Travelling to Rome: inflammation, endoplasmic reticulum stress and angiogenesis during atherosclerotic plaque development

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Dedicated to

Ariadni-Zoe & Theodosia-Penelope

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ABSTRACT

Cardiovascular diseases are the leading cause of death worldwide followed by cancer. Atherosclerosis, the major underlying cause of cardiovascular diseases, is a syndrome affecting medium-sized and large arteries. Progressive atherosclerotic disease and the development of acute lesion instability are linked with plaque angiogenesis. It is widely accepted as an inflammatory disease involving both innate and adaptive immune mechanisms. During the development of an atherosclerotic plaque, lesions are infiltrated by inflammatory cells and professional antigen presenting cells (APCs). Identifying the leukocyte populations and APCs involved during plaque maturation is of great interest for understanding the pathogenesis of the disease and providing targets for therapeutic interventions aimed at controlling the activation state of culprit cells.

Endothelial dysfunction (ED) is another key event in the initiation and progression of atherosclerosis and it serves as a risk factor for the development of cardiovascular events. Stimuli that cause oxidative stress, endoplasmic reticulum (ER) stress, metabolic stress and genotoxic stress may lead to ED through enhanced endothelial cell (EC) injury or death, conditions which are considered essential for plaque rupture. Unfolded protein response (UPR) is the front line of defense during ER stress, aiming to re-establish cellular homeostasis and rescue the cell from apoptosis. Although many steps of the ER stress signalling pathway have been elucidated, coordination between intracellular ER stress and cell-surface prompted survival signals has been poorly investigated.

Paraphrasing the modern version of the medieval sentiment “all roads lead to Rome” to “all cellular paths which lead to atherosclerosis”, my dissertation addresses the pathophysiology of atherosclerosis from two different aspects.

iNKT cells, inflammation and angiogenesis

In the present dissertation we provide evidence for the first time for the involvement of CD1d-expressing APCs and invariant natural killer T (iNKT) cells in disease progression in patients suffering from atherosclerosis. CD1d-expressing APCs are present in advanced atherosclerotic plaques and are more abundant in plaques with ectopic neovascularization. Patients with active disease have reduced numbers of iNKT cells

circulating in blood and the iNKTs present in plaques are more responsive to lipid antigens than the ones found in blood. The *in vitro* data demonstrate that lipid activation of plaque-derived iNKTs increases the migration capacity and angiogenic activity of EC in an IL-8 dependent manner. Further investigations revealed that the stimulatory effects of EC on migration, sprouting and actin reorganization from activated iNKT cells are driven through EGFR with selective downstream activation of focal adhesion kinase (FAK) and Src. These findings introduce iNKT cells as novel cellular candidates promoting plaque neovascularization and destabilization in human atherosclerosis. In addition the data demonstrate that EGFR inhibition may represents a novel therapeutic modality for the control of inflammation-associated neovascularization within developing atherosclerotic plaques.

ER stress and T-cadherin

T-cadherin is an unusual member of the cadherin superfamily of surface adhesion molecules. It is widely expressed in the cardiovascular system and is upregulated during proliferative vascular disorders such as atherosclerosis. This dissertation provides evidence for the importance of T-cadherin to influence UPR signalling and EC survival during ER stress. During UPR T-cadherin levels are significantly elevated. Overexpression or silencing of T-cadherin in EC respectively attenuated or amplified the ER stress-induced increase in phospho-eIF2alpha, Grp78, CHOP and active caspases. Upregulation of T-cadherin expression on EC during ER stress attenuates the activation of the proapoptotic PERK (PKR (double-stranded RNA-activated protein kinase)-like ER kinase) branch of the UPR cascade and thereby protects EC from ER stress-induced apoptosis.

1. INTRODUCTION

1.1 Atherosclerosis-the current view

Atherosclerosis is the principal cause of death in developed countries and emerging economies worldwide. Hypertension, endothelial injury, as well as dyslipidemia, diabetes, hyperhomocysteinemia, smoking, aging, and increased body mass index are major risk factors for the development of atherosclerosis. It is a disease of medium-sized and large arteries in which fatty material and plaque are deposited in the wall of an artery, resulting in narrowing of the arterial lumen and eventual impairment of blood flow (Figure 1).

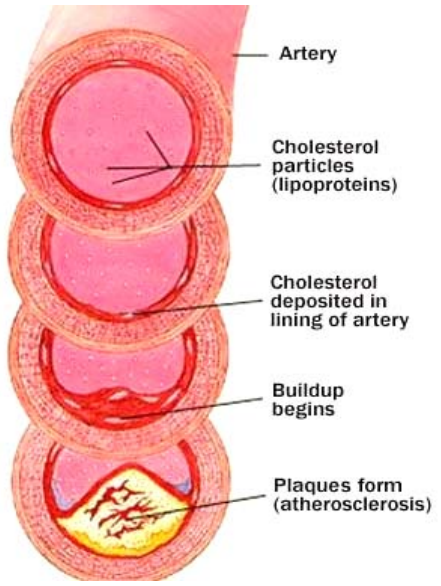

Figure 1. Schematic of arteriosclerosis

While the classic concept of atherosclerosis as a disorder of lipid metabolism and deposition is widely accepted, evolving understanding of the biology linking the lipid disorder to vascular involvement during atherogenesis and subsequent clinical manifestations indicates a far more complex pathophysiology than mere lipid storage. Atherosclerosis is today recognized as a sub-acute inflammatory condition of the vessel wall. Inflammation and infiltration of immune cells appear crucial in all stages of atherosclerosis, from the very initial phases through to the progression and finally to the clinical complications (Libby, 2008).

Animal models have been extensively used in order to clarify what causes atherosclerosis, even though there are limitations due to significant species differences compared to humans (Smithies and Maeda, 1995). Under physiological conditions leukocytes do not adhere to the endothelial monolayer. However, in circumstances of endothelial dysfunction adhesion molecules and chemotactic factors recruit and bind leukocytes (monocytes, T-cells and mast cells) (Figure 2). Vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), integrins, L- P- and E-selectins and cadherins are such adhesion molecules, which play an important role for the development of atherosclerosis (Table 1) (Collins et al., 2000; Dong et al., 1998; Ivanov et al., 2001; Shih et al., 1999).

Table 1. Adhesion molecules involved in atherosclerosis			
Selectins/ligands	Integrins	Immunoglobulins	Cadherins
P-selectin	Integrin α2β1	ICAM-1	VE-cadherin
E-selectin	Integrin α4β1	ICAM-2	T-cadherin
L-selectin	Integrin αDβ2	ICAM-3	N-cadherin
P-selectin ligand 1	Integrin αVβ3	VCAM-1	
E-selectin ligand 1	Integrin αVβ5	PECAM-1	

After adherence, chemoattractants prompt the leukocytes to penetrate into the arterial wall, at which point M-CSF can stimulate scavenger receptor expression to allow the cells to engulf to modified lipoprotein particles and become the foam cells that consist the so called ¨fatty streak¨, an aggregation of lipid rich macrophages and T lymphocytes within the innermost layer of the artery wall the intima (Figure 3) (Hansson and Libby, 2006) .

Fatty streaks are not clinically significant, but they are the precursors of the atherosclerotic plaques. Lesions consisting of a fibrous cap that encloses a lipid rich core and is the place where inflammation, lipid accumulation and cell death takes place (Figure 4).

Plaques mature over time and become extremely complex. Small vessels are prone to grow inside the lesion causing haemorrhage, but also calcification and ulceration are observed in advanced lesions, processes that make lesions extremely unstable and prone to rupture (Figure 4) showing that the quality of the plaque is more important than the size (Lusis, 2000). Intimal calcification is an active process in which pericyte-like cells secret a matrix scaffold which becomes calcified, akin to bone formation. The process is regulated by oxysterols and cytokines (Watson et al., 1994). When the plaque ruptures, tissue factor gains contact with the blood to set the stage for thrombosis and acute myocardial infarction (Lusis, 2000).

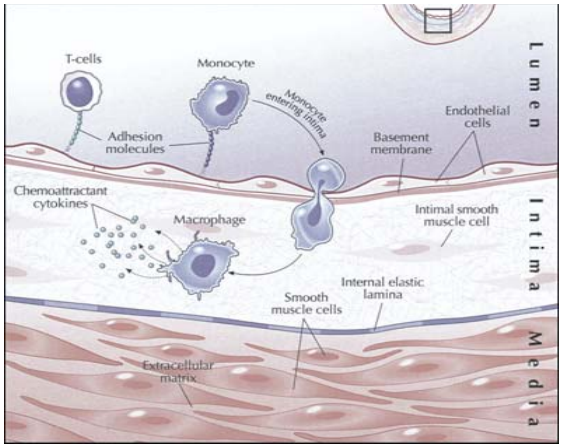

Figure 2. Adhesion and infiltration of immune cells. The three layers that comprise an artery are depicted (full cross-section depicted on the upper right). The endothelium lies over the intimal layer. The intima normally contains a few resident smooth muscle cells and a layer of extracellular matrix. The internal elastic lamina provides the boundary between the intimal layer and the tunica media, normally filled with quiescent smooth muscle cells in an elastin-rich extracellular matrix. Activating the endothelium leads to expression of adhesion molecules for leukocytes and chemoattractants, which bring the bound leukocytes into the intimal layer. (Libby, 2008).

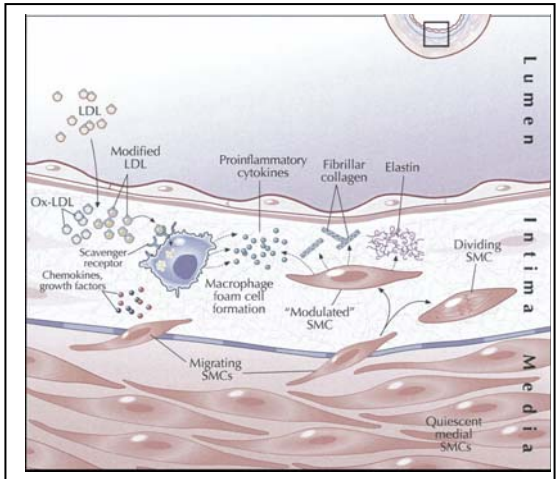

Figure 3. Fatty streak formation. Macrophages absorb modified lipoproteins such as oxidized low-density lipoprotein (ox-LDL) through scavenger receptors to produce foam cells. Macrophages in the lesions release chemoattractant cytokines, proinflammatory mediators, and small lipid molecules such as leukotrienes and prostaglandins. At this stage SMCs are also activated (Libby, 2008).

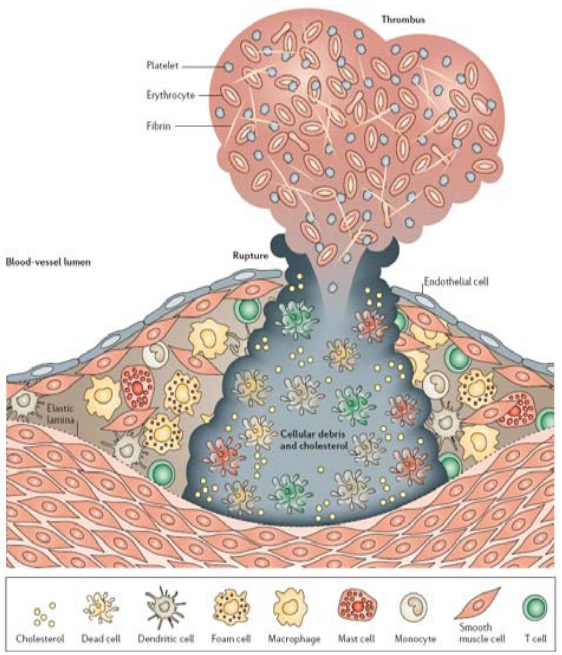

Figure 4. Plaque rupture and thrombosis. The core of the atherosclerotic plaque contains lipids and debris from dead cells. Immune cells are present in the plaque, which can affect inflammation and vascular function, by releasing pro-inflammatory cytokines, proteases, pro-thrombotic molecules and vasoactive substances. Endothelium is damaged and the weakened plaque ruptures and a thrombus is formed. The necrotic core is a key factor in plaque vulnerability. Macrophage debris promotes inflammation, plaque instability, and thrombosis. Plaque necrosis arises from a combination of lesional macrophage apoptosis and defective clearance of these dead cells, a process called efferocytosis (Hansson and Libby, 2006; Tabas, 2009).

