 1995; Korherr et al., 1997).

The IL-1 receptor type II (IL-1RII), a 68 kDa membrane protein, lacks a signal transducing cytosolic domain which makes it a functionally negative receptor **(scheme 5)**. It acts as a decoy molecule for IL-1 by preventing the binding of IL-1β to the type I receptor (Colotta et al., 1993a; Colotta et al., 1993b; Mantovani et al., 2001). In cells expressing both IL-I type I and type II receptors, there is competition to bind IL-1 first.

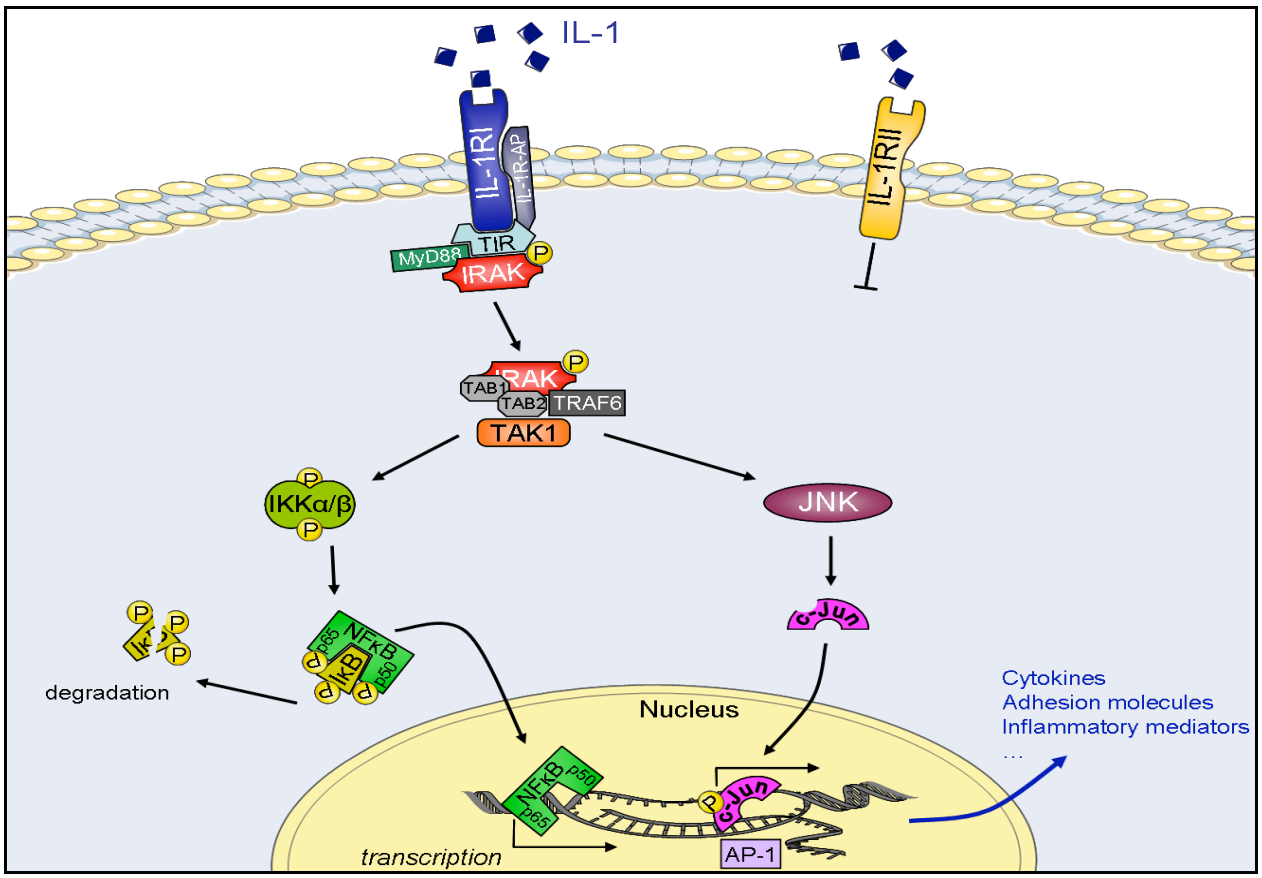
Proteolytical cleavage of the extracellular domains of IL-1RI and IL-1RII results in soluble

receptors in the circulation and extracellular fluids which represent a third binding target for IL-1. However, binding of IL-1 to the soluble receptors does not result in signal transduction (Dinarello, 1996; Orlando et al., 2000). Soluble type II IL-1 receptors bind IL-1β much more avidly than IL-1ra and function as inhibitors of IL-1 action (Smith et al., 2003). In contrast, soluble type I IL-1 receptors bind IL-1ra almost selectively (Svenson et al., 1995). Thus, soluble type I IL-1 receptors might block or neutralize the natural anti-inflammatory actions of IL-1ra in vivo (Burger et al., 1995).

The IL-1 receptor belongs to the IL-1/ Toll-like receptor superfamily which consists of a large number of receptors that all contain an intracellular Toll-IL-1RI receptor (TIR) domain (Dunne and O'Neill, 2003; Slack et al., 2000). Various signaling cascades are activated by IL-1RI stimulation, including three families of mitogen activated protein kinases (MAPK) activated by IL-1 binding to the IL-1RI: the Ras-Raf-p42/44 MAPK pathway, the p38 MAPK signaling and the p54 MAP kinase (responsible for phosphorylation of heat shock protein 27 (hsp 27)) (Freshney et al., 1994; Huwiler and Pfeilschifter, 1994; Kowluru and Odenbach, 2004; Kracht et al., 1994). Furthermore, phospholipase A2 (PLA2) signaling, the Jun kinase (JNK) pathway and, most importantly, the NF-κB signaling cascade are involved in mediating post-receptor effects of IL-1 (Dinarello, 1997a; Orelio and Dzierzak, 2003; Silverman and Maniatis, 2001). Within the IL-1 receptor signaling cascade the IL-1 receptor accessory kinase (IRAK), the transforming growth factor β activated kinase 1 (TAK1) and the regulatory TAK1 binding proteins (TABs) play important parts in the activation of NF-κb and JNK (Bol et al., 2000; Ghosh and Karin, 2002; Janssens and Beyaert, 2003; Kondo et al., 1998; Suzuki et al., 2002). Phosphorylation and dephosphorylation of transcription factors eventually enable the cells to transcribe genes controlled by the IL-1 activation pathways **(scheme 5)**. It emerged that most of the biologic effects of IL-1 take place in cells after nuclear translocation of NF-κB and JNK/activating protein-1 (AP-1) which then activate IL-1-inducible genes (Stylianou et al., 1992).

One peculiarity of IL-1 signal transduction is that a low number of receptors and correspondingly low concentrations of IL-1 can induce a relatively large biologic response (Orencole and Dinarello, 1989). This suggests the existence of intracellular amplification processes. The nature and regulation of these amplification pathways are not clear at present.

In conclusion, the division of the IL-1 signal into various intracellular signalling pathways with multiple postreceptor phosphorylations accounts for the fact that IL-1 induces a wide variety of genes in the same cell at the same time and leads to the complexity of cellular responses following stimulation by IL-1.



Scheme 5 Overview of IL-1 intracellular signalling

Upon IL-1 interaction with the IL-1RI, the accessory receptor protein IL-1R-AP is recruited and allows interaction of the adaptor protein MyD88 and the kinase IRAK with the receptor. Via the adapter proteins TRAF6, TAB1, TAB2 and the kinase TAK1 the signal is propagated to the kinase JNK which induces gene activation via the transcription factor c-jun. The signal cascade also relays a signal to the inhibitory κB (IκB) kinase complex which phosphorylates IκB and thereby targets it for degradation. Subsequently the transcription factor NF-κB is released and translocates into the nucleus in order to activate target genes. Upon binding of IL-1 to the IL-1RII no signal is propagated as the IL-1RII lacks an intracellular TIR (Toll-IL-1-Receptor) domain which is required for recruitment of signalings. The IL-1RII is therefore regarded as a decoy receptor.

1.5.3 Interleukin-1 in vascular inflammation

Interleukin-1 along with TNF-α is one of the key players in the pathogenesis of inflammatory vascular diseases. The findings of raised serum concentrations of IL-1α and IL-1β in patients with coronary artery disease (Galea et al., 1996; Hasdai et al., 1996; Waehre et al., 2004) and detection of increased synthesis of IL-1 in human arterial plaques (Barath et al., 1990; Dewberry et al., 2000; Gottsater et al., 2002; Tipping and Hancock, 1993) as well as in the damaged vessel wall of patients with autoimmune vasculitis (Weyand et al., 1994) first suggested a pivotal role of IL-1 in vascular inflammation. Further investigations verified the participation of IL-1 in the induction of intimal lesions in coronary arteries in pigs (Shimokawa et al., 1996) and demonstrated decreased severity of atherosclerosis in ApoE-deficient mice lacking the gene for IL-1β (Kirii et al., 2003). The predominant role of IL-1 in atherosclerotic processes has also been shown by Biasucci et al. who proved an association between elevated IL-1 levels and incidence of coronary events in patients with unstable coronary artery disease (Biasucci et al., 1999).

Various cell types produce IL-1 in response to vascular injury: During the vascular inflammatory process Interleukin-1 is mainly derived from endothelial cells, but also from smooth muscle cells in the vessel wall (Bevilacqua et al., 1984; Libby et al., 1986b) as well as from activated platelets, adhering leucocytes and lipid-filled invading macrophages (foam cells) (Holvoet and Collen, 1994; Libby et al., 1992). Interleukin-1 production is induced by deposition of oxidized LDL-particles in the vessel wall, adherence of thrombocytes and monocytes to endothelium, release of TNF-α by inflammatory cells and, in an autocrine way, by IL-1 itself (Libby, 1992; Libby et al., 1986a; Libby et al., 1986b; Warner et al., 1987).

The diverse effects of IL-1 during the development of atherosclerotic lesions in the vasculature are to a large part mediated by activation of the transcription factor NF-κB leading to multiple transcriptional regulations of genes involved in the inflammatory cascade within the arterial intima and media (Collins and Cybulsky, 2001). NFκB-activation accounts for the large variety of inflammatory responses of endothelial cells and has been identified in several cell types within human atherosclerotic lesions, including macrophages and vascular smooth muscle cells (Brand et al., 1996; Collins et al., 1995).

Endothelial cells represent a prime target for the action of Interleukin-1. IL-1 mediated induction of endothelial surface expression of ELAM-1, ICAM-1 and VCAM-1 is the major determinant of PMN adhesion and localization of leukocyte-vessel wall interactions at sites of inflammation and critically influences the development of vascular lesions (Bevilacqua et al., 1985; Eriksson et al., 2001; Huo et al., 2000; Pober, 1988; Price and Loscalzo, 1999; Ramos et al., 1999; Tamaru et al., 1998). Especially VCAM-1 seems to hold a major role in vascular inflammation leading to atherosclerotic lesions and neointimahyperplasia (Cybulsky et al., 2001; Dansky et al., 2001; Nakashima et al., 1998; Oguchi et al., 2000). The soluble forms of these adhesion molecules have additionally been associated with the severity of atherosclerosis and coronary artery disease and the risk of coronary events and myocardial infarction (Blankenberg et al., 2001; Haim et al., 2002; Malik et al., 2001; Mizia-Stec et al., 2002; Parker et al., 2001; Peter et al., 1997).

Further element of vascular inflammation is that IL-1 produced from either platelets, foam cells or the endothelium induces the chemokines Interleukin-8 (IL-8), monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein 1α (MIP-1α), which facilitate attraction, activation and transendothelial migration of inflammatory cells (Dickfeld et al., 2001). Transendothelial migration of monocytes is additionally promoted by an increase in endothelial cell barrier permeability due to an IL-1-mediated widening of junctional gaps (Brett et al., 1989; Moser et al., 1989).

Besides the effects of IL-1 on leukocytes and leukocyte-endothelial interactions, IL-1 induces release of the growth factors PDGF (Raines et al., 1989) and FGF by endothelial cells and platelets. These factors are implicated in smooth muscle cell (SMC) and fibroblast activation and proliferation (Beasley and Cooper, 1999; Dzau et al., 2002; Libby et al., 1988). Overgrowth of the damaged endothelial area by medial and advential SMC and synthesis of extracellular matrix by activated fibroblasts leads to thickening of the subendothelial vessel wall with subsequent stenosis (Filonzi et al., 1993). Thickness of the vessel wall seems to be correlated with elevated levels of IL-1 and distinct IL-1 gene variants (Kornman et al., 1999). This process, referred to as neointimahyperplasia, also applies for the formation of fibrous caps that overgrow the

subendothelial deposition of lipoproteins.

IL-1 additionally increases expression of the clotting factors tissue factor, thrombin and fibrinogen as well as thrombin receptor and plasminogen activator inhibitor (PAI-1) (McEver et al., 1989; Nachman et al., 1986; Ott, 2003; Ott et al., 2001). This gene regulation is another integral part in the development of inflammatory vascular lesions. Tissue factor, the main inducer of the extrinsic pathway of coagulation, is predominantly released from damaged endothelium and IL-1 was found to be one of the main inducers of tissue factor expression (Bevilacqua et al., 1986a; Bevilacqua et al., 1986b; Ott et al., 1996). Furthermore, IL-1 downregulates antithrombotic proteins such as thrombomodulin (Shebuski and Kilgore, 2002). The initiated procoagulatory process leads to thrombin formation, fibrin deposition and acute vascular occlusion (Zimmerman et al., 1992). Lastly, IL-1 is also mainly responsible for dramatic decreases in endothelial NO production. Besides a significant decrease of endothelium-dependent vasodilatation (Bhagat and Vallance, 1997; Hingorani et al., 2000) this results in further impairment of endothelium-dependent antiinflammatory and antithrombotic mechanisms as NO is a natural inhibitor of platelet and leukocyte adhesion and accumulation on the endothelial surface (Landmesser et al., 2004; Mazzone et al., 2001).

The multiple biologic effects of IL-1 as an inflammatory mediator define the crucial role of this cytokine in recruitment, local accumulation and transendothelial migration of inflammatory cells with subsequent activation of subendothelial tissue which constitute the hallmarks of any inflammatory process.

1.6 Interleukin-1 receptor antagonist - inhibition of IL-1 mediated proinflammatory activity

Interleukin-1 receptor antagonist (IL-1ra, IL1RN) is an antiinflammatory cytokine and acute-phase reactant, released during several inflammatory and infectious diseases and in traumatic conditions (Gabay et al., 1997). IL-1ra is a protein that binds to IL-1 receptors and competitively inhibits the binding of IL1-α and IL1-β. As a consequence, the biologic activity of IL-1 is diminished in physiologic and pathophysiological immune responses and inflammatory processes (Dinarello, 2002). The interleukin-1 receptor antagonist, first discovered in supernatants of human monocyte cultures (Arend et al., 1985; Arend et al., 1989) and in the urine of patients with fever or myelomonocytic leukaemia (Balavoine et al., 1986; Seckinger et al., 1987), was cloned by Carter et al. and is the first described naturally occurring specific receptor antagonist of any cytokine or hormone-like molecule in the human organism (Arend et al., 1998b; Carter et al., 1990).

1.6.1 General information

Apart from the IL-1R2 decoy receptor and the soluble IL-1 receptors, production of IL-1ra represents a third biologic mechanism to down-regulate responses to IL-1β. With 26% homology in amino acids, which is greater than that between IL-1α and IL-1β, IL-1ra is structurally similar to IL-1β (Carter et al., 1990; Eisenberg et al., 1990). IL-1ra binds to IL-1R1 and IL-1R2 with the same affinity as IL-1β but due to the lack of a second binding site for IL1R-AP intracellular signalling and biologic responses do not occur (Guo et al., 1995; Wesche et al., 1996). By

blockage of IL-1R1 IL-1ra is a competitive inhibitor of IL-1β; the biologic effects of IL-1β are therefore proportional to the ratio of IL-1β to IL-1ra (Langdahl et al., 2000).

IL-1ra exists in four different isoforms derived from the same gene which maps to chromosome 2q14-q21 in the region of the IL-1α and IL-1β loci (Arend et al., 1998b; Patterson et al., 1993; Steinkasserer et al., 1992). One isoform is secreted (sIL-1ra) while the three others (icIL-1ra 1/2/3) are intracellular due to a lack of a functional leader sequence. The sIL-1ra and icIL-1ra1 mRNAs are transcribed from different promoters and underwent alternative splicing of different first exons (Butcher et al., 1994). Secretory IL-1ra is synthesized as a 177 amino acid protein including a 25 amino acid leader sequence which is split of prior to secretion resulting in a 152 amino acid protein with a molecular weight of 17 kDa. Intracellular IL-1ra type 1 (icIL-1ra1) is a 159 amino acid protein of 18 kDa molecular weight (Haskill et al., 1991). The mRNA for icIL-1ra2 (long intracellular splice form) is transcribed from the icIL-1ra1 promoter, but contains an additional exon of 63 bp. Correspondingly, it has an additional insert of 21 amino acids and constitutes a 25 kDa protein (Muzio et al., 1999; Muzio et al., 1995).

The 16 kDa icIL-1ra3 (short intracellular splice form) is produced by alternative translation initiation from the sIL-1Ra transcript (Malyak et al., 1998a; Weissbach et al., 1998). Due to diverse posttranslational glycosylations there may be some variation in molecular weight of the different IL-1ra isoforms (Hannum et al., 1990).

The expression of the four isoforms of IL-1ra is cell-type specific and stimulus specific. Secreted IL-1ra is predominantly derived from peripheral blood mononuclear cells, lung, spleen and liver, where it is produced as an acute phase protein. IcIL-1ra1 is constitutively expressed in epithelial cells, and is inducible in monocytes and macrophages. The mRNA for icIL-1ra2 has been detected in monocytes, neutrophils, keratinocytes and activated fibroblasts and icIL-1ra3 is produced in lipopolysaccharide-stimulated neutrophils, monocytes and macrophages (Malyak et al., 1998a; Malyak et al., 1998b).

The major biological role of extracellular sIL-1ra is to block and to modulate the effects of IL-1 by binding to the type I IL-1 receptor at the cell membrane and microenvironment (Dinarello, 2000b).

The mode of action of the intracellular splice variants of IL-1ra is less clear and remains to be elucidated. IcIL-1ra might possibly exhibit an intrinsic autocrine role and provide a counterbalance for intracellular IL-1α by blocking the binding of IL-1α or proIL-1α to nuclear DNA within the cells that produce it (Haskill et al., 1991). Furthermore, it has been shown that icIL-1ra inhibits phosphorylation of p53, c-Jun and IκB, binds specifically to CSN3 (COP9 signalosome complex) and thereby acts as an inhibitor of the p38 MAPK signal transduction pathway and inhibits nuclear translocation of NF-κB (Banda et al., 2005; Garat and Arend, 2003). Under certain conditions icIL-1ra might also be released from the producing cells and block binding of IL-1 to cell surface receptors (Corradi et al., 1995; Levine et al., 1997; Wilson et al., 2004). IcIL-1ra binds to IL-1RI equally as avidly as sIL-1ra; therefore both isoforms may function as extracellular receptor antagonists of IL-1. It has been suggested that icIL-1ra might represent an intracellular reservoir of IL-1 receptor antagonist which is released from the cells under extreme inflammatory conditions and cell death in the sense of a potential additional rapid mechanism to inhibit IL-1 (Muzio et al., 1999).

This hypothesis is underscored by the finding that ovarian cancer epithelial cells, which express high levels of icIL-1ra, displayed lessened IL-1-induced IL-8 mRNA expression in comparison to cells that did not produce icIL-1ra (Watson et al., 1995). Moreover, in experimental studies it was demonstrated that transgenic retroviral expression of icIL-1ra in nonproducing cells led to a pattern of response analogous to that seen with cells spontaneously producing icIL-1ra (Arend and Guthridge, 2000). In another experimental study IL-1α stimulation was not able to induce detectable IL-6 and IL-8 production in keratinocytes exhibiting high levels of icIL-1ra (Banda et al., 2005). In addition, high levels of both constitutive and cytokine induced icIL-1ra in keratinocytes and in transfected fibroblasts were associated with decreased plasma membrane expression of ICAM-1 (Gabay et al., 1999; Norris et al., 1997).

Thus, all the isoforms of IL-1ra, either intracellular or secreted, share the ability to inhibit IL-1 activity (Bertini et al., 1992; Muzio et al., 1999). Furthermore, current literature data suggests that icIL-1ra might perform additional cell specific regulatory roles inside the cells synthesizing IL-1 without involvement of direct receptor binding (Banda et al., 2005).

1.6.2 Characteristics and regulation of Interleukin-1 receptor antagonist expression

Induction of IL-1ra is mediated via toll-like receptors (Carl et al., 2002) and in activated tissues (e.g. the vascular wall) both IL-1ra and IL-1β are coexpressed upon stimulation (Arend et al., 1991) so that levels of IL-1β and IL-1ra in vivo often vary in parallel (Chamberlain et al., 1999; Hirsch et al., 1996; Wang et al., 2000). Appearance of IL-1ra characteristically follows the upregulation of IL-1 synthesis (Hurme and Santtila, 1998). However, studies in both animal and human systems suggest that the levels of endogenous production of IL-1ra in local tissues are not sufficient to overcome the inflammatory effects of the IL-1 agonists and the balance of net biologic function remains shifted towards IL-1β and IL-1α (Arend, 1991; Arend, 2001; Firestein et al., 1994; Horai et al., 2004).

It has been found that despite the near equal affinities of IL-1 and IL-1ra for IL-1RI, a 10- to 100-fold molar excess of IL-1ra is required to achieve a 50% inhibition of IL-1 activity during in vitro experiments (Arend et al., 1990). However, in some experiments even a 1:1 molar ratio of IL-1ra to IL-1 significantly blocked the IL-1 induced activation of endothelial cells and monocytes (Arend et al., 1990; Granowitz et al., 1992a; Granowitz et al., 1992b; Kaplanski et al., 1993). In humans and other mammalian organisms a 100- to 1.000- fold molar excess of IL-1ra over that of IL-1 is actually needed to block systemic responses to IL-1 and to observe reduction in disease activity in vivo (Ohlsson et al., 1990).

The current knowledge about IL-1ra expression by endothelial cells is not yet conclusive. While Bertini et al. and other groups showed that human umbilical vein endothelial cells (HUVEC) do not express IL-1ra (Bertini et al., 1992), Dewberry et al. reported that IL-1ra is abundantly produced by both HUVEC and coronary artery endothelial cells (CAEC) stimulated with LPS, PMA and TGF-β (Dewberry et al., 2000). At present, one assumes that the intracellular splice variant is the exclusive IL-1ra isoform in mature endothelium, in contrast to the monocyte which produces both intracellular and secreted forms, and the hepatocyte that solely releases the secreted form of IL-1ra (Dewberry et al., 2000). IL-1ra expression by cord blood-derived EPC has not yet been

investigated.

1.6.3 Interleukin-1 receptor antagonist in vascular inflammation

Several lines of evidence underscore the importance of a role for IL-1ra in vascular diseases and the impact of endothelium-derived imbalance between IL-1 and IL-1ra on vascular physiology. In a study with 565 patients the presence of a single nucleotide polymorphism (SNP) of the IL-1ra gene, IL-1RN*2, was found to be associated with an increased prevalence of single vessel coronary artery disease (Francis et al., 1999; Momiyama et al., 2001). The allele IL-1RN*2 was found to be associated with a decreased production of IL-1ra protein in endothelial cells linked with a polymorphism of the IL-1β gene leading to increased IL-1β production (Dewberry et al., 1999; Santtila et al., 1998). In this way the IL-1RN*2 genotype causes relatively unopposed and thus enhanced IL-1 activation of endothelial cells (Dewberry et al., 2000; Dewberry et al., 1999; Santtila et al., 1998). Presence of IL-1RN*2 was also associated with significantly elevated ELAM-1 and vWF levels in acute coronary syndromes reflecting the proinflammatory changes in the endothelium due to an impaired production of IL-1ra (Blankenberg et al., 2001; Ray et al., 2002).

In an animal model of vascular disease, diminished levels of icIL-1ra could be detected in the endothelium of diseased coronary arteries in ApoE knockout mice and administration of IL-1ra was shown to inhibit the fatty streak formation. More importantly, treatment with IL-1ra was more efficient than TNF-α antagonism (Elhage et al., 1998). The interaction of ApoE and IL-1ra was further highlighted by results that mice both deficient for ApoE and IL-1ra showed early atherosclerotic lesions of the aorta that were significantly larger and contained decided more lipid deposits and macrophages than their control group. These morphological findings were associated with significantly increased expression of IL-1β, MCP-1, ICAM-1 and VCAM-1 mRNA within the diseased vasculature suggesting that deficiency of IL-1ra may induce the development of atherosclerosis and cause plaque instability by accumulation of numerous macrophages/monocytes in the lesions (Isoda et al., 2004). In another experimental model, mice lacking the IL-1ra gene developed lethal arterial inflammation, presenting as vessel wall collapse, stenosis, myocardial infarction and haemorrhage from ruptured aneurysms. This was the consequence of massive transmural infiltration of neutrophils, macrophages and lymphocytes (Nicklin et al., 2000). Furthermore, deficiency of endogenous IL-1ra in IL-1ra knockout mice was shown to promote neointimal formation after vascular injury in several experiments. This was found to be due to an unopposed action of IL-1 in the arterial intima and media with an increased inflammatory reaction in the vessel wall and subsequently enhanced proliferation of fibroblasts and SMCs compared to IL-1ra positive mice (Isoda et al., 2003; Loppnow et al., 1998; Wang et al., 2000). Complementary, in a LDL receptor knockout mouse model of atherosclerosis, IL-1ra overexpressing transgenic mice showed a significant decrease in atherosclerotic foam cell lesions compared to LDL-R knockout mice lacking the transgene (Devlin et al., 2002; Ishibashi et al., 1994). These observations suggest that disequilibrium between IL-1ra and IL-1 in the vessel wall strongly enhances the development of vascular inflammation and production of IL-1ra thus represents an indispensable protective mechanism of the vessel wall.

In summary, one can say that IL-1ra is produced by many tissues in the normal human

organism and that its production is upregulated in the host response to infection and acute or chronic inflammation. IL-1ra is an important negative regulator of the action of IL-1 that controls vascular participation in inflammation and immunity and provides local protection against the disease provoking effects of IL-1. Enhancing endogenous IL-1ra production or exogenous administration of IL-1ra - either systemically or locally - might become a therapeutic strategy to ameliorate the cytokine balance in inflamed tissues and attenuate adverse effects of IL-1 overproduction.

2 Objectives

Current data indicate that endothelial dysfunction caused by vascular injury or activation of endothelial cells by proinflammatory cytokines is the key event in the initiation of inflammatory vasculopathies such as atherosclerosis and neointimahyperplasia.

Interleukin-1 is a major player in the initiation of endothelial dysfunction and the formation of inflammatory lesions in the vasculature. It predominantly acts on endothelial cells and impairs their physiologic function in vascular homeostasis by rendering their phenotype proinflammatory and prothrombotic. Insufficient renewal of malfunctioning endothelium due to diminished recruitment of EPC to sites of vascular disintegration further contributes to endothelial dysfunction and promotion of vascular inflammation.

Based on these facts we hypothesized that enhancement of reendothelialization by transplantation of endothelial progenitors and inhibition of vascular inflammation by blocking the effects of Interleukin-1 at the vessel wall might be an effective strategy to reverse the characteristic pathogenetic processes of inflammatory vasculopathies.

Therefore, this study aims to evaluate the feasibility of a combined antiinflammatory and regenerative approach in treatment of inflammatory vasculopathies. Our purpose was to create cord blood-derived endothelial progenitor cells that carry the transgene Interleukin-1 receptor antagonist and thereby combine the ability to reendothelialize sites of vascular injury with the local delivery of an antiinflammatory drug at the vessel wall.

Prior to clinical implementation of this approach several questions have to be answered and several problems have to be resolved.

1. Endothelial progenitor cells must be reliably isolated from cord blood and expanded to clinically relevant numbers

2. The feasibility of gene transfer into these EPC must be examined.

Therefore I aimed to develop an optimized protocol for expansion and stable retroviral transduction of EPC. For transduction, I chose to use the icIL-1ra gene because it has similar binding properties as sIL-1ra, but would not be released in the supernatant so much, thereby minimizing direct effects of IL-1ra to endothelial cells.

To elucidate the effects of transgenic icIL-1ra production, we investigated the competence of IL-1ra transduced CBEC to inhibit the relevant mechanisms involved in inflammatory processes. Therefore I studied gene expression profiles of IL-1ra-transduced versus parental CBEC with regard to relevant pro- and anti-inflammatory genes. In addition, I established experimental models of adhesion and transendothelial migration processes as well as endothelial-monocyte cocultures to study endothelial activation.

These studies should elucidate the potential clinical applicability and antiinflammatory efficiency of icIL-1ra transduced endothelial progenitors from cord blood for the treatment of inflammatory vasculopathies.

3 Materials

3.1 Cells

- Primary Cells: Cord blood derived mononuclear cells *(from healthy newborns)*
  Peripheral blood leucocytes *(from healthy volunteers)*
- Cell lines: Mono-Mac-6 human AML FAB M5 derived mononuclear cells *(DSMZ-No. ACC 124, kind gift from AG Dr. Licht)*
  NIH 3T3 mouse fibroblast-derived PT67 retroviral packaging cells *(BD Biosciences Clontech, Palo Alto, CA)*
  293 T human embryonal kidney derived fibroblastoid cells *(DSMZ-No. ACC 305)*

3.2 Culture media and supplements

3.2.1 Culture media

Dulbecco`s modified Eagles Medium (DMEM) *(Gibco BRL [Karlsruhe])*

Endothelial Cell Basal Medium (EBM-2) *(Cambrex Bio Science [Verviers/Belgien])*

Iscove`s Modified Dulbecco`s Medium (IMDM) *(Gibco BRL [Karlsruhe])*

RPMI-1640 *(Gibco BRL [Karlsruhe])*

3.2.2 Supplements

EGM-2 Bullet Kit/ growth factor supplement *(Cambrex Bio Science [Verviers/Belgien])*

Fetal Bovine/Calf Serum (FBS/FCS) *(PAN Biotech [Aidenbach, Germany])*

Fibroblast Growth Factor (rh FGF-2) *(R&D [Wiesbaden])*

Geneticin (G-418 Sulphate) *(Gibco BRL [Karlsruhe])*

Horse serum (HS) *(Stem Cell Technologies [France/European office])*

L-Glutamine *(Gibco BRL [Karlsruhe])*

Non essential amino acids (NEAA) *(Gibco BRL [Karlsruhe])*

Penicillin/Streptomycin *(Gibco BRL [Karlsruhe])*

Sodium Pyruvate *(Gibco BRL [Karlsruhe])*

Stem Cell Factor (rh SCF) *(R&D [Wiesbaden])*

Stem Cell Growth factor (rh SCGF-ß) *(PeproTech [London/UK])*

Vascular Endothelial Growth Factor (rh VEGF) *(R&D [Wiesbaden])*

3.3 Chemicals and reagents

3.3.1 Cell isolation

Biocoll Separations Solution (Biochrom AG [Berlin])

Direct CD 34 Progenitor Cell Isolation Kit *(Miltenyi Biotec [Bergisch Gladbach])*

Ficoll-Hypaque *(Seromed [Berlin, Germany])*

HF/2+ buffer:

- HBSS 10x *(Gibco BRL [Karlsruhe])*	100 ml
- FCS *(PAN Biotech [Aidenbach, Germany])*	20 ml
- Hepes buffer 1M *(Gibco BRL [Karlsruhe])*	10 ml
- Pen/Strep *(Gibco BRL [Karlsruhe])*	10 ml
- Aqua distillate	ad 1000 ml

Heparin (Liquemin®) *(Roche AG [Grenzach-Wyhlen])*

3.3.2 Cell culture

Aqua distillate *(Delta-Pharma [Pfullingen])*

DMSO (Dimethylsulfoxide) *(Sigma-Aldrich Chemie [München])*

Fibronectin *(Cell Systems [St. Katharinen, Germany])*

Gelatine Type B, from bovine skin *(Sigma-Aldrich Chemie [München])*

Phosphate Buffered Saline (PBS) *(Gibco BRL [Karlsruhe])*

Trypan blue Solution *(Gibco BRL [Karlsruhe])*

Trypsin/EDTA-solution *(Gibco BRL [Karlsruhe])*

Türks Solution *(Merck Eurolab [Darmstadt])*

BSA (bovine serum albumine) *(Sigma-Aldrich Chemie [München])*

3.3.3 Plasmid preparation

Agarose (Ultra Pure) *(Life Technologies [Eggenstein])*

Ampicillin *(Sigma-Aldrich Chemie [München])*

DH5α Chemically Competent E.coli *(Gibco [Paisley, UK])*

DNA-Ladder (100bp / 1Kb plus) *(Invitrogen [Karlsruhe])*

Ethidiumbromide *(Sigma-Aldrich Chemie [München]), (Serva [Heidelberg])*

LB-medium:

- 1% Bacto Tryptone *(DIFCO [Kansas, US])*
- 1% Sodium Chloride *(DIFCO [Kansas, US])*

- 0.5% Yeast Extract *(DIFCO [Kansas, US])*
- Aqua distillate ad 100%
- 1.5% Bacto Agar (for agar-plates) *(DIFCO [Kansas, US])*

pLXSN retroviral vector construct (BD Biosciences Clontech [Palo Alto, CA])

pLXSN sequence primer: - sense (5`- CCC TTG AAC CTC CTC GTT CGA CC - 3`)
- antisense (5`- GAG CCT GGG GAC TTT CCA CAC CC- 3`)
(Sigma-Aldrich Chemie [München])

pLXSN-IL-1ra (long intracellular form) retroviral vector construct *(provided by Dr. M. Muzio [Instituto di Ricerche Farmacologiche, Milano]*

Quiaprep Spin Miniprep kit / Plasmid Maxi kit (Quiagen GmbH [Hilden, Germany])

Restriction buffer H 20 *(Amersham-Pharmacia Biotech Europe GmbH [Frankfurt])*

Restriction enzymes: - BamH I *(Boehringer [Mannheim])*
- EcoR I *(Amersham-Pharmacia Biotech Europe GmbH [Frankfurt])*
- Hind III *(Boehringer [Mannheim])*
- Sac I *(Boehringer [Mannheim])*

TAE-buffer (1x):
- 4.84 g Tris base [2 M] *(Carl Roth [Karlsruhe])*
- 1.14 ml Acetic acid (glacial) *(Carl Roth [Karlsruhe])*
- 2 ml EDTA sodium salt (0.5 M, pH 8.0) (0.05 M) *(Sigma-Aldrich Chemie [München])* - Aqua distillate ad 1000 ml

3.3.4 Transfection

Calcium Phosphate Transfection System *(Life Technologies [Eggenstein])*

Chloroquine diphosphate *(Sigma Chemicals [St Louis, MO, USA])*

Lipofectamine Plus reagent *(Invitrogen [Karlsruhe])*

Lipofectamine reagent *(Invitrogen [Karlsruhe])*

Polybrene *(Sigma-Aldrich Chemie [München])*

3.3.5 Transduction

DEAE-Dextran *(Amersham Pharmacia Biotech [Uppsala, Sweden])*

Chondroitinase ABC from Proteus vulgaris *(Sigma-Aldrich Chemie [München])*

3.3.6 Real-time-PCR

10 mM dNTP Mix *(Invitrogen [Karlsruhe])*

0.1M DTT *(Invitrogen [Karlsruhe])*

0.1M First-Strand-Buffer *(Invitrogen [Karlsruhe])*

10x PCR Buffer, Minus Mg^{2+} *(Invitrogen [Karlsruhe])*

50 mM Magnesium Chloride *(Invitrogen [Karlsruhe])*

Chloroform *(Sigma-Aldrich Chemie [München])*

Ethanol *(Carl Roth [Karlsruhe])*

Isopropanol *(Carl Roth [Karlsruhe])*

Molecular Water *(Gibco BRL [Karlsruhe])*

Oligo $(dT)_{12-18}$ Primer (500 µg/ml) *(Gibco BRL [Karlsruhe])*

RNaseOUT Recombinant Ribonuclease Inhibitor (40 units/µl) *(Invitrogen [Karlsruhe])*

RNAse-free DNAse I *(Invitrogen [Karlsruhe])*

Superscript RNase H^- Reverse Transcriptase (200 units/µl) *(Invitrogen [Karlsruhe])*

Sybr Green I *(Applied Biosystems [Darmstadt])*

Taq DNA Polymerase (5 units/µl) *(Invitrogen [Karlsruhe])*

Trizol reagent *(Invitrogen [Karlsruhe])*

Primers for semiquantitative PCR *(Sigma-Aldrich Chemie [München])*:

Molecule	Gene Bank ID	5`-Primer	3`-Primer	Product length	Reference
Angiopoetin-1	gi: 22203640	AGC AAC TGG AGC TGA TGG AC	CAT CTG CAC AGT CTC TAA ATG G	112 bp	Shan, Z.X., Yu, X.Y., 2002
ANPC-receptor (atrial natriuretic peptide clearance receptor)	gi: 178651	TCC AGG AGG AGG GTT TGC AC	ATT GCG CAC GAT GTC TTC CA	80 bp	Porter, J.G. et al., 1990
Caspase-1 (Interleukin-1 converting enzyme, ICE)	gi: 717039	CCC ACA TCC TCA GGC TCA GA	CAA GAC GTG TGC GGC TTG AC	124 bp	Alnemri, E.S. et al., 1995
Collagen type IV (alpha 1 chain)	gi: 29548	TAA GGT GGA CAT GGG CAG CA	TCG GGA TCC CTT CTC ACC AA	88 bp	Soininen, R. et al., 1987
COX-2 (Cyclooxygease-2)	gi: 181253	AAT TGC TGG CAG GGT TGC TG	CTG CCT GCT CTG GTC AAT GG	85 bp	Hla, T., Neilson, K., 1992
Cyclin A1	gi: 1753108	AGG CAC AGA CCC AAA GCA CA	ACC TCC ACC AGC CAG TCC AC	89 bp	Yang, R. et al., 1997
ELAM-1 (endothelial leukocyte adhesion molecule)	gi: 182047	ATC CAG CCA ATG GGT TCG TG	GAA GGC TCT GGG CTC CCA TT	114 bp	Hession, C. et al., 1990
EMAP-2 (endothelial-monocyte activating polypeptide II)	gi: 498909	TGG CTC CTC CAA ATG GGT CT	AGG CTG GAT CTG CTC CCA AAT	113 bp	Kao, J. et al., 1992
Endothelin-1	gi: 21359861	CCT GCT CGT CCC TGA TGG AT	GGA ACA ACG TGC TCG GGA GT	85 bp	Itoh, Y. et al., 1988
GAPDH (glyceraldehyde-3-phosphate dehydrogenase)	gi: 7669491	GAA GGT GAA GGT CGG AGT C	GAA GAT GGT GAT GGG ATT TC	225 bp	Hanauer, A., Mandel, J.L., 1984
ICAM-1 (intercellular adhesion molecule-1)	gi: 184534	CAG CCA GTG GGC AAG AAC CT	ACA GCT GGC TCC CGT TTC AG	116 bp	Staunton, D.E. et al., 1988
Common human **IL-1ra**	gi: 32577	GAC GAT CTG CCG ACC CTC TG	GAA GGC TTG CAT CTT GCT GGA	48 bp	Eisenberg, S.P. et al., 1990
Transgene human **intracellular IL-1ra**	gi: 1008970	TGT ATG AAG AAG GAG GTG GAG GAG G	GAA GGC TTG CAT CTT GCT GGA	106 bp	Muzio, M. et al., 1995
IL-1ß (Interleukin-1 beta)	gi: 27894305	CTG GAC CTC TGC CCT CTG GA	GGC AGG GAA CCA GCA TCT TC	130 bp	Auron, P.E. et al., 1984
IL-6 (Interleukin-6)	gi: 184628	ATC CTC GAC GGC ATC TCA GC	TTT CTG CCA G TG CCT CTT TGC	85 bp	May, L.T. et al., 1986
IL-8 (Interleukin-8)	gi: 28610153	CAC ACT GCG CCA ACA CAG AA	CCC TCT GCA CCC AGT TTT CC	87 bp	Matsushima et al., 1988)
NOS-3 (nitric oxide synthase)	gi: 189211	CCC GGG ACT TCA TCA ACC AG	CCA CCT CTT GAA GCC GCT GT	81 bp	Janssens, S.P. et al., 1992
Laminin 5 (beta 3 chain)	gi: 2429078	ACC AGT GCC AGC GAG GCT AC	TGG CAT TGC GGA GTC TAC CA	123 bp	Morishima, Y. et al., 1995
MCP-1 (monocyte chemoattractant protein)	gi: 34513	ATG CCC CAG TCA CCT GCT GT	TTG GGA CAC TTG CTG CTG GT	97 bp	Yoshimura, T. et al., 1989

PAI-1 (Plasminogen activator inhibitor 1)	gi: 189541	CCC AGC AGC AGA TTC AAG CA	ATC GCG TCT GTG GTG CTG AT	130 bp	Ginsburg, D. et al., 1986
PBGD (porphobilinogen deaminase)	gi: 35306	TTC ACC ATC GGA GCC ATC TG	CTC TTC GCA GGG AGC TGG TT	130 bp	Raich, N. et al., 1986
PDGF (platelet derived growth factor)	gi: 35371	TGA CGC TGG AAG ACC ACC TG	TCC GAA TGG TCA CCC GAG TT	126 bp	Collins, T. et al., 1985
Prostacyclin Synthase	gi: 537948	TGC TGC ACC TGG AGG AGA TG	AAG GCA GCG GGA CCC ATA TT	100 bp	Miyata, A. et al., 1994
PSGL-1 (P-Selectin glycoprotein ligand 1)	gi: 6031197	TGA AGC CGA GAA AGC CTT GG	TCT GGA GGC TCC GTT TCT GG	105 bp	Sako, D. et al., 1993
RPL-P0 (ribosomal protein, large, P0)	gi: 49087144	CAG ATC CGC ATG TCC CTT CG	CCG GAT ATG AGG CAG CAG TT	125 bp	Rich, B.E., Steitz, J.A., 1987
Thrombin receptor	gi: 339676	TTG ACC GGT TTC TGG CTG TG	AGC CCA GAT GGC CAG ACA AG	92 bp	Vu, T.K. et al., 1991
Thrombomodulin	gi: 339656	CGA TGG CTT CCT CTG CGA GT	TGC CGT AGG TGA TCG AGA CG	98 bp	Wen, D.Z. et al., 1987
Thromboplastin (tissue factor)	gi: 10518499	CTT GGC ACG GGT CTT CTC CT	TGT TGG CTG TCC GAG GTT TG	124 bp	Scarpati, E.M. et al., 1987
Thrombospondin-2	gi: 307505	ACT CGG TGG ACG GTG AGG AG	CCA AGC AGG TGT TGC TGG TG	123 bp	LaBell,T.L. et al., 1993
VCAM-1 (vascular cell adhesion molecule)	gi: 37648	TCG AAC CCA AAC AAA GGC AGA	CAG GAT GGA GGA AGG GCT GA	92 bp	Polte, T. et al., 1990

3.3.7 Western Blot

Antibodies (primary):

- Anti IL-1ra goat anti human antibody (biotinylated) *(R&D Systems [Wiesbaden])*
- Anti ß-Actin mouse anti human antibody *(R&D Systems [Wiesbaden])*

Antibodies (secondary):

- Streptavidin (horseradish peroxidase conjugated) *(Caltag Laboratories [Burlingame, CA])*
- Sheep anti mouse antibody (horseradish peroxidase conjugated) *(Amersham Biosciences [Freiburg])*

Benzamidine (Na_3VO_4) (100x / 0.1 M) in Isopropanol *(Fluka Chemie [Buchs, Switzerland])*

Chemoluminescent developing solution Super Signal West Pico/Dura/Femto Extended Duration Substrate *(Pierce [Rockford, IL])*

DC Protein Assay *(Bio Rad [München])*

Dual Colour Precision Plus Protein Standard *(BioRad [München])*

HCl *(Merck Eurolab [Darmstadt])*

Immobilon-P^{SQ} nitrocellulose/polyvinylidenfluoride transfer membrane (0.45 µm/0.2 µm pore size) *(Amersham-Pharmacia Biotech Europe GmbH [Frankfurt])*

Lysis buffer (5 x):

- 150 mM NaCl *(Merck Eurolab [Darmstadt])*
- 50 mM Tris-HCl (pH 7.5) *(Merck Eurolab [Darmstadt]) (Carl Roth [Karlsruhe])*
- 25 mM EDTA (pH 8.0) *(Sigma-Aldrich Chemie [München])*
- 5% Triton X 100 *(Sigma-Aldrich Chemie [München])*
- Aqua distillate ad 1000 ml

Methanol *(Merck Eurolab [Darmstadt])*

Milk powder *(Carl Roth [Karlsruhe])*

Natriumdodecylsulfate (SDS) *(Carl Roth [Karlsruhe])*

Natriumfluoride (NaF) (10x / 0.5 M) *(Fluka Chemie [Buchs, Switzerland])*

Natriumhydroxide (NaOH) *(Merck Eurolab [Darmstadt])*

Natriumpyrophosphate ($Na_4O_7P_2$) (5x / 0.5 M / pH 7.5) *(Fluka Chemie [Buchs, Switzerland])*

Phenylmethylsulfonylflourid (PMSF) (100x / 0.1 M) in Isopropanol *(Fluka Chemie [Buchs, Schweiz])*

Phosphate buffer (25x): - Dinatriumhydrogenphosphat (Na2HPO4) (0.5 M / pH 8.8)
- Natriumsodiumphosphat (NaH2PO4) (0.5 M / pH 4.4) ad pH 7.0
(both Fluka Chemie [Buchs, Switzerland])

Ponceau S *(Sigma-Aldrich Chemie [München])*

Protease-Inhibitor-Cocktail-Tablet *(Boehringer [Mannheim])*

Protein marker (molecular weight standard) *(BioRad [München])*

Recombinant human IL-1ra (positive control) *(R&D Systems [Wiesbaden])*

Sodium Orthovanadate (100x / 0.1 M) *(Sigma-Aldrich Chemie [München])*

Tween 20 *(Fluka Chemie [Buchs, Switzerland])*

Whatman blotting paper *(BioRad [München])*

X-ray film Reflection NEF-496 *(NEN Du Pont [Bad Homburg])*

Stacking gel:
- 0.26 ml Acrylamide 30%-bisacrylamide 0.8% solution *(Carl Roth [Karlsruhe])*
- 0.505 ml Stacking gel buffer (4 x) (0.5 M Tris/HCl, 0.4% SDS, pH 6.8) *(Merck Eurolab [Darmstadt]) (Carl Roth [Karlsruhe])*
- 1.235 ml H_2O
- 0.01 ml 10% APS (Ammoniumpersulfate) *(Sigma-Aldrich Chemie [München])*
- 0.002 ml TEMED *(Carl Roth [Karlsruhe])*

Resolving gel (12%):
- 1.6 ml Acrylamide 30%-bisacrylamide 0.8% solution *(Carl Roth [Karlsruhe])*
- 1.0 ml Resolving gel buffer (4 x) (0.5 M Tris/HCl, 0.4% SDS, pH 8.8) *(Merck Eurolab [Darmstadt]) (Carl Roth [Karlsruhe])*
- 1.39 ml H_2O
- 0.017 ml 10% APS (Ammoniumpersulfate) *(Sigma-Aldrich Chemie [München])*
- 0.003 ml TEMED *(Carl Roth [Karlsruhe])*

Loading buffer (2 x):
- 20 ml 10% SDS (Sodiumdodecylsulfate) *(Carl Roth [Karlsruhe])*
- 10 ml 15.5% DTT (Dithiothreitol) *(Sigma-Aldrich Chemie [München])*
- 10 ml Glycerol *(Carl Roth [Karlsruhe])*
- 5.0 ml 1 M Tris-HCl buffer (pH 6.8) *(Merck Eurolab [Darmstadt]) (Carl Roth [Karlsruhe])*
- 2.5 ml 1% Bromphenol blue *(Carl Roth [Karlsruhe])*
- 2.5 ml H_2O

Running buffer (10 x):
- 144 g Glycine *(Sigma-Aldrich Chemie [München])*
- 10 g SDS *(Carl Roth [Karlsruhe])*
- 30 g Tris base *(Carl Roth [Karlsruhe])*
- Aqua distillate ad 1.000 ml

Transfer buffer:
- 100 ml Methanol *(Merck Eurolab [Darmstadt])*
- 50 ml Running buffer (10 x)
- 350 ml Aqua distillate

Amido Black Destain solution:
- 10% Methanol *(Merck Eurolab [Darmstadt])*
- 10% Acetic acid *(Carl Roth [Karlsruhe])*
- 80% Aqua distillate

3.3.8 ELISA

Quantikine Immunoassay IL-1ß (human) *(R&D Systems [Wiesbaden])*

Quantikine Immunoassay IL-1ra (human) *(R&D Systems [Wiesbaden])*

3.3.9 FACS

Antibodies (mouse anti human) [10 µg/ml]:
- IgG_1-FITC isotype control (clone MOPC-21) *(Immunotech/BeckmanCoulter [Krefeld])*
- IgG_1-PE isotype control (clone 679.1Mc7) *(Immunotech/BeckmanCoulter [Krefeld])*
- anti-CD45-FITC (clone J.33) *(Immunotech/Beckman Coulter [Krefeld])*
- anti-CD45-PE (clone J.33) *(Immunotech/Beckman Coulter [Krefeld])*
- anti-CD11b (Mac-1)-PE (clone ICRF44) *(BD Biosciences/PharMingen [San Diego, USA])*
- anti-CD54 (ICAM-1)-PE (clone 84H10) *(Immunotech/Beckman Coulter [Krefeld])*
- anti-CD106 (VCAM-1)-PE (clone 51-10C9) *(BD Biosciences PharMingen [San Diego, USA])*
- anti-CD142 (tissue factor)-PE *(BD Biosciences Pharmingen [San Diego, USA])*

FACS buffer:
- 500 ml PBS *(Gibco BRL [Karlsruhe])*
- 5 ml BSA *(Sigma-Aldrich Chemie [München])*
- 2.5 ml Na-azide (20% in PBS) *(Merck Eurolab [Darmstadt])*

Flow Count Beads *(Beckman Coulter [Krefeld])*

Propidiumiodide *(Sigma-Aldrich Chemie [München])*

3.3.10 Functional assays

A-C-D Solution:
- Citric Acid (7.3 g)
- Glucose Monohydrate (24.5 g)
- Tri-Sodium Citrate Dihydrate (22.4 g)
- ad 1000 ml Aqua distillate

Cell Stain Solution *(Chemicon [Temecula, CA])*

Collagenase NB 5 sterile grade *(Serva [Heidelberg])*

Cytokines:
- Monocyte chemoattractant protein (MCP-1) *(R&D [Wiesbaden])*
- Macrophage inflammatory protein (MIP-1α) *(R&D [Wiesbaden])*
- Interleukin-1 (rh IL-1ß) *(R&D [Wiesbaden])*
- Tumour necrosis factor alpha (TNF-α) *(R&D [Wiesbaden])*
- Interleukin-1 receptor antagonist (rh IL-1ra) *(R&D [Wiesbaden])*

Extraction buffer *(Chemicon [Temecula, CA])*

24-well transwell polycarbonate membrane (6.5 mm diameter, 8 µm pore size) *(Costar, Corning Incorporated [Wiesbaden])*

Rhodamine
(Orange CMTMR (5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine)
(Molecular Probes, Invitrogen [Karlsruhe])

Collagen IV (from human placenta) *Sigma-Aldrich Chemie [München]*

Laminin (from human placenta) *(Sigma-Aldrich Chemie [München])*

anti-CD49b antibody (mouse anti human, clone Gi9) *(Immunotech/BeckmanCoulter [Krefeld])*

anti-CD49f (rat anti human, clone GoH3) *Sigma-Aldrich Chemie [München])*

3.4 Other material

0.45 µm cellulose-acetate-filter *(Schleicher & Schuell BioScience [Keene, USA])*

6-, 12-, 24-, 48-, 96-well culture plates *(Nunc [Wiesbaden])*

Cellscraper *(Nunc [Wiesbaden])*

Cloning discs *(Sigma-Aldrich Chemie [Steinheim])*

Cryotubes (1.0 ml, 1.8 ml) *(Costar, Corning Incorporated [Wiesbaden])*

FACS tubes Falcon 2052 Polystyrene, round bottom *(Becton Dickinson [NJ, USA])*

Glass plates (22 mm Ø) *(Kindler [Germany])*

LS columns for Quadro Mac *(Miltenyi Biotec [Bergisch Gladbach])*

Monoject blunt needles *(Sherwood-Davis & Geck [Gosport, UK])*

Optical well plate and Adhesive Cover *(Applied Biosystems [Darmstadt])*

Pipetboy *(Tecnorama [Zürich])*

Pipette tips *(Sarstedt [Nümbrecht])*

Pre-Separation Filters (30 µm pore size) *(Miltenyi Biotec [Bergisch Gladbach])*

Safe-Lock Tubes 1,5ml *(Eppendorf [Hamburg])*

Serological Pipettes *(Costar, Corning Incorporated [Wiesbaden])*

Steritop Filter (0.22 µm) *(Millipore [Schwalbach])*

Syringes (5, 10, 20 ml) *(Becton Dickinson [NJ, USA])*

Tissue culture dishes (100x20 mm, 145x20 mm) *(Cellstar, Greiner bio-one [Frickenhausen])*

Tissue culture dishes (35x10 mm, 60x15 mm, 92x17 mm) *(Nunc [Wiesbaden])*

Tissue culture flasks (50, 100, 250 ml) *(Cellstar, Greiner bio-one [Frickenhausen])*

Tubes (15 ml, 50 ml) *(Nunc [Wiesbaden])*

3.5 Laboratory equipment

ABI Prism 7700 Sequence Detector *(Applied Biosystems [Darmstadt])*

Centrifuges: - Megafuge 3.0 RS *(Kendro [Osterode])*

- Biofuge fresco *(Heraeus [Hanau])*

ELx800 Universal Microplate Reader *(Bio-Tek Instruments [Winooski, USA])*

EPICS XL flow cytometer *(Beckman Coulter [Hialeah, FL])*

Kodak Image Station *(Kodak digital science [Stuttgart])*

Microscope (Olympus CK 40) *(Olympus [Hamburg])*

Microscope (Zeiss AxioVert 25) *(Zeiss [Jena])*

Mini-PROTEAN 3 Electrophoresis chamber *(BioRad [München])*

Neubauer cell count chamber *(LO-Laboroptik GmbH [Friedrichsdorf])*

Flow chamber device w/ camera *(Zeiss [Jena])*

Perfusor Secura FT *(Braun [Germany])*

Pipettes *(Eppendorf [Hamburg])*

Power Pac 200 mains receiver *(BioRad [München])*

Quadro Macs Separation Magnet *(Miltenyi Biotec [Bergisch Gladbach])*

Shaker Polymax 1040 *(Heidolph [Schwabach])*

Sterile laminar flow workbench Steril-Antares *(Biohit [Italy])*

Thermomixer comfort *(Eppendorf [Hamburg])*

Trans-Blot SD Semi-Dry Transfer System *(BioRad [München])*

Ultra-spec Plus (spectral photometer) *(Amersham-Pharmacia Biotech Europe GmbH [Frankfurt])*

Incubator IR Senor *(Sanyo [München])*

3.6 Software

Microsoft Word, Excel, PowerPoint (2003) for Windows XP

EndNote 6.0

Primer 3 programme

McMolly Sequence Comparison

Summit III/IV FACS Analysis

Cricket Graph

SPSS 12.0

Adobe Photoshop 7.0

Applied Biosystems Sequence Detection

BioTek Instruments Microplate 96

4 Methods

4.1 Isolation of CD34+ Cells

Heparinized human umbilical cord blood (25 I.E. heparin/ml) (obtained from healthy newborn donors after informed parental consent) was mixed 1:1 with PBS (containing 2 mM EDTA) and 30 ml each were subjected to density gradient centrifugation on 15 ml Ficoll-Hypaque for 20 minutes at 2.100 rpm at 20°C without brake. Cell clumps were removed in advance by passing cells through a 30 µm pore size nylon filter After the upper layer was aspirated and discarded, the interphase containing the mononuclear cells was removed, washed with 50 ml PBS (containing 2 mM EDTA) and centrifuged for 10 min at 1.600 rpm. The pellet was resuspended in HF/2+ buffer at a concentration of 10^8 cells/ 300 µl and $CD34^+$ cells were recovered thereof by immunomagnetic separation. In detail, after mixing with 100µl FcR blocking reagent per 10^8 cells, the suspension was incubated for 30 min at 4°C under slight rotation in a ratio of 1:1 with anti-CD34-antibody-linked immunomagnetic microbeads. Cells were washed, filtered through a 30 µm pore size filter, and resuspended in $HF/2^+$ buffer at a concentration of max. $2x10^8$ cells/ml. $CD34^+$ cells were isolated by double flow through a magnetic column according to a protocol described by Murohara et al. (Murohara et al., 2000).

4.2 Differentiation and expansion of Endothelial Progenitor Cells

CD34+ cord blood (CB)-derived EPC were cultured using a protocol as described by Gehling et al. (Gehling et al., 2000) with slight modifications.

In detail, cord blood-derived $CD34^+$ cells of 2-3 donors were pooled and seeded on 1% gelatine- or 10 µg/cm^2 fibronectin- precoated plastic plates at a density of 10^6 cells per 10 cm^2 and cultured in IMDM with each 10% horse serum and fetal calf serum supplemented with penicillin/streptomycin, 50 ng/ml recombinant human stem cell factor (SCF), 50 ng/ml Vascular Endothelial Growth Factor (VEGF), 20 ng/ml Fibroblast Growth Factor-2 (FGF-2) and 100 ng/ml Stem cell growth factor (SCGF-β). Cells were grown at 37°C and 5% CO_2 in an incubator removing non-adherent cells and replacing medium with growth factors every 2 days. Culture plates were scored for the presence of endothelial cell colonies starting 5 days after onset of culture. With the appearance of adherent endothelial cell clusters at week 1-2 of culture cells were adapted to Endothelial cell basal medium (EBM-2) with 2% FCS and growth factor supplement (EGM-2) containing VEGF, EGF, FGF-2, R3-IGF-1, hydrocortisone, ascorbic acid, heparin, gentamycin and amphotericin B. Every 48 h $^2/_3$ of the medium was sucked off and replaced by fresh EBM-2. Reaching 70% confluence, cord blood derived endothelial cells (CBEC) were passaged by trypsin/EDTA dispersion. Cells were reseeded at a density of 0.8 $x10^3$ cells/cm^2 throughout the whole culture period and cultured until senescence. Cumulative growth curves were generated for 5 different pools of CBEC.

For long-time storage, CBEC were resuspended in freezing medium (DMEM with 50% heat-inactivated FCS and 10% DMSO) and stored in cryovials at -196°C in liquid nitrogen. For thawing, cells were warmed up at 37°C in a water bath, diluted at a ratio of 10:1 with EBM-2 containing 10% FCS and then centrifuged at 218 x g at 4°C for 10 min. The pellet was resuspended in low-serum EBM-2 with supplements and cells were seeded at a density of $0.8x10^3$ cells/cm^2 onto precoated plastic plates.

4.3 Transformation and amplification of retroviral vectors

The 578 b sequence for the long splice form of intracellular Interleukin-1 receptor antagonist including a 32 b signal sequence (gene bank ID: gi 1008970) was inserted between the restriction sites EcoR 1 and BamH 1 into the multiple cloning site of the retroviral vector pLXSN 3' of the internal Moloney murine leukaemia virus (MoMuLV) promoter. pLXSN additionally contains the retrovirus packaging sequence ψ^+ and a simian virus (SV40) early promoter controlled neomycin resistance gene (neo^r).

Transformation of vector constructs and propagation/amplification of transformed E.coli bacteria was performed using the following protocol. DH5α Chemically Competent E.coli (50 µl) were thawed on ice and mixed with 2µg of pLXSN-IL-1ra and pLXSN empty-vector plasmid DNA respectively. After 20 min incubation on ice cells were exposed to heat shock at 42°C for 30 sec in a thermomixer followed by 2 min cooling at 0°C. Afterwards, 100 µl LB-medium were added and the reaction was shaked at 37°C and 225 rpm for 1 hour. Various dilutions of this bacteria suspension in LB-medium (1:1 – 1:10) were prepared and 100 µl each were spreaded on LB-agar-plates containing 100 µg/ml ampicillin followed by overnight incubation at 37°C. Then single clones were picked from the agar-plates, transferred into 5 ml LB-medium containing 100 µg/ml ampicillin and multiplied at 37°C with vigorous shaking (~240 rpm) for approximately 6-8 h. Cells were harvested by centrifugation at 3000 x g for 15 min at 4°C and plasmid DNA was isolated by alkaline lysis, DNA-binding silica-matrix columns and isopropanol precipitation using the QIAprep Spin Miniprep Kit. Yield and purity of isolated DNA was determined by photometric measurement in a spectral photometer at 260/280 nm wavelength.

In order to examine integrity and completeness of vector and insert restriction digest of pLXSN-IL-1ra and pLXSN-empty vector was performed. 5 µg plasmid-DNA were mixed with 1 µl of each restriction enzyme, 1.5 µl buffer (adapted to the enzymes) and 6.5 µl aqua dist., incubated at 37°C for 3 h and then cooled down at -20°C overnight. Produced fragments were separated on a 0.8% agarose gel containing 10 µg/ml ethidiumbromide with TAE-buffer at 2-10 volts per cm gel length for 3 h and gels were photographed under UV-illumination (254 nm wavelength). Size of restriction products was determined by comparison to size markers with defined DNA fragments which were loaded onto the same gel. pLXSN empty-vector was cut with Hind III and Sac I as indicated by the manufacturer. Restriction digest of pLXSN-IL-1ra with EcoR 1 and BamH 1 aimed to obtain the 578 b fragment of the inserted sequence for ic-IL-1ra and a second 5.9 kb fragment of intact cut vector. Intactness of the vector-insert was proved in a sequencing reaction on an ABI 3700 sequencer by GATC-Biotech AG with the above-mentioned

sequencing primers for pLXSN.

E.coli clones with confirmed correct sequences of pLXSN and pLXSN-IL-1ra underwent further amplification by inoculation of 200 ml ampicillin-containing LB-medium with 200 µl bacteria suspension of the preculture and incubation for 48 - 62 h at 29°C while shaking at 200 rpm. Plasmid-DNA was isolated thereof with the Plasmid Maxi kit according to the manufacturer's instructions.

4.4 Transfection and retrovirus production

NIH 3T3 mouse fibroblast derived dualtropic PT67 packaging cells containing the MoMuLV gag, pol and env genes were grown on 60/100 mm culture plates in DMEM supplemented with 10% FCS, 2% glutamine, 1% penicillin/streptomycin, and 1% sodium pyruvate. Prior to transfection experiments optimal concentration of antibiotic selection agent (kill curve) and optimal plating density was determined following BD Biosciences protocol PT3132-1. Reaching 80% confluence cells were stably transfected with the retroviral constructs pLXSN-IL-1ra and empty vector pLXSN respectively using either Calcium Phosphate (Pham et al., 2001) or Lipofectamine/Lipofectamine Plus-reagent. The Calcium Phosphate Transfection System is based on coprecipitation of vector DNA with both calcium phosphate crystals and a carrier DNA followed by adherence of the complexes to cell membranes and entering into the cytoplasm of target cells by phagocytosis. Lipofectamine as a cationic lipid reagent forms unilamellar liposomes with a positively charged surface which is attracted electrostatically both to the phosphate backbone of the DNA and the negatively charged cell membrane surface. Nucleic acid delivery is then effected through endosomes and lysosomes.

After 2h pre-treatment with 50 µM Chloroquine diphosphate (Pear et al., 1993) transfection was performed according to the manufacturer's instructions. Subsequent to the transfection procedure fresh medium was added and cells were allowed to grow for further 24 hours before stably transfected packaging cells were isolated by antibiotic selection with neomycin (G-418) at a concentration of 800 µg/ml for at least 2 weeks of culture. Stably transfected producer cells were collected and frozen at -80°C until use for retrovirus production.

4.5 Retroviral transduction of cord blood-derived endothelial cells

Transductions were performed with cord blood-derived EPC from 5 different donors based on a DEAE-Dextran transduction protocol by Dichek (Dichek, 1991) and Kahn (Kahn et al., 1992) with modifications. Prior to transduction experiments optimal concentration of antibiotic selection agent (kill curve) and optimal plating density were determined in accordance with the proceedings for retroviral producer cells. All transductions were carried out within 21 to 30 days after seeding of $CD34^+$ cells and 6 to 11 days after appearance of endothelial cell clusters respectively.

In detail, target cells were plated 24h prior to infection at a density of $1x10^5$ cells per 100 mm culture dish. 2h before transfection medium was replaced by serum-free EBM-2 containing Chloroquine diphosphate (100µM). Retroviral supernatant was harvested each 24h after replacement of culture medium from confluent producer cells. Virus-containing culture medium was subjected to chondroitinase treatment (1 h, 37°C, 0.2 U of chondroitinase ABC per ml in 0.01% bovine serum albumin (BSA)) in order to remove chondroitinsulfate proteoglycanes from the virus surface and filtered through a 0,45 µm pore size cellulose acetate filter. After medium was removed from the endothelium, cells were washed once with PBS and incubated with 10 ml of a DEAE-Dextran solution in serum-free DMEM (1mg/ml) for 1 minute. Then endothelial cells were washed three times with 3 x 10ml of PBS and thereafter incubated with 8 ml of freshly produced retroviral supernatant containing 50 ng/ml VEGF and 20 ng/ml FGF-2 in a 32°C CO_2-incubator (Bunnell et al., 1995; Kaptein et al., 1997) at a multiplicity of infection (MOI) of 80. After 8 h, retrovirus-containing supernatant was replaced by normal EBM-2. Transduction was performed twice in an interval of 24 hours (Morgan et al., 1995). Two days after completion of the transduction process endothelial cells were subjected to antibiotic selection with 400µg/ml neomycin (G-418) which was kept throughout the whole expansion period. According to parental CBEC transduced endothelial cells were cultured until senescence and cumulative growth curves were generated for all 5 pools.

4.6 Detection and quantification of transgenic IL-1ra expression

4.6.1 Real-time-PCR

CBEC were grown until confluence and supplied with fresh medium 24h before total RNA was isolated. Cells were lysed using Trizol Reagent (containing phenol and guanidine isothiocyanate), lysates were collected and incubated for 5 min at 30°C before 1 ml each were transferred into RNase-free tubes and vigorously mixed with 0.2 ml of chloroform for 15 sec. After a second incubation step at 30°C for 3 min samples were centrifuged at 11.000 g for 15 min at 4°C. The emerging colourless upper aqueous phase (containing the RNA) was transferred into fresh tubes and the RNA was precipitated by mixing with each 0.5 ml isopropyl alcohol. After a third incubation step at 30°C for 10 min RNA precipitates were centrifuged down at 11.000 g for 10 min at 4°C and supernatants were removed. RNA pellets were washed once by addition of 1 ml ethanol (75%) with subsequent centrifugation at 7.000 g for 5 min at 4°C. The pellets were allowed to air-dry for 5 min and RNA was redissolved in 20 µl RNase-free water. Residual DNA was removed by incubation with RNAse-free DNAse I. RNA concentration of the samples was determined by measuring the OD at 260/280 nm with a spectrophotometer.

Reverse Transcription of extracted RNA was performed using Superscript Reverse Transcriptase kit as described in the manufacturer's instructions. Yielded cDNA was used for amplification in Real-time-PCR after concentrations of the samples were determined by measuring the OD at 260/280 nm. Primers were diluted to a final concentration of 10 µM. Mastermix was prepared on ice, containing each 0.225 µl of sense- and antisense-primer with

11.25 µl Sybr Green (containing 10x PCR Buffer Minus Mg2+, 10mM dNTP mixture, 50 µM MgCl2, 2.5 units Taq DNA Polymerase) and 8.3 µl molecular water for 1 sample. Then 20 µl Mastermix and 2.5 µl cDNA [40-372 ng/µl] each were transferred into single wells of an optical 96-well plate and covered by an adhesive film followed by a short centrifugation. All samples were measured in triplicates.

PCR was performed in an ABI Prism 7700 Sequence Detector under the following conditions:

	pre-treatment	denaturation	annealing	extension	post-treatment	cycles
temperature	1. 50°C / 2. 95°C	95°C	60°C	72°C	72°C	40x
duration	1. 2 min / 2. 10 min	15 sec	60 sec	0 sec	10 min	

Intensity of Sybr Green fluorescence, which is proportional to the amount of amplificated PCR product during the exponential amplification phase, was detected in every cycle after the extension phase. A threshold value of signal intensity was set in the exponential phase of all signal curves and C_T - values of each sample were measured. C_T - values (crossing points) are defined as the cycle at which the regression line through the linear segment of the logarithmic signal curve (corresponds to the phase of exponential increment) intersects the threshold value of the fluorescence intensity. C_T - values show linear proportionality to the logarithm of the initial amount of template DNA. In order to achieve semiquantitative determination of gene expression levels, C_T - values of every single gene were compared to C_T - values of the housekeeping genes GAPDH (Glyceraldehydphosphatdehydrogenase), PBGD (Porphobilinogendesaminase) or RPL-P0 respectively:

$\mathbf{Ct}_{\text{Test}} - \mathbf{Ct}_{\text{housekeeping gene}} = \mathbf{x} \quad \rightarrow \quad {}^{1}\!/\!_{2^x} =$ relative gene expression level compared to housekeeping genes (Heid et al., 1996).

4.6.2 Western Blot

For Western Blot analysis, approximately $6x10^6$ CBEC were grown to near confluence on culture dishes. Cells were thoroughly detached with 1 ml PBS comprising 10 mM EDTA using cell scrapers. Cell suspensions were collected, centrifuged at 1.800 rpm at 4°C for 5 min and supernatants were discarded. 10 ml of Lysis buffer was prepared on ice immediately before use by mixing 2 ml of 5x Lysis buffer, 2 ml Natriumpyrophosphate (5x), 1 ml Natriumfluoride (10x), 0.4 ml Phosphate buffer (25x), 0.1 ml of each Benzamidine (100x), Sodium Orthovanadate (100x) and PMSF (100x), 1 Protease Inhibitor Cocktail Tablet and 4.4 ml Aqua distillate. Pellets were resuspended in 50 µl Lysis buffer and incubated on ice for 20 min with intermittent mixing. The homogenized samples were clarified by centrifugation at 14.000 rpm at 4°C for 20 min and the resulting supernatants were collected. Included proteins were quantified by the method of Lowry as described previously (Lowry et al., 1951). Protein lysates were aliquoted and stored at -80°C until further analysis.

Protein separation was carried out by denaturing SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (Laemmli, 1979). According to the molecular weight of IL-1ra (21 kDa) and ß-Actin (42 kDa), resolving gels with an acrylamide concentration of 12% were used. Stacking and resolving gels were composed as indicated in the section Materials. Protein

samples of 40 µg each and a concentration of no fewer than 1.8 µg/µl were mixed with 2x or 5x loading buffer and heated to 95°C for 5 min in order to reduce and denaturate the proteins. Then protein samples (25 µl) and rhIL-1ra positive control (15 ng) were applied to the gel and electrophoresis was carried out at 100-120 V for 1.5 to 2.5 hours. Molecular weight of the separated proteins was determined by comparison with a fluorescent molecular weight standard.

Using a trans-blot semidry system, the separated proteins were electrophoretically transferred to a polyvinylidenfluoride (PVDF) membrane as described by Towbin et al. (Towbin et al., 1979). Blotting was carried out for 1-2 h at 100 mA and 25 V. Even transference of the proteins was confirmed by staining of the membrane with Ponceau S. The blotted membranes were shaked for 1h at RT in PBS-Tween (0.1%) containing 10% milk powder in order to saturate unspecific binding sites. Subsequently, incubation with primary anti-IL-1ra antibody (200 ng/ml in PBS containing 0.1% BSA; 2h at RT or 12h at 4°C) and peroxidase-conjugated secondary antibody (1:10.000 in PBS containing 10% milk powder; 1h at RT) was carried out. Protein bands were visualized by a chemoluminescence reaction of a developing solution containing the enzyme substrate luminol and stable peroxide solution. Luminol is oxidized following the peroxidase-mediated decomposition of H_2O_2 resulting in the release of light (428 nm) which was photographed in an image station. Afterwards, membrane bound secondary antibodies were detached (stripped) by incubation with Amido Black Destain. Following another blocking step membranes were incubated with anti ß-Actin antibody and processed as described above. ß-Actin is integral part of the cytoskeleton of all cells and serves as a marker for total protein load of every sample on the membrane. Independent of the activation state, detection of ß-Actin thus constitutes an internal control for even protein load of all samples.

4.6.3 ELISA

Production of human Interleukin-1 receptor antagonist and Interleukin-1ß was quantified by determination of intracellular and secreted cytokine levels.

Cell culture supernates were collected from confluent endothelial cell monolayers growing for 24 h in fresh endothelial cell basal medium (EBM-2). After removal of particulates by centrifugation, samples were aliquoted and stored at -20°C until measurement.