1.2. Endothelial dysfunction in atherosclerosis

Loss of normal endothelial function (endothelial dysfunction, ED) is a hallmark for vascular diseases. ED has long been recognized as an integral component of atherosclerotic vascular disease and its presence is a risk factor for the development of clinical events. It is the earliest measure of a functional abnormality in the blood vessels and precedes the anatomic lesions in the development of atherosclerosis. ED is usually caused by endothelial cell (EC) injury or death. In the most extreme case, significant injury leads to EC desquamation from the vessel lining. The pathophysiology of ED is very complex, involving several factors which, while etiologically distinct, frequently share common mechanisms of action (Pober et al., 2009; Roquer et al., 2009). ED should not be confused with endothelial activation which is defined as the acquisition of a new endothelial function that benefits the host, and represents the normal homeostatic functions of the endothelium.

The endothelium is composed of a thin layer of EC that cover the inner surface of blood vessels (Figure 5). It is no longer considered as just a passive barrier which separates the blood vessels and the blood. Endothelial tissue is on the contrary a very active and specialized organ. EC are quiescent *in vivo* under physiological conditions, but in case of injury or any kind of ED, EC change their phenotype and migration and proliferation rates in order to heal the lesion and maintain the homeostasis (Bachetti and Morbidelli, 2000). Its total weight in a healthy adult man is comparable to that of the liver, and when extended will cover a surface area of several tennis courts. This renders the endothelium one of the biggest and most important glands of the body (Rubanyi, 1993). Endothelial function is not only autocrine, but also paracrine and endocrine (Esper et al., 2006). It is very sensitive organ responding to any physical or chemical stimulus. Accordingly it releases the corresponding biochemical substances in order to maintain homeostasis. A functional endothelium maintains the balance between vasodilation and vasoconstriction, growth inhibition and growth promotion, anti-thrombosis and pro-thrombosis, anti-inflammation and pro-inflammation, anti-oxidation and pro-oxidation, *inter alia* (Luscher, 1990; Vallance et al., 1989; Vane et al., 1990; Vanhoutte, 1989). It has both sensory and executive functions, releasing molecules that regulate all the

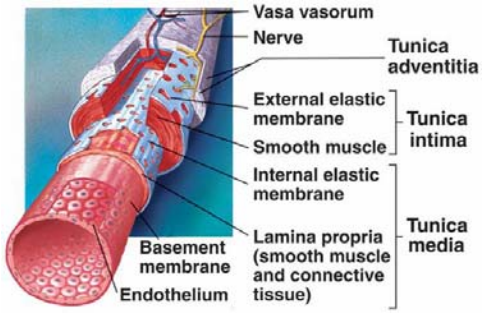

Figure 5. Monolayer of EC lining on the luminal surface of a vessel.

biological processes mentioned above. Upsetting this tightly regulated balance leads to ED (Ross, 1999).

The term “endothelial dysfunction” was coined in the eighties by Furchgott and Zawadzki who discovered that acetylcholine requires the presence of the EC to relax the underlying vascular smooth muscle. The relaxing factor, first termed as endothelium-derived relaxing factor, was identified as nitric oxide (NO) (Furchgott and Zawadzki, 1980). NO is a small molecule that can easily diffuse across the intima and induce smooth muscle fiber relaxation and finally vasodilation (Loscalzo and Welch, 1995). In addition to its potent vasodilator actions, NO also reduces tissue oxidation and inflammation, causes inactivation of prothrombotic factors, modulates cell growth, proliferation and migration and favors fibrinolysis. Thus NO is considered an important anti-atherogenic molecule (Cooke et al., 1992; Cooke and Tsao, 1994; Libby, 2001; Marx et al., 2001; Tak and Firestein, 2001).

ED portrays a disturbance in the physiological protective regulatory balance of the endothelium and is manifest as a loss of endothelium-dependent vasodilation because of reduced endothelial availability of NO. In addition to loss of physiologic dilation and appearance of inappropriate constriction, loss of NO activity results in increased platelet adhesion and aggregation, increased leukocyte adhesion and migration into the subendothelial space, formation of lipid-laden foam cells, and propagation of atherogenesis. While loss of EC generated NO is considered as the primary causative event of ED, conditions of sustained injury to the endothelium may culminate in cell death (by apoptosis) of insulted cells. EC apoptosis represents an irreversible state of EC injury/dysfunction that compromises vessel wall permeability to cytokines growth factors, lipids and immune cells, increases coagulation and also contributes to further downregulation of vasohomeostatic regulators such as NO, thus further propagating progression of atherosclerosis. ED is caused by situations such as the impact of the classical atherosclerotic risk factors, genetic alterations, elevated plasma homocysteine concentrations, infectious microorganisms (e.g. herpes viruses or *Chlamydia pneumoniae*) as well as the imbalance between the magnitude of injury and the capacity for endothelium repair (Pober et al., 2009; Roquer et al., 2009).

ED, which interrupts vascular homeostasis, is characterized by an imbalance between relaxing and contracting factors, pro-coagulant and anti-coagulant substances, and between pro-inflammatory and anti-inflammatory mediators (Figure 6) (Roquer et al., 2009). When ED becomes evident, there is vasoconstriction, increased leukocyte adherence, upregulation of adhesion molecules, increased chemokine secretion and cell permeability, enhanced LDL oxidation, cytokine production, platelet activation, mitogenesis, thrombosis, impaired coagulation, vascular inflammation, vascular smooth muscle cell proliferation and migration, and atherosclerosis. ED contributes to the initiation and progression of atherosclerotic plaque formation as well as the triggering of thrombotic complications in late stages of the disease. The exposure of the endothelium to risk factors leads to ED with reduced NO bioavailability, increased oxidant excess, and the expression of adhesion molecules. This is followed sequentially by intimal-medial thickening, overt manifestations of atherosclerosis, development of arterial stenosis and, ultimately, plaque rupture and endovascular thrombosis (Roquer et al., 2009).

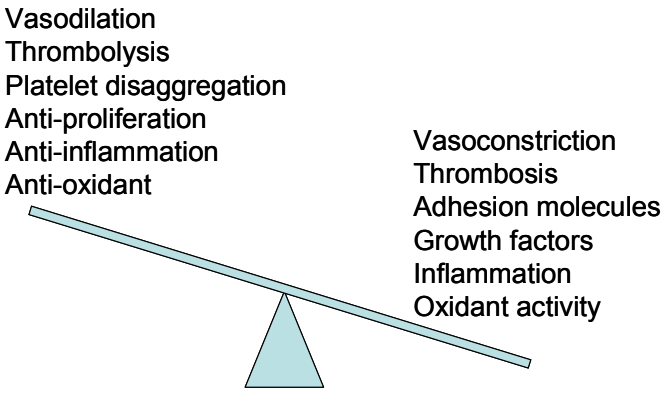

Figure 6. Regulatory functions of the endothelium.
Normal or anti-atherogenic versus dysfunctional or atherogenic properties.

1.3. Mechanisms promoting endothelial dysfunction in atherosclerosis

Stimuli that can cause endothelial dysfunction include environmental stresses such as oxidative stress, endoplasmic reticulum stress, metabolic stress and genotoxic stress, as well as pathways of injury mediated by the innate and adaptive immune systems.

1.3.1. Oxidative stress in endothelial dysfunction

Oxidative stress is a harmful condition that occurs when there is an excess of free radicals. It can be defined as an imbalance between the production of endogenous reactive oxygen species (ROS) and the presence of antioxidant molecules that scavenge and metabolize those reactive species (Figure 7). Free radicals are formed when oxygen interacts with certain molecules and a

chain reaction starts. The different oxidants can originate principally from cellular and extracellular sources, and from enzymatic and nonenzymatic paths (Table 2). Recent studies suggest that ED may be caused by accelerated inactivation of NO by ROS (Figure 7) (Victor, Rocha et al. 2009).

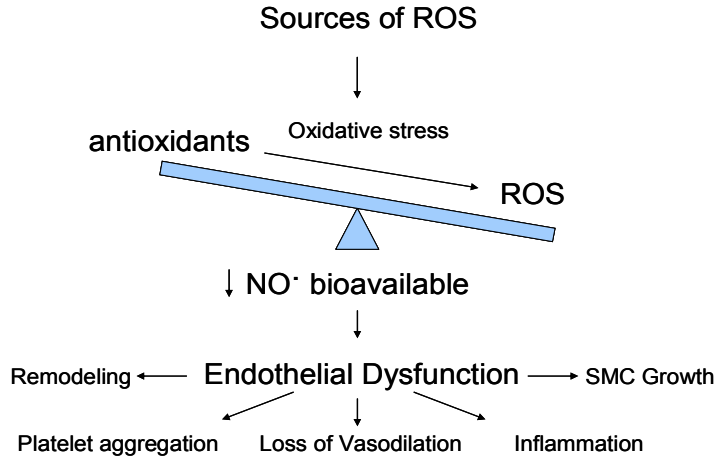

Figure 7. Oxidative stress-induced endothelial dysfunction in cardiovascular diseases.

Table 2. Sources of oxidative stress in vascular wall
NAD(P)H oxidase
Nitric oxide synthases
Myeloperoxidase
Xanthine oxidase
Lipoxygenase / cyclooxygenase
Mitochondrial respiratory chain / oxidative phosphorylation

It is believed that inflammation and oxidative process are interconnected since inflamed cells produce free radicals, which are involved in cell degradation. Within an inflamed vascular setting, LDL becomes oxidized, contributing to atherosclerosis by triggering more inflammation, disrupting normal vascular function and resulting in the accumulation of plaque. LDL in its native state is not atherogenic. It can be oxidatively modified by all major cells of the arterial wall (Keaney 2005; Singh, Devaraj et al. 2005). It can be found in a mildly oxidized form that binds to the LDL-receptor and does not lead to foam-cell formation. On the other hand, mildly oxidized LDL stimulates the production of monocyte chemotactic protein (MCP-1) which promotes monocyte chemotaxis and the production of monocyte colony stimulating factor (M-CSF), promoting the differentiation and proliferation of monocytes into macrophages (Singh, Devaraj et al. 2005). Extensively oxidized LDL (Ox-LDL) is not recognized by the LDL-receptor and is instead ingested by the macrophages *via* the scavenger receptor, leading to foam-cell formation (Rosenfeld, Khoo et al. 1991). Ox-LDL thus importantly contributes to the initiation and progression of the atherosclerotic plaque (Figure 8).

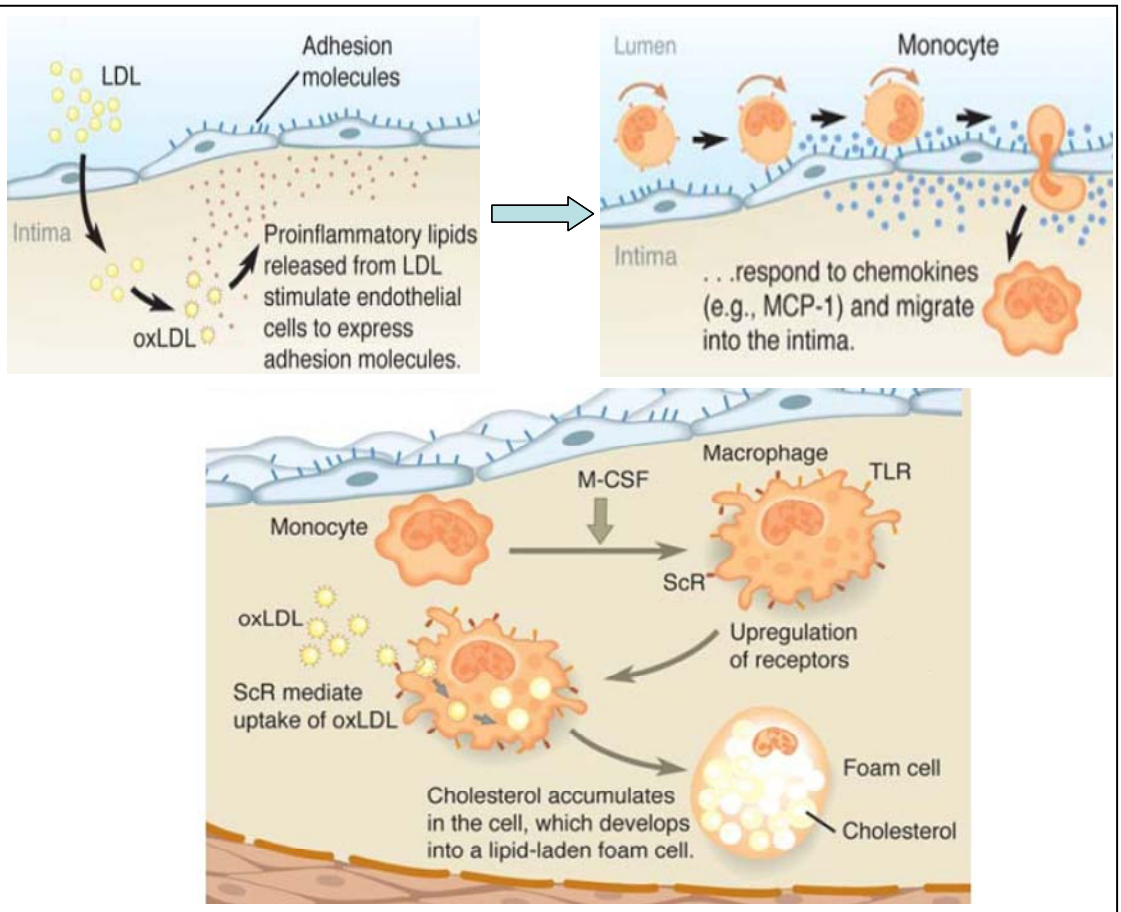

Figure 8. The role of lipid oxidation in initiation and progression of atherosclerosis. LDL transits into the intima where it undergoes oxidation. Oxidized lipids activate EC which start to express adhesion molecules which attract monocytes to adhere and transmigrate into the intima. Monocytes-derived macrophages ingest oxidized-LDL through scavenger receptors (ScR) and lipid-laden foam cells are formed. (Hansson et al., 2006).

ROS may further contribute to the atherogenic process *via* the induction of pro-inflammatory mediators. Upregulation of cell adhesion molecules such as VCAM-1, intercellular adhesion molecule-1, and E-selectin also occur through oxidant-sensitive mechanisms involving peroxynitrite-dependent activation of pro-inflammatory transcription factors such as NF-κB, AP-1, and egr-1 (Napoli et al., 2001). ROS can impair neurovascular coupling and vasodilation mediated by activation of potassium channels, induce vasoconstriction, induce EC apoptosis, promote vascular remodeling through enhancement of smooth muscle cell proliferation and through upregulation and activation of matrix metalloproteinases (MMPs), which may also increase vascular permeability and weaken the thrombus fibrous cap (Touyz, 2006).

1.3.2. Endoplasmic reticulum stress

Another type of stress response activated by disturbances in cell function is the endoplasmic reticulum (ER) stress response, or unfolded protein response (UPR). The accumulation of unfolded proteins in ER represents a stress that is induced by multiple stimuli and pathological conditions. Hypoxia, redox changes, glucose deprivation, Ca^{2+} aberrancies, protein inclusion bodies and viral infections can cause accumulation of unfolded proteins in the ER and eventually ER stress (Marciniak and Ron, 2006). Upon ER stress, evolutionary conserved signalling events are initiated aiming to ameliorate the accumulation of unfolded proteins in the ER. The cellular ER stress/UPR signalling cascade falls into three major phases with different effector functions, namely adaptation, alarm and apoptosis (Figure 9) (Malhotra and Kaufman, 2007; Ron and

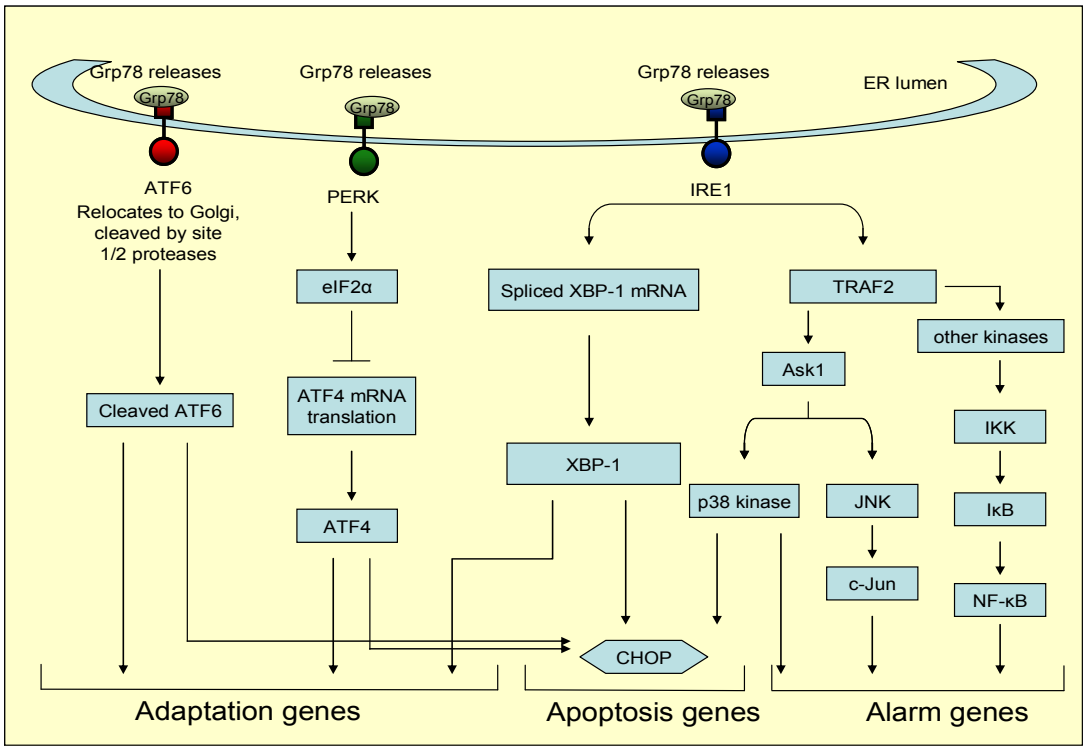

Figure 9. Tripartite stress signalling from the ER. Upon release of Grp78 from the sensor proteins Ire1 and PERK oligomerize in ER membrane. Oligomerized Ire1 binds TRAF2, activating Ask1 other downstream kinases and eventually p38, c-Jun and NF-κB causing expression of genes associated with host defence. The intrinsic ribonuclease activity of Ire1 results in production of XBP-1, a transcription factor that induces genes involved in restoring protein folding or removal of unfolded proteins. PERK oligomerization induces phosphorylation of eIF2α which attenuates mRNA translation. Paradoxically phospho-eIF2α preferentially translates selected mRNAs such as ATF4 mRNA. ATF4 plays a role in restoring ER homeostasis. ATF6 translocates to the Golgi apparatus, where the active protein is formed in order to express UPR genes. The signalling pathways activated by each of these sensors require unique lag times. If ER stress persists a delayed pathway takes over, leading to apoptosis.

Walter, 2007; Wu and Kaufman, 2006; Xu et al., 2005).

The molecular mechanisms by which ER stress promotes cell protection or death pathways have largely been described in cell types other than EC (e.g. epithelial tumor cell lines, pancreatic β cells, macrophages) (Boyce and Yuan, 2006; Kim et al., 2008; Lin et al., 2008; Malhotra and Kaufman, 2007; Ron and Walter, 2007; Schroder, 2008; Wu and Kaufman, 2006). ER stress is triggered by an accumulation of misfolded proteins in the ER which bind to ER chaperone Grp78, causing dissociation of Grp78 from the three major ER stress sensors PERK, ATF6 and IRE1 and a resultant launching of the UPR (Figure 9). The first response aims at re-establishing homeostasis and normal ER function. PERK-dependent phosphorylation of eIF2α results in translational attenuation reducing the load of new protein synthesis on the ER. IRE1 induces expression of X-box binding protein XBP-1, while ATF6 is translocated to the Golgi and activated by proteolysis. XBP-1 and ATF6 act together with eIF2α-downstream target ATF4 as transcription factors activating expression of ER chaperones and components of ER associated degradation system (ERAD) eliminating misfolded proteins. In a later phase, immune and anti-apoptotic responses are activated *via* the NFκB pathway. When the adaptive mechanisms fail to compensate in the face of protracted or excessive ER stress apoptotic cell death is induced to protect the organism by eliminating the damaged cells. Several apoptosis pathways are known to be involved, the central role being played by the proapoptotic transcription factor CHOP which blocks expression of antiapoptotic protein Bcl-2. Transcriptional induction of CHOP mostly depends upon activation of PERK/eIF2α; however, IRE1 and ATF6 pathways also stimulate CHOP transcription, meaning that CHOP operates at the convergence of all UPR branches. ER stress-induced apoptosis can also occur *via* IRE1-dependent activation of TRAF2/ASK1/JNK cascade and *via* Ca^{2+}-dependent activation of caspase-12.

Malfunctions of the ER stress response are associated with a variety of diseases, including neurodegenerative disorders, diabetes mellitus, ischemic injury, cancers, inflammation, infection, and psychotic diseases (Yoshida, 2007; Zhao and Ackerman, 2006). There is a growing body of evidence suggesting that ER stress response also occurs during many processes associated with ED and cardiovascular diseases. ER stress and protein misfolding are rapidly induced in several cell types including EC and cardiomyocytes by tissue ischemia, hypoxia and reactive oxygen species. Upregulation of Grp78, Ask1 and other ER stress-related genes were detected in hypoxic cultured myocytes, ischaemic hearts and in a murine model of myocardial infarction (Azfer et al., 2006; Szegezdi et al., 2006; Thuerauf et al., 2006), while $Ask1^{-/-}$ mice show reduced cardiomyocyte apoptosis rates and better preservation of ventricular function (Yamaguchi et al.,

2003). In EC, activation of UPR genes causes a concomitant increase in VEGF and IL-8 production which lead to stimulation of angiogenesis (Marjon et al., 2004). Accumulation of free cholesterol in macrophages causes calcium release, UPR activation and CHOP-induced apoptosis (Feng et al., 2003). NF-κB, p38 and JNK are activated through PERK and IRE1 inducing the expression of inflammatory cytokines (Li et al., 2005). In addition, oxidized lipids can result in ER stress and UPR activation in human aortic ECs (Gargalovic et al., 2006). Grp78 silencing decreases cell tolerance to oxidative stress (Liu et al., 1998), while Grp78 overexpression inhibits homocysteine-induced ER stress in EC (Werstuck et al., 2001) and inhibits procoagulant activity of tissue factor, a membrane glycoprotein essential for initiation of the coagulation cascade on the cell surface in response to ROS, homocysteine and other stimuli (Watson et al., 2003). Interestingly, there is a cross-talk between ER stress and oxidative stress: ER stress may result in accumulation of ROS and initiate expression and activation of oxidation-related signalling mediators (Cullinan and Diehl, 2006). Importantly, Grp78 protein has been detected on the surface of EC and monocyte/macrophage-like cells in atherosclerotic lesions (Bhattacharjee et al., 2005), on endothelial-derived procoagulant membrane microparticles (Banfi et al., 2005) and in the peripheral circulation of healthy donors (Delpino and Castelli, 2002).

1.3.3. Metabolic Stress

EC supply nutrition and oxygen to tissues and can themselves experience stresses from abnormal metabolism (e.g., high glucose and high lipid levels), energy depletion, or hypoxia.