For quantification of intracellular IL-1ra, $4x10^5$ cells of subconfluent monolayers each were lysed in 50 µl lysis buffer and further processed as described in section 4.6.2. Protein lysates were stored at -80°C and diluted with Calibrator Diluent RD5 at a ratio of 1:8 before use.

The ELISA is a quantitative sandwich enzyme immunoassay with a specific monoclonal antibody for IL-1ra or IL-1ß respectively, which is precoated onto a microplate. All standards and samples were assayed in duplicate strictly following the manufacturer's instructions. The optical density of the emerging dyed solutions was measured at 450 nm with a wavelength correction set to 540 nm using a microplate reader. The duplicate readings for each standard, control, and sample were averaged, standard curves were generated, and sample concentrations of IL-1ra and IL-1ß, respectively, were determined. Minimum detectable dose was approximately 14 pg/ml for IL-1ra and 2 pg/ml for IL-1ß, as stated by the manufacturer.

4.7 Functional assays

4.7.1 Activation of Endothelial Cells

Basal expression of Tissue Factor and PDGF was ascertained during the course of endothelial cell expansion. Therefore, RNA was isolated and semiquantitative real-time PCR was performed 3 to 4 times between day 30 and day 110 of endothelial cell culture.

In order to determine the effect of transgenicly produced Interleukin-1 receptor antagonist on endothelial cell activation by Interleukin-1ß and TNF-α CBECs at passages 2 through 5 – parental, empty-vector and IL-1ra-transduced – were subjected to stimulation experiments. Cells were seeded onto culture dishes in 8 ml EBM-2 (containing 1% FCS) and grown to near confluence over 24 h. After this time, IL-1ß and TNF-α, respectively, were added to the medium in a final concentration of 1 ng/ml. In parallel, a part of the cells remained unstimulated aiming to acquire basal levels of gene expression. After 4h of stimulation at 37°C both control and treated cells were collected in Trizol reagent as aforementioned and stored therein at -80°C until further analysis. RNA isolation, reverse transcription, and real time PCR were performed as previously described. RNA was examined for transcripts of decisive genes in vascular physiology and hemostasis, endothelial-leukocyte interaction, signal transduction and extracellular matrix composition.

4.7.2 Rolling of peripheral blood mononuclear cells on endothelial monolayers

In order to study the role of transgenic endothelial IL-1ra expression rolling adhesion of whole blood peripheral mononuclear cells (PBMC) was assayed in a parallel flow chamber system as described previously (Kuijpers et al., 2000; Lawrence et al., 1994; Luscinskas et al., 1994; Renard et al., 2003). In brief, empty-vector and IL-1ra transduced CBEC cultured less than 4 weeks were seeded onto round glass plates and grown to near confluence in EBM-2 (containing 1% FCS). Cells were subjected to 6 h of prestimulation with Interleukin-1ß at a concentration of 0.2 ng/ml. 30 min before completion of the prestimulation 30 ml blood taken from healthy volunteers was heparinized (20 U/ml), gently mixed with rhodamine (at a final concentration of 5 µM) and - while incubating - prewarmed to 37°C in a water bath. After integrity of CBEC monolayers was checked under a light microscope the glass dishes were immediately placed and fixed in the flow chamber device. The blood-containing syringe was then inserted into a perfusor and the entire flow chamber was placed under a fluorescence microscope connected to a camera. Flow of rhodamine-stained, heparinized whole blood at 20 ml/h and 37°C was started, resulting in defined shear forces by means of a parallel flow of 10 dyne/cm^2, which comes near to the range of wall shear stress in medium-sized human venous vessels (Renard et al., 2003). Laminar flow was maintained for 10 min followed by another 5 min of flow with prewarmed PBS to rinse off non-adherent blood cells. Finally, digital photos were taken by light fluorescence colour photography of 4-6 arbitrarily chosen fields of the endothelial monolayer of 1 mm^2 each using a total magnification of 400 x. Again the analysed fields were checked for endothelial monolayer integrity before adherent leucocytes were counted on the digital photographs.

4.7.3 Adhesion of monocytic cells on endothelium

Adhesion assays were conducted aiming to examine the effect of transgenic endothelial IL-1ra production on adhesion properties towards monocytic cells. Following Taekema-Roelvink et al. (Taekema-Roelvink et al., 2001) adhesion assays were adjusted to our needs and performed as described below.

In detail, parental, empty-vector and IL-1ra-transduced CBECs at passages 2 through 6 were seeded onto 24-well plates at a final density of $8x10^3$ cells per cm^2. Seeding of cells was followed by a centrifugation step at 100 x g for 2 min at 37°C to ensure even sticking on the growth surface. CBECs were allowed to reach confluence during overnight incubation. Mono-Mac-6 human monocytic cell starvation was started one day prior to experiments by culturing in low-serum RPMI-1640 (containing 1% glutamine, 1% penicillin/streptomycin and 1% FCS). After 24 h, Interleukin-1ß was added to the wells of the 24-well culture plates, resulting in increasing concentrations in the range of 0.3 to 5.0 ng IL-1ß per ml culture medium. CBECs were stimulated in that differently conditioned medium for 4.5 h. In some experiments, recombinant human IL-1ra (100 ng/ml) was added to the cells 0.5 h prior to stimulation with IL-1ß. Subsequently, conditioned medium was removed and $5.0x10^5$ Mono-Mac-6 cells (resuspended in low-serum RPMI-1640) were layered onto the CBECs of every single well. Monocytes were centrifuged down at 100 x g and 37°C for 2 min and allowed to be in contact with the endothelial monolayer for 30 min. Then medium was removed from all wells followed by 3 washes and gentle sweeping with prewarmed RPMI-1640. Persistently adherent cells were finally harvested by addition of 200 µl Collagenase (1 mg/ml in PBS) per well and rinsing with 200 µl of RPMI-1640 (containing 10% FCS). Cells were washed once with 0.5 ml of FACS-buffer and then resuspended in 0.2 ml thereof. Afterwards, all samples were mixed with 8 µl of FITC-conjugated anti-CD45 antibody and incubated at 4°C for 15 min, followed by 2 washes with 1 ml FACS buffer each, centrifugation and resuspension of the pellet in 0.5 ml FACS buffer. All washing and staining steps were performed in the presence of A-C-D Solution (added to a final concentration of 0.1%) in order to prohibit formation of cell clusters. For control measurements, additional $1.5x10^6$ monocytes were washed with 2 ml of FACS buffer, centrifuged down, and resuspended in 600µl FACS buffer. One third hereof was used as undyed blank value; the rest of the cells were stained with FITC-conjugated IgG_1 isotype control and FITC-conjugated anti-CD45 antibody, respectively, in order to serve as negative and positive control.

Proceeding to flow cytometry, blank value and controls were measured first in order to adjust instrument settings. Immediately before determination of specimen fluorescence intensity 50µl FACS bead suspension (correspondent to 50.000 beads) was added to every sample. As the CD45 antigen – a monocytic marker protein – is only expressed by Mono-Mac-6 but not by endothelial cells, fluorescence intensity of the samples is proportional to the number of monocytic cells in every single experiment. Every single measurement was carried out until registration of 10.000 FACS beads was completed and each sample was analyzed in duplicate. Total and relative number of adherent monocytes on endothelium was calculated from the ratio of CD45-FITC counts to FACS bead counts in relation to CBECs count.

4.7.4 Transendothelial migration of monocytic cells

In transmigration assays chemoattracted monocytes were allowed to pass through differently prestimulated endothelial cell monolayers on 8 µm pore size polycarbonate membranes.

The transmigration assay was build up following other investigators (D'Amico et al., 1998; Taekema-Roelvink et al., 2001; Weber et al., 1999) but was modified in several points as described in the following. We used collagen-coated transwell membranes in order to achieve proper adherence of CBEC. Therefore, a 0.3% solution of collagen in 0.1% acetic acid was diluted 1:1 with an equal volume of absolute ethanol and 30 µl thereof were added to each insert of the transwell plates followed by drying overnight. Monocytes were grown in serum-free RPMI-1640 for 12 h before use in transmigration experiments.

After filling the lower chambers with 500 µl EBM-2 each, transwell inserts were placed into the wells and $1x10^5$ CBEC in 200 µl EBM-2 containing 1% FCS were seeded into every collagen-coated transwell insert (6.5 mm diameter). Endothelial cells were allowed to grow to confluence within 24h. Afterwards, progressional amounts of IL-1ß (ranging from 0.0 to 5.0 ng/ml) were added to the CBECs in the upper chamber and endothelial prestimulation was performed for 4h. To ensure that these treatments did not change the confluence of the endothelial monolayer, medium of two transwell inserts per plate was sucked off and cells were dyed by incubation with 100 µl Cell Stain Solution (CSS) for 25 min. After removal of CSS and triple washing with aqua distillate, integrity of the endothelial monolayer was determined by microscopic examination.

After completion of endothelial cell prestimulation medium from the upper and lower chambers was sucked off and the lower chambers were filled with 500 µl RPMI-1640 (containing 5 % FCS, 100 ng/ml MCP-1 and 20 ng/ml MIP-1α) each. The upper chambers were rinsed with 200 µl EBM-2 and then filled with $3.0x10^6$ Mono-Mac-6 in a total volume of 200 µl serum-free RPMI-1640 each. Transmigration of monocytes through endothelial monolayers was allowed to proceed for 24 h. After this time, transwell inserts were lifted and the undersurface was rinsed several times with medium of the bottom chamber in order to recover cells eventually bound to the underside of the membrane. In this manner, cells that had migrated to the lower chamber were combined with those detached from the filter. Cells were washed once with 0.5 ml of FACS-buffer and then resuspended in 0.2 ml thereof. Staining, FACS analysis (including flow beads) and control measurements were carried out as described in the previous section. Total and relative number of transmigrated monocytes was calculated from the ratio of CD45-FITC counts to FACS bead counts.

4.7.5 Endothel – Monocyte Coculture

Focussing attention on surface adhesion molecules and endogenous IL-1ß secretion by CBEC during direct contact with monocytes, a model of endothelial-monocytic coculture was established and expression of ICAM-1 and VCAM-1 and Tissue Factor was determined by FACS analysis. Secreted IL-1ß in coculture supernatants was quantified by ELISA.

In detail, empty-vector and IL-1ra-transduced CBEC at passages 2 through 6 were seeded into 6-well culture plates at a density of $1.7x10^5$ cells. Additional CBEC (pLXSN and IL-1ra

transduced, respectively) were seeded simultaneously in order to obtain basal (unstimulated) protein expression levels. Cells were grown to 80% confluence over 40h. Monocytes were adapted to low-serum medium (RPMI-1640 with 1% FCS) and starved for 24h before onset of coculture. Six hours of endothelial-monocytic coculture were started by addition of $1.3x10^6$ monocytes to each well. Half of the series of experiments were additionally stimulated by addition of IL-1ß (0.5 ng/ml) for ½h prior to the coculture. After the incubation time supernatants were gently removed from all wells and stored at -80°C until use for detection of secreted IL-1ß by ELISA. Cells of all samples were detached by trypsin/EDTA dispersion and rinsing with RPMI-1640 containing 10% FCS. After washing twice in 2 ml cold FACS-buffer cell mixtures were each resuspended in 400µl thereof.

For control measurements and instrument adjustments $2.5x10^6$ monocytes were washed with 2 ml of FACS buffer, centrifuged down, and resuspended in 1 ml of FACS buffer. Cells were distributed equally into 5 FACS tubes, one of whom was used as blank value. The others were stained with FITC- or PE-conjugated IgG_1-antibodies and FITC- or PE-conjugated anti-CD45 antibodies serving as negative and positive controls for FITC and PE staining, respectively.

For quantification of basal cytokine expression levels of monocytes and endothelial cells, both separately cultured CBEC and Mono-Mac-6 were harvested, counted, washed twice, and resuspended in FACS buffer. Afterwards, CBEC-pLXSN and CBEC-IL-1ra were each mixed with the 10-fold number of monocytes, resulting in a total volume of 1.6 ml of endothel-monocyte suspension which was used for four independent measurements.

All samples of unstimulated, cocultivated and additionally IL-1ß-stimulated cell mixtures were then incubated for 15 min at 4°C with 8 µl of two antibodies each; FITC-conjugated anti-CD45 antibody (in order to detect monocytic cells) and at the same time either PE-conjugated anti-CD54, anti-CD106 or anti-CD142 antibody (to quantify endothelial and monocytic surface expression of the indicated proteins). Staining was finished by addition of 1 ml FACS buffer, centrifugation, and a second washing with another 1 ml of FACS buffer followed by resuspension of every sample in 0.5 ml thereof. Immediately before measurement 1 µl Propidiumiodide [1mg/ml] was mixed into every sample aiming to preclude mortified cells from sample fluorescence detection.

Following measurement of blank values, positive and negative controls, and adjustment of instrument settings basal, coculture-activated and IL-1ß-induced surface marker expression on endothelial and monocytic cells was determined by FACS analysis.

4.8 Statistical analysis

All experiments were performed at least in triplicate and differences were analyzed by unpaired Student's t-test or ANOVA as appropriate. Significance level was set $p< 0.05$ in the two-tailed test. Values in text, tables and figures are given as average (mean) with standard error of the mean (SEM) unless indicated otherwise.

5 Results

5.1 Differentiation and expansion of cord blood derived Endothelial Progenitor Cells

Using the aforementioned protocol, cord blood derived $CD34^+$ cells of all 5 donor pools could be differentiated into endothelial cells. Purity of initially recovered CD34+ cells ranged between 85 and 99% as assessed by flow cytometry. Outgrowth of colonies of adherent spindle-shaped cells was observed between day 7 and 14 of culture. Phenotypic characterization and immunocytological proof of endothelial cell surface markers was performed previously by our group and confirmed the endothelial characteristics of the obtained cells (Ott et al., 2005b).

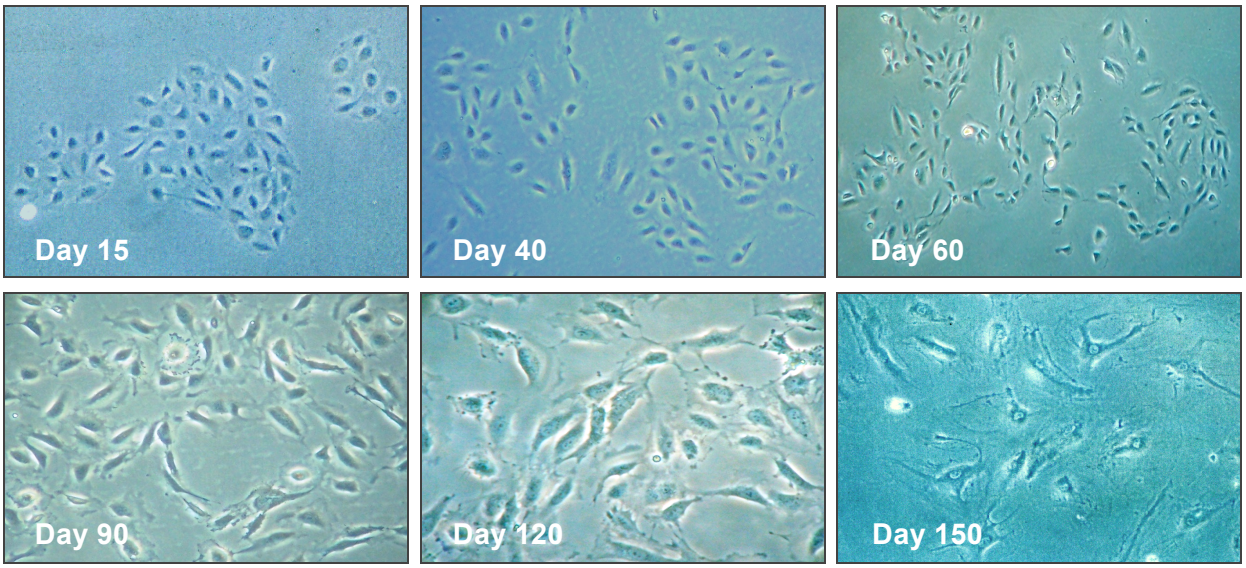


Figure 1 Outgrowth of Cord Blood-derived Endothelial Progenitor Cells.
After enrichment of CD34+ cells from cord blood endothelial progenitors were differentiated into endothelial cells. First clusters of adherent spindle-shaped endothelial cells emerged after 1 to 2 weeks of culture (top left). Cells were grown in endothelial cell basal medium with the addition of growth factors and expanded until senescence. Pictures show morphological changes during endothelial cell expansion and senescence.

As shown in **figure 1**, cord blood-derived endothelial cells (CBEC) underwent distinct morphological changes from the emergence of first progenitor cell colonies (day 15) over the period of the fastest growth of endothelial cells in the first two to three months of culture to the time of proliferation deceleration at the end of CBEC life span (day 150). Senescent CBEC are shown in **figure 2**. Eventually, they remarkably increase in size, show irregular cell borders, accumulate cell detritus, and develop nuclear polymorphisms.

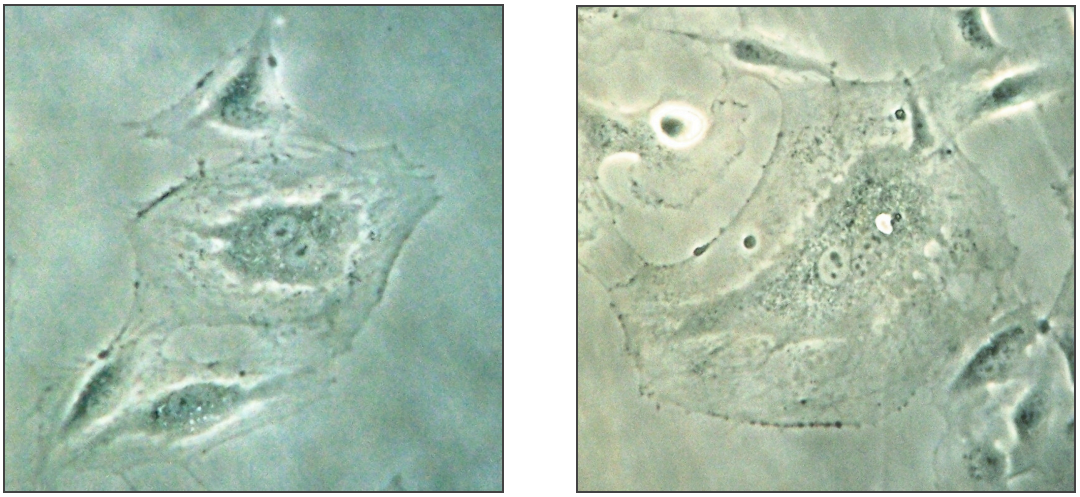


Figure 2 Endothelial cell morphology and senescence.

CBEC grow as monolayers of spindle-shaped flat cells with diameters up to 50 µm. Senescent endothelial cells increase in size, flatten, and spread on their growth surface. To an increasing degree they contain granules with cell detritus which are transported to the cell membrane. Furthermore, senescent endothelial cells progressively show nuclear polymorphism, disturbed nucleo-plasmic ratio, and eventually karyopyknosis.

The number of endothelial cell colonies emerging from 10^6 $CD34^+$ cells ranged from 8 to 14 (mean ± SD: 11.4 ± 2.2), implying that only about 0,0012% of the $CD34^+$ cells differentiated into endothelial cells. Passaged between 16 and 32 times (mean ± SD: 23.6 ± 5.7), CBEC from 5 donor pools were cultured continuously until senescence, resulting in an average life span of over 4 months (mean ± SEM: 127.2 ± 14.8 days) with one pool of cells growing over 6 months.

With an input of 10^6 $CD34^+$ cells and averagely 56.5 ± 1.4 population doublings (calculated from the number of CFU-EC) CBEC could be expanded 10^{16}-fold to a mean number of $8.4x10^{20}$ ($7.0x10^{15}$ - $3.9x10^{21}$) cells. During the first 60 days of culture CBEC doubling time ranged between 34.4 and 47.4 h (mean ± SEM: 41.6 ± 2.8 h). However, after the first two months growth decelerated in all five cell pools, resulting in an overall doubling time of 53.5 ± 4.5 h (mean ± SEM).

Figure 3 shows the growth curves of all 5 CBEC pools that we cultured and expanded during this work. Starting at 10^6 $CD34^+$ cells each, two pools of cells (NB 937-938 and NB 989-991) showed faster proliferation rates than the other three and reached total cell numbers of 10^{13} CBEC within the first six weeks of culture. To yield this number of cells, CBEC of the slower proliferating pools had to be cultured over 2 months. However, the longest life span and the largest yield of endothelial cells were observed in a pool of cells (NB 892-894) that belonged to the fraction of CBEC with a slower proliferation rate. With the least number of initially outgrowing CBEC colonies cell pool NB 882-884 also yielded the lowest total count of CBEC and showed the shortest life span in culture. This suggests that the initial number of EPC colonies largely determines the proliferation and expansion properties of CBEC.

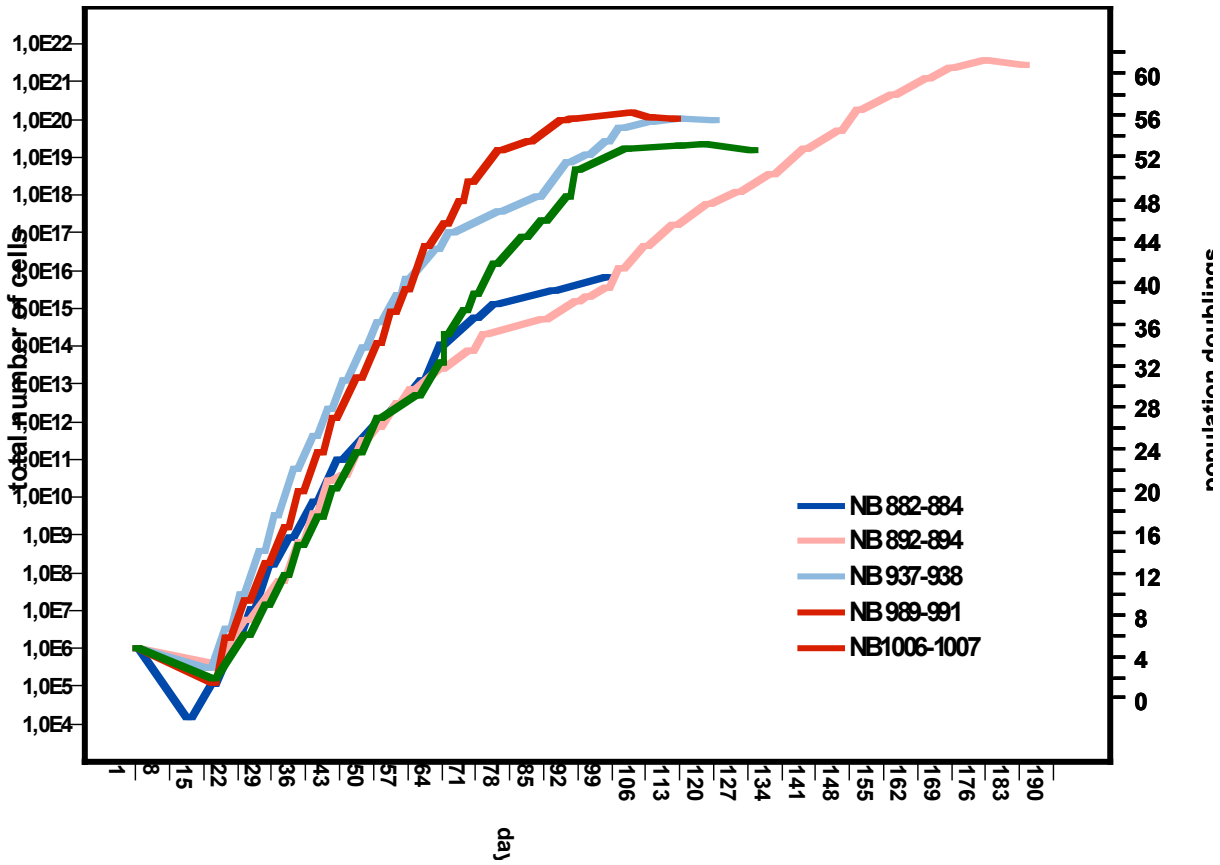


Figure 3 Cumulative growth curves of Cord Blood-derived Endothelial Cells (CBEC).
Cord blood was obtained from 5 different pools of donors. $CD34^+$ cells were isolated and $1x10^6$ each were grown in endothelial differentiation medium containing VEGF, FGF-2, SCF, and SCGF-β and thereby differentiated into CBEC. After 3 weeks and appearance of clusters of adhering endothelial cells, cultures were adapted to EGM-2 with supplements. Proliferating adherent cells with endothelial morphology were perennially passaged before reaching confluence and cultured until senescence. The graphs show the number and the cumulative population doublings of CBEC plotted against the time after enrichment of $CD34^+$ cells.

5.2 Verification of retroviral vector constructs

Two procedures - restriction digest and DNA-sequencing - aimed to confirm correctness and integrity of the vector construct on the one hand and the vector insert on the other hand. Restriction digest of pLXSN empty vector with restriction enzymes Hind III and Sac I resulted in fragments of 3.1, 1.4, and 1.3 kb as indicated by the manufacturer. Restriction digest of pLXSN-IL-1ra with restriction enzymes EcoR 1 and BamH 1 resulted in a 578 b fragment of the inserted sequence for ic-IL-1ra and a single 5.9 kb fragment of cut vector. **Figure 4** shows the electrophoretical separation of the pLXSN-IL-1ra and empty-vector pLXSN fragments on an agarose gel subsequent to restriction digest. Correctness of the vector-insert was proved in a sequencing reaction on an ABI 3700 sequencer by GATC-Biotech AG with the appropriate sequencing primers for pLXSN. The sequencing reaction confirmed the 578 b sequence for the long splice form of intracellular Interleukin-1 receptor antagonist including a 32 b signal sequence (gene bank ID: gi 1008970).

In summary, restriction digest and sequencing reaction showed intactness of both empty-vector

pLXSN and construct pLXSN-IL-1ra and confirmed the right sequence of the intracellular Interleukin-1 receptor antagonist DNA insert.

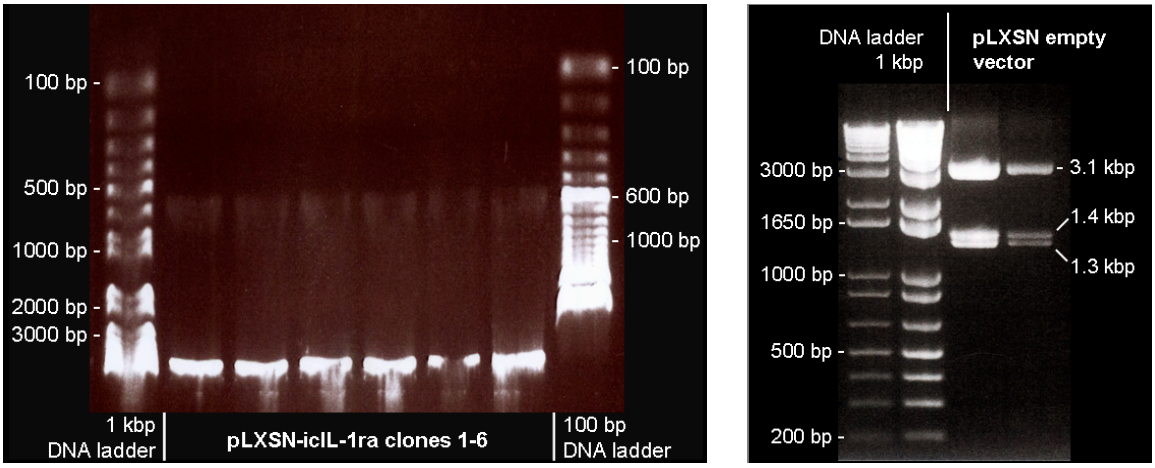


Figure 4 Restriction digest of retroviral vector constructs.

After transformation and initial amplification by DH5α E.coli restriction digest of plasmid constructs was carried out in order to confirm integrity and completeness of both vector and insert. Restriction digest of pLXSN-IL-1ra with restriction enzymes EcoR 1 and BamH 1 resulted in a 578 b fragment of the inserted icIL-1ra sequence and a single 5.9 kb fragment of cut vector (left). Restriction digest of pLXSN empty vector with restriction enzymes Hind III and Sac I resulted in fragments of 3.1, 1.4 and 1.3 kb as indicated by the manufacturer (right). One clone each of pLXSN and pLXSN-IL-1ra containing E.coli was then subjected to further amplification.

5.3 Transfection and retrovirus production

Two different methods of transfection – Calcium Phosphate and Lipofectamine plus based - were tested out to introduce the retroviral vector construct pLXSN-IL-1ra and empty vector pLXSN into mouse fibroblast-derived PT67 packaging cells.

Compared to Calcium Phosphate coprecipitation, the Lipofectamine based transfection showed various advantages such as simpler and faster performance, more consistently reproducible results, and higher transfection efficiency over a broader range of conditions with lower requirement of plasmid DNA.

In most of the transfected cells the plasmid DNA is transported to the nucleus for transcription and mRNA is subsequently transcribed in the cytoplasm. However, most of the foreign DNA is degraded by nucleases or diluted by cell division within a few days and transgene expression is no longer detectable. The small fraction of cells that integrates the plasmid DNA into their genome by recombination and replicates the transgene in synchrony with the cell cycle represents a stable source for retrovirus production. As the vector pLXSN contains the aminoglycoside phosphotransferase gene coding for APH enzyme that confers resistance to Geneticin (G 418 Sulphate) antibiotic (by rendering the drug inactive through phosphorylation) stably transfected cells could be selected by addition of Geneticin to the culture medium. Death of non-transfected cells usually started within 4-6 days after antibiotic selection medium was added and was complete after approximately 10 days.

Infectious titer of PT67 packaging cells was 4-6 x 10^6 U (293T-transducing units) / ml as determined by control transduction of 293T fibroblast cells in a diluted titer assay. As freezing and rethawing did not affect the infectious titer this method of transfection and selection of stably transfected cells was suitable to obtain a reliable, constant, easily accessible, and efficient source of retrovirus production for subsequent transduction assays.

5.4 Retroviral transduction of cord-blood derived endothelial cells

Various transduction experiments using Calcium Phosphate, Lipofectamine or Polybrene as additives (Davis et al., 2002; Porter et al., 1998), concentration of retroviral supernates by centrifugation and spinoculation (centrifugation during transduction to enhance retrovirus binding to target cells) resulted in poor endothelial cell viability after transduction and consistently failed to lead to any verifiable retrovirus-mediated gene transfer into endothelial cells. Eventually, conversion to a DEAE-Dextran-based transduction method including chloroquine preincubation, chondroitinase treatment of retrovirus-containing medium and double transduction process with freshly produced, filtered, and undiluted retroviral supernates at 32°C led to efficient transduction of CBEC without major impairment of cell viability and growth. **Figure 5** shows both PT67 retrovirus packaging cells and newly seeded CBEC which were cultured simultaneously in order to ensure availability of fresh retrovirus supernatant for transduction procedures.

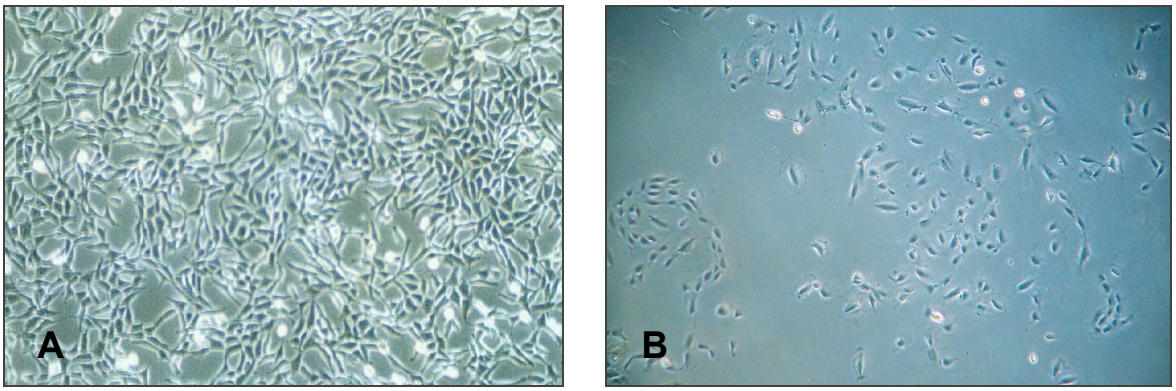
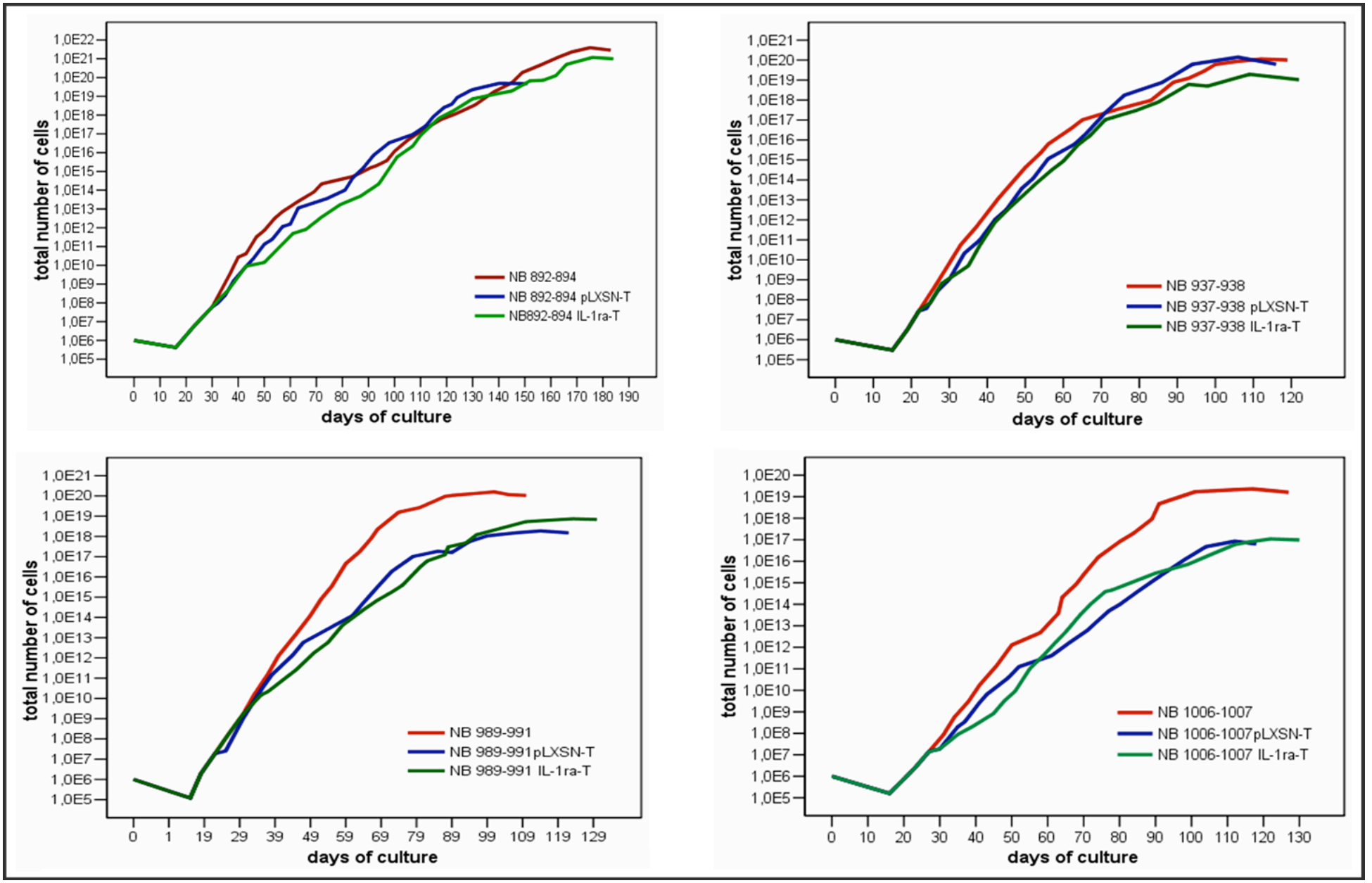


Figure 5 Transduction of CBEC by packaging cell-derived retrovirus.
PT 67 mouse fibroblast derived-packaging cells were transfected with the retroviral constructs pLXSN-IL-1ra and empty-vector pLXSN, respectively. After selection of stably producing packaging cells retrovirus-containing supernatants were harvested from nearly confluent PT 67 monolayers. 30-40 % confluent endothelial cells were then transduced with freshly produced retrovirus using the modified DEAE-Dextran method described above. Following transduction cells were subjected to antibiotic selection in order to obtain stably transduced CBEC carrying the transgene IL1ra or the pLXSN plasmid, respectively. **A** packaging cells. **B** CBEC.