Insulin resistance, defined as decreased sensitivity and/or responsiveness to metabolic actions of insulin that promote glucose disposal, is an important consequence of metabolic stress to EC. Insulin resistance is observed in diabetes, glucose intolerance, and dyslipidemias, as well as in oxidative stress and inflammation settings. Normal insulin receptor/IGF1 receptor signal transduction is well characterized (Saltiel and Kahn, 2001). These receptors have intrinsic protein tyrosine kinase activity that is activated by ligand binding. The activated receptors phosphorylate several substrates, including insulin receptor substrate 1 (IRS1), which leads to activation of phosphatidylinositol-3-kinase (PI3K)-Akt, and Shc, which leads to activation of the Ras-Raf-ERK signalling cascades. The PI3K-Akt pathway is the major branch of insulin signalling that regulates metabolic function, triggering translocation of glucose transporter 4 (GLUT4) to the plasma membrane where it mediates glucose uptake in skeletal muscle and adipocytes. Thus, impaired IRS-1/Akt activation underlies the molecular mechanism for insulin

resistance and diabetes. Another important target of Akt in EC is eNOS. Akt phosphorylates human eNOS at Ser-1177 and activates the enzyme, leading to NO release (Dimmeler et al., 1999; Fulton et al., 1999). Mice lacking insulin signalling components (e.g., IRS1) have impaired endothelium-dependent vasodilation (Abe et al., 1998). The linkage of IRS1/Akt/eNOS to EC function is supported by studies from insulin sensitizers known as thiazolininediones, which have been shown to improve EC function and to strongly inhibit the development of atherosclerosis in mouse models (Li et al., 2000). In addition to inhibiting eNOS, metabolic stresses such as hyperglycemia may induce expression of extracellular matrix and procoagulant proteins, increase EC apoptosis, decrease EC proliferation, and inhibit fibrinolysis. Similarly, free fatty acids (FFAs) also inhibit EC proliferation and increase EC apoptosis. Mechanistic studies suggest that hyperglycemia and FFAs elicit common oxidative stress and proinflammatory signalling as well as unique signalling.

Hypoxia is another source of metabolic stress that has not been extensively studied in EC. Recent reports suggest that hypoxia promotes mitochondrial ROS production, paradoxically producing oxidative stress (Quintero et al., 2006).

1.3.4. Genotoxic Stress

Cells are constantly exposed to both extrinsic and intrinsic DNA damage signals. Irradiation and chemical mutagens are the extrinsic sources of damage, while intrinsic damage is self generated. Anti-cancer treatments rely in part on ionizing radiation or systemic administration of genotoxic chemotherapeutics that promote stress-mediated apoptosis of tumor cells in response to DNA damage. ROS may also damage DNA, leading to an alternative activation of death responses (Norbury and Zhivotovsky, 2004). The DNA damage–dependent response to ROS depends on induction of the protein p53, which regulates the transcription of many different genes involved in DNA repair, cell-cycle arrest, senescence, and apoptosis (Figure 10) (Riley et al., 2008). These pathways have been extensively studied in tumor cells, but effects of

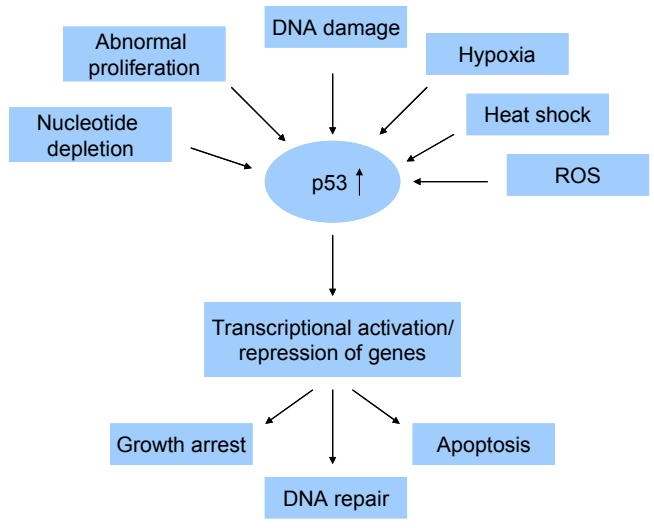

Figure 10. Induction of p53 by diverse genotoxic signals leading to growth arrest and apoptosis.

genotoxic stress on EC are less well characterized. Proliferating vascular cells within a tumor microenvironment do become exposed to chemotherapeutic agents, which may sensitize EC to other agents. In addition, cytokines such as TNF can also cause genetic damage, by generating free-radicals, which may eventually cause genomic instability (Pober et al., 2009).

1.3.5. Inflammatory cells and inflammation

Inflammation is an essential component of the immune response to pathogens and damaged cells. Infectious agents, oxygen radicals, mechanical factors heat shock proteins (HSP) are some of the stimuli causing inflammation. It can be classified as either acute or chronic. The initial response against the stimuli that is harmful for the tissue is known as acute inflammation, and it is achieved by the recruitment of leukocytes from the blood to the injured tissue. Chronic inflammation is the prolonged inflammation which leads to a progressive destruction and healing of the damaged tissue. Recent epidemiological studies demonstrate a significant link between coronary artery disease (CAD) and chronic inflammation (Asanuma et al., 2003; Gelfand et al., 2006; Nicola et al., 2005).

In the presence of risk factors (including oxidative, endoplasmic, metabolic and genotoxic stresses) abnormal activation of the endothelium begins with mechanisms that signal the onset of inflammation. Two examples include the activation of protein kinase C and nuclear translocation of nuclear factor-κB (NF-κB) in EC. These lead to their production of mediators that amplify inflammation (cytokines, interleukin-1β, tumor necrosis factor-α) and EC membrane expression of adhesion molecules that bind monocytes and lymphocytes. This process is amplified by the loss of NO release from the activated endothelium. If the inflammatory “insult” to the endothelium persists, the state of reduced NO bioavailability cannot be reversed, ED becomes aggravated and endothelial apoptosis will ensue (Galkina and Ley, 2009).

Interaction of EC expressed adhesion molecules with integrins expressed on leukocytes facilitates adhesion and extravasation of the leukocytes. Within the lesion, activation of T-cells by local antigens (e.g. peptides, lipids), monocyte-to-macrophage transformation with subsequent foam cell formation and/or mast cell degranulation lead to an accumulation of proinflammatory cytokines and ROS which amplify the local inflammatory response. Concomitant production of metalloproteinases eventually weakens the leading edge of plaques, facilitating stress-induced fracture (Lessner and Galis, 2004; Newby, 2005).

An important aspect of extravasation concerns the antigen specificities of T cells migrating in the early lesions. Several types of leukocytes have an important role during atherogenesis, including both pro- and anti-inflammatory subtypes. Those cell types include dendritic cells, mast cells, B cells and natural killer cells (NKT) cells (Figure 11) (Bobryshev and Lord, 1995; Galkina and Ley, 2007; Jonasson et al., 1986; Kovanen et al., 1995). A series of investigations suggest that activated T-cells are the first inflammatory cells observed in the arterial intima (Wick et al., 2004; Wick et al., 1995). There is evidence in rabbit and mouse models that these early infiltrating T cells recognize heat-shock protein 60 (HSP-60) (George et al., 1999; Xu et al., 1993). Studies have not been conducted in early human lesions, but data obtained in late lesions confirm the presence of T cells with these specificities, albeit at low frequencies. It is not yet clear whether there is local expansion of T cells specific for other antigens accumulating in the lesions. Candidate antigens stimulating these T cells are oxidized lipoproteins, microbial antigens released after infection and locally accumulated modified lipids. Identification of the antigen specificities of plaque-infiltrating T cells is important for understanding this key aspect of plaque evolution. Knowledge of antigen specificities can be used to perform appropriately targeted vaccination, which is a new emerging concept in preventing tissue lesions in atherosclerosis (Hansson, 2002).

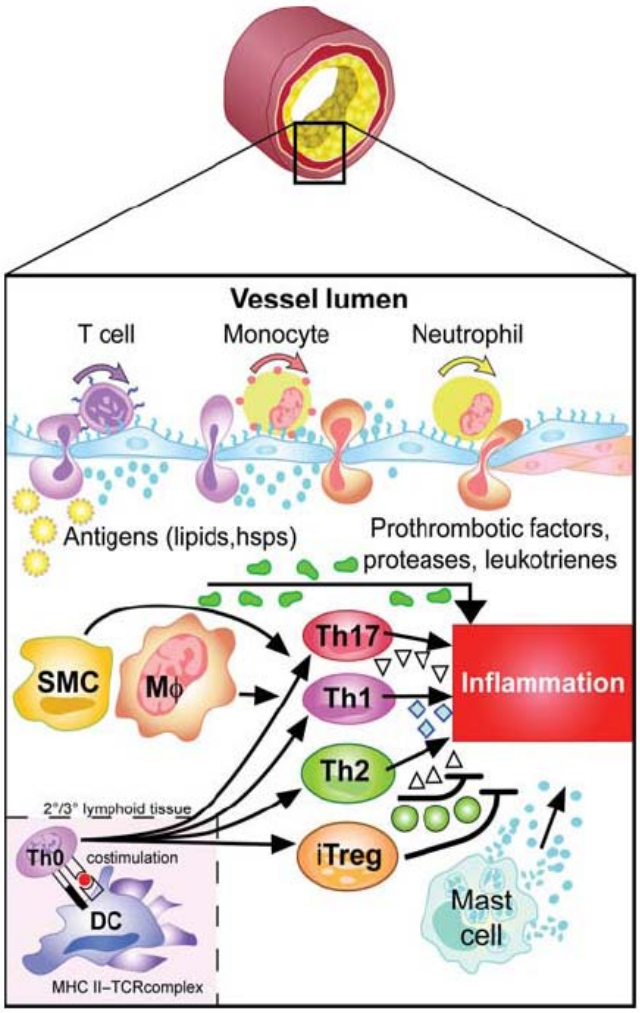

Figure 11. Leukocyte diversity in atherosclerosis (Klingenberg and Hansson, 2009)

NKT cells are a distinct subtype of T lymphocytes, which express markers of natural killer cells as well therefore function as a 'bridge' between innate and adaptive immunity. Their major difference from T cells is that they posses the unique ability to respond to lipid antigens. In order to get activated the lipids have to be presented to them by antigen presenting cells that express CD1d molecules. CD1d molecule is a member of the CD1 family of glycoproteins and they are related to the class I major histocompatibility complex (MHC) molecules (Figure 12). Lipids are recognized through the semi-invariant T-cell receptor (TCR) Vα24Jα18/Vβ11 in humans (Vα14Jα18/Vβ8 in mice) (Kronenberg, 2005).

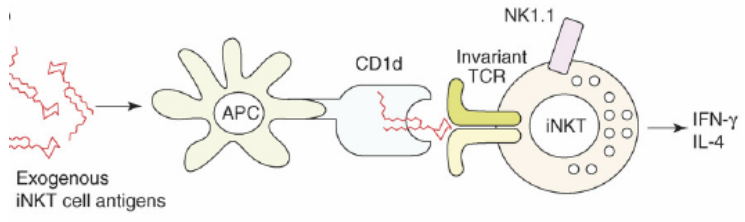

Figure 12. iNKT cell activation via uptake of exogenous lipid antigens by antigen presenting cells (APCs). A CD1d-restricted antigen-presentation mechanism (Van Kaer, 2007)

It is only relatively recent that NKT cells have been implicated in pathological conditions and more specifically in atherosclerosis. NKT cells were demonstrated to localize in advanced lesions and specifically in the rupture-prone shoulders of atherosclerotic plaques and in plaque in patients with abdominal aortic aneurysm (Bobryshev and Lord, 2005; Chan et al., 2005). Several studies in murine models demonstrate that NKT cell activation has the potential to accelerate the atherogenic process. It has been shown that α-Galactosylceramide (αGalCer), a glycolipid antigen and potent activator of iNKT cells, accelerates atherosclerotic lesion formation in apoE$^{-/-}$ and Ldlr$^{-/-}$ mice and in C57B1/6 mice that are on an atherogenic diet; CD1d-deficient and TCR Vα14-deficient mice, which both lack iNKT cells, are protected from development of atherosclerosis (Major et al., 2004; Nakai et al., 2004; Tupin et al., 2004). Moreover, adoptive transfer of iNKT cells markedly increases plaque burden (VanderLaan et al., 2007). Taken together, these animal studies provide strong evidence to suggest that iNKT cells are involved in atherosclerotic plaque development. However, no detailed investigations were performed yet on iNKT cells in human atherosclerosis. Although CD1d protein is expressed in human atherosclerotic lesions (Melian et al., 1999) it remains unknown whether CD1d expression correlates with lesion severity or disease activity.

1.4. The role of neovascularization in atherogenesis and development of the vulnerable plaque

Angiogenesis is defined as the formation of new vessels. The angiogenic process is either physiological (e.g. wound healing) or pathophysiological (e.g. neoplasia, inflammation) and it is the most dynamic process of the endothelium.

Angiogenesis is today a recognized feature of the atherogenic process. Angiogenesis in the developing lesion serves primarily to provide nutrients to the developing and expanding intima and therefore, may prevent cellular death and contribute to plaque growth and stabilization. However, angiogenesis in the setting of the vulnerable plaque is a double-edged sword and underlies many of the clinical complications associated with atherosclerosis (Doyle and Caplice, 2007; Herrmann et al., 2001; Khurana et al., 2005; Mause and Weber, 2009). Cardiovascular events complicating atherosclerosis are most commonly the result of sudden arterial thrombosis in the heart, brain, legs, and other organs. Causes of the acute arterial thrombosis are plaque rupture and erosion, and atherosclerotic plaques which are prone to precipitate acute thrombotic occlusions are unstable, vulnerable plaques. Intraplaque neovascularization significantly contributes to plaque instability.

In the absence of disease, adventitial blood vessels originating from the vasa vasorum (Vv) penetrate into the vessel wall. Those microvessels provide vessel wall nutrients to the tunica media, while the intima is fed by oxygen diffusion from the lumen. Microvessels are not usually present in the normal human intimal layers (Ritman and Lerman, 2007). The Vv remains in a dormant state probably due to the expression and synthesis of anti-angiogenic factors e.g. thrombospondin and endostatin which more than counterbalance the presence of low quantities of pro-angiogenic factors in the micro-environment. However during intimal plaque development, and initially triggered by tissue hypoxia and insufficiency of nutritional supply, the balance between the angiogenic and anti-angiogenic factors becomes altered with increased production of growth factors and cytokines together with a reduction in negative modulators. Hence the angiogenic switch shifts to “on”, resulting in adventitial vessel angiogenesis at the site proximal to internal vascular damage and plaque growth (Slevin M., 2009).

Neovascularization of the atherosclerotic plaque (Figure 13) has important clinical consequences. The new microvessels are immature and prone to leak and serve as a port of entry for inflammatory cells, from the systemic circulation to the nascent atherosclerotic lesion (Moulton, 2001; Moulton et al., 2003) (Figure 13). Microfocal accumulations of blood vessels potentiate inflammatory reactions and encourage matrix remodelling which is in many ways analogous to wound healing (Ross, 1993; Ross, 1999). However, despite the contribution of angiogenesis to reparative processes, the excessive neovascularization of such vulnerable plaque regions as the shoulder and fibrous cap contributes to local plaque destabilization and rupture. Neoformed microvessels in the plaque are devoid of basement membrane and easily leak lipids, proteins, and blood cells in the surrounding interstitium. They are inherently weak, and prone to disruption especially in response to the haemodynamic forces of the vessel lumen (Folkman, 1995; McCarthy et al., 1999; Mofidi et al., 2001). The ensuing intraplaque haemorrhage predisposes the plaque to rupture, thereby causing local thrombosis and subsequent ischemic clinical manifestations such as stroke, unstable angina or myocardial infarction (Barger and Beeuwkes, 1990; Folkman, 1995; McCarthy et al., 1999; Mofidi et al., 2001; Moulton, 2001). The presence of adventitial Vv, intimal angiogenesis and plaque neovascularization are increasingly considered as predictors of instability in atheromatous lesions of cerebrovascular and cardiovascular patients.

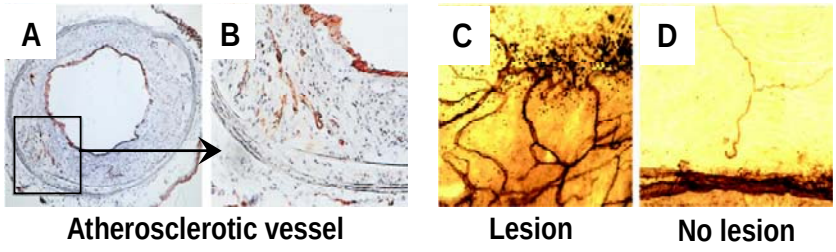

Figure 13. Neovascularization within the atherosclerotic lesion. A,B – Staining for endothelial cells in a section from lesional tissue. C, Strong inflammatory infiltrate is present in neovascularized lesional tissue. D, non-lesional tissue

The mechanisms of microvessel formation in atheroma probably resemble those common to other sites of angiogenesis. Tissue hypoxia and insufficiency of nutritional supply are well known triggers. The correlation of focal collections of inflammatory cells with areas of intraplaque neovascularization and haemorrhage, suggests that release of growth factors and cytokines by macrophages and leukocytes may also have a key role in modulating the vascularization process. Inflammatory protagonists and mechanisms underlying intraplaque neovascularization are not well understood. The inflammatory triggers of the early phases of atherogenesis are relatively well known, but few direct mediators of angiogenesis have been identified and

associated with plaque instability *in vivo*. Oxidized lipids (e.g. 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine) that are prevalent in atherosclerotic plaque have been shown to stimulate EC proliferation and migration and angiogenesis *in vitro* (Ross, 1999). Inflammatory cells residing in the plaque, including macrophages, can also produce a number of pro-angiogenic cytokines and chemokines (e.g. IL-1α, IL-6, IL-8, bFGF, VEGF). *In vivo* studies have shown that blocking blood vessel formation can significantly reduce plaque size (Stefanadis et al., 2007). Pharmacological inhibition of angiogenesis and therefore inhibition of plaque development can be achieved by targeting key molecules and cells. Key molecules involved in initiation and maintenance of the angiogenic process involve the angiopoietin signalling pathways. Ang-1 induces formation of stable blood vessels, whereas Ang-2 destabilizes the interaction between EC and their support cells. Ang-2 is also correlated with the activity of matrix metalloproteinase-2 (MMP-2) expression suggesting a role in development of unstable plaque microvessels. Furthermore Ang-1 is an anti-inflammatory cytokine which can reduce neovessel leakage and vascular permeability (Slevin M., 2009).

1.5. Cell adhesion molecules in atherosclerosis

A wide variety of cell-cell interactions take place during inflammation. These interactions include leukocyte-leukocyte, leukocyte-endothelium, leukocyte-vascular smooth muscle cell, leukocyte-extracellular matrix and leukocyte-interstitial cell interaction. The proteins mediating these interactions are the adhesion molecules. Cell adhesion molecules play numerous crucial functions at the interface of a cell and its environment, whether this environment is another cell, from a similar or different cell type, the extracellular matrix or even sometimes soluble elements. Adhesion molecules are widely distributed and virtually every cell expresses cellular adhesion molecules. There are four major families of cell adhesion molecules, namely the selectins (and selectin ligands), the immunoglobulins, the integrins and the cadherins.

Several lines of evidence support a role for cell adhesion molecules in atherogenesis. A major function of these adhesion molecules is to promote leukocyte recruitment from the vasculature into the tissue. Increased expression of adhesion molecules was detected on established lesions in animal models of atherosclerosis and more importantly in humans. In human coronary atherosclerotic plaques, expression of adhesion molecules on plaque neovasculature was two fold higher than on an arterial luminal endothelium. Increased leukocyte infiltration on plaque sites is correlated with expression of specific adhesion molecules (Huo and Ley, 2001). Levels of soluble adhesion molecules have been postulated to be useful risk predictors of cardiovascular

events in healthy populations and various settings of disease, even though their pathological role remains uncertain.

1.5.1. The role of cell adhesion molecules in mediating leukocyte recruitment and extravasation

The recruitment of leukocytes from the circulation and their subsequent influx into surrounding tissues at sites of inflammation or injury requires multistep adhesive and signalling events, including selectin-mediated capture and rolling, leukocyte activation, integrin-mediated firm adhesion and their subsequent transendothelial migration (Figure 14) (Blankenberg et al., 2003).

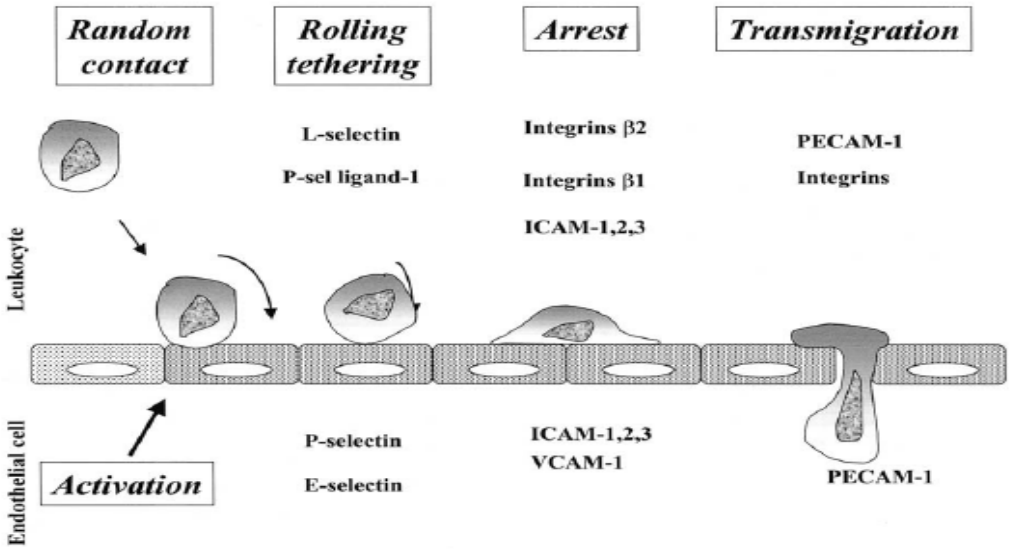

Figure 14. Adhesion molecules promote leukocyte recruitment through a series of events. Leukocyte rolling, firm adhesion, activation and extravasation into the tissue are the main events and are controlled by adhesion molecules.

1.5.1.1. Selectins and their ligands

Selectins are a family of three C-type lectins sharing a conserved structure and are expressed exclusively by bone-marrow-derived cells and EC. The three members of the selectin family are named according to their main expression site: L-selectin is expressed in leukocytes, E-selectin is specific to EC and P-selectin is mainly found on platelets but is also expressed on EC. They are carbohydrate-binding proteins and the extracellular domain of each consists of a

carbohydrate recognition motif, an epidermal growth factor (EGF)-like motif, and varying numbers of a short repeated domain related to complement-regulatory proteins (CRP).

The main physiological function of all selectins is in mediating leukocyte adhesion under flow, but both selectins and their ligands have signalling functions (Ley and Kansas, 2004). The selectins have each a specific pattern of expression. L-selectin is present in almost all leukocytes, on some T and B cells and some NK cells depending on their activation state, and is constitutive. P-selectin is found in secretory granules of platelets and is expressed on the platelet surface after activation. It is also inducible expressed by inflamed EC in many diseases including atherosclerosis. In human atherosclerotic lesions, strong expression of P-selectin was detected on the endothelium overlying active atherosclerotic plaques but not on normal arterial endothelium or on endothelium overlying inactive fibrous plaques (Johnson-Tidey et al., 1994; Ley, 2003). This availability of P-selectin molecules at the cell surface is achieved within a few minutes. E-selectin is expressed by acutely inflamed EC in most organs and in non-inflammed skin microvessels. The NF-κB transcription factor seems crucial in the activation of this gene. This induction takes a few hours and decreases rapidly after a peak.