About 20 to 40 % cell death and a lag phase in growth was observable during and after transduction (due to toxicity of transduction reagents and antibiotic selection of non-transduced


total number of cells
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1,0E21
1,0E20
1,0E19
1,0E18
1,0E17
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1,0E14
1,0E13
1,0E12
1,0E11
1,0E10
1,0E9
1,0E8
1,0E7
1,0E6
1,0E5
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190
days of culture
NB 892-894
NB 892-894 pLXSN-T
NB892-894 IL-1ra-T
total number of cells
1,0E21
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1,0E5
0 10 20 30 40 50 60 70 80 90 100 110 120
days of culture
NB 937-938
NB 937-938 pLXSN-T
NB 937-938 IL-1ra-T
total number of cells
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days of culture
NB 989-991
NB 989-991pLXSN-T
NB 989-991 IL-1ra-T
total number of cells
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1,0E6
1,0E5
0 10 20 30 40 50 60 70 80 90 100 110 120 130
days of culture
NB 1006-1007
NB 1006-1007pLXSN-T
NB 1006-1007 IL-1ra-T

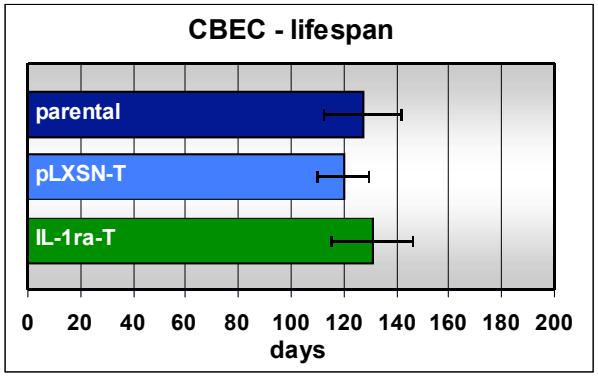
Figure 6 Cumulative growth curves of retrovirally transduced and parental CBEC.

CBEC were differentiated from CD34+ cells positively selected from cord blood of 4 different pools of donors and grown in endothelial cell basal medium-2 (EGM-2) supplemented with 2% FCS and growth factors as described. 20 to 30 days after onset of culture (correspondent 1-2 weeks after emergence of endothelial cell clusters) a part of the cells was transduced with the retroviral constructs pLXSN-IL-1ra and pLXSN empty-vector, respectively. Transduced cells underwent antibiotic selection for at least 2 weeks starting 4 to 6 days post-transduction and were cultured until senescence. At each passage, cells were counted and the total number of cells was calculated. Figure shows expansion graphs of 4 different transduction experiments; the cumulative number of CBEC is plotted against the time after the enrichment of CD34+ cells.

cells) before the cells recovered and proliferation rates approached those of untransduced cells.

However, transduction efficiency did not exceed 20-50 % as determined by comparison of growth curves and total yield of cells of transduced and parental endothelium. **Figure 6** shows cumulative growth curves of 4 pools of CBEC and compares parental cells with empty-vector pLXSN transduced and transgenic CBEC-pLXSN-IL1ra, respectively. While in two pools proliferation and expansion potential was very similar in the subtypes of cells (CB 892-894 and CB 937-938), the two other cell pools showed perceptible differences in growth. In these two cell pools (CB 989-991 and CB 1006-1007) parental CBEC grew faster and finally yielded about 10- to 100-fold more cells than CBEC-pLXSN or CBEC-pLXSN-IL-1ra. However, expression of the transgene icIL-1ra obviously did not alter expansion capacities of CBEC compared to empty-vector transduced cells as very similar growth behaviour was observable throughout the expansion period in both subtypes of cells that underwent the transduction procedure, actually in all 4 pools of CBEC **(figure 6)**. Thus, rather the retroviral transduction procedure than transgenic expression of icIL-1ra had major impact on CBEC proliferation and total yield of cells albeit endothelial cell morphology was not altered in cells that underwent transduction procedures. Exact data of endothelial cell proliferation and expansion have been calculated during CBEC culture and are described in the following.

The charts of **figure 7** display the characteristic hallmarks of in vitro culure of CBEC in our laboratory. Expansion of transduced endothelial cells resulted in median total cell numbers of 1.9×10^{18} (5.2×10^{15} – 1.4×10^{20}) and 7.4×10^{18} (7.1×10^{14} – 1.2×10^{21}) for CBEC-pLXSN and CBEC-pLXSN-IL-1ra, respectively, and showed no significant difference to parental CBEC (1.1×10^{20} cells, ranging from 7.0×10^{15} to 3.9×10^{21} cells). Furthermore, average life span of empty-vector pLXSN- and pLXSN-IL-1ra- transduced endothelial cells (mean ± SEM: 119.8 ± 9.4 and 130.6 ± 15.5 days respectively) was not significantly different compared to parental cells (mean ± SEM: 127.2 ± 14.8 days). Absolute number of population doublings during endothelial cell culture was slightly, but not significantly lower in transduced than in parental cells (mean ± SEM: 48.8 ± 3.1 / 49.6 ± 3.9 for CBEC-pLXSN / CBEC-pLXSN-IL-1ra versus 56.5 ± 1.4 for parental CBEC). However, translated into relative numbers, population doublings of parental CBEC (mean ± SEM: 105.5 ± 2.0 %) significantly exceeded the values for both CBEC-pLXSN and CBEC-pLXSN-IL-1ra (96.7 ± 1.9 % and 97.8 ± 1.4 %, $p < 0.05$).



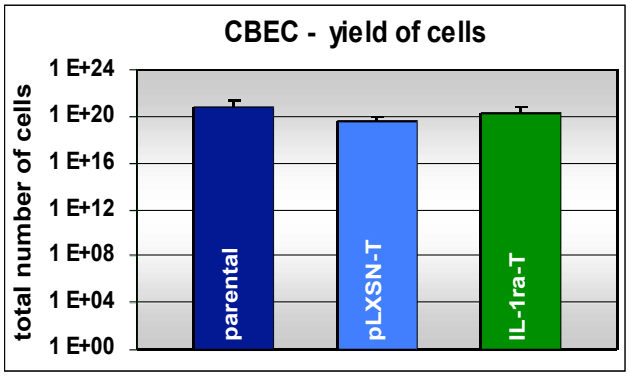
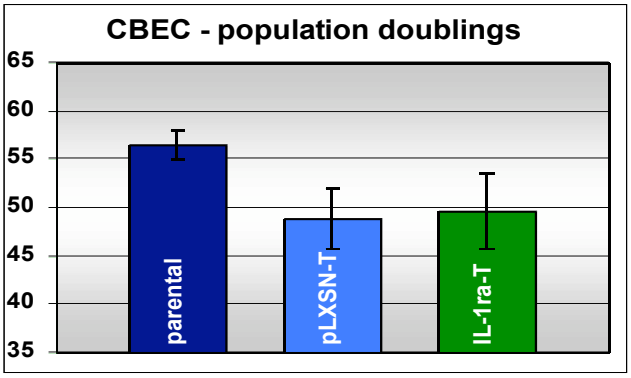
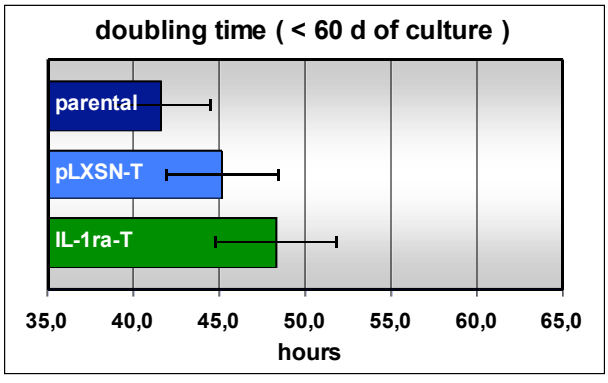


Figure 7 Expansion properties of CBEC.

Cord blood-derived endothelial cells were grown in EBM-2 on gelatine coated culture dishes. Cells were passaged, counted and given fresh medium every 2-4 days before reaching confluence until cell number no longer increased. Data are averaged from 5 different pools of CBEC and show means ± SEM. Differences in lifespan, total amount of cells and population doublings between transduced and untransduced cells did not show significance in this setting.



With an average doubling time of 53.5 ± 4.5 h and 54.8 ± 3.0 / 59.5 ± 3.9 h (parental CBEC versus CBEC-pLXSN / CBEC-pLXSN-IL-1ra) during endothelial cell in vitro culture parental and transduced cells proliferated without any significant distinction **(see figure 8)**. However, referring to the first 60 days of endothelial cell culture, all cells proliferated distinctly faster compared to overall doubling time (41.6 ± 2.8 / 45.1 ± 3.3 / 48.3 ± 3.5 hours; CBEC-par, -pLXSN and –pLXSN-IL-1ra, respectively) **(figure 8)**.



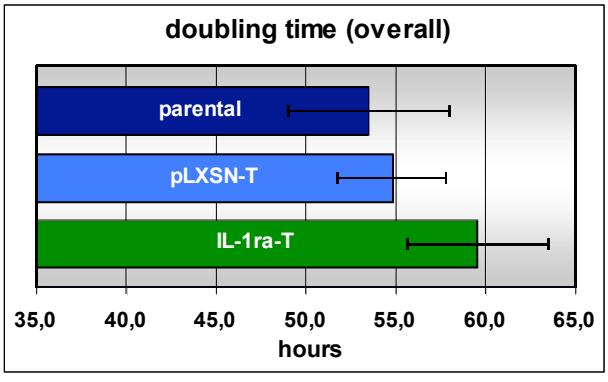


Figure 8 Doubling time in the course of CBEC culture.

Cord blood-derived endothelial cells were cultured in low-serum EBM-2 with supplementation of growth factors and passaged before reaching confluence as mentioned under *Materials and Methods*. Cells were counted within the passaging procedure and doubling time was calculated from total population doublings and endothelial cell life span. CBEC exerted highest proliferation capabilities during the first two months of culture. Despite a trend towards longer doubling times in transduced CBEC no significant differences between CBEC parental, CBEC-pLXSN and CBEC-pLXSN-IL-1ra could be observed. Displayed data are averaged from 5 different pools of CBEC and show means ± SEM.

Translated into relative numbers, proliferation rate of all three cell types showed significant differences within the first 2 months of culture. Parental CBEC grew faster than both transduced CBEC types (92.5 ± 1.7 % versus 100.2 ± 0.7 % (CBEC-pLXSN) and 107.3 ± 1.6 % (CBEC-pLXSN-IL-1ra), $p<0.01$) and empty-vector-transduced CBEC showed better relative proliferation properties than CBEC carrying the transgene IL-1ra. This might be due to chemical stress and suboptimal growth conditions during transduction procedures and the supervening demands on CBEC-pLXSN-IL-1ra to replicate more genetic material and translating/transcribing the transgene into an additionally produced protein. Nonetheless, both transduced and parental cells showed similar physiological characteristics and expansion capacities. Appraising these features, both parental and transgenic cord blood derived endothelial cells can be expanded into clinically relevant numbers and their proliferation properties seem to be equal with regard to a potential use of CBEC in clinical applications.

5.5 Detection and quantification of transgenic IL-1ra expression

Transgenic expression of Interleukin-1 receptor antagonist was determined on the posttranscriptional level by detection of mRNA using semiquantitative polymerase chain reaction with preceeding reverse transcription (RT-PCR) and on the posttranslational level by verification of protein synthesis through Western Blot analysis and Enzyme linked Immunosorbent Assay (ELISA).

About 2-3 weeks after retroviral transduction - ensuring completed antibiotic selection of resistant, transduced endothelial cells - and repetitively during endothelial cell culture RNA was isolated of CBEC-pLXSN-IL-1ra, CBEC-pLXSN, and parental CBEC, respectively, and semiquantitative RT-PCR for Interleukin-1 receptor antagonist was performed under the aforementioned conditions. Aiming to facilitate distinction between genetically introduced ic(intracellular)IL-1ra and possibly naturally occurring endogenous isoforms of Interleukin-1 receptor antagonist two different primer pairs were designed as described in **figure 9**. In order to detect potential induction of IL-1ra gene expression in parental and empty-vector transduced CBEC in response to proinflammatory stimuli and aiming to guarantee constant expression of transgenic icIL-1ra by retrovirally transduced CBEC-pLXSN-IL-1ra even in an activated state semiquantitative RT-PCR was additionally carried out after endothelial cell prestimulation with IL-1ß and TNF-α, respectively.

As determined at several time points throughout the expansion period by semiquantitative RT-PCR with both primer pairs described above, parental CBEC and empty-vector transduced CBEC-pLXSN did not express detectable amounts of transcripts of any IL-1ra isoforms neither in a resting state nor in response to cytokine stimulation **(figure 10)**. In contrast, with a theoretical 2^{13} -fold overexpression of icIL-1ra, genetically transduced CBEC showed a high level of transgene expression that remained stable under stimulation conditions in activated endothel. This indicates transgenic transcriptional independence of regulation through natural promoters. Moreover, in CBEC retrovirally transduced with the pLXSN-icIL-1ra construct, the transgenic long intracellular variant of Interleukin-1 receptor antagonist was found to be the

exclusive isoform expressed by these cells in our experiments.

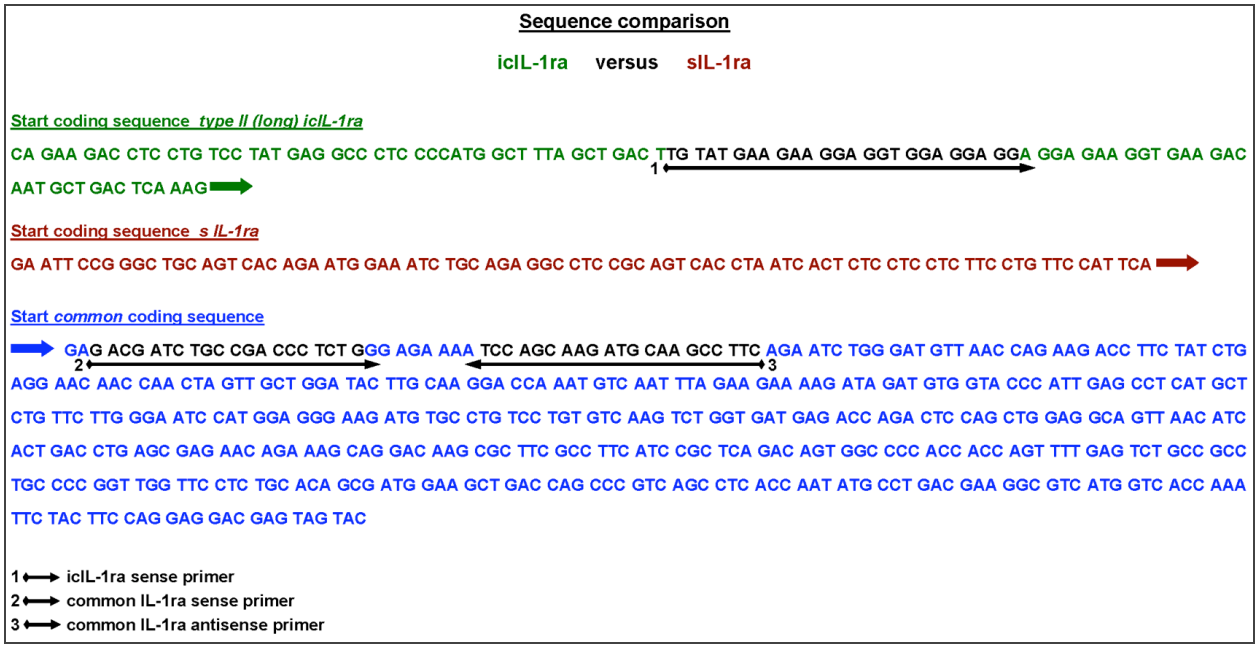


Figure 9 Sequence comparison of IL-1ra isoforms and primer design.

IL-1ra exists in four different isoforms derived from the same gene. One isoform is secreted (sIL-1ra) while the three others (icIL-1ra 1/2/3) are intracellular due to a lack of a functional leader sequence. The mRNA for icIL-1ra type 2 contains an additional exon of 63 bp in its signal sequence which distinguishes it from the other isoforms. In order not only to detect transgenic icIL-1ra but also to distinguish it from possibly naturally expressed receptor antagonist in CBEC two different forward primers were designed and used with a common backward primer. The first primer binds to the signal sequence of icIL-1ra type II and therefore exclusively detects the transgenic variant. The second primer binds to the common coding sequence of IL-1ra and in this manner detects any transcript variant of IL-1ra.

Compared to PBGD, a housekeeping gene involved in hem synthesis of human cells, transduced CBEC showed an 8.5 -fold overexpression of icIL-1ra. This level is comparable to the expression of genes that are characteristic for endothelial cells and play an important role in endothelial cell physiology such as collagen type IV, thrombin receptor, Interleukin-6 and -8 in resting endothel and VCAM-1 - one of the most important adhesion receptors - in activated endothel.

In conclusion, the results of semiquantitative RT-PCR firstly indicate that cord blood-derived endothelial cells do not express IL-1ra by nature - neither unstimulated nor in an activated state - and therefore lack a naturally occurring antagonist of IL-1 mediated processes. Secondly, retroviral transduction could efficiently be performed on CBEC and achieved high levels of transgenic icIL-1ra expression. Moreover, the transgenic expression of icIL-1ra was not altered during endothelial cell stimulation by IL-1β and TNF-α, indicating that one can reckon on stable levels of icIL-1ra expression even under proinflammatory conditions (e.g. in the following stimulation experiments).

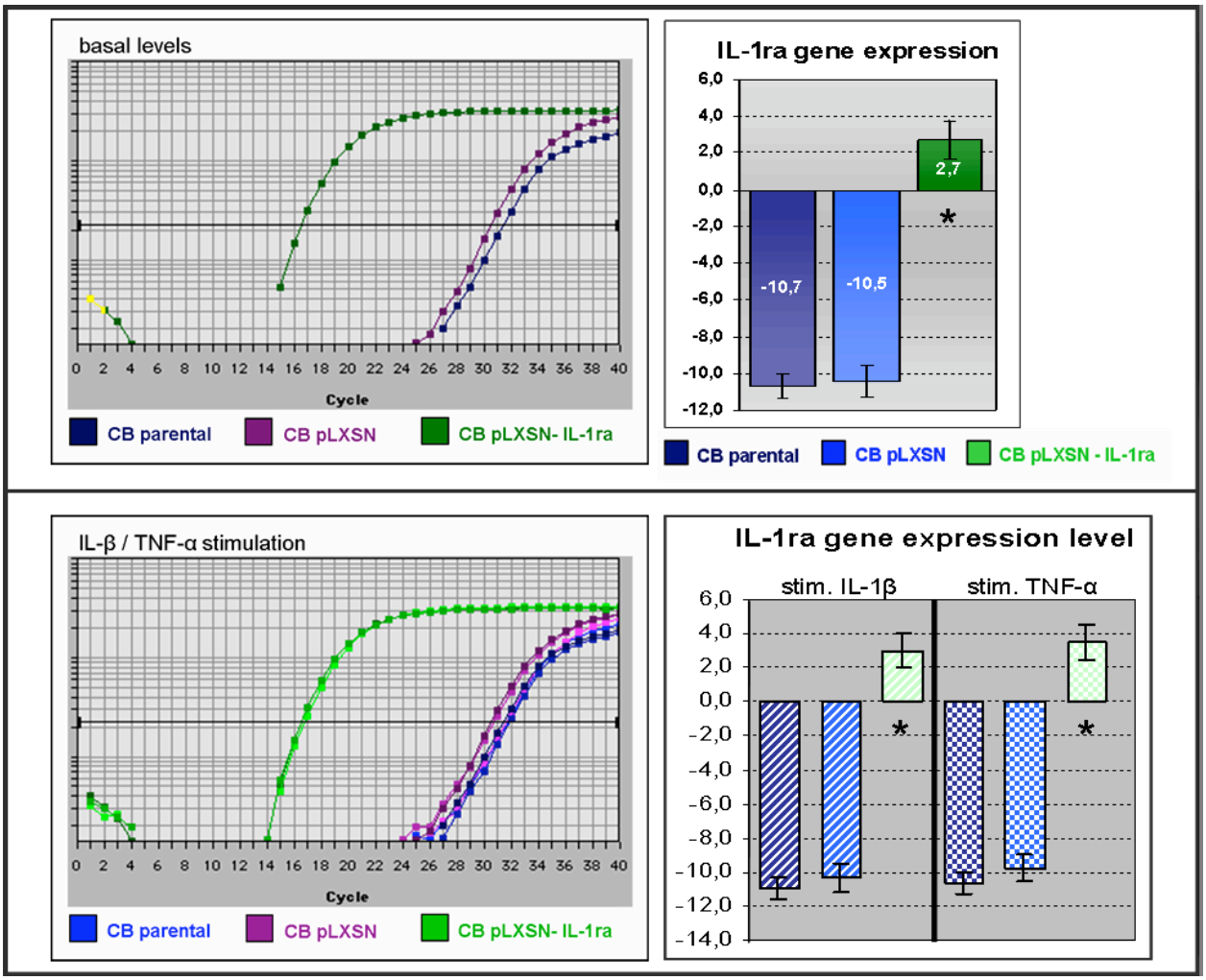


Figure 10 **Efficiency of retroviral transduction of Cord Blood-derived Endothelial Progenitor Cells with the transgene icIL-1ra**

Following an initial expansion period of 1-2 weeks CBEC from 5 different pools of donors were transduced with the retroviral constructs pLXSN (empty vector) and pLXSN-IL-1ra, respectively. Efficiency of IL-1ra gene transfer was determined by semiquantitative RT-PCR 4 weeks after transduction experiments. Upper row shows basal IL-1ra mRNA expression of transduced CBEC versus parental and empty-vector transduced cells. Lower row indicates IL-1ra transgene expression under the influence of IL-1β and TNF-α stimulation, respectively, compared to basal conditions. Graphs on the left show representative RT-PCR measurements out of 4 independent measurements; diagrams on the right show cumulative data of all 4 measurements in relation to PBGD housekeeping gene expression levels. Results are shown as mean ± SEM. * p< 0.001 as determined by Students t-test.

Following mRNA detection expression of IL-1ra protein was proved by Western Blot analysis. Western Blot of whole cell lysates of CBEC parental, -pLXSN and -pLXSN-IL-1ra was performed every third to fourth passaging time (every two to three weeks) during endothelial cell culture as described in *Materials and Methods*. After protein separation and semidry immunoblotting membranes were successively incubated with anti-IL-1ra and anti-ß-Actin primary antibodies and corresponding secondary antibodies. Importantly, the anti-IL-1ra antibody was capable of recognizing any isoform of IL-1ra.

In this manner, icIL-1ra was detected for at least 3 months in 4 pools of transgenic CBEC. In one pool of cells (CB 892-894) icIL-1ra protein production could be demonstrated up to 145 days or 5 months, respectively. According to Western Blot results, IL-1ra signal showed no drop

compared to the constitutively expressed protein ß-Actin. This suggests that IL-1ra protein production by CBEC-pLXSN-IL-1ra remained stable over the whole expansion period and no transcriptional or translational switching off or elimination of the retroviral vector construct from the original CBEC genome occurred. In contrast, parental and empty-vector pLXSN transduced CBEC never produced detectable amounts of any isoform of Interleukin-1 receptor antagonist protein during the expansion period. **Figure 11** summarizes the results of Western blot analysis of 4 pools of cord blood-derived endothelial cells cultured in our laboratory.

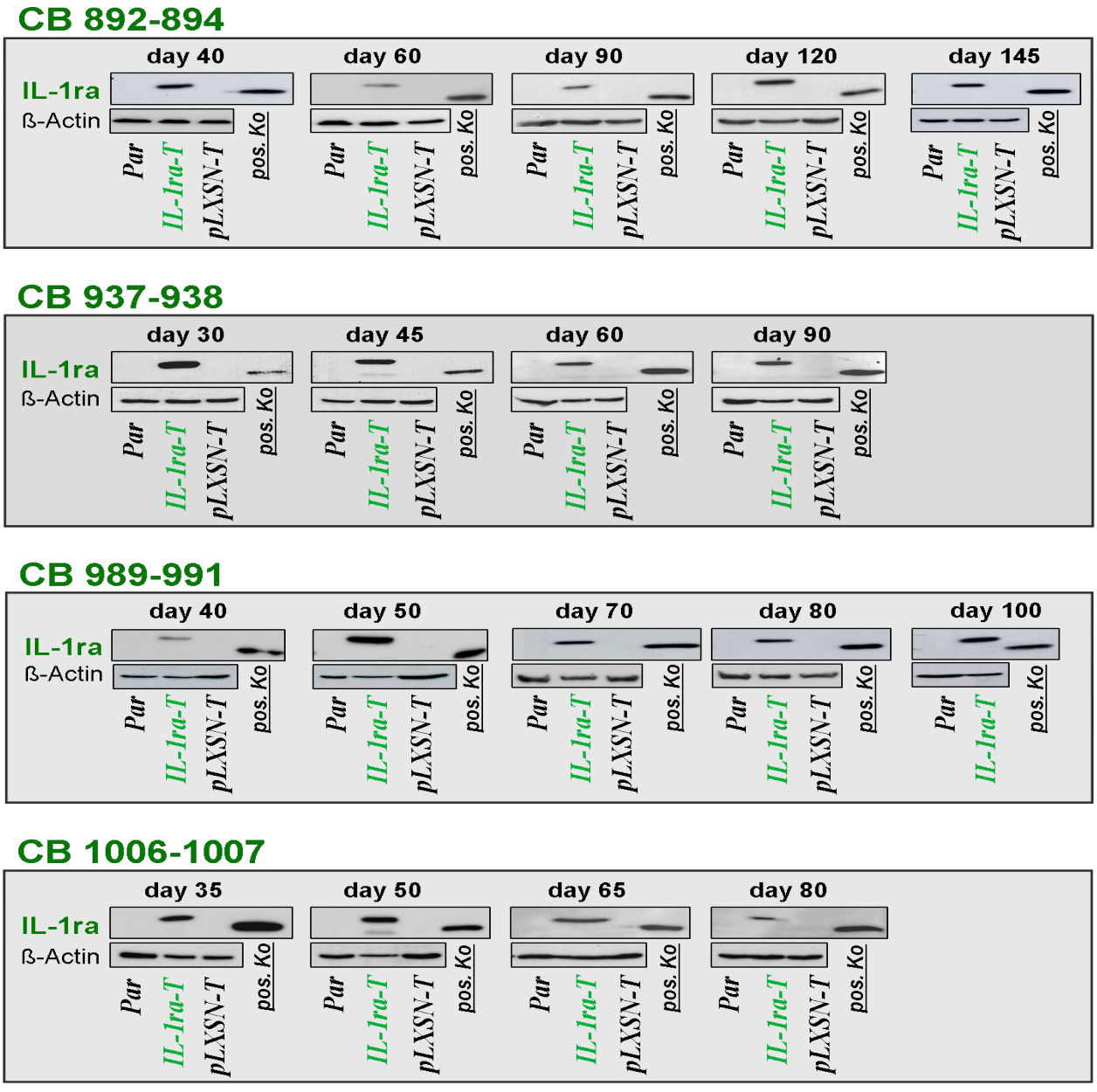


Figure 11 Transgenic CBEC show stable long-term expression of IL-1ra protein.
At indicated time points cultured CBEC were suspended in PBS/EDTA, resolved in lysis buffer and cell proteins were separated. Expression of IL-1ra protein was analyzed by Western blot with an antibody recognizing every isoform of IL-1ra as described under *Materials and Methods*. Recombinant human IL-1ra served as positive control (right lanes of every blot) and ß-Actin detection assured even total protein load of all samples (lower row of every blot). Blots show IL-1ra protein expression of 4 pools of transduced CBEC-pLXSN-IL-1ra in comparison to untransduced and empty-vector transduced control cells, respectively. No IL-1ra protein could be detected in control cells at any time.

While Western Blot analysis was exclusively used to detect intracellular IL-1ra protein on a semiquantitative basis exact levels of both the intracellular and secreted portion of transgenic IL-1ra production were quantified by ELISA. Cell culture supernates and cell lysates of parental, empty-vector and pLXSN-IL-1ra transduced CBEC of all 5 cell pools were collected five to seven times during the culture period as described under *Materials and Methods*.

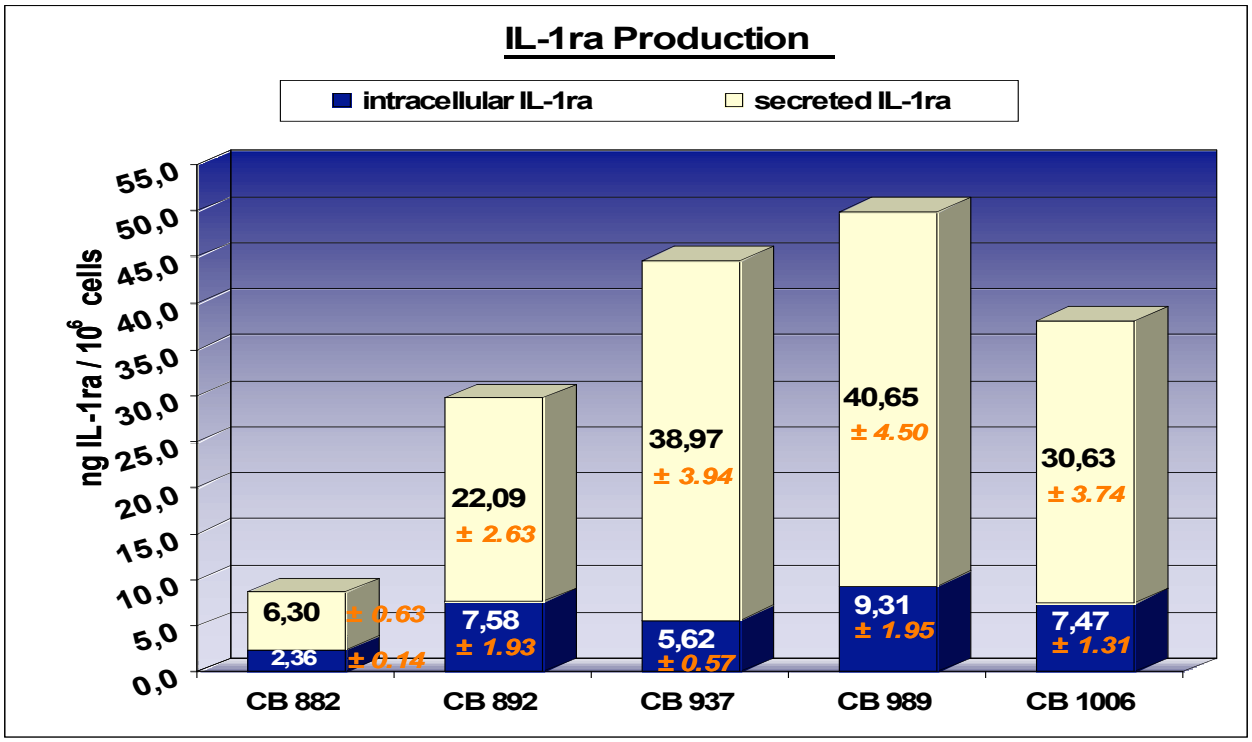


Figure 12 Quantification of IL-1ra production by transgenic CBEC-pLXSN-IL-1ra.
CBEC-pLXSN-IL-1ra generated from 5 different donor pools were grown to near confluence, supplied with fresh medium and incubated for 24 h at 37°C and 5% CO_2. Medium was then taken off and cell detritus was removed by centrifugation. CBEC were detached by trypsin/EDTA dispersion and resolved in lysis buffer. The secreted portion of IL-1ra protein in supernatants and the intracellular portion in cell lysates were detected by ELISA and calculated in relation to 10^6 cells. Untransduced CBEC and empty-vector transduced CBEC-pLXSN did never produce detectable amounts of IL-1ra, neither intracellular nor secreted. Measurements were performed in triplicate and data shown are means ± SEM.

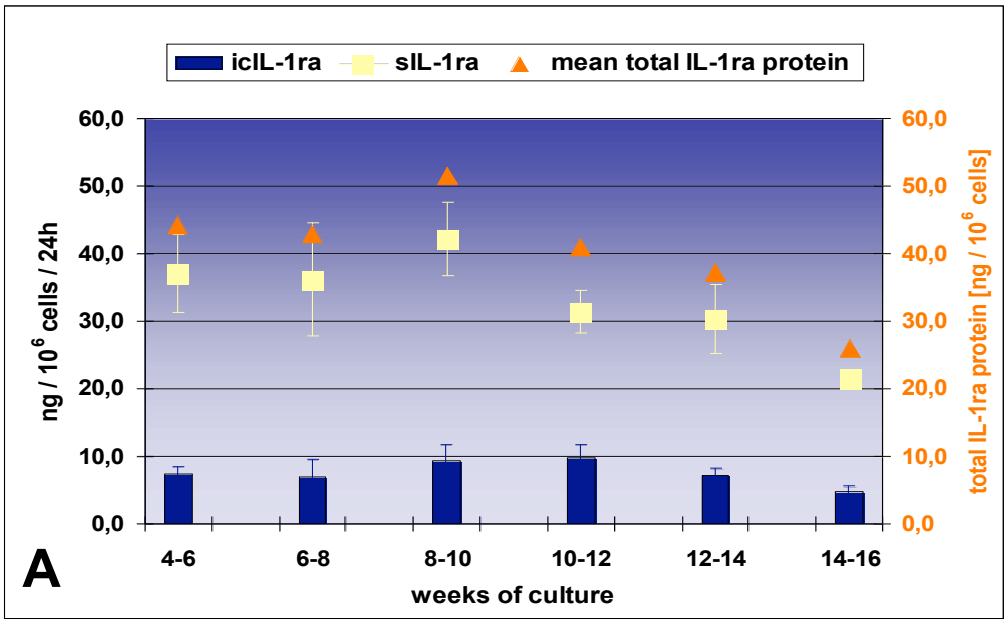
Apart from NB 882-884-pLXSN-IL-1ra - the cell pool that was used for the first transduction - total Interleukin-1 receptor antagonist production of all CBEC-pLXSN-IL-1ra ranged between 30 and 50 ng per 10^6 cells per 24 h (mean ± SEM: 40.6 ± 4.4 ng/10^6cells/24h (excluding NB 882-884-pLXSN-IL-1ra) and 34.2 ± 7.2 ng/10^6cells/24h (incl. NB 882-884-pLXSN-IL-1ra)). The amount of the intracellular portion of IL-1ra ranged between 2.4 and 9.3 ng/10^6cells/24h with an average of 7.5 ± 0.8 ng/10^6cells/24h (6.5 ± 1.2 incl. NB 882-884-pLXSN-IL-1ra). Averagely detected secreted IL-1ra was 33.1 ± 4.3 ng/10^6cells/24h (27.7 ± 6.3 incl. NB 882-884-pLXSN-IL-1ra) and ranged from 22.1 to 40.7 ng/10^6cells/24h. On average, secreted IL-1ra exceeded 4.2 ± 0.8 -fold the amount of intracellular IL-1ra. **Figure 12** lists all the 5 CBEC pools that were retrovirally transduced with the pLXSN-IL-1ra construct and indicates amounts of intracellular and secreted Interleukin-1 receptor antagonist produced by these cells. Once again, no Interleukin-1 receptor antagonist protein - either intracellular or secreted - was detectable in

parental and control-vector pLXSN transduced CBEC. CB 882-884 was the first CBEC pool which underwent the transduction procedure and produced the least IL-1ra protein in this comparison. As described in **figure 3**, parental cells (as well as transduced CBEC, data not shown) of cord blood pool CB 882-884 showed considerable restraints in their expansion properties, possibly due to a lower number of endothelial progenitors in this batch of cord blood derived mononuclear cells or to an inferior viability compared to the other cell pools. Therefore, low levels of transgenic IL-1ra production might not only be the result of a not yet tried-and-tested transduction performance but might also have their roots in proliferation and expansion disadvantages.

The capacity of transgenic CBEC for stable expression of IL-1ra protein during endothelial cell culture was additionally investigated at 6 distinctive time points throughout the expansion period. With mean levels of 37.2 – 51.5 ng/10^6 cells/24h total IL-1ra protein production remained relatively stable during the first 8-10 weeks after transduction with peak levels occurring at week 4-6 post transduction. The proportion of secreted to intracellular IL-1ra turned out fairly constant and ranged from 3.2 to 5.2 with a mean ratio of 4.5 ± 0.3. After 10-12 weeks of culture - and thereby 2-4 weeks earlier than intracellular IL-1ra production - secretion of IL-1ra into the culture medium decreased which was accompanied by reduced proliferation and increasing senescence of CBEC. Production and secretion of IL-1ra was eventually provable for up to 4 months of culture in four different cell pools. Characteristics of the time course of transgenic IL-1ra production by CBEC-pLXSN-IL-1ra shown in the two charts of **figure 13**.