The mechanisms by which these molecules are downregulated after activation are important: it is necessary to clear them from the cell surface after activation in order to limit/stop the inflammatory process. Over-expression or maintenance of these molecules may have critical consequences on inflammation and on the integrity of the vascular wall. After cell activation, selectin molecules are rapidly removed from the cell surface, by a mechanism of internalization and lysosomal targeting (P- and E-selectin) or by shedding/proteolytic cleavage (L-, E-selectin). Soluble isoforms of E- and L-selectins, measurable in blood, are the results of this latter process (Hafezi-Moghadam et al., 2001). A soluble isoform of P-selectin is also observed, and is the product of an alternatively spliced messenger lacking the transmembrane domain (Ishiwata et al., 1994). Shedding, although not confirmed, might also contribute to a shorter soluble isoform of P-selectin (Berger et al., 1998). These soluble molecules are not only degradation products: they can have their own function, potentially as competitors interacting with their normal counter-receptors without triggering cell recruitment, as inhibitors or as agonists. All selectins participate in different, albeit overlapping, ways to the early steps of leukocyte recruitment at the endothelial surface under shear forces: leukocyte rolling and tethering (Figure 14). By interactions with their ligands, selectins create weak bonds between activated EC (E- and P-selectin) and leukocytes (L-selectin). P-selectin/PSGL-1 binding triggers leukocyte activation, integrin mobilization and induces inflammation and thrombosis (Blankenberg et al., 2003).

1.5.1.2. Immunoglobulin (Ig) adhesion molecules

The immunoglobulin (Ig) –like large family of adhesion molecules are membrane glycoprotein receptors. Ig superfamily cell adhesion molecules have an extracellular domain, which contains a variable number of Ig-like intrachain disulfide-bonded loops with conserved cysteine residues, a transmembrane domain, and an intracellular domain that interacts with the cytoskeleton (Blankenberg et al., 2003). Members of the Ig superfamily include the intercellular adhesion molecules (ICAMs), vascular-cell adhesion molecule (VCAM-1), platelet-endothelial-cell adhesion molecule (PECAM-1), and neural-cell adhesion molecule (NCAM). ICAM-1 is widely expressed at a basal level and can be up-regulated by pro-inflammatory cytokines in leukocytes and EC (Manka et al., 1999), whereas ICAM-2 is present on leukocytes, platelets and endothelium but is rather down-regulated by inflammatory mediators. ICAM-3 is detected in EC and leukocytes and is the only ICAM molecule on neutrophils. Soluble forms of ICAM-1 and ICAM-3 have been shown to result from shedding (Fiore et al., 2002). VCAM-1 is transcriptionally induced on activated EC but can also be expressed by other cell types like macrophages, myoblasts, dendritic cells (Blankenberg et al., 2003). VCAM-1 participates to the recruitment of blood cells by allowing them to firmly adhere on activated EC. PECAM-1 is expressed by leukocytes, platelets and EC. It is mainly found at adherent junctions of EC where it participates in homophilic binding between adjacent cells (Newton et al., 1997). In humans, plaque neovasculature shows increased levels of ICAM-1 and VCAM-1 expression. In addition macrophages and T-lymphocytes are efficiently recruited in plaques when ICAM-1 and VCAM-1 are expressed, suggesting that inflammatory cell recruitment through activation of intimal neovasculature may participate in the pathogenesis of atherosclerosis (Huo and Ley, 2001).

1.5.1.3. Integrins

Integrins are heterodimeric transmembrane glycoproteins resulting from the non-covalent association of an α chain and a β chain. Eighteen different α chains and 8 β chains have been described, whereas only 24 different heterodimers have been observed. Alternative splicing is common in this family and increases the complexity/specificity of associations (Armulik, 2002). Both the α and β subunits contain two separate tails, both of which penetrate the plasma membrane and possess small cytoplasmic domains. Soluble forms of integrins have not yet been reported. Most integrins function as receptors for ECM proteins, however some family members mediate heterotypic cell-cell adhesion. Integrins are expressed in a large variety of cells and have various conformational states. In resting cells, they are usually non-adhesive,

present on the cell surface but in a conformation that provides low affinity for ligands. The mechanism by which a cell regulates the affinity state of its integrin receptor is called “inside-out” signalling. It involves the propagation of conformational changes from the cytoplasmic domains of integrins to the extracellular binding sites in response to intracellular signalling events. “Outside-in” signals are delivered within the cell after ligation between an integrin and its ligand (Abram and Lowell, 2009; Hughes and Pfaff, 1998; Qin et al., 2004). Integrin-mediated cellular interactions initiate signalling pathways that regulate a plethora of responses including cell morphology, differentiation, survival, proliferation, migration, and invasion. Integrin mediated anchorage is also a key regulator of anoikis, a programmed cell death (apoptosis) caused by loss of anchorage. Normal cells are dependent on anchorage not only for growth, but also for survival.

In atherosclerosis, integrins have distinct roles in inflammatory cell recruitment to the damaged vessel wall. β2 integrins are strictly expressed in leukocytes, the α chains giving more subtle patterns of expression to the functional dimeric protein. For example, αLβ2 is expressed in almost all leukocytes whereas αXβ2 is more restricted to monocytes/ macrophages (Lopez-Rodriguez et al., 1995). This class of integrins interacts with molecules of the ICAM subfamily; after cell activation, they become themselves activated and interact with ICAMs to induce a firm arrest at the surface of the activated endothelium (Lynam et al., 1998). Cells can then change their shape and migrate upon the surface of the endothelium to reach a junction. The interaction VCAM-1/integrin α4β1 is also important for this step (Rose et al., 2001). Integrins from the β3 family are expressed in platelets: α2bβ3, also known as glycoprotein (GP) IIb/IIIa, is specific for this cell type and plays a role in fibrin formation, whereas αVβ3 is more widely expressed and is involved in cell survival, migration and proliferation (Desgrosellier and Cheresh; Schwartz et al., 1999).

A subset of integrins contributes to the regulation of angiogenesis. In atherosclerotic plaques, αvβ3 is highly expressed by medial and intimal SMCs and by EC of angiogenic microvessels (Hoshiga et al., 1995). In a rabbit model of atherosclerosis, targeting of EC expressed αvβ3 with nanoparticles containing antiangiogenic fumagillin was shown to markedly reduce plaque neovascularization (Winter et al., 2006). In addition selective αvβ3 blockage, limited macrophage infiltration and neovascularization in the vessel wall (Bishop et al., 2001). Integrin α7 expression was shown to be enhanced in a rat model of atherosclerosis (Chao et al., 2004). Experimental studies have demonstrated that engagement of α5β1, α1β1, α2β1 and αvβ5 integrins promote

tumor growth and angiogenesis (Avraamides et al., 2008). However, their precise roles in the context of plaque neovascularization are not yet understood.

1.5.2. The role of cadherins in regulating endothelial function

Cadherins play an important role in endothelial cell integrity and growth and vascular morphogenesis. The large cadherin superfamily includes calcium dependent cell adhesion molecules responsible for cell-cell recognition and adhesion (Figure 15).

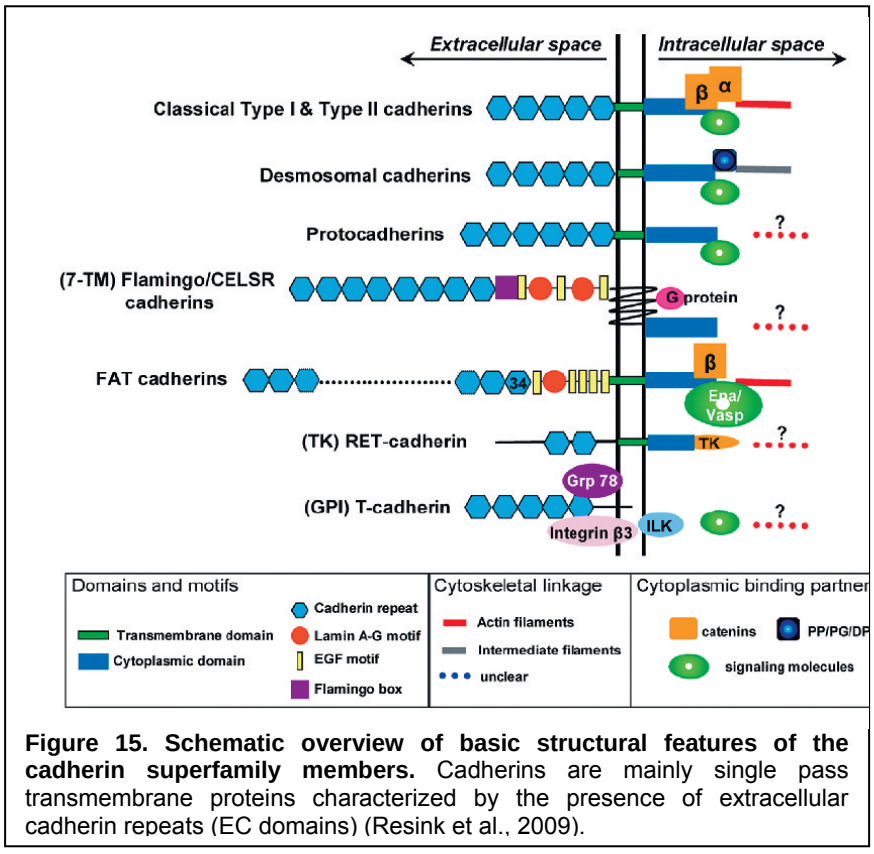

Figure 15. Schematic overview of basic structural features of the cadherin superfamily members. Cadherins are mainly single pass transmembrane proteins characterized by the presence of extracellular cadherin repeats (EC domains) (Resink et al., 2009).

Besides their adhesive properties, cadherins may act by transferring intracellular signals though interactions with complex network of cytoskeleton and signalling molecules. They are expressed in several types of tissues with some specificity. EC express VE-cadherin, N-cadherin and T-cadherin. VE-cadherin is unique to EC and is the cadherin present in the adherens junctions of EC (Dejana, 2004). A specific feature of VE-cadherin as compared with all other classical cadherins is that it may also behave as a desmosomal-like cadherin, whereby through plakoglobin VE-cadherin can recruit desmosomal proteins desmoplakin and vimentin at the membrane. This desmosomal-like structure (*complexus adhaerentes*) is specific for EC (Bazzoni and Dejana, 2004). N-cadherin is excluded from adherens junctions in EC and is diffusely distributed on the cell membrane (Navarro et al., 1998; Resink et al., 2009; Salomon et al., 1992). It is also expressed at contact zones between pericytes and EC (Gerhardt et al., 1999). T-cadherin in EC is globally distributed on the cell surface with only very minor enrichment at cell-cell borders and polarizes to the leading edge of migrating EC (Philippova et al., 2003).

Regulation of EC adhesive and signalling functions by VE-cadherin has been the subject of intensive study for decades. N-cadherin and T-cadherin are relative newcomers to investigations on EC biology and their roles in vascular diseases are poorly defined.

1.5.2.1. VE-cadherin

A wealth of *in vitro* and *in vivo* studies have established that VE-cadherin plays essential roles in controlling vascular permeability, integrity, and remodeling, leukocyte transmigration, vasculogenesis and angiogenesis (Bazzoni and Dejana, 2004; Carmeliet and Collen, 2000; Cavallaro et al., 2006; Dejana, 2004; Dejana et al., 2008; Lampugnani and Dejana, 2007; Vestweber, 2007; Vestweber, 2008; Wallez and Huber, 2008; Wallez et al., 2006). Not surprisingly therefore, alterations in VE-cadherin expression and distribution contribute to atherogenesis. In the mouse has been shown that VE-cadherin is very important during angiogenesis, and inactivation of the corresponding gene leads to a lethal phenotype. Although the EC that do not express VE-cadherin are able to form vascular networks, indicating that it is not essential for this process, vascular remodelling is affected. EC tend to detach from one another and vessels regressed and collapsed leading to early embryonic lethality in mice, a consequence of vascular insufficiency (Carmeliet et al., 1999; Gory-Faure et al., 1999). Different mechanisms may account for the role of VE-cadherin in angiogenesis. Apart from its adhesive properties it is suggested that it is also implicated in intracellular signalling. VE-cadherin associates to VEGF-R2 and modulates its signalling pathway, leading to alterations of vascular development with aberrant lumen, hemorrhages and eventually vascular regression (Grazia Lampugnani et al., 2003). Additionally VE-cadherin has a key role in vascular permeability when is associated with β-catenin (Ukropec et al., 2000). In the intima of healthy human arteries VE-cadherin is not expressed (Bobryshev et al., 1999). However during atherosclerosis it is found within the intima with a frequency and intensity that increases in advanced lesions and reflects the level of neovascularization (Bobryshev et al., 1999; Sigala et al., 2003). *In vivo,* VE-cadherin expression at cell-cell junctions is weaker in atherosclerosis-susceptible sites (Miao et al., 2005). The expression of VE-cadherin was associated with plaque instability, degree of stenosis and clinical events (Sigala et al., 2003). Neovessels surrounded by inflammatory cells had irregular or reduced levels of VE-cadherin in association a breakdown of endothelial integrity, favouring further infiltration of inflammatory cells into plaque tissue (Bobryshev et al., 1999). “Soluble” VE-cadherin can be detected in human plasma (Koga et al., 2005; Soeki et al., 2004). Plasma levels of VE-cadherin were correlated with the degree of coronary atherosclerosis, independently of classical atherosclerotic risk factors (Soeki et al., 2004).

1.5.2.2. N-cadherin

Expression of N-cadherin on EC has not yet been studied in human atherosclerosis or experimental restenosis and its role has remained largely elusive. However, its pro-angiogenic properties suggest that it is likely that an increase might be detected in association with neovascularization. In contrast with the typical localization of VE-cadherin at cell-cell junctions, N-cadherin is distributed over the whole cell surface of cultured EC (Navarro et al., 1998). N-cadherin has been shown to play a critical role in angiogenesis, although its function differs from that of VE-cadherin. Whereas VE-cadherin mostly promotes the homotypic interaction between EC, N-cadherin is responsible for the formation of heterotypic ablumenal adherens junctions between EC and pericytes (Gerhardt et al., 1999; Gerhardt et al., 2000; Liebner et al., 2000) or myoendothelial junctions between EC and underlying VSMC (Isakson et al., 2008; Paik et al., 2004). N-cadherin-dependent pericyte coverage is critical for stabilization and of maturation of newly-formed endothelial sprouts. N-cadherin blockage *in vivo* results in defective pericyte adhesion accompanied by vascular dysmorphogenesis and hemorrhage (Gerhardt and Betsholtz, 2003; Gerhardt et al., 2000). Recent data suggest that N-cadherin may also influence angiogenesis by directly regulating EC function and influencing VE-cadherin expression levels. Knockdown of N-cadherin *in vivo* and *in vitro* caused a significant decrease in VE-cadherin (Luo and Radice, 2005). A synthetic peptide capable of antagonizing N-cadherin-mediated adhesion disrupted angiogenesis *in vitro* (Devemy and Blaschuk, 2008). Furthermore, neutralization of N-cadherin with a cyclic peptide containing the HAV motif was shown to induce apoptosis through inhibition of cadherin-mediated activation of FGFR signalling (Erez et al., 2004). Soluble N-cadherin (consisting of the extracellular domain) stimulated angiogenesis *in vivo* and migration *in vitro* (Derycke et al., 2006). Soluble N-cadherin did not affect intercellular adhesion, and its effects on EC migration are mediated through complex formation with fibroblast growth factor receptor (FGFR), which has been implicated as an important partner of N-cadherin in a number of cell types, including EC (Derycke et al., 2006). Possibly metalloproteinase-mediated shedding of soluble N-cadherin from VSMC during proliferation may have a knock-on pro-angiogenic effect (Uglow et al., 2003).

1.5.2.3. T-cadherin

T-cadherin is an atypical cadherin. It is the only one member of the cadherin superfamily which lacks both the transmembrane and cytoplasmic regions, but it is instead anchored to the membrane via a glycosylphosphatidylinositol (GPI) anchor (Figure15)(Philippova et al., 2009).

Therefore it cannot interact with the classical intracellular partners and with the cytoskeleton. T-cadherin on EC is globally distributed on the cell surface with only very minor enrichment at cell-cell borders, and it polarizes to the leading edge of migrating EC (Philippova et al., 2003). It is suggested that T-cadherin is rather involved in cell signalling and it is suggested that perhaps it role is in sensing neighbouring cells and informing its host cell of the microenvironment, thus functioning as a guidance receptor (Ivanov et al., 2004b; Joshi et al., 2007). *In vitro* T-cadherin is upregulated on proliferating EC (Ivanov et al., 2004a) or EC exposed to oxidative stress (Joshi et al., 2005). T-cadherin overexpression on EC *in vitro* induces proliferation and motility (Ivanov et al., 2004b) and protects EC from oxidative stress-induced apoptosis (Joshi et al., 2005; Philippova et al., 2008). Together the data suggest that T-cadherin is both a marker of EC activation/stress and an inducer of an activated EC phenotype. Anti-adhesive/repulsive functions for T-cadherin in the vasculature emerged from studies showing that homophilic ligation in EC is rapidly followed by the acquisition of a less-adhesive, motile or pro-migratory, pro-angiogenic phenotype (Ivanov et al., 2004b; Philippova et al., 2005). These functions of T-cadherin require the activity of the small GTPases RhoA and Rac1 (Philippova et al., 2005). GPI-anchored T-cadherin lacks transmembrane and cytosolic domains, and thus its effects on EC behaviour require association with molecular adaptors to mediate inward signalling. A number of membrane adaptors including integrin β3, Grp78/Bip and integrin linked kinase have been identified (Joshi et al., 2007; Philippova et al., 2008). *In vivo* T-cadherin is up-regulated in atherosclerotic lesions (Ivanov et al., 2001), during restenosis (Kudrjashova et al., 2002) and on EC from tumour vasculature (Wyder et al., 2000). Proangiogenic properties for T-cadherin have been demonstrated using *in vitro* models of angiogenesis (Philippova et al., 2006). Using a model of myoblast-mediated gene transfer to mouse skeletal muscle delivery of soluble T-cadherin potentiated VEGF effects on neovascularization in a manner that involved an increase in vessel caliber (Philippova et al., 2006). Another study using the Matrigel implant model reported that ectopic delivery of T-cadherin inhibited neovascularization (Rubina et al., 2007). However, specific confirmation of proangiogenic functions for T-cadherin was recently provided through use of T-cadherin null mice whereby T-cadherin deficiency was found to limit angiogenic responses (Hebbard et al., 2008).

2. AIMS

2.1. Inflammation and angiogenesis in plaque instability

Increasing evidence has suggested that the extent of intraplaque neovascularization is directly related to the infiltration of inflammatory cells into the vessel wall, the stage of plaque development, the risk of plaque rupture, and subsequently, the presence of symptomatic disease, the timing of ischemic neurological events and myocardial/cerebral infarction. We aim to identify relevant inflammatory cells and pro-angiogenic soluble factors released by inflammatory cells that might contribute to development of unstable atherosclerotic plaque by activation of intraplaque angiogenesis.

The first objective (Project 1) was to investigate $CD1d^+$ APC and iNKT cells in human atherosclerotic lesions, correlate them with disease severity and activity, and elucidate potential mechanisms by which these cells might be involved in plaque formation and/or destabilization.

The second objective (Project 2) was to identify key signal transduction mechanisms for the angiogenic response elicited in response to iNKT cell activation.

2.2. Regulating endoplasmic reticulum stress in endothelial cells: the role of T-cadherin

Accumulating data suggest some relationship between T-cadherin and ER stress. T-cadherin is both a marker of EC activation/stress and facilitates acquisition of an activated, proangiogenic EC phenotype. In EC, activation of UPR genes causes a concomitant increase in VEGF and IL-8 production which lead to stimulation of angiogenesis. Overexpression and ligation of T-cadherin on the EC surface activate cell survival and proliferation signalling pathways (PI3-kinase, Akt, GSK-3β) common to those activated by chaperon Grp78, a crucial regulator of UPR signalling during ER stress. We aimed (Project 3) to identify the relationship between T-cadherin expression and ER stress response in EC. Here we investigate the ability of T-cadherin to influence UPR signalling and endothelial cell survival during ER stress.

Project 1

The role of lipid activated inflammatory cells in the pathogenesis of atherosclerosis

The results of this project have been published.

Invariant NKT cells: Linking inflammation and neovascularization in human atherosclerosis.

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(The paper is appended)

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Invariant natural killer T cells: Linking inflammation and neovascularization in human atherosclerosis

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Abbreviations used: αGalCer, α-galactosylceramide; iNKT cells, invariant natural killer T cells; CM, conditioned medium; EC, human microvascular endothelial cell line HMEC-1.

Key words: Angiogenesis, CD1 molecules, iNKT cells, Cell migration, Inflammation.

Summary

Atherosclerosis, a chronic inflammatory lipid storage disease of large arteries, is complicated by cardiovascular events usually precipitated by plaque rupture or erosion. Inflammation participates in lesion progression and plaque rupture. Identification of leukocyte populations involved in plaque destabilization is important for effective prevention of cardiovascular events. This study investigates CD1d-expressing cells and invariant natural killer T (iNKT) cells in human arterial tissue, their correlation with disease severity and symptoms, and potential mechanisms for their involvement in plaque formation and/or destabilization. CD1d-expressing cells were present in advanced plaques in patients who suffered from cardiovascular events in the past and were most abundant in plaques with ectopic neovascularization. Confocal microscopy detected iNKT cells in plaques, and plaque-derived iNKT cell lines promptly produced proinflammatory cytokines when stimulated by CD1d-expressing APC presenting α-galactosylceramide lipid antigen. Furthermore, iNKT cells were

diminished in the circulating blood of patients with symptomatic atherosclerosis. Activated iNKT cell-derived culture supernatants showed angiogenic activity in an endothelial cell (EC)-spheroid model of *in vitro* angiogenesis and strongly activated EC migration. This functional activity was ascribed to IL-8 released by iNKT cells upon lipid recognition. These findings introduce iNKT cells as novel cellular candidates promoting plaque neovascularization and destabilization in human atherosclerosis.

Introduction

Atherosclerosis is complicated by cardiovascular (CV) events, which usually occur when plaques rupture or erode. Vulnerable plaques prone to rupture are characterized by inflammation, plaque hemorrhage and abnormal apoptosis [1, 2], three processes that are spatially and temporally interconnected. Both innate and acquired immune responses can modulate atherosclerotic plaque development [3]. Macrophages and T lymphocytes infiltrating the arterial wall during atherosclerosis [2, 4] produce proinflammatory cytokines, chemokines, metalloproteinases and mesenchymal growth factors that are all potentially involved in plaque growth and rupture but might also contribute to plaque remodeling and stabilization. A histopathological quantitative analysis has suggested that macrophages in the arterial wall seem to be protective in the early, but deleterious in the late stages of disease [5]. T-cell populations with different functional capacities have been identified within atherosclerotic lesions [4] and contribute to the pathogenic complexity of the inflammatory process [6]. In addition to inflammation, other mechanisms such as lipid retention [7], neovascularization [1, 5, 8] and tissue remodeling [9, 10] support plaque growth. How different leukocyte populations contribute to or are affected by these additional mechanisms remains elusive. T cells recognizing protein or lipid antigens within plaques are likely involved. Invariant natural killer T cells (iNKT) cells, which express a semi-invariant TCR made by Vα24 and Vβ11 chains, have attracted attention as lipid-responsive cells [11]. These cells recognize lipid antigens presented by CD1d, a member of the CD1 family of antigen-presenting molecules [12]. α-Galactosylceramide (αGalCer), a glycolipid antigen and potent activator of iNKT cells, accelerates atherosclerotic lesion formation in the ApoE$^{-/-}$ mouse model [13-15]. CD1d-deficient and TCR Vα14-deficient mice, both lacking iNKT cells, are protected in this model of atherosclerosis [15-17]. Moreover, in this model adoptive transfer of iNKT cells markedly increases plaque burden [18]. In contrast, in the LDL receptor$^{-/-}$ mouse model an atheroprotective role for iNKT cells has been described [19]. Taken together, these animal studies provide strong evidence that iNKT cells are involved in atherosclerotic plaque development and progression.