Aiming to elucidate whether diminishing of IL-1ra protein production in late endothelial cell culture was due to transcriptional downregulation or cancelling of the integrated transgenic vector construct expression or occurred as a part of physiological endothelial cell senescence and reduction of protein synthesis, quantity of intracellular Interleukin-1 receptor antagonist was related to concurrently determined total endothelial cell protein content.

As shown in **figure 14**, IL-1ra expression of all four investigated cell pools remained quite steady in a range from 0.001 to 0.005 % of total cell protein quantity with only one pool of trans-



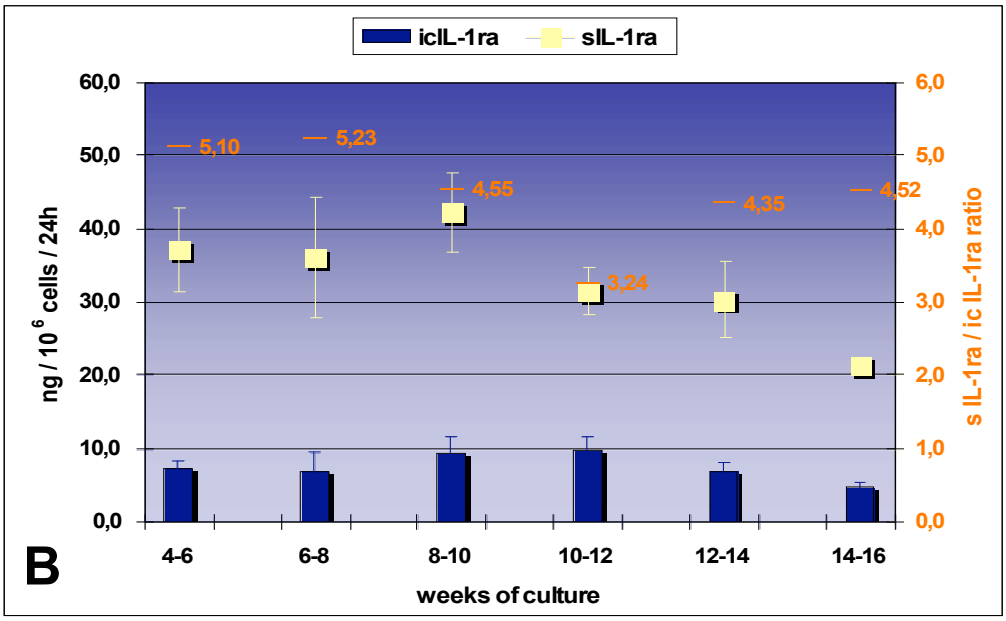


Figure 13 A + B Time course of transgenic IL-1ra production by CBEC-pLXSN-IL-1ra.

Transduced and untransduced CBEC were cultured in EBM-2 medium with growth supplements. In order to determine levels of transgenic IL-1ra expression during endothelial cell culture supernatants and cell lysates from confluent endothelial monolayers were obtained after incubation periods of 24 h with fresh medium at indicated times throughout the expansion period. IL-1ra protein was determined by ELISA and plotted against the time of endothelial cell culture. Intracellular and secreted IL-1ra is itemized separately in the diagrams. Orange triangles in the upper diagram (A) additionally show total amount of IL-1ra whereas dashes in the lower diagram (B) indicate the ratio of intracellular to secreted IL-1ra. Untransduced CBEC and empty-vector transduced CBEC-pLXSN did never produce detectable amounts of IL-1ra during their life span. Measurements were performed in triplicate at each time point and data shown are means ± SEM.

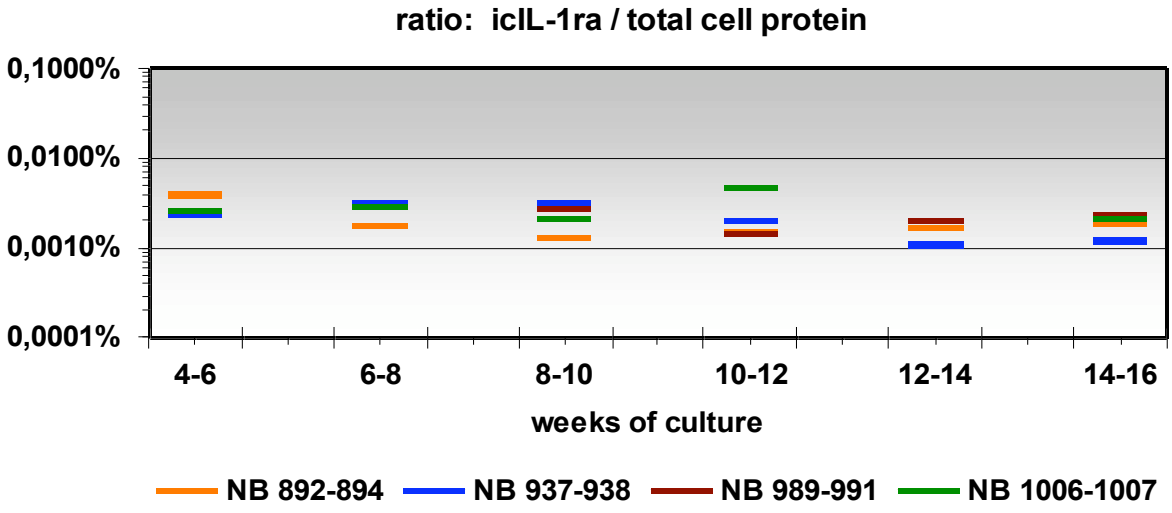


Figure 14 Ratio of icIL-1ra production of transgenic CBEC to total protein content.

Transgenic icIL-1ra production was determined throughout the expansion period by ELISA of cell lysates of 4 pools of pLXSN-IL-1ra transduced CBEC. Total protein load of CBEC was concurrently quantified by the method of Lowry as described under *Materials and Methods*. In this manner quantity of intracellular Interleukin-1 receptor antagonist was related to total endothelial cell protein content as shown in the chart.

duced cells showing a decrease of that ratio at the end of the observation period. These results confirmed and specified more precisely the findings of Western Blot analysis. All in all these data suggest that pLXSN-IL-1ra vector construct was stably integrated into the host genome and retroviral transduction of CBEC led to long lasting high-level expression of the icIL-1ra transgene.

With each of the 5 cord blood donor cell pools holding individual expansion features, proliferation capabilities of CBEC were related to total transgenic IL-1ra expression. As illustrated in **figure 15**, a negative linear correlation - yielding Pearson's correlation coefficient of approximately -0.83 (R^2 = 0.69) - could be conjectured between IL-1ra production and doubling time of CBEC (with highest levels of IL-1ra found in fastest growing CBEC). However, with a p-value of 0.17 in the two-tailed tests this correlation failed to reach significance.

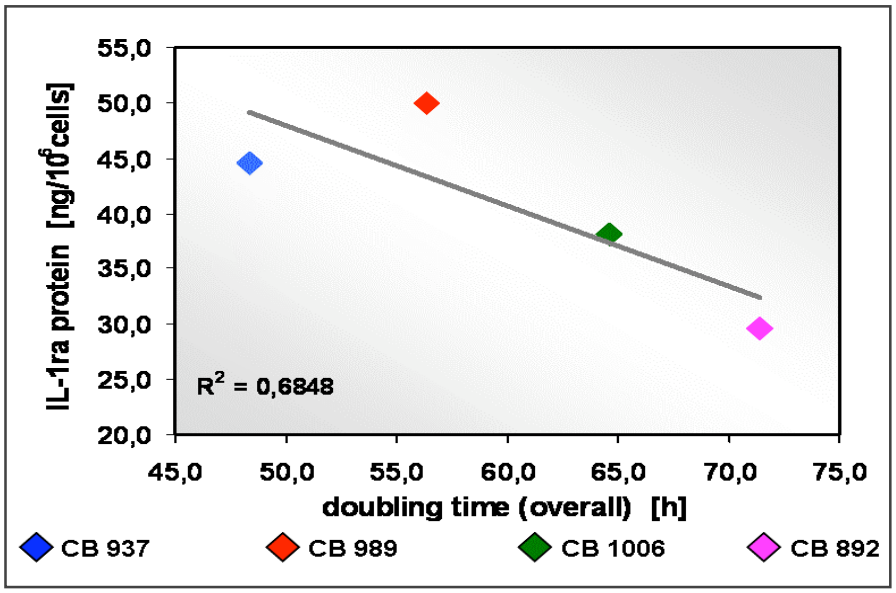


Figure 15
Correlation between CBEC growth and IL-1ra protein production.
Total IL-1ra protein expression by transgenic variants of 4 CBEC pools as determined by ELISA is plotted against average endothelial cell doubling time. The better the proliferation properties the more IL-1ra protein is produced by CBEC.

5.6 Activation of Endothelial Cells

Activation of endothelial cells is central to the pathogenesis of inflammatory vasculopathic processes as it profoundly changes endothelial cell function. In the early phase of endothelial cell activation various genes undergo transcriptional regulation resulting in changes in protein expression and thereby alterations of the endothelial cell phenotype.

Activation of CBEC has been tested in different settings in order to elucidate the effect of retroviral transduction and transgenic icIL-1ra expression on endothelial gene expression patterns.

a) Time course of PDGF and Tissue Factor expression during CBEC culture

Both Tissue Factor (TF) and Platelet-derived Growth Factor (PDGF) are increasingly produced by activated and dysfunctional endothelium and render its phenotype procoagulative and proadhesive and promote migration of subendothelial smooth muscle cells (Annex et al., 1998; Cunningham et al., 1999; Randolph et al., 1998).

In order to determine the effect of retroviral transduction with either pLXSN-IL-1ra or empty-vector pLXSN and the consequences of long-term in vitro expansion of CBEC on mRNA expression of TF and PDGF semiquantitative RT-PCR was performed at 4 points during the expansion period. As indicated in **figure 16**, expression of TF was at very low levels (only about 1.5% compared to PBGD expression) and did not increase during endothelial cell culture. Obviously, neither empty-vector transduction nor transgenic production of icIL-1ra had any significant effect on TF expression compared to parental CBEC. In the case of PDGF, expression levels were found to be comparable to those of the housekeeping gene PBGD and did not show any significant changes throughout the expansion period. Despite a trend towards lower levels of PDGF expression in empty-vector pLXSN transduced CBEC no significant differences were found between CBEC transgenic for IL-1ra and untransduced or empty-vector transduced control cells, respectively.

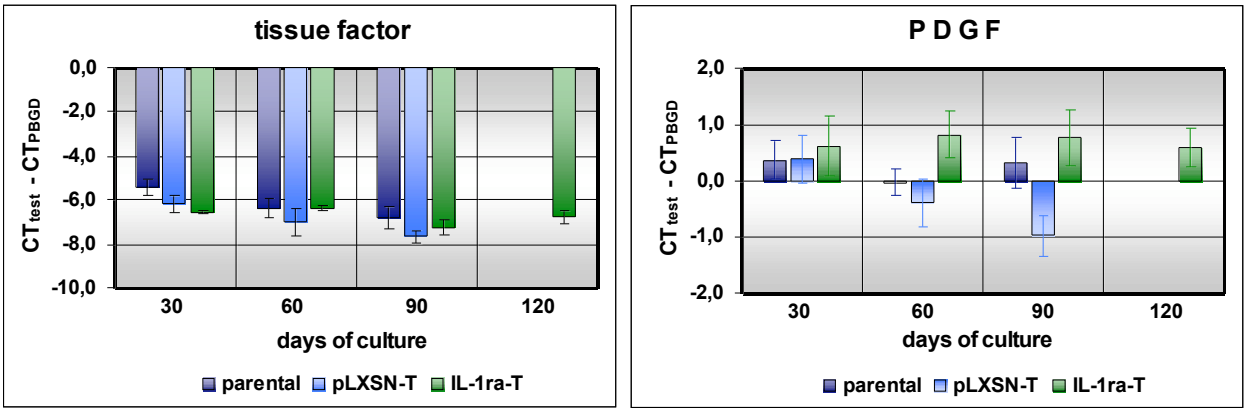


Figure 16 Time course of Tissue Factor and Platelet derived Growth Factor gene expression during endothelial cell life span.

TF and PDGF gene expression levels were determined every month during endothelial cell culture. Therefore, CBEC were grown to near confluence, supplied with fresh EBM-2 medium and total RNA was isolated after another 24 h of culture. Following reverse transcription into cDNA RT-PCR was performed with specific primers for TF and PDGF as listed under *Material and Methods*. Signal intensities of the target genes were determined as Ct values and compared to the Ct value of PBGD in each sample. Charts show relative gene expression data averaged from 4 independent measurements in different cell pools.

b) Activation of CBEC by the proinflammatory cytokines IL-1β and TNF-α

Among the vast amount of proinflammatory cytokines that impact on the endothelium IL-1β and TNF-α are the most important ones as they predominantly occur at sites of vascular inflammation and induce a wide variety of proinflammatory transcriptional responses in endothelial cells that strongly influence the further development of inflammatory processes.

Therefore we tested the gene expression pattern of CBEC either parental, empty-vector transduced or transgenic for icIL-1ra in response to stimulation by the proinflammatory cytokines IL-1β and TNF-α, respectively. We elucidated transcriptional regulations of distinct endothelial genes involved in leucocyte-endothelial interaction, vascular physiology, signal transduction, and extracellular matrix modification which thereby hold important functions in vascular inflammatory processes. Thereby we characterized the impact of transgenic production

of icIL-1ra on phenotypical changes of CBEC in response to proinflammatory stimulation.

Figure 17 shows the endothelial expression profile of all the genes tested in this assay and indicates significant effects of transgenic IL-1ra production on transcriptional responses compared to empty-vector pLXSN transduced CBEC. Firstly, basal gene expression showed distinct differences: For example, transcription of endothelial-leukocyte adhesion molecules ICAM-1, VCAM-1, and ELAM-1 as well as IL-1β, Tissue Factor, and thrombospondin was at very low levels in unstimulated CBEC, whereas endothelial monocyte activating protein (EMAP-2), endothelin-1, plasminogen activator inhibitor (PAI-1) and thrombin receptor were found to be highly expressed in resting endothelium. Other proinflammatory molecules (e.g. IL-6, IL-8, MCP-1, COX-2, and PDGF) showed intermediate gene expression levels under unstimulated conditions compared to the housekeeping gene PBGD. With the exception of EMAP-2 and ANPC-receptor only being upregulated by TNF-α stimulation and two genes (endothelin-1 and thrombin receptor) with high basal expression levels that did not show further upregulation by either IL-1β or TNF-α stimulation, all the genes examined by RT-PCR showed transcriptional responses to both IL-1β and TNF-α stimulation. Expression levels of any of the genes tested in this set of experiments did not differ significantly between parental and empty-vector pLXSN transduced CBEC in our measurements neither in unstimulated state nor subjected to IL-1β and TNF-α stimulation. Therefore, further comparisons of pLXSN-IL-1ra transduced CBEC are related to empty-vector pLXSN transduced CBEC. As shown in **figure 17**, endogenous transgenic production of icIL-1ra changed the gene expression profile of CBEC. In CBEC-pLXSN-IL-1ra several genes showed reduced transcriptional upregulation following cytokine-mediated stimulation compared to CBEC-pLXSN. However, even though many trends became noticeable only a part of the effects of transgenic icIL-1ra expression on CBEC transcriptional regulation reached statistical significance.

The adhesion molecule ICAM-1 and the monocyte chemoattractant MCP-1, strongly expressed by cytokine-activated CBEC, show reduced basal levels of the corresponding mRNA (-3.4-fold and -13.6-fold, respectively) as well as diminished transcriptional upregulation in response to IL-1β stimulation (-2.9-fold and -8.2-fold, respectively). In addition, transgenic expression of icIL-1ra was also capable of attenuating TNF-α mediated upregulation of ICAM-1 mRNA transcripts in CBEC (reduction by factor 13.2) and therefore ICAM-1 is the only molecule in this set of experiments with lowered transcription levels in CBEC-pLXSN-IL-1ra both under unstimulated conditions and following IL-1β and TNF-α stimulation, respectively.

Moreover, the high basal mRNA levels of thrombin receptor were significantly reduced in CBEC-pLXSN-IL-1ra although expression of thrombin receptor did not rise importantly after IL-1β or TNF-α stimulation. Thereby thrombin receptor represents a third gene with a diminished basal expression in CBEC transgenicly producing icIL-1ra.

Figure 18 shows the real-time PCR measurements and results for ICAM-1 analysis of parental, empty-vector pLXSN transduced CBEC and transgenic CBEC-pLXSN-IL-1ra and **figure 19** indicates the corresponding results for MCP-1. Significant effects of endogenous production of icIL-1ra by CBEC on ICAM-1 and MCP-1 mRNA expression are summarized in **figure 20**.

function	gene	basal level	Regulation by		Inhibitory effect of ic IL-1ra on...		
			IL-1ß	TNF-α	basal levels	IL-1ß stimulation	TNF-α stimulation
leucocyte-endothelial interaction	ICAM-1	low	⇑	⇑	- 14,6 x *	- 8,2 x *	- 13,2 x *
	VCAM-1	low	⇑	⇑	-	-	-
	ELAM-1	low	⇑	⇑	+	- 13,8 x *	+
	IL-6	medium	⇑	↑	-	-	-
	IL-8	medium	⇑	⇑	+	-	-
	EMAP-2	high	-	↑	-	-	+
	MCP-1	medium	⇑	⇑	- 3,4 x *	- 2,9 x *	+
	PSGL-1	low	↑	↑	-	-	-
vascular physiology	Angiopoetin-1	low	↓	↓	-	+	+
	ANPC-receptor	low	-	↑	+	+	-
	COX-2	medium	⇑	↑	+	- 5,2 x *	-
	Endothelin-1	high	-	-	-	-	-
	IL-1ß	low	⇑	↑	+	- 2,8 x *	-
	NOS-3	medium	↓	↓	-	+	-
	tissue factor	low	⇑	⇑	-	+	-
	PAI-1	high	↑	↑	-	+	-
	PDGF-b	medium	↑	↑	-	+	-
	Prostacyclin-Synthase	low	↑	↑	-	- 3,9 x *	-
	Thrombinreceptor	high	-	-	- 3,2 x *	+	-
	Thrombomodulin	low	⇓	↓	+	- 5,0 x *	-
	Thrombospondin	low	↑	↑	-	-	-
cytoskeletal and ECM	Collagen IV	medium	⇑	↑	-	- 3,8 x *	-
	Laminin 5	low	⇑	↑	+	- 5,2 x *	-
signal transduction	Cyclin A1	medium	↓	-	+	++	+
	Caspase-1 (ICE)	medium	↑	↑	-	-	-

basal levels: - *low* ≙ △CT < - 4
- *medium* ≙ - 4 < △CT < 4
- *high* ≙ △CT > 4

regulation: ↑ ≙ △CT = 1-2 ⇑ ≙ △CT > 2
↓ ≙ △CT = -(1-2) ⇓ ≙ △CT < -2

* p < 0.05, significant inhibition compared to CBEC-pLXSN control cells

Figure 17 Gene expression profile of Cord Blood-derived Endothelial Cells.
Endothelial cell RNA was examined for transcripts of decisive genes in vascular physiology and hemostasis, endothelial-leukocyte interaction, signal transduction and extracellular matrix composition. Therefore, CBEC were grown to 80-90% confluence, supplied with fresh medium and cultured for another 24 h before IL-1ß or TNF-α (1 ng/ml) respectively were added to the medium. Unstimulated cells provided basal levels of gene expression. After an incubation period of 4 h cells were lysed and total RNA was isolated. Following reverse transcription into cDNA gene expression was determined by RT-PCR using specific primers for the indicated molecules as listed in *Materials and Methods*. Signal intensities were determined as Ct values and compared with the Ct value of PBGD in each sample whereby relative (semiquantitative) expression levels were obtained. Data shown are combined results of 4 independent measurements and compare gene expression levels of empty-vector transduced CBEC-pLXSN to that of CBEC-pLXSN-IL-1ra. Significant effects of transgenic IL-1ra production on endothelial gene expression are marked with *. Trends ($p<0.10$) towards inhibitory effects of transgenic IL-1ra are marked with +.

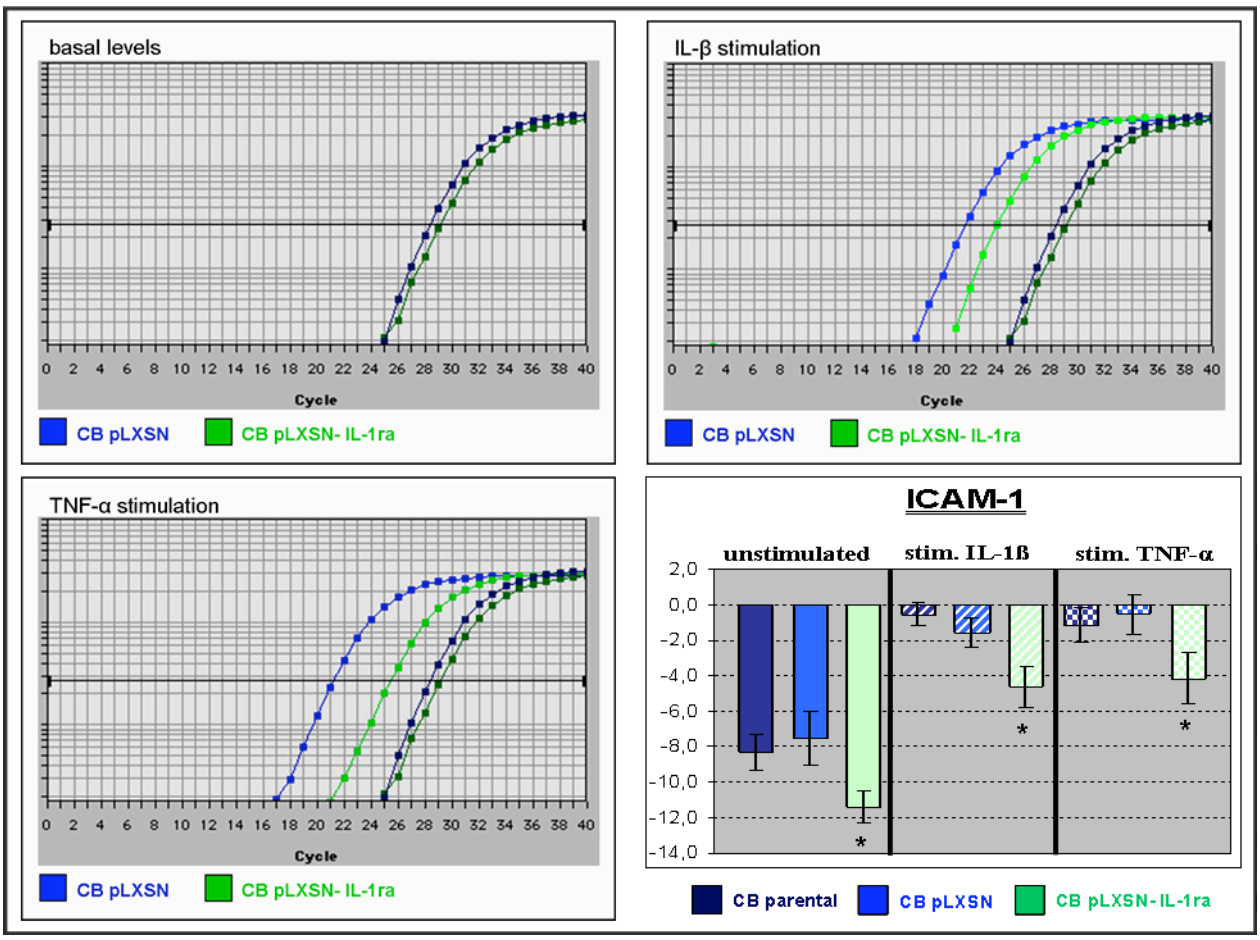


Figure 18 Transgenic icIL-1ra reduces induction of ICAM-1 gene expression in CBEC.

Endothelial cells were stimulated with 1 ng/ml IL-1β or TNF-α, respectively. After an incubation period of 4 h mRNA was isolated from the cells and real-time PCR was performed in an ABI Prism 7700 (Applied Biosystems) with primers for ICAM-1 and the housekeeping gene PBGD. Signal intensities were determined as Ct values and compared with the Ct value of PBGD in each sample (see diagram). Unstimulated cells provided basal levels of endothelial ICAM-1 mRNA expression. In this manner relative expression levels of the ICAM-1 gene in endothelial cells in comparison to the PBGD expression level were obtained. Data are averaged from 4 independent measurements and graphs show representative results. * P< 0.05 versus pLXSN empty vector group by Student's t-test.

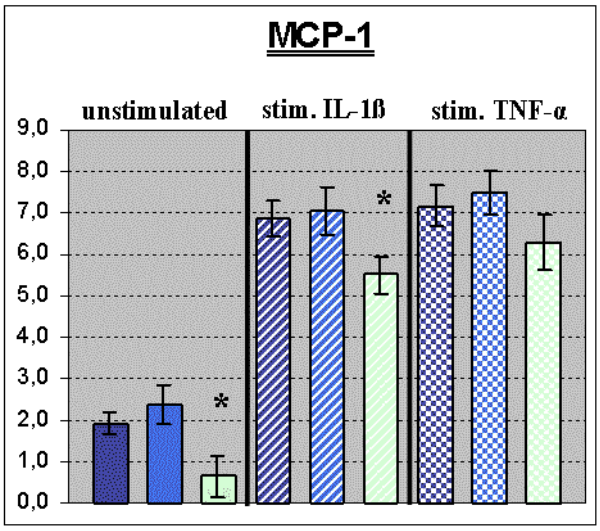


Figure 19 MCP-1 gene expression is attenuated in CBEC-pLXSN-IL-1ra in response to IL-1ß stimulation and under basal conditions.

Endothelial cells were stimulated with 1 ng/ml IL-1β or TNF-α, respectively. Induction of MCP-1 gene expression was determined by RT-PCR as described before. Unstimulated cells provided basal levels of endothelial MCP-1 mRNA expression. Ordinate indicates the difference between the Ct values for MCP-1 and those for the housekeeping gene PBGD. In this manner, the diagram shows relative expression levels of the MCP-1 gene compared to PBGD. * P< 0.05 versus pLXSN empty vector group by Student's t-test.

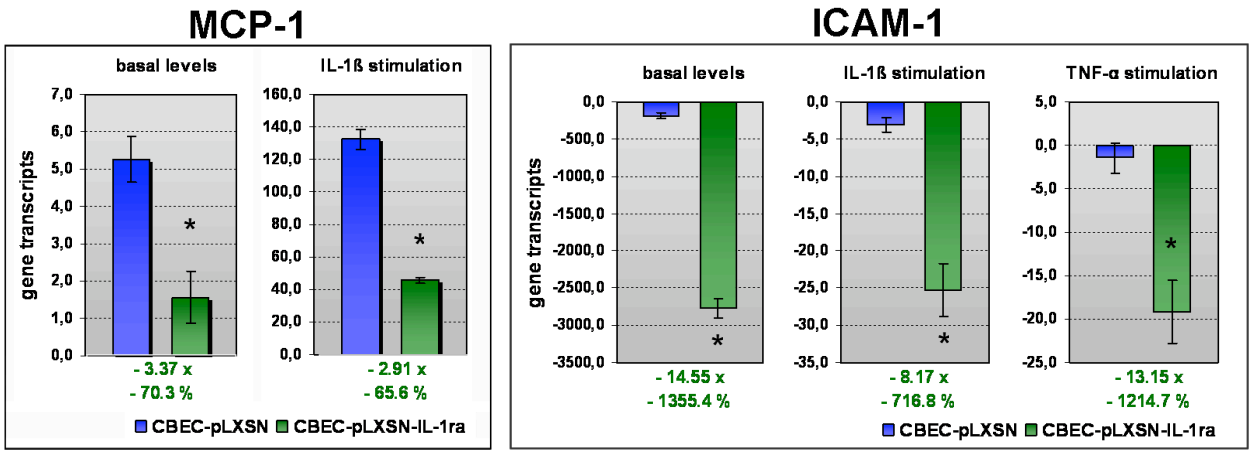


Figure 20 Attenuation of transcriptional upregulation of MCP-1 and ICAM-1 in CBEC-pLXSN-IL-1ra in response to inflammatory stimuli.

Endothelial cell stimulation, RNA isolation, and RT-PCR were performed as described previously. Based on the measurement of Ct values for the target molecules and GAPDH, gene transcripts of MCP-1 and ICAM-1 were calculated (as described in *Methods*) and related to those of the housekeeping gene. The chart shows multiplication of mRNA transcripts of the target genes in response to IL-1β and TNF-α and compares transcriptional regulation in CBEC-pLXSN-IL-1ra (green bars) to empty-vector transduced control cells (blue bars). * P< 0.05 versus pLXSN empty-vector group by Student's t-test.

Furthermore, several other genes involved in leukocyte-endothelial interactions, regulation of vascular homeostasis and extracellular matrix production showed attenuated upregulation selectively in response to IL-1β stimulation. Among those are the genes for ELAM-1, COX-2, prostacyclin synthase and the extracellular matrix components collagen IV and laminin.

Especially ELAM-1 upreglation, which besides ICAM-1 induction determines for the most part the adhesivity of endothelial cells towards peripheral blood mononuclear cells, was considerably attenuated in CBEC transgenic for icIL-1ra. Transcriptional upregulation of the ELAM-1 gene in CBEC-pLXSN-IL-1ra in response to IL-1β stimulation was merely just under 10% of that in CBEC-pLXSN **(figure 22)**.

A reduced increment of mRNA levels in response to IL-1β stimulation in CBEC-pLXSN-IL-1ra could also be observed for endogenous expression of IL-1β though basal levels of IL-1β gene expression were not different to those of empty-vector transduced CBEC. IL-1β mRNA expression in transgenic CBEC was reduced by factor 2.8; the graphs of RT-PCR analysis of endogenous IL-1β expression are shown in **figure 21**.

Among the genes that showed significantly diminished transcriptional response to IL-1β stimulation the thrombomodulin gene holds different properties compared to the other genes tested as it is not up- but downregulated through IL-1β **(see figure 17)**. Under physiologic conditions thrombomodulin is partly responsible for the anticoagulative character of endothelial cells as it functions as an activator of protein C. As itemized in **figure 22**, transgenic expression of icIL-1ra partially protected CBEC from transcriptional downregulation of thrombomodulin so that the decline of thrombomodulin expression in CBEC-pLXSN-IL-1ra following IL-1β stimulation was only 20% of that in CBEC-pLXSN control cells.

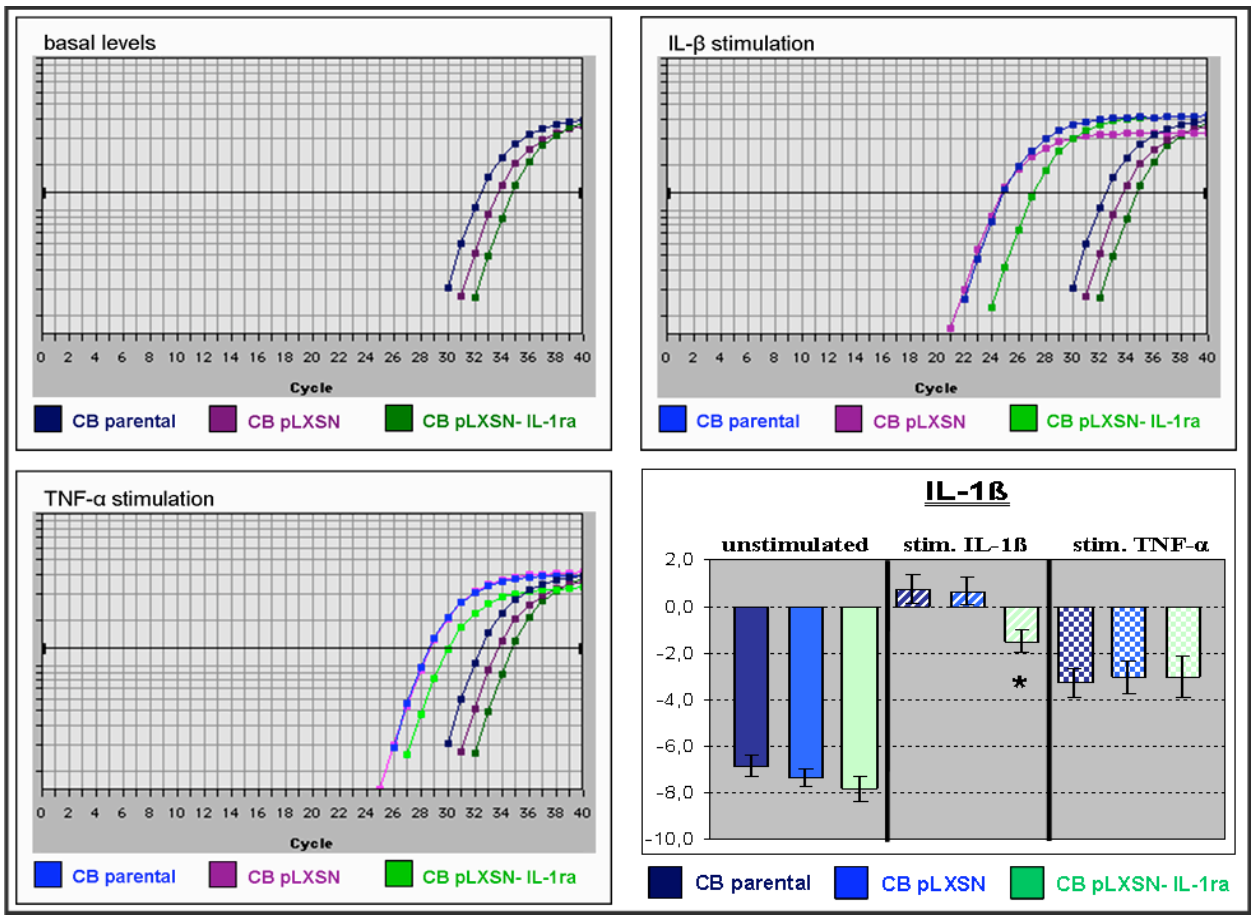


Figure 21 **Inhibition of IL-1-induced IL-1β gene expression in Cord Blood-derived Endothelial Cells transgenic for icIL-1ra.**

Endothelial cells were stimulated with 1 ng/ml IL-1β or TNF-α, respectively. After an incubation period of 4h mRNA was isolated from the cells and reverse transcribed into cDNA. Semiquantitative RT-PCR was performed with primers for IL-1β and related to the housekeeping gene PBGD. Unstimulated cells provided basal levels of endothelial IL-1β mRNA expression. Signal intensities were determined as Ct values (cycle at which signal intensity reaches half maximum value) and compared with the Ct value of PBGD in each sample. In this manner relative expression levels of the IL-1β gene in endothelial cells in response to inflammatory stimuli were obtained. Data are averaged from 4 independent measurements and graphs show representative results. * P< 0.05 versus pLXSN empty vector group by Student's t-test.

Figure 22 summarizes the significant inhibitory effects of icIL-1ra in transgenic CBEC on IL-1β induced transcriptional regulation of genes tested in our stimulation experiments. Together with **figure 17** these charts display the alterations of the CBEC expression profile of decisive genes of involved in vascular homeostasis and regulation of inflammatory reactions and give prominence to the effect of genetic modification of these cells by retroviral transduction with the transgene icIL-1ra. Of the 25 genes examined in this activation assay 10 (40%) were significantly differently regulated in CBEC-pLXSN-IL-1ra compared to empty-vector transduced CBEC-pLXSN control cells.

In summary, transgenic expression of icIL-1ra in CBEC significantly reduced expression of important genes involved in attraction and adhesion of monocytes and leukocytes on endothelial cells (MCP-1, ELAM-1, ICAM-1), production of extracellular matrix components (collagen, laminin) as well as genes responsible for regulation of coagulation and inflammation (thrombin receptor, thrombomodulin, IL-1β, prostacyclin synthase, COX-2).

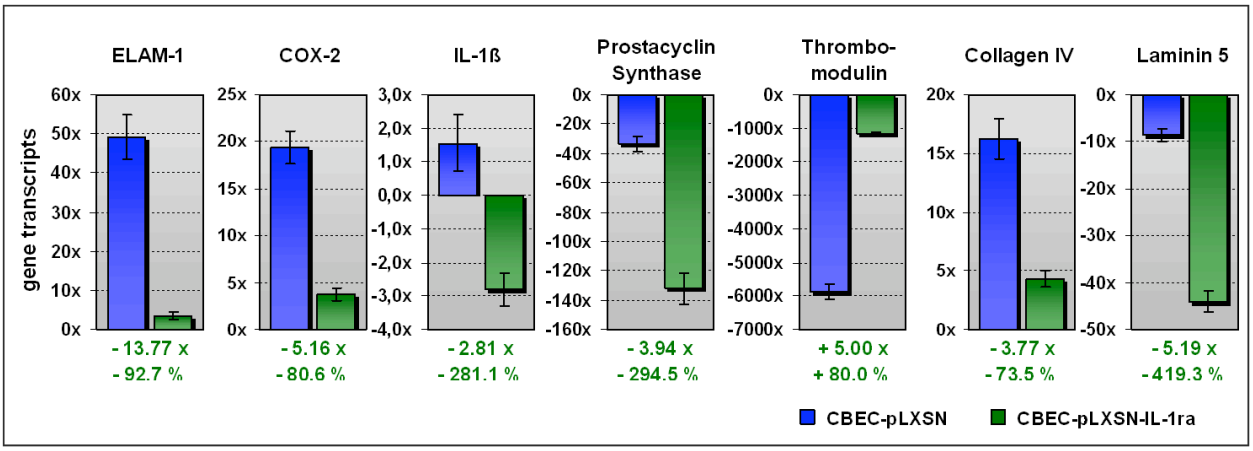


Figure 22 Inhibition of mRNA expression in transgenic CBEC in response to IL-1β-stimulation.
As a supplement to figure 17, this chart compares changes in transcription of distinct endothelial genes in response to IL-1β stimulation. Data are calculated as described in section *Methods*. Based on the measurement of Ct values for the target molecules and GAPDH relative gene transcripts were determined (as described in *Methods*) and related to those of the housekeeping gene. The chart shows multiplication of mRNA transcripts of the target genes in response to IL-1β and compares transcriptional regulation in CBEC-pLXSN-IL-1ra (green bars) to empty-vector transduced control cells (blue bars), indicated in percentages and x-fold induction. All the genes itemized in this diagram showed significantly ($p < 0.05$) diminished transcriptional responses to IL-1β stimulation in transgenic CBEC-pLXSN-IL-1ra compared to empty-vector transduced CBEC-pLXSN. Basal expression levels of these genes under unstimulated conditions did not differ significantly between transgenic and control cells (**see figure 17**).

Upregulation of these genes is a characteristic of endothelial dysfunction during vascular inflammatory processes. In the following, we determined the effect of transgenic expression of icIL-1ra by CBEC on these processes in different in vitro studies.

5.7 Rolling of peripheral blood mononuclear cells on endothelial monolayers

Rolling of monocytes and leukocytes on activated endothelium of the vessel wall is a crucial step of leukocyte-endothelial interactions and initiates the vascular inflammation cascade at sites of inflamed tissue. Rolling mononuclear cells eventually stick loosely on the endothelial layer which leads to further activation of both the endothelium and the inflammatory cells of the blood stream. In order to determine the effect of transgenic production of IL-1ra by CBEC on the initial adhesion of mononuclear cells onto activated endothelium under conditions similar to the blood stream, flow chamber experiments were set up as described under *Materials and Methods*. Under controlled conditions peripheral blood of healthy volunteers was allowed to stream on resting and prestimulated monolayers of empty-vector transduced CBEC-pLXSN and transgenic CBEC-pLXSN-IL-1ra, respectively.

As shown in **figure 23**, only few PBMC adhered on the non-activated endothelial monolayers. However, prestimulation of CBEC prior to flow chamber studies resulted in a marked increase of PBMC adhesion. Regarding adhesion on unstimulated monolayers, CBEC with transgenic expression of IL-1ra showed significantly lower numbers of sticking PBMC compared to CBEC-

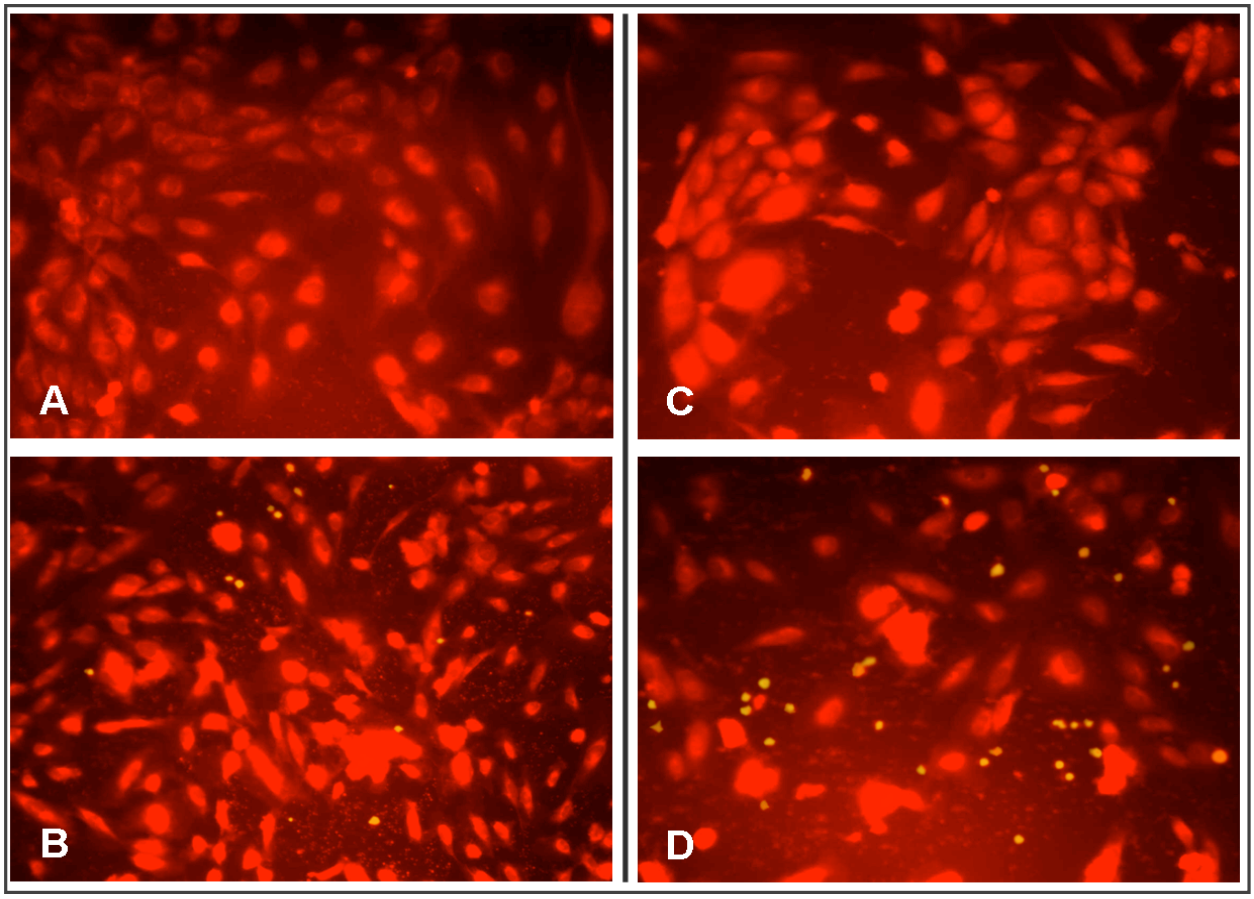
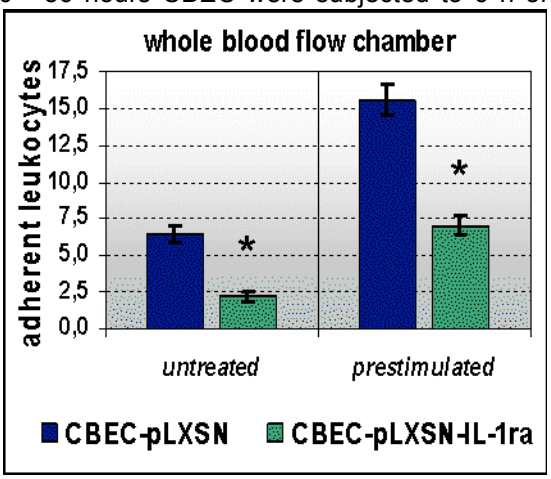


Figure 23 **Diminished rolling and adhesion of peripheral blood leukocytes on CBEC-pLXSN-IL-1ra monolayers under flow conditions.**

After growing to near confluence on glass plates within 30 - 36 hours CBEC were subjected to 6 h of prestimulation with 0.2 ng/ml Interleukin-1ß or left unstimulated and thereafter subjected to whole blood flow in a flow chamber device. Digital photos were taken by light fluorescence colour photography of 4-6 arbitrarily chosen fields of the endothelial monolayer. Exact staining, measuring and quantification method in *Materials and Methods*. **A** Whole blood flow over unstimulated CBEC-pLXSN-IL-1ra. **B** Blood flow over prestimulated CBEC-pLXSN-IL-1ra. **C** and **D** apply likewise for empty-vector transduced CBEC-pLXSN. Notice adhering yellow-stained peripheral blood leukocytes. Pictures show one representative experiment whereas the diagram combines data from 3 independent experiments. * p< 0.01 significant difference as determined by Students t-test.



pLXSN (2.2 ± 0.3 versus 6.5 ± 0.5, consistent with a reduction of adhering PBMC on CBEC-pLXSN-IL-1ra by 66%). Similar results were obtained following prestimulation with 0.2 ng/ml IL-1β; with averagely 15.6 ± 1.1 adhering PBMC on CBEC-pLXSN monolayers and 7.0 ± 0.6 on CBEC-pLXSN-IL-1ra the level of rolling adhesion was reduced by 55% (p< 0.01) in CBEC carrying the transgene IL-1ra.

Thus, transgenic expression of icIL-1ra is capable of effectively attenuating rolling and subsequent loose adhesion of PBMC on the endothelial surface and thereby inhibits an

important early step of leukocyte-endothelial interactions in vascular inflammatory processes.

5.8 Adhesion of monocytic cells on endothelium

Protracted contact between monocytic cells and the endothelium and intensified activation of the endothelium during vascular inflammatory processes lead to firm adhesion and accumulation of inflammatory cells at the vessel wall. Aiming to elucidate the potential of CBEC expressing the transgene icIL-1ra to inhibit firm adhesion of monocytic cells on endothelial monolayers, the flow chamber experiments were supplemented by an adhesion assay under steady conditions.

Figure 24a shows representative photographs of Mono-Mac-6 monocytic cells firmly adhering on sequentially prestimulated CBEC monolayers after completion of the adhesion assays. While, as expected, only few monocytic cells adhered on unstimulated resting CBEC monolayers, IL-1β prestimulation of CBEC proved to act as a potent inducer of endothel-monocyte adhesion and significantly increased count of firmly adhering Mono-Mac-6 on both CBEC-pLXSN and CBEC-pLXSN-IL-1ra monolayers. FACS measurements **(figure 24b)** revealed that prestimulation with 0.3 ng/ml IL-1β (which was the lowest concentration used in this set of experiments) induced nearly maximum adhesivity of parental and empty-vector transduced CBEC monolayers for monocytic cells. Further increase of IL-1β concentrations during prestimulation of parental CBEC and CBEC-pLXSN resulted only in slight increments of adhering monocytic cells. No significant variances in adhesion between parental CBEC and CBEC-pLXSN became apparent in these series of experiments. In contrast, CBEC-pLXSN-IL-1ra showed notable differences in IL-1β induced adhesivity as maximum levels of adhering monocytic cells did not occur until the transgenic endothelium was stimulated with 3.0 ng/ml IL-1β. Prestimulation with lower concentrations of IL-1β indeed sequentially increased adhesivity of CBEC-pLXSN-IL-1ra, but the number of adhering monocytes was significantly diminished in the range of 0.3 to 1.0 ng/ml IL-1β prestimulation compared to parental and empty-vector transduced CBEC. With 60.1 ± 9.3, 72.0 ± 7.9 and 79.5 ± 11.7 % of adhering monocytic cells on CBEC-pLXSN-IL-1ra prestimulated with 0.3, 0.6 and 1.0 ng/ml, compared to 93 - 100% adhering Mono-Mac-6 on parental CBEC and 99 to 114% on CBEC-pLXSN , transgenic expression of icIL-1ra reduced monocyte adhesion by 37.6%, 22.9% and 21.1%, respectively, compared to parental CBEC ($p < 0.01$) **(figure 24)**. Adhesion assays were additionally performed with exogenous addition of 100 ng/ml recombinant IL-1ra protein to empty-vector transduced CBEC while keeping up same concentrations of IL-1β prestimulation. These experiments resulted in inhibition of monocytic adhesion following prestimulation with 0.3 and 0.6 ng/ml IL-1β at levels comparable to those in CBEC-pLXSN-IL-1ra but failed to reduce adhesivity subsequent to 1.0 ng/ml IL-1β prestimulation (data not shown).

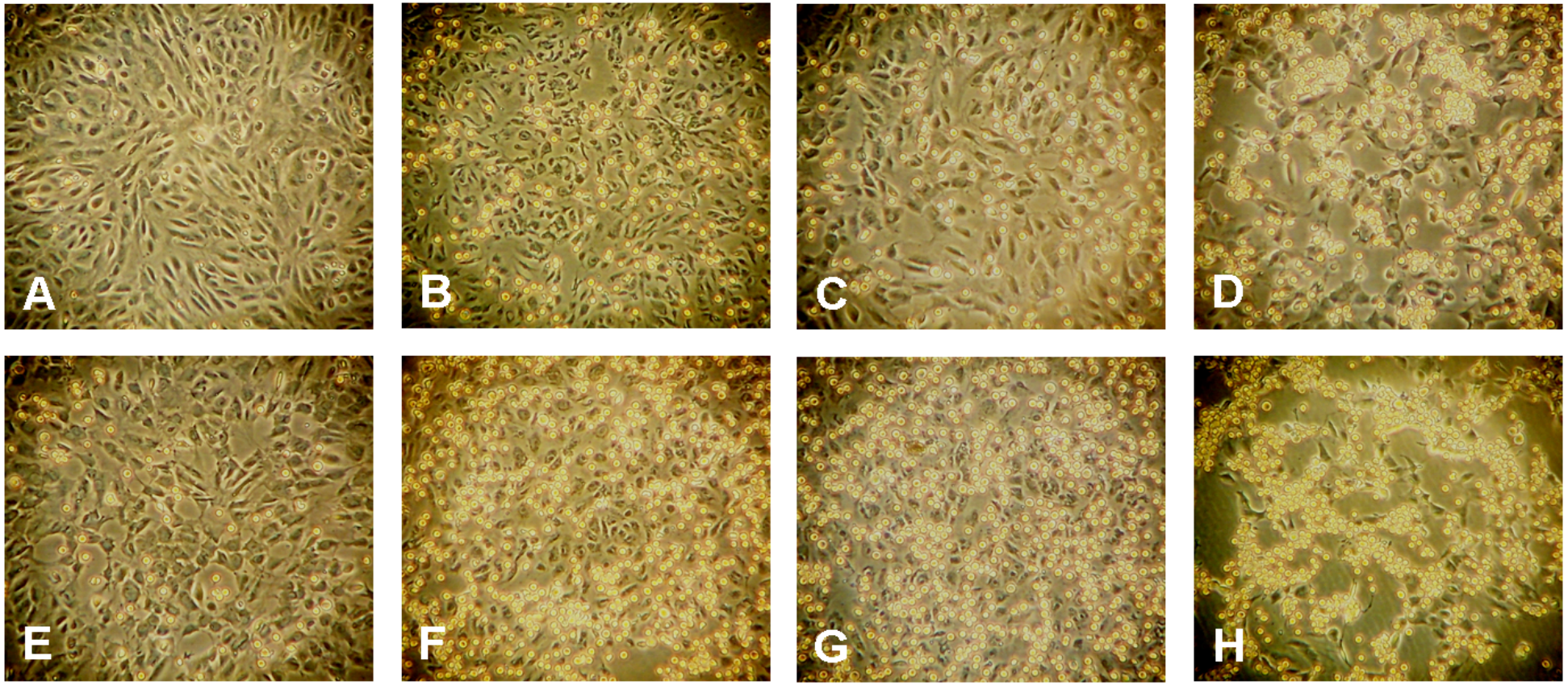
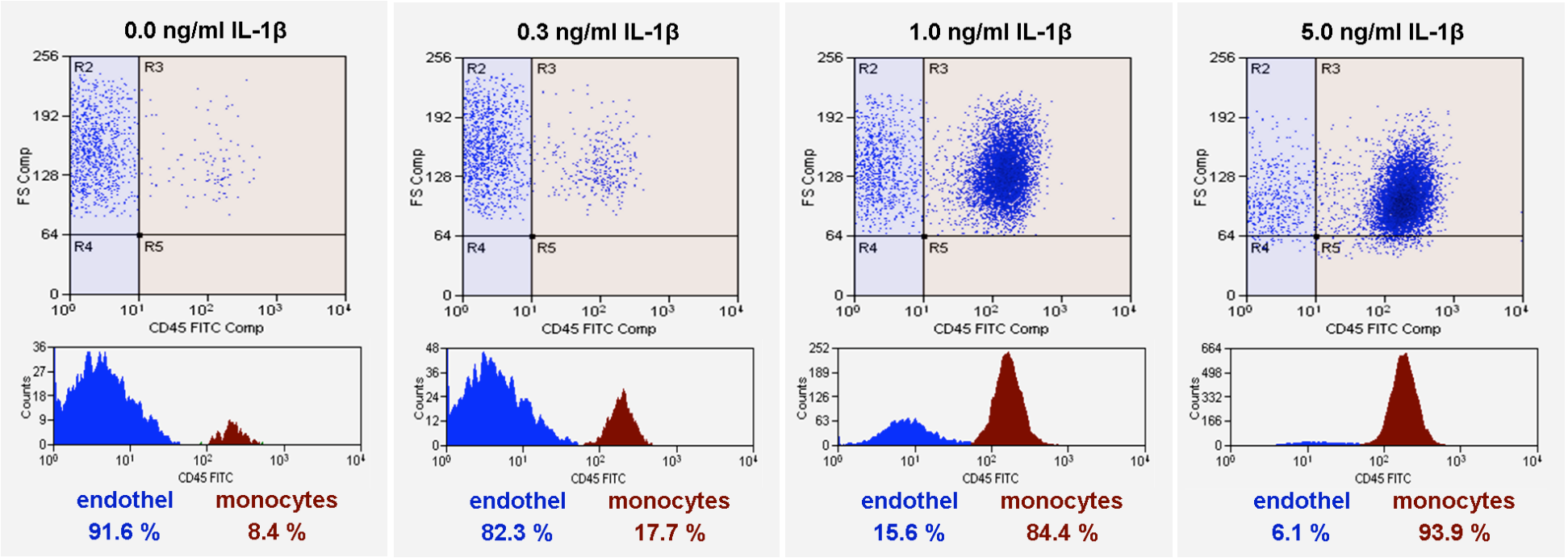


Figure 24a Adhesion of monocytes to endothelial cell monolayers.
CBEC were seeded onto 24 well culture plates and grown to near confluence (1). Endothelial cells were then stimulated with varying concentrations of IL-1β and 4.5 h later $0.5x10^6$ Mono-Mac-6 monocytic cells were centrifuged onto the CBEC monolayers and allowed to be in contact with the endothelial cells for 30 min (2). Afterwards, all non-adhering monocytic cells were removed within 3 washing steps. Further proceedings were taken as described previously. Upper row of photos (A-D) show CBEC-pLXSN-IL-1ra, lower row (E-H) shows CBEC-pLXSN. A,E Occasional monocytes adhering on unstimulated endothelium. B,F Monocytes firmly adhering on CBEC prestimulated with 0.6 ng/ml IL-1β. C,G Adhesion of monocytes on 1 ng/ml IL-1β prestimulated CBEC. D,H As determined on loosely seeded subconfluent monolayers, monocytes exclusively adhered to the endothelial cells and not to the underlying collagen-coated surface (prestimulation with 3 ng/ml IL-1β).



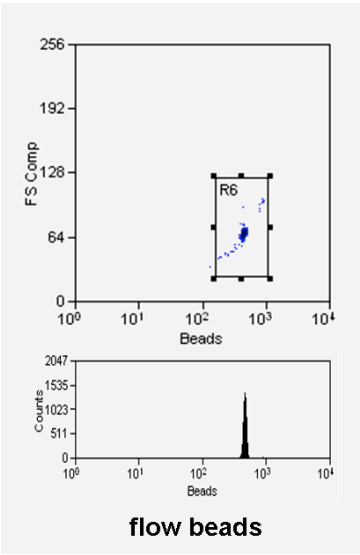


Figure 24b Monocyte adhesion to endothelium - quantification by FACS and flow beads.

Upper row shows FACS results of one representative experiment of monocyte adhesion to CBEC pLXSN-IL-1ra either unstimulated (left histogram) or prestimulated with 0.3, 1.0 and 5.0 ng/ml IL-1β, respectively. Adhesion assay was performed as mentioned previously and persistently adherent monocytes were harvested along with the underlying endothelial cells by collagenase treatment. Cells were labelled with FITC-conjugated CD45 antibodies and thereby monocytic cells (CD45 positive) were separated from CBEC (CD45 negative) as shown in the charts above. Scatter plots and histograms of FACS analysis indicate the relation of adherent Mono-Mac-6 monocytic cells to endothelial monolayers in dependency of the indicated concentrations of IL-1β during the prestimulation period. Quantification and determination of absolute numbers of adherent monocytic cells was achieved by simultaneous count of appointed amounts of flow beads with each measurement (left chart) as described under *Materials and Methods*.

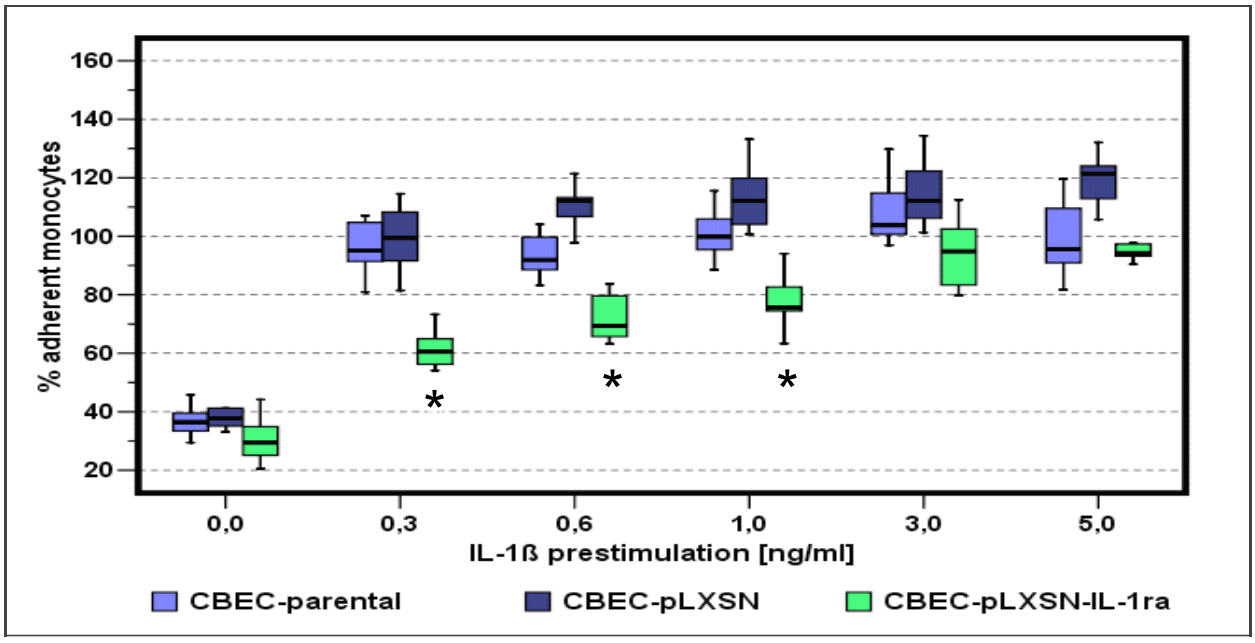


Figure 24 Decreased adhesivity of CBEC-pLXSN-IL-1ra towards monocytic cells.

CBEC (parental, pLXSN- and pLXSN-IL-1ra transduced, respectively) were seeded onto 24 well culture plates and allowed to reach confluence during overnight incubation. After 24 h CBEC prestimulation was started by establishing indicated concentrations of IL-1β in the endothelial cell culture medium. 4.5 h later $0.5x10^6$ Mono-Mac-6 monocytic cells were layered onto the CBEC and allowed to be in contact with the endothelial monolayer for 30 min. The adhesion assay was terminated by removal of all the non-adhering monocytic cells within 3 washing steps. Persistently adherent cells - comprising both monocytes and CBEC - were finally harvested with collagenase treatment. Quantification of adherent monocytes was achieved by FACS analysis by means of CD45 antibodies and FACS beads as described under *Materials and Methods*. In the chart, number of adherent monocytes on untransduced CBEC following prestimulation with 5 ng/ml was set 100% and all other measurements are related to that value. Box plots show means ± SD and 25/75 percentile. * P< 0.01 versus pLXSN empty vector group by Student's t-test.

The results of the endothel-monocyte adhesion assays provide evidence for an important role of icIL-1ra in inhibition of IL-1β mediated monocytic adhesion and show the possibility of reduction of endothelial adhesivity by means of transgenic expression of icIL-1ra protein by retrovirally transduced CBEC.

5.9 Transendothelial migration of monocytic cells

Transendothelial migration of monocytes and leukocytes through the vascular endothelial lining is a hallmark of inflammatory processes and constitutes a complex interplay of endothelial and monocytic activation, release of chemotactic factors by the endothelium and the subendothelial tissue, upregulation of endothelial adhesion receptors and both structural and functional injury of the endothelial layer. Transmigrated inflammatory cells lead to activation and proliferation of the subendothelial lining and thereby damage the inflamed tissue. The intact endothelium is the first line of defense against transmigrating monocytes and leukocytes and impairment of the endothelial function results in enhanced adhesion with subsequent transmigration through the endothelium. Similar to monocyte adhesion, activation of endothelial cells by proinflammatory

cytokines (IL-1β is among of the most important ones) represents a crucial step in initiation of transmigration processes. Performing transmigration studies in a modified Boyden chamber assay we aimed to determine potential benefits of CBEC producing the transgene icIL-1ra in reducing transmigration of monocytic cells through endothelial monolayers.

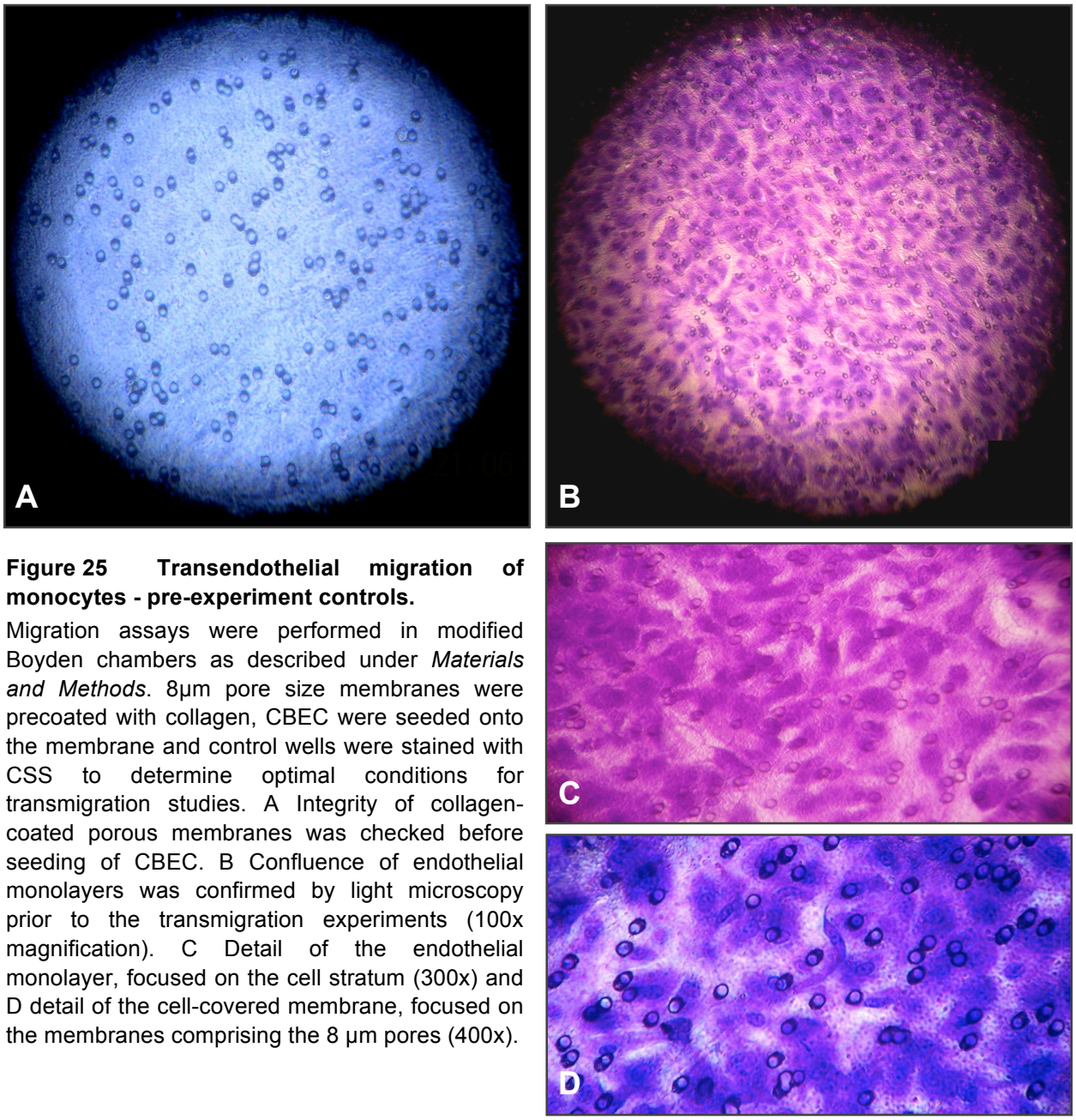


Figure 25 Transendothelial migration of monocytes - pre-experiment controls.
Migration assays were performed in modified Boyden chambers as described under *Materials and Methods*. 8µm pore size membranes were precoated with collagen, CBEC were seeded onto the membrane and control wells were stained with CSS to determine optimal conditions for transmigration studies. A Integrity of collagen-coated porous membranes was checked before seeding of CBEC. B Confluence of endothelial monolayers was confirmed by light microscopy prior to the transmigration experiments (100x magnification). C Detail of the endothelial monolayer, focused on the cell stratum (300x) and D detail of the cell-covered membrane, focused on the membranes comprising the 8 µm pores (400x).

Figure 25 explains pre-experimental preparations and controls while **figure 26** shows structure and experimental design of the transmigration assay. In order to ensure unimpaired permeability of the preformed pores of the membrane after the coating procedure, transmigration experiments with Mono-Mac-6 monocytic cells were also carried out without seeding of endothelial cells onto the membrane. **Figure 25 A** shows the transmigration membrane post coating with collagen. Control experiments revealed that collagen coating did not alter permeability of the transmigration membrane (data not shown). As determined by light

microscopy following staining (shown in **figure 25 B** and **C)**, it was possible to establish confluent endothelial monolayers on the collagen-coated porous membranes of the chambers.

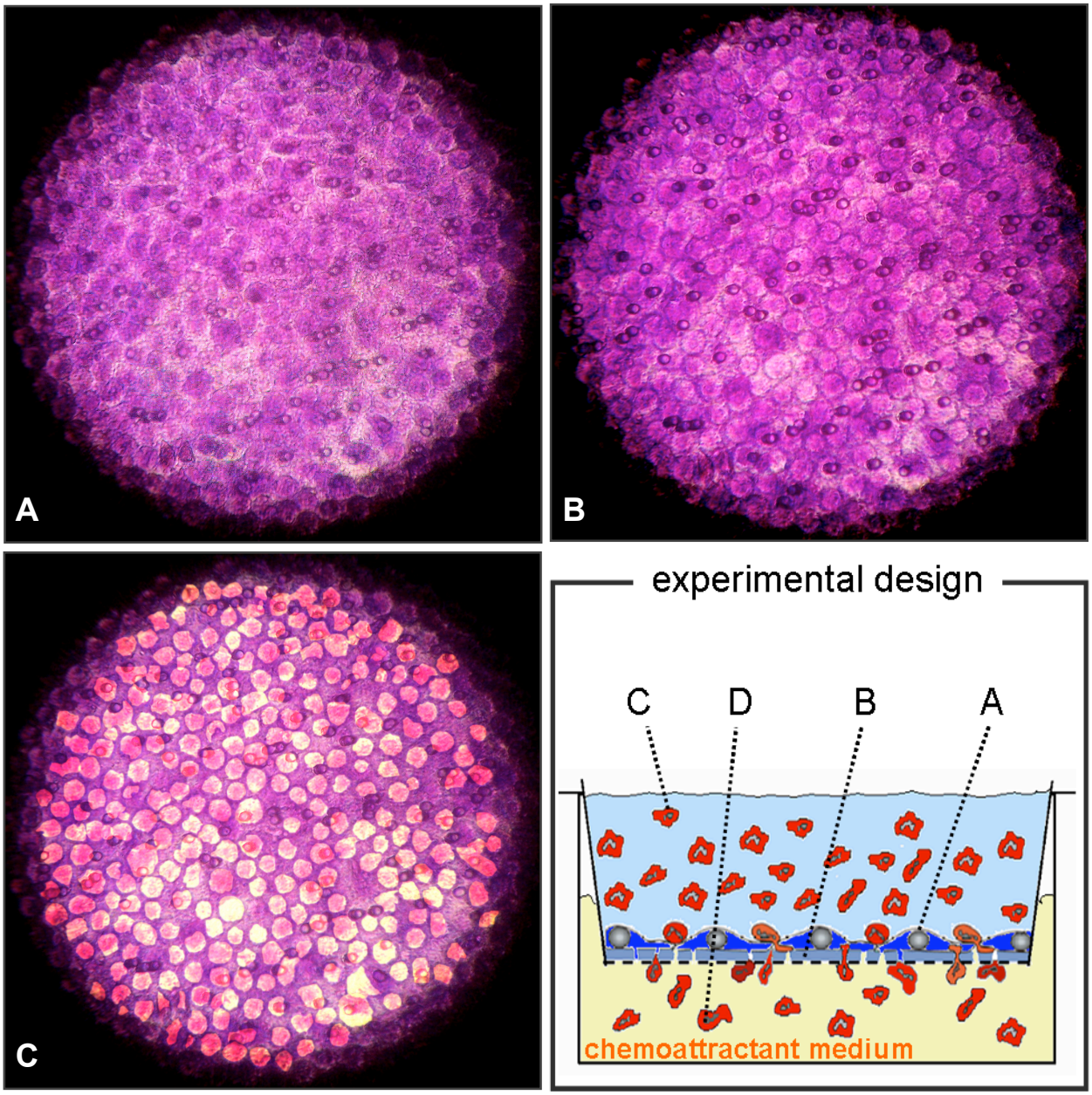


Figure 26 Structure of the transmigration assay.

Three strata of the modified Boyden chamber: **A** CSS stained endothelial monolayer **B** Focus on the 8 µm pore size membrane **C** Overlying monocytes in the upper chamber during transmigration procedure **D** Transmigrated monocytes in lower chamber.

Furthermore, pre-experimental controls proved the cytokines MCP-1 and MIP-1α to be potent chemoattractants for monocytic cells in our set of experiments (data not shown). Taken together, control experiments ascertained controlled and reproducible conditions for undisturbed performance of transmigration studies.

Results of monocytic cell migration through CBEC monolayers are shown in **figure 27**. Firstly, non-activated endothelial monolayers on the semipermeable membranes constituted

functionally efficient barriers for monocytic cell transmigration as merely less than 60% of the monocytic cells of the upper chamber transmigrated through the unstimulated endothelium. In that, CBEC producing icIL-1ra showed significant inhibition of basal transmigration rates of monocytic cells (38.5 ± 7.8 % (CBEC-pLXSN-IL-1ra) versus 57.1 ± 10.3 % and 52.3 ± 6.6 % for CBEC parental and CBEC-pLXSN, respectively).

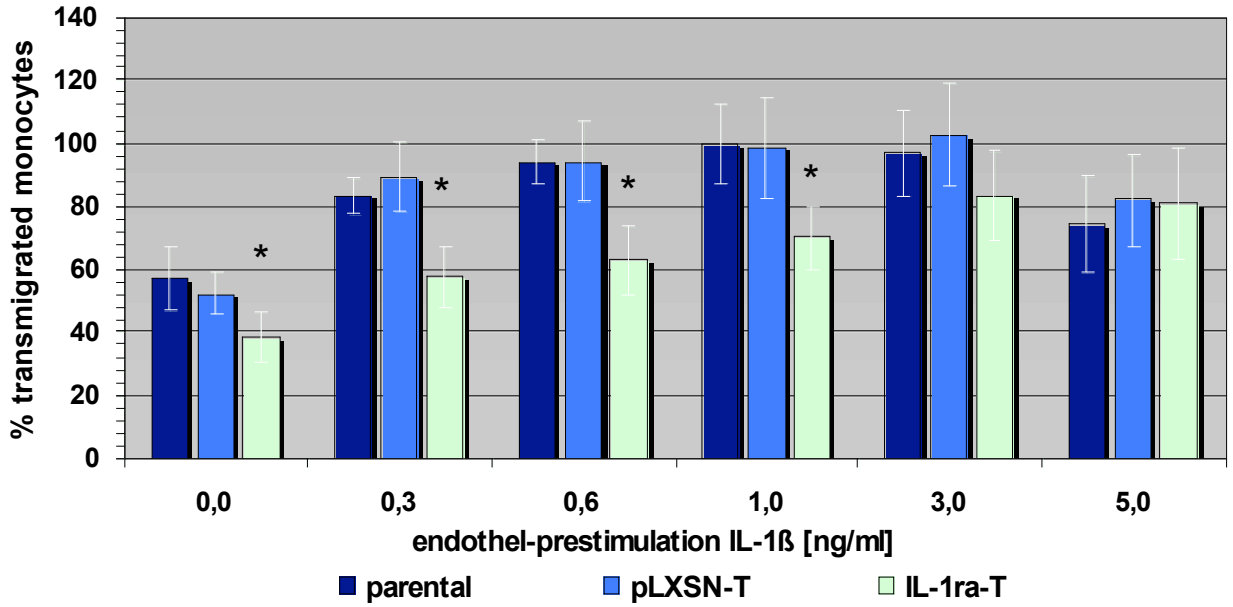


Figure 27 Migration of monocytic cells through activated endothelial monolayers is diminished in CBEC transgenic for icIL-1ra.

CBEC were grown to a confluent state on collagen-coated 8 µm pore size polycarbonate membranes. Prestimulation of the endothelial monolayer was carried out by establishing progressional amounts of IL-1β in the modified Boyden chambers as indicated in the abscissa. After 4 h of prestimulation the state of endothelial cell confluence was confirmed by staining and light microscopy of the monolayers and the upper chambers were thereupon filled with equal amounts of Mono-Mac-6 monocytic cells. Lower chambers were filled with the chemoattractants MCP-1 and MIP-1α whereon transendothelial migration of the monocytic cells along this cytokine-gradient was allowed for 24 h. Transmigrated monocytic cells that had come down to the lower chambers and to the undersurface of the membranes were recovered and subsequently detected and counted by means of staining with CD45 antibodies and FACS with the aid of FACS beads as previously described (see figure 24b). The diagram shows relative numbers of transmigrated monocytes through differently prestimulated monolayers of CBEC - either parental, empty-vector transduced or IL-1ra transduced, respectively. Number of transmigrated monocytes through parental CBEC prestimulated with 1 ng/ml IL-1β was set 100%. Data shown are means ± SD and represent 4 independent experiments. * P< 0.05 versus pLXSN empty vector group by Student's t-test.

Secondly, prestimulation of the endothelial monolayers with sequentially increased amounts of IL-1β resulted in a marked increase of transmigrating monocytic cells reaching its maximum at a concentration of 1 ng/ml IL-1β on parental CBEC and CBEC-pLXSN. However, prestimulation with 0.3 ng/ml IL-1β already resulted in 83.4 ± 5.6 % and 89.2 ± 11.0 % and thereby near maximum transmigration rates through parental and empty-vector transduced CBEC. In contrast, CBEC monolayers transgenicly producing icIL-1ra showed different properties in transendothelial migration assays with monocytic cells as shown in **figure 27**. With 57.8 ± 9.7 %, 62.9 ± 10.8 % and 70.0 ± 9.9 % of transmigrating Mono-Mac-6 cells through CBEC-pLXSN-

IL-1ra monolayers prestimulated by 0.3, 0.6 and 1.0 ng/ml IL-1β, respectively, transgenic expression of icIL-1ra significantly reduced transendothelial migration of monocytic cells by 35.2%, 33.2% and 29.0% compared to empty-vector pLXSN transduced CBEC.

In conclusion, transgenic expression of icIL-1ra by CBEC remarkably inhibited transendothelial migration of monocytic cells and thus preserved the natural function of the endothelium as a barrier for inflammatory cells even under influence of IL-1β stimulation in this in vitro assay.

5.10 Endothel - Monocyte Coculture

Rolling, adhesion, and transendothelial migration of mononuclear blood cells is strongly influenced by the interactions between the endothelium on the one hand and monocytes or leukocytes, respectively on the other hand. Endothelial activation by proinflammatory cytokines (mainly IL-1β and TNF-α) or by direct contact with the inflammatory cells is a major determinant of these interactions. Since activation studies revealed attenuated upregulation of distinct genes involved in vascular inflammatory processes in CBEC carrying the transgene icIL-1ra (see chapter 5.6 b) and functional in vitro assays proved transgenic production of icIL-1ra by retrovirally transduced CBEC to inhibit rolling, firm adhesion and transmigration of monocytic cells on/through endothelium (chapter 5.7, 5.8 and 5.9) we wanted to confirm the differential phenotype of transgenic CBEC-pLXSN-IL1ra on the protein level. Therefore, we investigated expression of ICAM-1 and VCAM-1 (constituting the most important adhesion molecules involved in adhesion and transmigration processes between endothelial and mononuclear blood cells) and Tissue Factor (the main inducer of the extrinsic coagulation pathway and therefore responsible for prothrombotic processes at sites of vascular inflammation) in an experimental model of endothel-monocyte coculture. Surface expression of the mentioned molecules was analysed by fluorescence activated cell sorting (FACS) analysis whereas production of IL-1β in coculture experiments was simultaneously detected by enzyme linked immunosorbent assay (ELISA).

Figure 28 A shows one representative measurement of ICAM-1 (CD54) expression on CBEC and Mono-Mac-6 monocytic cells during endothel-monocyte coculture and **figure 28 B** gives cumulative coculture results for ICAM-1, VCAM-1 and tisse factor expression on CBEC-pLXSN-IL-1ra versus CBEC-pLXSN.

ICAM-1 was found to be markedly increased on the surface of both transgenic and empty-vector transduced CBEC during endothel-monocyte coculture (mean ± SEM: 85.5 ± 2.4 % and 58.8 ± 4.5 % in coculture with monocytic cells versus 60.6 ± 5.8 % and 23.5 ± 2.5 % under basal conditions in monoculture for CBEC-pLXSN and CBEC-pLXSN-IL-1ra, respectively). As shown in **figure 28 A**, additional stimulation of the coculture with 0.5 ng/ml IL-1β resulted in further increase of ICAM-1 surface expression with nearly all empty-vector transduced CBEC staining positive for CD54 (98.8 ± 0.2 % and 82.0 ± 3.1 % positive CBEC-pLXSN and CBEC-pLXSN-IL-1ra, respectively). Thereby transgenic expression of icIL-1ra by retrovirally transduced CBEC significantly decreased ICAM-1 surface expression at both stages of endothel-monocyte coculture as well as under basal conditions in endothelial monoculture (-

61.3 % (basal), -31.2 % (coculture), -17.2 % (coculture stimulated) compared to CBEC-pLXSN). Further observations have been made in the monocytic cell fraction of the cocultures. While endothel-monocyte coculture itself did not upregulate ICAM-1 expression on Mono-Mac-6, additional stimulation with IL-1β caused a notable increase of ICAM-1 surface expression on the monocytic cells (83.9 ± 4.1 % in stimulated coculture versus 66.7 ± 4.4 % in unstimulated coculture). However, the monocytic induction of ICAM-1 was far less than the induction in endothelial cells and production of icIL-1ra by transgenic CBEC failed to reduce increased ICAM-1 expression on monocytic cells in this set of experiments.

Different results were found in coculture FACS analysis of VCAM-1 and Tissue Factor as coculture of monocytic cells with CBEC alone did not result in enhanced expression of any of these molecules **(figure 28 B)**. Only additional stimulation with IL-1β manifestly upregulated both VCAM-1 and tissue factor expression solely on the endothelial cell fraction but not on the monocytic cells. IL-1β mediated increase in VCAM-1 surface expression was less prominent in CBEC-pLXSN-IL-1ra (25.3 ± 2.2 % versus 40.3 ± 1.5 %, $p < 0.01$) as well as tissue factor levels were significantly lower in CBEC carrying the transgene icIL-1ra when cultured with monocytic cells and stimulated with 0.5 ng/ml IL-1β (20.7 ± 2.5 % versus 27.6 ± 1.8%, $p < 0.05$) compared to empty-vector transduced CBEC.

Regarding the release of IL-1β during endothel-monocyte coculture we observed very low levels in culture supernatants in monoculture of CBEC and Mono-Mac-6 as well as in cocultures either additionally stimulated or not. Coculture resulted in noticable increases of IL-1β release whereas stimulation of cocultures did not induce further increase in IL-1β production compared to unstimulated cocultures **(figure 29)**. Nonetheless, CBEC-pLXSN-IL-1ra showed significantly lower levels of IL-1β production in monoculture and both unstimulated and stimulated coculture (0.8 ± 0.2 vs. 2.6 ± 0.2 (-69.2%) in monoculture, 2.0 ± 0.1 vs. 3.4 ± 0.4 (-41.2%) and 2.1 ± 0.3 vs. 3.1 ± 0.4 ng/ml (-32.3%) in cocultures, respectively) compared to CBEC-pLXSN.

In conclusion, we found upregulation of ICAM-1, VCAM-1, Tissue Factor, and IL-1β expression during endothel-monocyte coculture. In case of ICAM-1 and IL-1β coculture alone induced upregulation of these molecules, whereas increased VCAM-1 and tissue factor expression was exclusively due to additional stimulation by IL-1β. Under the aforementioned conditions transgenic production of icIL-1ra by transduced CBEC was able to inhibit the increased expression of these molecules. These results supplement our RT-PCR activation analysis (chapter 5.6 b) as ICAM-1 and IL-1β expression patterns of CBEC-pLXSN and CBEC-pLXSN-IL-1ra are confirmed and specified whereas VCAM-1 and tissue factor FACS analysis over and above RT-PCR results showed significantly attenuated expression of these molecules in transgenic CBEC during endothel-monocyte coculture. Moreover, this coculture model shows that increased expression of ICAM-1 and Tissue Factor during coculture studies was mainly due to upregulation on the endothelial cell fraction though inducible in both monocytic cells and endothelial cells.

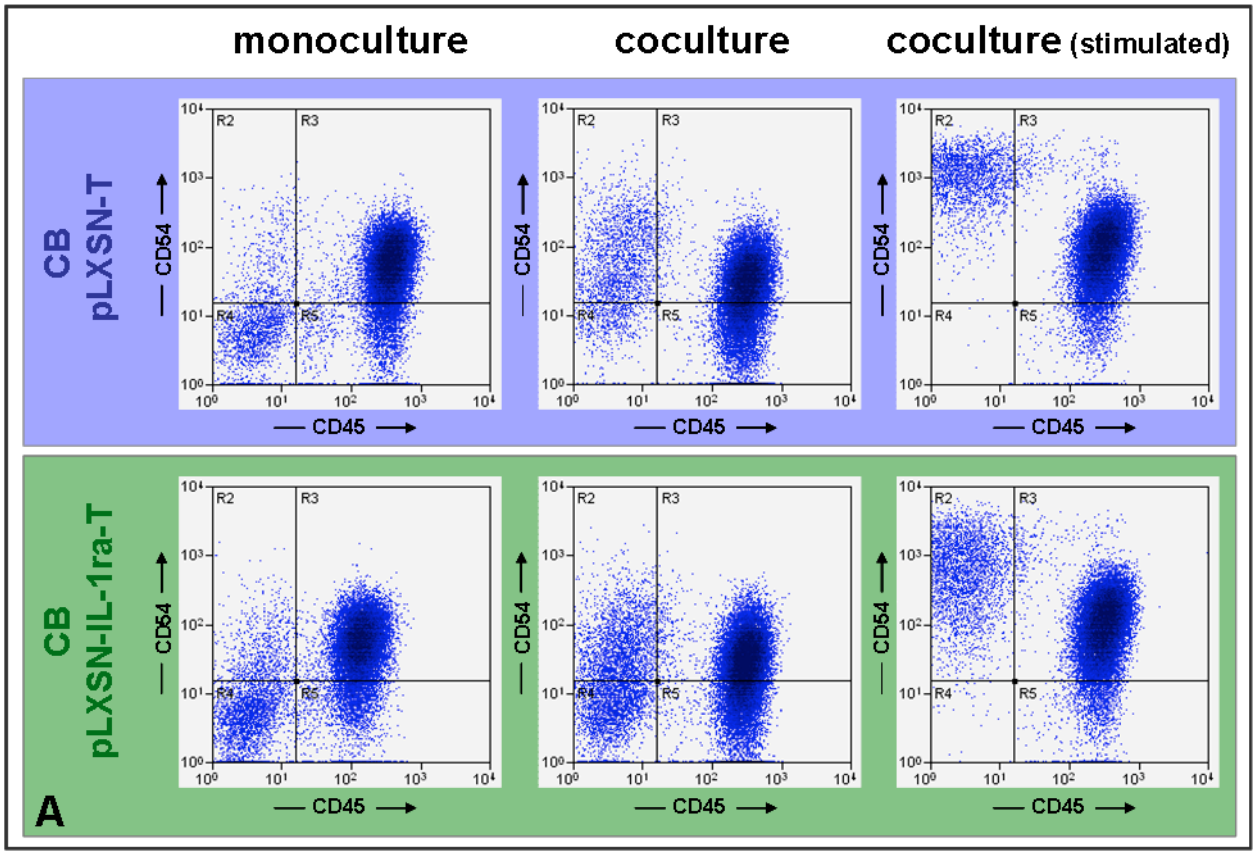
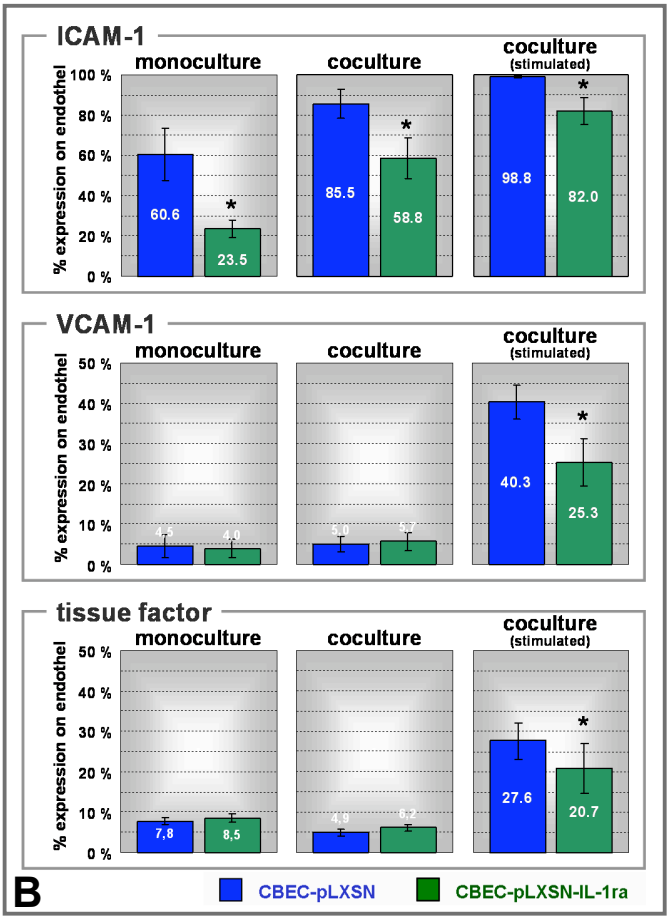


Figure 28 Endothel - Monocyte Coculture.

Basal expression of ICAM-1 and VCAM-1 adhesion molecules on the endothelial and monocytic surface as well as Tissue Factor expression by endothelial cells was determined by FACS analysis of both CBEC and monocytic cells growing in separate cultures (results shown in column monoculture, diagram on the left). Monocultures were allowed to grow to near confluence in fresh medium before measurements were performed. In addition, expression of ICAM-1, VCAM-1, and tissue factor was determined during endothel-monocyte coculture either with or without additional stimulation by 0.5 ng/ml IL-1β. Coculture was carried out for 6 h after which cells were harvested and prepared for FACS. Endothel-monocyte cell suspensions underwent double staining: FITC-conjugated anti-CD45 antibody facilitated distinction between $CD45^+$ monocytic cells and $CD45^-$ CBEC; PE-conjugated anti-CD54, anti-CD106, or anti-CD142 antibodies were used to detect endothelial and monocytic surface expression of the corresponding proteins ICAM-1, VCAM-1, or Tissue Factor, respectively. Figure at the top shows one representative measurement of ICAM-1 expression. Abscissa gives fluorescence intensity of bound CD45 antibodies on the cells. Consequently, clouds on the left represent CBEC and dots on the right correspond to monocytic cells. Ordinate indicates intensity of ICAM-1 expression on CBEC and monocytes. Charts show cumulative results of 4 independent experiments. Data are presented as means ± SD and compare CBEC-pLXSN-IL-1ra to CBEC-pLXSN. * P< 0.01 significant diff. as determined by Students t-test.



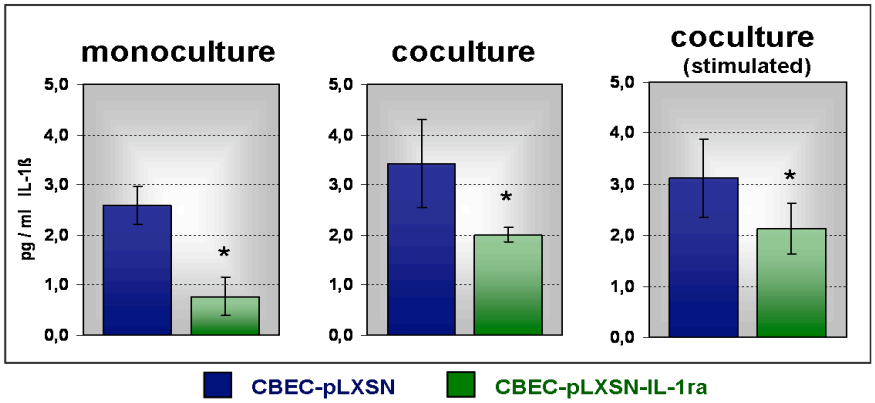


Figure 29 Transgenic production of icIL-1ra reduces release of IL-1β during endothel-monocyte coculture.

Endothel-monocyte coculture was carried out as described before. Prior to harvesting of cells after termination of coculture supernatants were removed, freed from cell detritus and present IL-1β was determined by ELISA according to the manufacturers instructions. Data are derived from 4 independent measurements and show means ± SD. * P<0.01 versus pLXSN empty vector group by Student's t-test.

Thereby our results give further proof of the outstanding role of the endothelium in regulation of molecules predominantly involved in inflammatory processes. Moreover, they provide additional evidence for the feasibility of reducing CBEC activation through retroviral transduction with the transgene icIL-1ra.

6 Discussion

The endothelium, innermost stratum of all blood vessels, physiologically holds important antiinflammatory and antithrombotic functions in vascular homeostasis and constitutes the first line of defense against detrimental influences of the blood flow. Thereby the lining of endothelial cells is to a main part responsible for maintenance of regular blood circulation and protection of the integrity of the vessel wall.

Endothelial dysfunction is caused by activation of endothelial cells by proinflammatory cytokines, mechanical damage by the blood flow (e.g. high blood pressure or flow turbulences) or its harmful contents (e.g. oxLDL or infectious agents) or by contact with inflammatory cells of the blood stream (thrombocytes, leukocytes and monocytes). Insufficient self-renewal of senescent, damaged, and malfunctioning endothelium caused by impaired angiogenesis and vasculogenesis are other causes of endothelial dysfunction. Impaired vasculogenesis results from a lack of endothelial progenitor cells capable of differentiating into mature endothelium and thereby able to reendothelialize sites of functional or structural endothelial damage.

Endothelial dysfunction is characterized by a change in the endothelial phenotype rendering it proinflammatory, proadhesive for peripheral blood mononuclear cells, prothrombotic and vasoconstrictive. Due to the strong influence of endothelial cells on vascular homeostasis and due to their exposed localization in the circulatory system endothelial dysfunction plays a crucial role in the development and regulation of inflammatory vascular processes.

Upon activation of the endothelium the vascular inflammation cascade with a multitude of leukocyte/monocyte-endothelial interactions is initiated. Characterized by rolling, firm adhesion and transmigration of monocytes and leukocytes it leads to activation and proliferation of subendothelial tissue and eventually results in acute processes such as neointimahyperplasia, thrombosis and acute vessel occlusion or chronic processes such as atherosclerosis, stenosis and tissue ischemia.

The proinflammatory cytokine Interleukin-1 is one of the most important mediators of vascular inflammatory processes. It is produced by nearly all the cells involved in the pathogenesis of inflammation (mainly thrombocytes, monocytes, leukocytes, endothelial cells, smooth muscle cells) and exerts detrimental effects on a wide variety of target cells. In vascular inflammatory processes the endothelium represents the main target of IL-1 mediated actions. The most important effects of IL-1β on the vascular endothelium are upregulation of adhesion molecules, release of chemoattractant and prothrombotic substances, induction of smooth muscle and fibroblast proliferation, endothelial apoptosis and production of further inflammatory cytokines.

Based on this background the present work pursued two trains of thoughts to develop new strategies for future treatment of inflammatory vasculopathies:

Firstly, delivery of endothelial progenitors capable of reendothelializing sites of vascular injury and differentiation into mature endothelium might be suitable to replace dysfunctional endothelium of the vasculature and thereby re-establish the natural antiinflammatory properties

of intact endothelium and restore integrity of the vessel wall resulting in inhibition of vascular inflammation.

Secondly, as the distinctive processes of the vascular inflammation cascade - activation of endothelial cells with subsequent rolling, adhesion and transmigration of mononuclear blood cells on/through the endothelium - are strongly influenced by the proinflammatory cytokine Interleukin-1, local delivery of Interleukin-1 receptor antagonist, a natural occurring inhibitor of Interleukin-1 mediated actions, might be a useful instrument to inhibit the distinctive processes of vascular inflammation.

By transduction of Endothelial Progenitor Cells derived from human umbilical cord blood with the transgene Interleukin-1 receptor antagonist our purpose was to combine the possibility of enhancement of reendothelialization with local antiinflammatory drug delivery eventually aiming to specifically inhibit inflammatory vascular processes.

In order to examine the feasibility of this approach several investigations had to be made. First of all, consistency of endothelial progenitor cell isolation from human umbilical cord blood and possibility of expansion to clinically relevant numbers had to be examined. Secondly, aiming to achieve stable gene transfer into cord blood-derived endothelial cells, an effective and reliable transduction method had to be developed and long-term transgene expression had to be proven.

In the third part of our studies we wanted to determine the effect of transgenic expression of icIL-1ra by transduced CBEC on the distinct mechanisms of vascular inflammation. Therefore we investigated endothelial activation by gene expression analysis as well as in a model of endothel-monocyte coculture. Furthermore, we performed flow adhesion experiments and developed in vitro models of monocyte adhesion and transendothelial migration in order to elucidate the capability of CBEC expressing the transgene icIL-1ra to inhibit these processes.

Our investigations show that endothelial progenitor cells can consistently be isolated from human umbilical cord blood and regularly be expanded up to 10^{20} cells within up to 6 months of culture. Furthermore, we show feasibility of stable gene transfer into CBEC by a modified retroviral transduction method and prove long-term gene expression of the transgene intracellular Interleukin-1 receptor antagonist.

Activation assays revealed attenuated induction of distinct genes playing major parts in vascular inflammation in CBEC carrying the transgene icIL-1ra in response to stimulation by proinflammatory cytokines. Among those are the adhesion molecules ICAM-1 and VCAM-1, the procoagulative substance tissue factor, the monocyte chemoattractant MCP-1 and other proinflammatory agents including IL-1β and COX-2.

Moreover, CBEC-pLXSN-IL-1ra exert inhibitory effects on the critical mechanisms of vascular inflammation as they reduced monocyte rolling, adhesion and transendothelial migration during in vitro assays.

The results of our study are discussed in the following.

6.1 Isolation and expansion of cord blood-derived endothelial progenitor cells

Endothelial Progenitor Cells (EPC) have been isolated and expanded from peripheral blood (Asahara et al., 1997; Lin et al., 2000), bone marrow (Lin et al., 2000; Shi et al., 1998) and umbilical cord blood (Murohara, 2001; Nieda et al., 1997). Due to their angiogenic potential EPC are of major interest in research on the field of vascular repair and therapeutic vasculogenesis in ischemic tissues (Asahara et al., 1997). Limited quantities of EPC are a main factor impeding effective clinical application (Masuda and Asahara, 2003; Rafii and Lyden, 2003). Deduced from animal experiments it can be assumed that no fewer than 1x 10^7 cells per kg bodyweight are required for efficient therapeutic vasculogenesis in humans. However, using peripheral blood-derived progenitors a volume of more than 10 l may be necessary to obtain sufficient numbers for clinical applications (Masuda and Asahara, 2003) which limits the use of peripheral blood as a source for EPC in patients. Further disadvantages of peripheral blood are a decrease of total EPC numbers and functional impairment associated with several risk factors present in patients requiring therapeutic vasculogenesis, such as aging, diabetes, and hypercholesterolemia. Alternatively, the use of autologous or perhaps even allogenic EPC from cord blood and their efficient in vitro expansion prior to transplantation might constitute a feasible strategy for delivery of cells capable of vascular repair and neovasculogenesis.

Compared to the other accessible sources, cord blood contains the largest number of progenitor cells and shows the most rapid outgrowth and proliferation of EPC (Murohara et al., 2000; Vaziri et al., 1994) up to a mean of 56.5 ± 1.4 (mean ± SEM) cumulative population doublings **(this study, figure 7)**.

These observations show that cord blood-derived EPC might have distinct advantages regarding experimental transplantation studies and possible clinical applications. Therefore, optimization of isolation and expansion of EPC from cord blood has been subject of numerous investigations.

We isolated and differentiated endothelial progenitor cells from cord blood (CB) in the presence of FGF-2, SCF, SCGF-β and VEGF based on a protocol described by Gehling et al. (Gehling et al., 2000). Using this protocol, our group could expand an input of 10^5 -10^7 CB-derived $CD34^+$ progenitors up to numbers of 10^{15} endothelial cells during a total culture time of 8 weeks (Herder et al., 2003). These growth characteristics resemble those shown in **figure 3**. However, with a total yield of averagely 8.4 x 10^{20} endothelial cells emerging from 10^6 CB-derived $CD34^+$ cells and an average life span of over 4 months **(figure 7)** our present results surpassed those of our earlier cultures (Ott et al., 2005a). In fact, the number of CBEC expanded following our protocol surpasses the total pool of endothelial cells in the vasculature of a human individual (approx. 10^{13} cells) by far (Cines et al., 1998).

It has been reported that expansion of cord blood $CD34^+$ progenitor cells in coculture with autologous human umbilical vein endothelial cells (HUVEC) is more efficient compared to cytokine-supplemented liquid culture (Yildirim et al., 2005). This strategy might represent a feasible alternative approach for ex vivo expansion of cord blood-derived EPC.

6.2 Retroviral transduction of cord blood-derived endothelial cells

Present strategies in treatment of inflammatory processes and restoration of vascular homeostasis mainly focus on influencing thrombocytes, monocytes, leukocytes, smooth muscle cells and the coagulative system. Substances used in current therapeutic schemes are anticoagulants (e.g. heparin and cumarins), antithrombocytica such as GPIIb/IIIa (CD41) blockers, lipid-lowering drugs (e.g. statins), NSAR (e.g. aspirin) and anti-hypertensive drugs such as ACE-inhibitors. However, during the last years the protective influence of ACE-inhibitors, statins and antioxidants such as Vitamin C and E on endothelial cell function has become more and more apparent and endothelial dysfunction increasingly became focus of attention as a potential new therapeutic drug target in cardiovascular disease and inflammation (Drexler and Hornig, 1999; Landmesser and Drexler, 2005; Landmesser et al., 2004).

Due to the prominent role of endothelial dysfunction in the pathogenesis of inflammatory vascular diseases and the exposed localization of the endothelium in the vascular system with immediate contact to the blood stream, vascular endothelial cells imply the potential to serve as vehicles for therapeutic drug delivery and constitute an attractive target for gene transfer approaches for future therapies (Koning et al., 2002). A number of candidate genes have already been employed in gene transfer studies of endothelial cells, including plasminogen activator (Dichek et al., 1996; Griese et al., 2003a), hirudin (Griese et al., 2003a; Lundell et al., 1999), nitric oxide synthase (Kong et al., 2004b) and VEGF (Iwaguro et al., 2002).

Regeneration of vascular physiology by transferring IL-1ra has been subject of several gene transfer experiments in mature endothelial cells. In our study, we first describe transduction of cord blood-derived endothelium with an antiinflammatory agent.

Two feasible approaches of genetic modification of the vascular endothelium have been pursued so far: The first is direct in vivo intravascular gene transfer to the mature endothelial cell lining (Lemarchand et al., 1993; Schulick et al., 1995; Smith and Walsh, 2001). However, this strategy has distinct disadvantages such as relatively uncontrolled systemic distribution of the vectors (Melo et al., 2004), low tissue concentration of infectious particles and inappropriate conditions during transduction (e.g. temperature, incubation time, transduction media etc) which usually cut back gene transfer efficiency. In vivo viral gene transfer may also result in acute of chronic immune responses of the host organism (Chirmule et al., 1999) and proinflammatory activation of the endothelium rendering it dysfunctional and causing neointimahyperplasia (Dengler et al., 2000; Newman et al., 1995). The second approach comprises ex vivo culture, expansion and transduction of endothelial cells with subsequent transplantation into autologous or allogenic organisms either by simple intravascular injection (Iwaguro et al., 2002; Parikh and Edelman, 2000) or by accurate delivery by means of vascular grafts (Dunn et al., 1996; Griese et al., 2003b; Panetta et al., 2002) and catheter-based techniques (Tahlil et al., 1997).

Gene transfer can be achieved by various transfection methods with nonviral vectors (including cationic liposomal transfection, crystal coprecipitation and physical methods such as electroporation or ultrasound) as well as by viral transduction of target cells using adenoviral, retroviral and lentiviral vector constructs (Koning et al., 2002; Melo et al., 2004; Parikh and Edelman, 2000).

Although naked plasmid transfer is an easy, time-sparing procedure these vectors usually yield low gene transfer efficiency because of lack of genomic integration and rapid degradation of the vector and thereby do not result in stable or long-term transgene expression (Niidome and Huang, 2002). Generally, transfection procedures are often detrimental to cell viability and impair further cell expansion and survival. This knowledge led to the development of numerous viral transduction techniques. These involve packaging of adeno-, retro- or lentiviral vector constructs into viral particles by a helper cell line and the use of these viruses to transduce target cells. Advantages of this method are effective gene transfer and long-term transgene expression, as the transgene is stably incorporated into the genome of the target cells and therefore constantly inherited on subsidiary cells (with the exception of adenoviral gene transfer, as episomal localization of the viral genomes causes rapid loss of transgene expression (Chen et al., 2003)). On the other hand retrovirus-based methods require proliferating target cells as only dividing cells are able to incorporate the transgene into the genome, rendering these vectors inefficient for transduction of relatively quiescent cells such as normal endothelium (Daly and Chernajivski, 2000a). In contrast, lentiviral vectors can infect both dividing and quiescent cells which should therefore favor their use for gene transfer to mature endothelium (Dishart et al., 2003). However, transgene expression following retro- or lentiviral transduction is mostly not as high as achieved using transfection methods for transient transgene expression. Furthermore, the transgene is occasionally eliminated from the target cell genome or promotors of the transgene are switched off resulting in drop of transgene expression (Daly and Chernajivski, 2000b). One major note of concern is that integration of the transgene into the host genome occurs randomly. This could result in interference with other promoters of the target cell and cause uncontrolled proliferation and oncogenesis (Hu and Pathak, 2000). A finite proliferative potential may therefore be a prerequisite for in vivo applications following vector-mediated gene transfer.

We chose to use retroviral gene transduction. As gene transfer into endothelial cells is often limited by a low transduction efficiency and a high sensitivity to toxic effects exerted by the substances used for transduction (Melo et al., 2004; Tanner et al., 1997), we developed a modified transduction protocol for cord-blood derived endothelial cells by combining results of the research of several groups. The basis of our modifications formed a DEAE-Dextran transduction method described by Kahn et al. (Kahn et al., 1992). Le Doux et al. revealed that retroviral infection of target cells is often inhibited by proteoglycans simultaneously secreted by retrovirus-producing packaging cells (Le Doux et al., 1998). Pretreatment of retrovirus stocks with chondroitinase ABC was reported to enhance transduction efficiency by more than twofold which led us to include this aspect in our optimizations. Furthermore, pretreatment of target cells with chloroquine diphosphate supplemented our transduction protocol (Pear et al., 1993). Morgan et al. revealed that during retroviral transductions concentration of viral particles on the target cells is much more important for transduction efficiencies than is the multiplicity of infection (MOI) (Morgan et al., 1995). Therefore we used least possible volumes of culture medium in order to obtain maximal concentrations of retroviral supernatant. For the same reason, following a publication by Kaptein et al., we abstained from freezing and rethawing of retroviral supernatants and confined to the use of freshly produced retrovirus (Kaptein et al., 1997). Further improvement of retrovirus concentration might arise from a reduction of the period of retrovirus

production from 24h to 6h as described by Dando et al. (Dando et al., 2001). Moreover, maximal infection of target cells was reported to occur after 24 h of virus exposure, with 70% of that level reached after 8h (Morgan et al., 1995). As prolonged duration of retrovirus exposure exerts detrimental effects on target cells (partly due to the fact that the retroviral supernatants do not produce the appropriate growth conditions for target cells) and retroviruses loose activity at 37°C with half-lifes of 6 to 9 hours (Paul et al., 1993), we decided to perform double transduction with a period of 8 hours each in an interval of 24 hours. Furthermore, as effective in increasing half-life of retroviruses and transduction efficiency, production of retroviral supernatants as well as transduction procedures were carried out at 32°C (Bunnell et al., 1995; Kaptein et al., 1997).

Our collaborators Herder et al. described lentiviral transduction of cord blood-derived endothelial progenitor cells with coagulation factor VIII (Herder et al., 2003). With some cell death during and after transduction and a lag phase before cells recovered to take up again the proliferation rate of untransduced controls observations of growth characteristics of transduced cells resembled those in our studies **(figure 6)**. Retroviral transduction did not affect expansion of CBEC as neither endothelial cell life span was shortened significantly nor total yield of cells was reduced in our studies **(figure 7)**. The trend towards a slight decrease in cumulative population doublings and modest prolongation of doubling time did not bear any significance in our in vitro endothelial cell culture **(figure 7 and 8)**. Importantly, post-transduction growth of CBEC of the 5 different donors we transduced did not give any hint on adverse effects such as insertional mutagenesis with uncontrolled proliferation and abolishment of the finite expansion potential of CBEC, as previously described by others (Hu and Pathak, 2000). However, as illustrated in **figure 6**, comparative growth curves of IL-1ra and empty-vector transduced versus parental cells of two pools of CBEC (CB 989-991 and CB 1006-1007) showed distinct differences in proliferation especially in the late phase of endothelial life span with markedly impaired growth of both transgene and empty-vector transduced CBEC. Similarly, Baer et al. observed inhibition of proliferation of late passage retrovirally transduced endothelial cells with concomitant impaired vascular graft endothelialization in vivo (Baer et al., 1996). However, other investigators could did not confirm these results and described normal growth of endothelial cells post retroviral transduction (Inaba et al., 1998; Jankowski et al., 1997). In our view, impaired growth of transduced CBEC from the two pools of donors mentioned above most likely results from the fact that these CBEC were the first pools subjected to the transduction procedure which was further optimized and therefore less cytotoxic in the following transduction procedures.

6.3 Transgenic expression of IL-1ra in cord blood-derived endothelial cells

Vascular inflammation threatens integrity of the vessel wall and bears the risk of tissue damage, thrombosis, and ischemia. The prominent role of IL-1 in vascular inflammation is indisputable (Dinarello, 1997a). IL-1ra is the first described naturally occurring specific receptor antagonist of any cytokine or hormone-like molecule in the human organism (Arend et al., 1998a; Carter et al., 1990)

and inhibits IL-1-dependent mechanisms both in vitro and in vivo (Dinarello, 1998).

Different observations are reported concerning expression of IL-1ra by endothelial cells: While Bertini and colleagues did not detect any isoforms of IL-1ra in HUVEC (Bertini et al., 1992; Haskill et al., 1991), Dewberry et al. demonstrated that CAEC of diseased coronary arteries express both icIL-1ra1 and sIL-1ra and detected exclusively icIL-1ra1 in cell lysates of HUVEC and thereby propagated important protective effects of endothelial IL-1ra expression against chronic vascular inflammation and atherosclerosis (Dewberry et al., 2000; Francis et al., 1999). However, IL-1ra production in these cells was very low, not sufficient to inhibit effects of locally produced IL-1 and did not increase upon IL-1 stimulation (unlike in many other cell types, e.g. chondrocytes (Palmer et al., 2002; Tominagaa et al., 2004)), indicating that the endothelium might lack an important inhibitory mechanism at sites of vascular inflammation.

Production of IL-1ra by cord blood-derived endothelial cells has not been investigated so far. Using the primer design described in **figure 9** we aimed to detect transgenicly expressed icIL-1ra2 as well as any other isoform of IL-1ra in CBEC. As shown in **figure 10**, RT-PCR revealed an extremely weak signal of IL-1ra transcripts in parental and empty-vector transduced CBEC which was over 2^{10}-fold less than that of the housekeeping gene PBGD. There was no upregulation of this signal in response to the proinflammatory stimuli IL-1β and TNF-α **(figure 10)**, which would have been anticipated in case of real expression of IL-1ra, as IL-1β and IL-1ra mostly vary in parallel within the same cell type (Hirsch et al., 1996) and IL-1β mRNA was clearly upregulated in our experiments **(figure 21)**. As parental and empty-vector CBEC never produced detectable amounts of IL-1ra protein neither secreted nor cell-associated as determined by Western blot analysis **(figure 11)** and ELISA **(figure 12)** of cell lysates and supernatants we consider the RT-PCR signal as an unspecific amplification of mRNA below the threshold value.

In contrast, retrovirally transduced CBEC-pLXSN-IL-1ra abundantly produced IL-1ra protein with total levels reaching averagely 40.6 ± 4.4 ng/10^6 cells. With an IL-1ra/total endothelial cell protein ratio ranging between 1:10^4-10^5 in transduced CBEC **(see figure 14)** levels of IL-1ra transgene expression exceeded those of endogenous IL-1ra production by HUVEC (0.5 to 1.5 x10^{-9}, according to the findings of Dewberry et al. (Dewberry et al., 2000)) by factor 10^4 to10^5.

The biological effect of IL-1 is determined by the ratio between IL-1 and IL-1ra and a manifold surplus of IL-1ra is actually needed to markedly inhibit IL-1-mediated mechanisms (Arend, 2002). In order to estimate the physiological significance of transgenic icIL-1ra production by transduced CBEC, monocytes and polymorphonuclear cells (PMN) from human peripheral blood may serve as a reference as they constitute the main source of IL-1ra in the circulation in humans. Expressing both the mRNA for intracellular and secreted IL-1ra varying amounts of cell-associated and secreted IL-1ra are produced by these cells. Levels of intracellular IL-1ra range between 1 to 3 ng/10^6cells and secreted IL-1ra is usually below 1 ng/10^6cells/ml in both resting monocytes and PMN. Upon stimulation, intracellular and secreted IL-1ra levels in monocytes rise up to an average of 8 -16 ng/10^6cells and 14 -22 ng/10^6cells/ml (72% of total IL-1ra), respectively (Haskill et al., 1991; Kline et al., 1994; Muzio et al., 1994). Corresponding amounts of intracellular and secreted IL-1ra in stimulated PMN were found to be 3.7 ng/10^6cells and 1.7 ng/10^6cells/ml (32% of total IL-1ra) (Muzio et al., 1994). Production of IL-1ra has also been proven

for several other cell types. Keratinocytes are known to express predominantly the intracellular isoform 1 of IL-1ra and produce high levels of cell-associated IL-1ra up to 100 ng/10^6cells (Haskill et al., 1991), whereas chondrocytes predominantly produce the secreted isoform of IL-1ra and levels reach 1.2 ng/10^6cells/ml in response to proinflammatory stimuli (Palmer et al., 2002).

In conclusion, the amounts of IL-1ra transgenicly produced by CBEC are comparable to total IL-1ra production of stimulated peripheral blood monocytes as described by Muzio et al. (Muzio et al., 1994), and exceed levels of stimulated peripheral blood polymorphonuclear cells and neutrophils (Malyak et al., 1998b). As monocytes and PMN are regarded as the main source of IL-1ra in the human circulation (Arend et al., 1998a) and transgenic CBEC equal their production of this cytokine antagonist one can assume that the phenotypic character of CBEC-pLXSN-IL-1ra is shifted towards antiinflammatory properties with these cells potentially capable of inhibiting IL-1 mediated processes.

6.3.1 Effectiveness of retroviral transduction of CBEC

Two main issues are important when evaluating transduction efficiency: transgene expression level and persistence of transgene expression. A general and frequently described problem of retroviral gene transfer is that retrovirally delivered transgenes are prone to transcription silencing which may successively decrease the amount and shorten the duration of transgene expression in the target cells (Daly and Chernajivski, 2000b; Zwiebel et al., 1990).

In our experiments total expression of IL-1ra ranged between 30 and 50 ng per 10^6 cells per 24 h (mean ± SEM: 40.6 ± 4.4 ng/10^6 cells/24h) and remained stable over at least 12 weeks of endothelial cell culture **(see figures 11, 12 and 13)**. Thereby our retroviral transduction protocol establishes much higher and more durable expression of transgenic IL-1ra than other protocols. For instance, direct in vivo injection of naked IL-1ra plasmid into mice hearts resulted in maximum IL-1ra levels of 2 ng/ml/mg total protein, consistent with only 0.0005 % of total protein production. Moreover, expression of IL-1ra protein persisted only for 2 weeks (Lim et al., 2002). Comparing these results to a 10-fold higher transgene/total cell protein production ratio **(figure 14)** and up to 12-fold longer persistence of transgene expression **(figures 11 and 13)** in our experiments, in vitro transduction of target cells appears to be much more effective than direct in vivo gene transfer using plasmid DNA.

Adenoviral transduction of chondrocytes resulted in peak secreted IL-1ra levels of approximately 25 ng/10^6 cells/ml (Haupt et al., 2005) which comes near to the amount of secreted icIL-1ra in our experiments. However, transgene expression was only detected for one week during in vitro culture, and in vivo delivery of the IL-1ra gene in horses led to elevated intra-articular expression of interleukin-1 receptor antagonist solely for 28 days (Frisbie et al., 2002). Regarding efficiency of transduction on the mRNA-level (serving as a representative of number of copies of the transgene incorporated into the host genome) Haupt et al. reported a multiplication of the original IL-1ra copy number by factor $6x10^4$ post transduction. By means of retroviral transduction theoretical IL-1ra copy number in CBEC increased by factor 10^{13} in our experiments **(figure 10)**. Adenoviral transduction of the porcine kidney epithelial cell line PK15 resulted in concentrations of about 55 pg/10^6 cells/ml secreted IL-1ra in which is approximately

$5x10^2$-fold less compared to our method. In vivo transduction of swine lung alveolar epithelial cells with the same viral construct achieved transgene expression only for 10 days (Morrison and Murtaugh, 2001).

Considering these findings, the modified DEAE-Dextran-based retroviral transduction method performed by our group guarantees effective genetic modification of CBEC and seems superior to using adenoviral vectors for transduction of IL-1ra. Actually, the use of DEAE-Dextran in gene transfer experiments has previously been described as only suitable for transient transfections but as non-effective for stable integration of plasmids into the host genome (Tanner et al., 1997). However, the results of our study indicate that it is possible to achieve stable long-term and high yield IL-1ra transgene expression in cord blood-derived endothelial cells by means of our modified retroviral transduction technique.

6.3.2 Extracellular release of intracellular IL-1ra isoform type 2

The biological role of the intracellular forms of IL-1ra remains speculative. It has previously been shown that icIL-1ra2, when added exogenously, inhibits IL-1-mediated actions (Bertini et al., 1992; Muzio et al., 1995) albeit binding type 1 IL-1 receptor up to fivefold less avidly than do sIL-1ra and icIL-1ra1 (Malyak et al., 1998a). However, when produced endogenously icIL-1ra2 in contrast to sIL-1ra was reported to remain completely intracellular (Muzio et al., 1999). Moreover, Muzio et al. reported that cells carrying the transgene icIL-1ra2 unlike those transgenicly producing sIL-1ra failed to exert any inhibitory effects on cytokine expression induced by endogenous or exogenous IL-1 stimulation. Concerning this issue, Watson et al. reported different results in ovarian cancer cell lines. Using retroviral transduction to express icIL-1ra in an originally icIL-1ra-negative cell line they obtained transgenic cells displaying a profile of IL-1β-induced genes analogous to that found in cells spontaneously expressing icIL-1ra (Watson et al., 1995). Thereby they demonstrated intrinsic biologic activity of an intracellular splice variant of IL-1ra and suggested inhibitory effects of icIL-1ra on IL-1-mediated responses downstream of the initial IL-1/IL-1-receptor interaction. In contrast, Muzio et al. supposed that icIL-1ra might solely function as an intracellular reservoir of IL-1ra that is rapidly released into the extracellular space upon apoptosis or necrotic cell death in order to limit excessive IL-1-mediated inflammatory processes (Haskill et al., 1991; Muzio et al., 1999). It may then bind to type I cell surface receptors and block the stimulatory effects of IL-1. However, our studies and reports of other investigators indicate that icIL-1ra may also be released under physiologic conditions during cell culture (Corradi et al., 1995; Levine et al., 1997; Wilson et al., 2004). Thus, the exact underlying mechanisms of icIL-1ra release require further clarification. Conceivably, separate from binding to cell surface IL-1 receptors, icIL-1ra may also exert intracellular/intranucleic effects and thereby play a counterregulatory part to the intracellular effects of IL-1 and provide a counterbalance for intranuclear IL-1α. One possible mechanism of intracellular IL-1ra actions might consist in the binding to the COP9 signalosome complex (CSN3) with subsequent inhibition of the p38 MAPK signal transdction pathway as proposed by Banda et al. (Banda et al., 2005).

In conclusion, localization of icIL-1ra to the subcellular compartiments might be a useful approach to reveal the intracellular inhibitory mechanims of transgenicly produced IL-1ra. Especially the question wether icIL-1ra is packed into granules and transported to the cell

surface may be of special interest.

6.3.3 Impact of IL-1 and IL-1ra on CBEC expansion and proliferation

Interleukin-1 is known to hold an important role in human hematopoiesis. Both IL-1 and the IL-1 receptors are expressed in hematopoietic stem cells (HSC) and IL-1 either alone or in combination with other growth factors induces proliferation and promotes expansion of haematopoietic and myeloid precursors (Brugger et al., 1993; Crown et al., 1993; Majka et al., 2001). Likewise, IL-1 and its receptors are expressed on endothelial progenitors and mature endothelial cells (Dinarello, 2002). However, in contrast to its influence on HSC, IL-1 exerts antiproliferative effects on endothelial cells (Cozzolino et al., 1990) and acts as an inducer of endothelial cell apoptosis (Choy et al., 2001; Guevara et al., 2001). These findings point to a potential relevance of IL-1 in regulating vasculogenic/angiogenic properties of EPC and endothelial cells.

Apart from its antiproliferative effects, IL-1 has also been linked with decreased endothelial cell life span in culture, possibly by acting in the nucleus (Maier et al., 1994; Mantovani et al., 1998). It has been shown that IL-1α accumulates in aging endothelial cells and antisense oligonucleotides to IL-1α extended endothelial cell life span in vitro (Garfinkel et al., 1994; Maier et al., 1990). In addition to these findings and in correspondence with the knowledge that the biological effect of IL-1 depends on the balance between IL-1 and IL-1ra, studies of Dewberry et al. revealed that HUVEC carrying the allele IL-1RN*2*2, associated with decreased levels of intracellular IL-1ra in endothelial cells (Francis et al., 1999), show a markedly reduced proliferation and significantly diminished cumulative population doublings with an attenuated life span (Dewberry et al., 2003). Continuous exogenous addition of recombinant IL-1ra throughout endothelial cell life span restored the proliferative potential of HUVEC (IL-1RN*2*2) and extended cumulative population doublings in selected HUVEC cultures. Interestingly, this effect was only observed at concentrations of 1 ng/ml and not at 100 ng/ml IL-1ra.

Whether endogenous (transgenic) production of intracellular IL-1ra influences proliferation and expansion of cord blood-derived endothelial cells has not been investigated so far. In our hands, CBEC do not express detectable amounts of any isoform of IL-1ra **(figures 10 and 11)**. As transgenic icIL-1ra was found to be secreted to a large part with mean levels of 33.1 ± 4.3 ng/10^6 cells/ml in culture medium of IL-1ra transduced cells **(figure 13)** and cell cultures were carried out in a total volume of 10 ml growth medium one can conclude that (taking into account temporarily subconfluent states) 1 to 3 ng/ml icIL-1ra were persistently present in CBEC-pLXSN-IL-1ra culture supernatants throughout the whole expansion period. This is comparable to the culture conditions described by Dewberry et al. Taking further into consideration that in the mentioned study IL-1ra was only added to the culture medium every 2-3 days (with a known IL-1ra plasma half-life of 4-6 hours (Gouze et al., 2003)) it would not have been surprising to come into a position to confirm the growth promoting effects of IL-1ra reported by Dewberry et al. for cord blood-derived endothelial cells. However, though subcomparison of CBEC transgenic for icIL-1ra revealed a correlation between icIL-1ra protein expression and CBEC doubling time in culture (with the shortest doubling time in CBEC producing highest levels of icIL-1ra **(figure 15)**) we found no statistically significant differences in cellular proliferation between CBEC-pLXSN-IL-1ra and CBEC-pLXSN **(figure 7, 8 and 15)**. Thus, our studies do not support a role for Ic-IL-

1ra in cellular senescence or proliferation. Whether this property of icIL-1ra distinguishes it from sIL-1ra remains to be established.

6.4 Antiinflammatory properties of IL-1ra- transgenic cord blood-derived endothelial cells

Inflammation is an integral part of a wide array of human diseases. Its initiation is characterized by the sequential events of the vascular inflammation cascade (Butcher, 1991).

The purpose of retroviral transduction of cord blood-derived endothelial cells with the IL-1ra transgene was to inhibit activation of endothelial cells and thereby to reduce the ensuing proinflammatory processes that are emblematic for the inflamed vasculature in diseases such as atherosclerosis.

6.4.1 CBEC-IL-1ra show attenuated proinflammatory gene expression and reduced activation in response to proinflammatory stimuli

In order to investigate the influence of IL-1ra transgene expression on endothelial cell activation we determined the regulation of genes involved in acute and chronic inflammatory processes as well as we studied the crosstalk between CBEC and peripheral blood mononuclear cells in a model of endothel-monocyte coculture.

6.4.1.1 Influence of transgenic icIL-1ra on gene expression in CBEC

In a first step activation studies were carried out aiming to characterize the gene expression pattern of cord blood-derived endothelial cells and to determine differences in transcriptional regulation in parental and retrovirally transduced CBEC either transgenicly producing icIL-1ra or carrying the empty-vector construct pLXSN. Generally, regarding basal expression levels and the transcriptional response to the proinflammatory stimuli IL-1β and TNF-α, cord blood-derived endothelial cells showed a similar gene expression pattern to endothelial cells from other sources, e.g. HUVEC and HCAEC (Bandman et al., 2002; Bertini et al., 1992; Buzby et al., 1994). As adenoviral transduction has frequently been reported to result in activation of the target cells, revealed by upregulation of cytokines involved in immune responses (e.g. IL-1β and TNF-α) (Haupt et al., 2005) or increased expression of endothelial adhesion molecules (Channon et al., 1998; Newman et al., 1995), we were also interested whether such effects might also arise following retroviral transduction. However, in our studies none of the investigated proinflammatory molecules was upregulated in empty-vector transduced CBEC compared to parental CBEC. We deduce from these findings that neither in vitro culture conditions nor retroviral transduction influenced the state of CBEC activation. Therefore CBEC as well as their transduced derivates may pass through ex vivo expansion without gaining procoagulative or proinflammatory activity.

As illustrated in **figures 17-22** CBEC transgenicly producing IL-1ra showed distinct alterations in IL-1 regulated gene expression compared to untransduced control cells. Most importantly, expression levels of ICAM-1 and ELAM-1, major determinants of leukocyte adhesion to the endothelial lining (Dagia and Goetz, 2003; Luscinskas and Gimbrone, 1996; Renard et al., 2003), were

markedly reduced in CBEC-IL-1ra at basal levels as well as following stimulation by the proinflammatory cytokine IL-1β. Transgenic expression of IL-1ra additionally reduced the expression of further genes predominantly involved in inflammatory and procoagulative processes, such as MCP-1 (the major chemotactic molecule generated within the vessel wall (Namiki et al., 2002; Ross, 1999)), COX-2, IL-1ß, prostacyclin-synthase (a key enzyme in production of proinflammatory prostaglandins) and thrombin receptor. However, attenuation of proinflammatory activation of CBEC was not only mediated by transcriptional downregulation of proadhesive, chemotactic and procoagulative genes.

From the gene expression analyses shown in **figures 17-22**, we observed two different interesting and not altogether anticipated phenomena. Firstly, transgenic expression of IL-1ra upregulated thrombomodulin, an integrative component of the protein C anticoagulant pathway and essential antithrombotic moderator of the vascular endothelium (Esmon, 2001; Esmon, 2003). The increased expression of thrombomodulin in CBEC-IL-1ra displays a less coagulative phenotype upon stimulation compared to untransduced CBEC. This might have importance in the setting of vascular disorders such as atherosclerosis and neointima formation as local thrombosis due to activation of coagulation at sites of vascular inflammation is a crucial collateral risk of inflammatory vasculopathies and endothelial damage in the context of vascular injury. The second effect was that the transgenic icIL-1ra not only inhibited ICAM-1 upregulation in response to IL-1, but also to TNF-α stimulation. In contrast, exogenous addition of icIL-1ra did not inhibit TNF-α induced ICAM-1 expression in previous studies by other authors (Bertini et al., 1992). Therefore, the inhibitory effect of endogenously produced icIL-1ra might either be due to common intracellular signal pathways of IL-1 and TNF-α or to a reduced autocrine stimulation of the cells by attenuated secretion of IL-1. Indeed, it has recently been demonstrated that cells respond to TNF-α both directly, via activated TNF receptor, and indirectly, via the sequential release of transforming growth factor-a (TGF-a), interleukin-1α (IL-1α) and IL-1 receptor antagonist (IL-1ra) (Janes et al., 2006; Janes and Yaffe, 2006). Therefore, extracellular autocrine crosstalk may play as important a role as intracellular crosstalk in cellular responses to TNF.

Platelet derived growth factor (PDGF), mainly secreted by endothelial cells and thrombocytes, induces chemotaxis, migration and proliferation of subendothelial fibroblasts and smooth muscle cells and increases tissue factor (TF, CD142) production in monocytes and endothelial cells (Ernofsson and Siegbahn, 1996; Ross, 1999). Both PDGF and TF are increasingly expressed by activated and dysfunctional endothelium and render its phenotype procoagulative, proadhesive and hyperproliferative (Annex et al., 1998; Cunningham et al., 1999; Ikeda et al., 2001; Massberg et al., 2003; Randolph et al., 1998; Sano et al., 2001; Tremoli et al., 1999). Furthermore, paracrine signaling via platelet-derived growth factor b (PDGF-b), expressed by endothelial cells, and its receptor PDGFR-β on the surface of pericytes and vascular smooth muscle cells (VSMC), plays a central part in blood vessel maturation and constitutes one of the molecular pathways of VEGF-induced endothelial cell tube formation (Carmeliet, 2003; Jain, 2003). The interaction between endothelial PDGF-b and the PDGFR-β not only mediates recruitment of pericytes and VSMC but also produces a close association between the endothelium and the mural cells, thereby stabilizing blood vessels and preventing leakiness due to a lack of mural cell coverage (Lindblom and al., 2003). Contact of PDGFR-β-positive mural cells to PDGF-b-expressing endothelium thereby

plays an essential role in generation of new vessels (Carmeliet, 2003).

Endothelial cell culture and stimulation experiments revealed that PDGF-b and tissue factor expression did not differ between CBEC, CBEC-pLXSN and CBEC-icIL-1ra and that gene transcription was not upregulated in the course of CBEC in vitro expansion **(figure 16)**. Also, PDGF-b expression was not reduced in CBEC transgenic for icIL-1ra upon exposure to IL-1β (**figure 17)**. As levels of PDGF-b remain substantially unchanged in transgenic CBEC compared to untransduced controls we expect no obvious alteration of the PDGF-b-mediated crosstalk between endothelial and mural vascular cells.

6.4.1.2 Changes in ICAM-1, VCAM-1, IL-1β and TF expression in CBEC-monocyte cocultures and influence of transgenic icIL-1ra

FACS analysis and ELISA of CBEC and monocytes after in vitro coculture revealed reduced levels of ICAM-1 and IL-1β in the endothelial cell fraction transgenicly producing IL-1ra. In addition, CBEC-IL-1ra displayed reduced expression of VCAM-1 and tissue factor when cocultured with stimulated monocytes.

Tissue factor, the main inducer of the extrinsic coagulation pathway, is to a large part expressed by damaged and dysfunctional endothelium (Ott, 2003). TF is mainly responsible for initiation of coagulation at sites of vascular inflammation and injury and therefore plays a pivotal role in blood clotting especially in atherothrombosis (Giesen et al., 1999; Grybauskas, 2003). We therefore investigated tissue factor expression during endothel-monocyte coculture and found significantly decreased TF on the surface of CBEC transgenic for icIL-1ra compared to control cells **(see figure 28)**. Thus, CBEC-pLXSN-IL-1ra would express less procoagulative activity under inflammatory conditions.

Tissue factor is also known to be induced in peripheral blood mononuclear cells and macrophages by proinflammatory cytokines and thereby exerts a part of the procoagulative activity of the blood under inflammatory and ischemic conditions (Ott et al., 2001). However, in our experiments we did not observe any increase in monocytic tissue factor expression suggesting that endothelial cells are more sensitive to IL-1β stimulation and therefore represent the main determinants of procoagulative activity at sites of vascular inflammation.

VCAM-1 is involved in leukocyte adhesion to endothelial cells and production of IL-1 during endothel-monocyte interactions leading to further activation and proinflammatory cytokine release of both endothelial cells and monocytes (Zohlnhofer et al., 2000). The upregulation of VCAM-1 during endothel-monocyte coculture was significantly reduced in the presence of CBEC producing IL-1ra.

Similarly, IL-1β cytokine synthesis was significantly reduced in cocultures of monocytes with CBEC-icIL-1ra (-41.2% and -32.3% in unstimulated and stimulated cocultures, respectively, **figure 29**) compared to CBEC-pLXSN. Basal secretion of IL-1β was actually reduced by 69.2% in CBEC transgenicly expressing icIL-1ra. Initial findings of other groups suggested that equimolar concentrations of IL-1ra and IL-1β were sufficient to reduce IL-1β secretion by 20%, whereas twofold and 10-fold molar excess of exogenously added IL-1ra inhibited synthesis of IL-1β by monocytes by 50% and 73%, respectively (Granowitz et al., 1992a). With average levels of secreted IL-1ra of 6.6 ± 2.2 ng/ml/2x10^5 CBEC-pLXSN-IL-1ra and exogenous addition of 0.5

ng/ml IL-1β stimulated endothel-monocyte coculture experiments were carried out at IL-1ra/IL-1β ratios of about 10-15:1. In case of CBEC monoculture and in non-stimulated coculture experiments IL-1ra/IL-1β ratios were solely determined by endogenously produced IL-1β (2.6 ± 0.2 and 3.4 ± 0.4 pg/ml, respectively) and transgenic IL-1ra released by CBEC reaching levels of over 10^3 : 1. Though operating with markedly higher IL-1ra/IL-1β ratios we could not confirm the data obtained by Granowitz et al. in our experimental coculture model. Especially the IL-1 induced activation of monocytic cells (indicated by monocytic ICAM-1 expression and IL-1β secretion) was not attenuated by IL-1ra transduced CBEC as could have been expected with this excess of receptor antagonist. However, IL-1β levels during this assay were generally very low and levels of secreted IL-1β are not a reliable measure for total IL-1 protein synthesis, as proIL-1β and simultaneously produced IL-1α and its functionally active precursor proIL-1α are to a main part retained intracellularly (Borth et al., 1990). That is why it is difficult to make solid statements about the influence of transgenic IL-1ra expression on total IL-1 protein synthesis by CBEC and monocytic cells. However, diminished levels of IL-1β protein secretion by CBEC carrying the transgene icIL-1ra suggest that these cells might exert a less proinflammatory influence on their environment.

In summary, our stimulation assay and the coculture of transgenic CBEC with monocytes revealed that transgenic expression of icIL-1ra in CBEC alters their phenotype towards a more antiinflammatory, vasculoprotective state. Moreover, CBEC-IL-1ra exert protective effects against proinflammatory activation by monocytes.

6.4.2 IL-1ra transduced cord blood-derived endothelial cells inhibit vascular inflammatory processes

The activation assay and our coculture experiments revealed that transgenic production of IL-1ra in CBEC significantly attenuated IL-1β mediated increase in ICAM-1, ELAM-1 and VCAM-1 as well as MCP-1 expression and IL-1β secretion. These are major determinants of accumulation and adhesion of inflammatory cells at the vessel wall. In the course of vascular inflammatory processes in humans, ELAM-1 is expressed early and correlates with neutrophil influx, whereas ICAM-1 and VCAM-1 expression increases with a more prolonged time course and correlates with monocyte and lymphocyte influx (Bevilacqua, 1993; Bevilacqua et al., 1994). Firm adhesion of peripheral blood mononuclear cells to endothelial cells causes increasing activation of the endothelium and leads to accumulation of inflammatory cells of the blood stream at sites of vascular inflammation and disturbed endothelial integrity (Bevilacqua et al., 1994). Therefore, reduction of endothelial adhesion molecule expression constitutes a major target for strategies to inhibit leukocyte-endothelial interactions (Panés et al., 1999). Using a flow chamber device as well as experimental models of adhesion and transmigration, we aimed to find out whether CBEC-IL-1ra might present a reduced adhesivity towards the main cells involved in vascular inflammatory processes, namely leukocytes and monocytes.

As described in the Materials and Methods chapter, we used Mono-Mac-6 cells, a monocytic cell line, for our experimental studies. It has previously been demonstrated that Mono-Mac-6 cells express a similar repertoire of integrins (e.g. Mac-1 and VLA-4) and chemokine receptors

(e.g. the MCP-1 receptor CCR2) as mature monocytes. Furthermore, adhesion of Mono-Mac-6 cells to endothelium has been proven to show the same dependency on ICAM-1 and VCAM-1 mediated bindings and exhibit similar adhesion characteristics as mature human monocytes isolated from peripheral blood. Therefore Mono-Mac-6 cells are considered to be a cell line well suited for studying monocyte-endothelial cell interactions (Erl et al., 1995).

Indeed, our results demonstrate that the different phases of the early vascular inflammatory process (rolling, firm adherence, and transmigration, **scheme 4**) are all inhibited by transgenic expression of icIL-1ra in CBEC **(figures 23-27)**. These findings are of great importance for the estimation of the potential of transgenic CBEC in prevention of early inflammatory vascular lesions.

In conclusion, CBEC transgenicly producing IL-1ra clearly reduce levels of endothelial cell activation and significantly diminish recruitment of inflammatory cells to the endothelial surface indicating that these cells might be capable of attenuating already early stages of vascular inflammatory lesion formation. Once transplanted and engrafted to the vessel wall, CBEC transgenicly producing IL-1ra might not only restore the structural and functional integrity of the diseased vasculature but also rebuild a vascular lining less susceptible for adhesion, accumulation, and subsequent transmigration of inflammatory cells. This potentially beneficial effect should be subjected to further investigation during in vivo experiments in animal models of atherosclerosis, neointimahyperplasia, and ischemia.

6.5 Conclusions and future opportunities

6.5.1 Cord blood derived endothelial cells might constitute a useful tool for postnatal neovascularization and vascular repair in humans

Disease- and age-related dysfunction of endothelial cells including their progenitors may render the endothelium incapable of vascular repair during acute and chronic inflammatory vasculopathies such as ischemia/reperfusion injury, neointimahyperplasia, and atherosclerosis (Dong et al., 2005). Therefore, the provision of autologous or allogeneic endothelial progenitors to reconstitute the repair potential of the diseased vasculature combined with the restitution of intrinsic antiinflammatory functions through local drug therapy by means of transgenic expression of disease-modifying agents might be a useful new strategy of vascular repair. In our studies we show that CBEC can be reliably isolated and expanded to clinically relevant numbers. We found that cord blood-derived endothelial cells do not express IL-1ra by nature and therefore lack a naturally occurring antagonist of IL-1 mediated processes. However, with our retroviral transduction method CBEC could efficiently be genetically modified and high levels of transgenic icIL-1ra expression could be achieved for up to 5 months. Overexpression of IL-1ra in CBEC resulted in a less immunogenic phenotype and was capable of inhibiting the decisive processes of vascular inflammation *in vitro*. These results indicate they might be suitable for *in vivo* transplantation with the purpose of vascular repair and protection at sites of vascular damage and inflammation. As treatment of inflammatory vasculopathies requires long-

term expression of a therapeutic gene, ex vivo viral transduction, expansion and subsequent transplantation of progenitor cell-derived endothelial cells seems to be a feasible strategy of local delivery of therapeutic transgenes into the diseased vasculature.

Though several aspects concerning engraftment, long-term survival, proliferation, and immunogenicity in allogenic organisms still have to be clarified, endothelial cells differentiated from cord blood-derived progenitors seem to constitute a very suitable target for autologous or even allogenic transplantation into the vasculature. Compared to other sources, cord blood-derived endothelial cells show the best proliferative potential and the longest life span during in vitro culture which facilitates not only expansion to clinically relevant numbers but also efficient genetic modification of the cells prior to transplantation (Herder et al., 2003; Ott et al., 2005a). Furthermore, CBEC outlive long-term storage which makes it possible to isolate and freeze the cells at the date of birth and use them for therapeutic vasculogenesis/reendothelialization whenever needed in the course of the patients life and disease.

In experimental studies in immunodeficient nude rats, human CBEC have already been used successfully to colonize vascular grafts and to augment neovascularization in models of hind limb ischemia (Murohara et al., 2000). Results of our group give further prove that human CBEC contribute to postischemic neovascularization after experimental myocardial infarction in immunodeficient rats (Ott et al., 2005a). One of the most promising future applications for CB-derived EPC and outgrowing endothelial cells might be the treatment of chronic inflammatory vasculopathies such as atherosclerosis as there is mounting evidence that these diseases are accompanied by the senescence of mature endothelium and obolescence of corresponding progenitors (Dong et al., 2005; Kravchenko et al., 2005).

Therefore, transplantation of cord blood-derived endothelial cells might represent a promising strategy for the modulation of postnatal neovascularization and restoration of structural and functional vascular integrity in adults. The results of the present work clearly demonstrate the feasibility of this approach and provide the basis for future in vivo studies both in animals as well as in humans.

6.5.2 Local delivery of IL-1ra by genetically modified endothelial cells might attenuate inflammatory processes of the diseased vasculature

The ability to target appropriate molecules specifically to sites at increased risk represents a purposeful approach to the treatment of inflammatory vasculopathies (Koning et al., 2002). Genetic modulation of endothelial function might offer a new opportunity to influence both the blood stream and the vessel wall and thereby to modify the course of several haematological or vascular diseases such as atherosclerosis, thrombosis, coagulation disorders and ischemic artery disease (Kong et al., 2004b; Melo et al., 2004).

The feasibility of genetic modification of endothelial cells in order to exert direct influence on vascular homeostasis has previously been shown by other groups who described positive effects on anticoagulant and fibrinolytic pathways. For instance, retroviral transduction of endothelial cells with recombinant plasminogen activator (Dichek et al., 1996) and hirudin (Lundell et

al., 1999) proved to exert beneficial effects on vascular homeostasis in animal models. By lentiviral transduction of cord blood-derived EC with the coagulation factor VIII Herder et al. showed the attractiveness of CBEC as target cells for the treatment of inherited coagulation disorders such as haemophilia A (Herder et al., 2003).

Much interest is currently focused on determining the therapeutic value of inhibitors of endothelial-leukocyte interaction. Attenuation of endothelial cell activation, especially inhibition of adhesion molecule expression and chemokine release is considered as a novel target for future anti-inflammatory therapeutic strategies.

In this regard, IL-1ra plays a major role in the control of spontaneous inflammation in the vasculature and inhibits the development of endothelial dysfunction and vascular lesions. The findings that mice lacking the interleukin 1 receptor antagonist gene develop lethal arterial inflammation (Nicklin et al., 2000) and show increased neointimal formation after vascular injury (Isoda et al., 2003) give further prove of the important impact of IL-1ra on the attenuation of inflammatory processes in the vascular system. In humans, deficiency of IL-1ra leads to an increased prevalence of single vessel coronary artery disease (Francis et al., 1999; Momiyama et al., 2001). It is also known from experimental studies that administration of IL-1ra exerts beneficial effects in the modulation of vascular inflammation (Elhage et al., 1998; Suzuki et al., 2001).

However, interpreting the current knowledge about IL-1ra expression in the vascular wall, it appears that production of IL-1ra by mature endothelial cells apparently to low to effectively block the inflammatory consequences of local IL-1 production. Furthermore, there is a delay in the release of IL-1ra in the vascular lining compared to the initial upregulation of proinflammatory cytokines such as IL-1β and TNF-α (Dewberry et al., 2000; Hurme and Santtila, 1998; Lin et al., 2003). The consequence of this may be that decisive steps of the vascular inflammatory process (e.g. induction of leukocyte-endothelial adhesion molecules) have yet been initiated making it impossible for IL-1ra to counteract the ongoing inflammatory action.

The results of our study show that several key steps in the inflammatory cascade appear amenable to pharmacologic inhibition by IL-1ra produced by retrovirally transduced cord blood-derived endothelial cells. Considering these findings, intravascular transplantation of CBEC continuously producing IL-1ra might establish a constitutive protective action against IL-1-mediated disturbances of the healthy vascular endothelium. The local delivery of IL-1ra by engrafted CBEC in the vessel wall, the spot where vascular inflammation is initiated, might prove to be beneficial in treatment of inflammatory vascular diseases. Apart from direct intravascular transplantation of CBEC into the diseased vasculature one feasible approach may be the implantation of CBEC-coated vascular stents. In this manner, it appears possible to achieve high local concentrations of IL-1ra exceeding those of endogenous IL-1 production by factor 100-1.000 which is actually necessary to inhibit biologic activities in vivo.

In conclusion, local delivery of IL-1ra at sites of damaged vessels by means of genetically modified cord blood-derived endothelial cells might constitute a specific strategy to restore indispensable antiinflammatory properties of the endothelium, inhibit vascular inflammatory processes at sites of endothelial damage and diminish the detrimental consequences of vascular inflammatory diseases. Although the development of therapeutic strategies using EPC is very promising, mechanisms of local engraftment and survival at sites of vascular injury are

not yet thoroughly understood. It should be subject of future investigations to elucidate the factors that favour the persistence and allow optimal local engraftment of ex vivo-modified EPC, whether following localized or systemic intravascular delivery.

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8 Appendix

8.1 List of figures

8.2 Acknowledgements

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8.3 Curriculum vitae

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Hannover, Februar 2007

Publikationen und Kongreßbeiträge

Philipp Fischer, Ilka Ott, Katja Urlbauer, Chrisian Schulz, Sandra Hippauf, Marcus Niemeyer, Volker R. Jacobs, Christian Peschel, Robert A.J. Oostendorp. **Stable expression of icIL-1ra protects cord blood-derived endothelial-like cells against inflammatory responses.**
In progress

P. Fischer, I. Ott, C. Schulz, K. Urlbauer, E. Kolka, A. Baer, D. Hilfiker, R. Oostendorp. **Stable expression of icIL-1ra protects cord blood-derived endothelial cells against inflammatory responses.** European Society of Cardiology (ESC) Congress 2007, Vienna – oral presentation.

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P. Fischer, I. Ott, U. Keller, M. Wimmer, K. Urlbauer, C. Peschel, R.A.J. Oostendorp. **Endothelial Cells grown from $CD34^+$ Progenitors from mobilized peripheral blood, cord blood or bone marrow.**
Cellular Therapy 2003 - Second International Symposium on the Clinical Use of Cellular Products Regensburg, Germany, March 27-28, 2003 - oral presentation.

8.4 Affirmation

Hiermit erkläre ich, daß die vorliegende Dissertation von mir selbstständig und nur unter Verwendung der angegebenen Hilfsmittel angefertigt wurde.

Hannover, Februar 2007 Philipp Fischer