No detailed investigations on iNKT cells in human atherosclerosis have yet been performed. Although CD1d protein is expressed in human atherosclerotic lesions [20] it remains unknown whether CD1d expression correlates with lesion severity or disease activity. This study examines CD1d-expressing cells and iNKT cells in human atherosclerotic lesions, their correlation with disease severity and activity, and potential mechanisms for their involvement in plaque formation, progression and/or destabilization.

Results

CD1d$^+$ cells in human atherosclerotic lesions are a sign of arterial vulnerability

We quantified intimal macrophages and CD1d$^+$ cells in arterial tissue obtained from asymptomatic (ASA) patients who never experienced CV events previously (n = 21) and patients with symptomatic (SA) atherosclerosis who developed CV events in the past (n = 15) using human arterial tissue microarrays (Fig. 1). Definition of CV events is given under Materials and Methods. This approach permits correlation of histomorphological findings with disease activity and lesion severity. We analyzed a total of 108 arterial sectors obtained systematically from three different vascular beds (carotid, renal and iliac artery) of 36 patients (clinical characteristics shown in Table.1). Plaque type according to the American Heart Association (AHA) classification [21], and numbers of CD1d-expressing cells, CD68$^+$ macrophages and vWF-positive microvessels per intima area were determined in serial histopathological sections. In a per sector analysis, both CD68$^+$ macrophages and CD1d$^+$ cells were found more commonly in advanced lesions than at early plaque stages (Fig. 1A; Supporting Information Fig. 1 for CD1d$^+$ staining controls). In a per patient analysis, *i.e.* when the three observations in the iliac, renal and carotid artery for each patient were averaged, the density (number of cells per mm^2) of CD68$^+$ or CD1d$^+$ cells did not differ between ASA and SA patients (Fig. 1B). On the contrary, when signs of ectopic neovascularization were also considered as a variable, SA patients had on average the highest numbers of CD1d$^+$ cells ($P < 0.05$) (Fig. 1B). It is remarkable that CD1d$^+$ cells were virtually absent from lesions

without signs of ectopic neovascularization (early lesions) and low in asymptomatic patients. For the tissue microarray analysis, arterial rings were harvested on average 24h after death. We tested whether the number of detectable $CD1d^+$ cells would fade with time after death but found no such correlation (data not shown).

iNKT cells are found in atherosclerotic lesions

The presence of CD1d-expressing cells in advanced, unstable atherosclerotic lesions prompted a search for iNKT cells. Due to the predicted scarcity of these cells, different approaches were applied to investigate their presence in atherosclerotic plaques. Lesional arterial intima from 5 SA patients was examined by confocal microscopy. We demonstrated the presence of $CD3^+/V\alpha24^+$ and $CD3^+/V\beta11^+$ cells, which represented up to 3% of total infiltrating $CD3^+$ T cells in all lesions analyzed (Fig. 2A and Table 2). These findings suggest but do not prove the presence of iNKT cells. We therefore prepared cell suspensions from thrombendarterectomy specimens and performed co-staining with anti-Vα24 and anti-Vβ11 mAb *ex vivo* (Fig. 2B). The identification of Vα24/Vβ11 double-positive cells with fluorescent microscopy provided evidence that iNKT cells are present in the diseased arterial wall.

Next we performed dual fluorescence confocal microscopy of lesional tissue from 8 SA patients using anti-CD1d and anti-TCR Vα24-Jα18 (6B11) mAb, which recognizes the iNKT-specific invariant TCR Vα chain [22, 23]. Representative micrographs unequivocally demonstrating the presence of iNKT cells in atherosclerotic lesions are presented in Fig. 3. In some instances there was evidence of co-localization of the iNKT TCR with CD1d and even iNKT TCR and CD1d polarization towards each other. These findings could indicate an ongoing activation of iNKT cells within the atherosclerotic tissue.

To confirm and formally prove that iNKT cells reside in atherosclerotic lesions we isolated and expanded iNKT cells from thrombendarterectomy specimens obtained from SA patients and performed phenotypic and functional studies. We stimulated plaque-derived T cells with αGalCer and CD1d-expressing cells to facilitate the selective expansion of iNKT cells and succeeded in establishing 6 bulk T cell lines. Flow cytometry analysis using 5 color

staining showed that 60-90% of $CD3^+$ cells were co-expressing TCR Vα24 and Vβ11 chains (Fig. 4A). In all lines, $Vα24^+Vβ11^+$ cells were also stained with αGalCer-loaded soluble human CD1d dimers, thus confirming the lipid specificity and CD1d restriction of their TCR. Five of the 6 iNKT cell isolates were CD4 positive (representative shown in Fig. 4A) and all 6 were CD8 negative. Plaque-derived iNKT cells stimulated with αGalCer produced large amounts of IL-4, TNF-α, IFN-γ and GM-CSF (Fig. 4B). We compared the 6 plaque-derived iNKT cell lines and 66 blood-derived iNKT cell clones with respect to their responsiveness to αGalCer. The ED50 was calculated after measurement of IFN-γ (Fig. 4C), TNF-α, IL-4, and GM-CSF (data not shown) release. For all cytokines plaque-derived iNKT cells exhibited ED50 values at least 10-fold lower than peripheral blood-derived iNKT cells. Taken together, these results prove that the iNKT cells present within atherosclerotic lesions have phenotypic and functional features of *bona fide* iNKT cells [24] and react to αGalCer with unusual high efficiency.

Circulating iNKT cell numbers are reduced in patients with symptomatic atherosclerosis

Next we investigated iNKT cells in the blood from three groups of donors, namely SA patients, age-matched control patients free of CV events in the past (*i.e*. ASA) and young healthy individuals. iNKT cells were detected in PBMC with 4 color immunofluorescence analysis using anti-CD3ε, anti-TCR Vα24, anti-TCR Vβ11 mAb and αGalCer-loaded CD1d dimers. We detected a significant ($P \leq 0.001$) reduction of circulating iNKT cells in SA patients compared with either ASA patients or young healthy individuals (Fig. 5). A reduction was also observed in the ASA patients as compared with the young healthy individuals ($P < 0.01$), possibly reflecting an age-related effect on this lymphocyte subset [25]. These findings raise interesting issues regarding the fate of iNKT cells in peripheral blood of symptomatic atherosclerotic patients: are they reduced because of lack of proliferative responsiveness to stimulatory lipids, increased apoptosis or increased extravasation into tissues?

Characterization of proatherosclerotic activity of iNKT cells

The presence of $CD1d^+$ cells and iNKT cells within advanced atherosclerotic lesions, particularly in patients with symptomatic disease, led us to investigate whether this T lymphocyte population has a role in key processes of plaque formation and destabilization. Following αGalCer stimulation plaque-derived iNKT cells release proinflammatory and potential angiogenic modulators (Supporting Information Fig. 2). Both plaque- and blood-derived iNKT cells secreted the same type of cytokines (data not shown).

Since neovascularized arterial sectors had the highest numbers of $CD1d^+$ cells, subsequent investigations focused on effects of iNKT activation on angiogenic behavior of EC. We examined angiogenic potential of conditioned medium (CM) derived from iNKT cell cultures stimulated with (CM+) or without (CM-) αGalCer using the EC-spheroid model of *in vitro* microvascular sprout formation as a global functional test for angiogenesis. Visualization of spheroids indicated that CM+ induced greater sprout outgrowth than CM- (Fig. 6A). Morphometric analysis showed a significant increase in both the number (Fig. 6B) and length (Fig. 6C) of sprouts. CM collected from cultures containing αGalCer but lacking either $CD1d^+$-APC or iNKT cells, or both, failed to enhance sprout outgrowth (Supporting Information Fig. 3). Taken together, these data confirm that antigen-stimulated iNKT cells can promote angiogenesis *in vitro*.

Soluble factors released by iNKT cells promote endothelial cell migration

Angiogenesis is a complex process and both proliferation and migration of EC contribute to this phenomenon [8, 26]. To identify which of these activities is modulated in response to iNKT cell activation, we compared effects of CM on proliferation and migration of EC in monolayer cultures. CM+ derived from different iNKT cells did not activate EC proliferation (Supporting Information Fig. 4) but did induce cell migration. Two methods were used to evaluate migration. In the first, confluent EC monolayers were scrape-wounded and migration into the wound was recorded over a 12h period by time-lapse videomicroscopy. This wound-healing assay showed more rapid migration for EC cultured in the presence of

CM+ (Fig. 7A and B, and Supporting Information Fig. 5). Representative videos showing EC motility in the presence of CM- (Supporting Information Fig5video1-CM-.avi) and CM+ (Supporting Information Fig5video2-CM+.avi) are given in the Supporting Information. The second assay quantified transmigration of EC in a Boyden-chamber and also demonstrated enhanced migration of EC toward CM+ (Fig. 7C). These data suggest a chemokine-like effect on EC angiogenic behavior.

IL-8 is produced by iNKT cells and induces endothelial cell migration

IL-8, a pleiotropic chemokine with known angiogenic activity *in vitro* and *in vivo* [27, 28], was amongst numerous factors released by activated iNKT cells (Supporting Information Fig. 2). iNKT cells isolated from plaques (Fig. 8A) and peripheral blood (Supporting Information Fig. 6) showed strong intracellular staining for IL-8 when stimulated with αGalCer proving that they readily produce this chemokine. To determine the contribution of iNKT cell-released IL-8 to the angiogenic potential of CM+, wound-healing assays were conducted in the presence of anti-IL-8 blocking antibodies or using CM which had been immunodepleted of IL-8 prior to assay. Both treatments completely abrogated the enhanced EC migration (Fig. 8B). Basal EC migration was not affected by inclusion of anti-IL-8 antibodies or IL-8-depletion, excluding non-specific inhibitory effects of the antibodies. Therefore, the enhanced migration response of EC to CM+ is dependent upon IL-8 released by activated iNKT cells.

Discussion

Our study investigated iNKT cells in human atherosclerosis. We found that cells expressing CD1d are present in advanced atherosclerotic plaques and lesions from patients with active, symptomatic disease. In patients with symptomatic atherosclerosis, vascularized plaques had the highest number of $CD1d^+$ cells. We identified the presence of iNKT cells in atherosclerotic lesions and characterized their function after isolation from plaques. iNKT cells from plaques show a high reactivity to the αGalCer antigen and may promote neovascularization in an IL-8-dependent manner. Our study suggests that iNKT cells contribute to the predisposition of atherosclerotic plaques to rupture.

In order to perform quantitative immunohistochemical analysis of inflammatory cells in atherosclerotic plaques, we took advantage of the arterial tissue microarray technique which permitted us to compare serial sections of 108 arterial sectors from 36 patients. Our approach, recently reproduced [29], facilitated evaluation of associations between the presence of $CD68^+$ macrophages and $CD1d^+$ cells and disease activity, lesion severity (*i.e*. plaque stage) and plaque neovascularization. $CD68^+$ macrophages were found in all samples analyzed, even in those without lesions, as reported [5], and were slightly increased in very advanced plaques. In contrast, $CD1d^+$ cells were virtually absent from the normal arterial intima or in early plaque stages, whereas they were increased in advanced lesions particularly in the presence of neovessels. Expression of CD1d in human atherosclerotic plaques has been reported in two studies [20, 30]. However, sample numbers were small and no correlations were made with clinical stage, disease activity or histological hallmarks, leaving open the question of whether CD1d expression correlates with lesion grade. In our study, a substantial number of $CD1d^+$ cells was observed in advanced lesions (AHA type >IV) and particularly in lesions with signs of neovascularization, thus demonstrating a close correlation with advanced disease. The preferential localization of $CD1d^+$ cells in areas with neovascularization could be explained by their efficient recruitment into vascularized plaques [31] and/or by their capacity to promote plaque neovascularization. Our data are in accordance with the concept that CD1d may present lipid antigens locally to specific T cells,

including iNKT cells, which in turn may release angiogenic factors and contribute to neovascularization.

In the diseased arterial wall we found T cells expressing Vα24 or Vβ11 TCR chains, which are used by iNKT cells. Flow cytometric analysis of freshly isolated iNKT cells was not possible due to small biopsy size and the minute number of resident iNKT cells. This technical limitation prevented exact quantification of iNKT cells and analysis of expressed activation markers. Therefore, the activation status of iNKT cells within lesions remains unknown. However, we could identify iNKT cells in lesional tissue by several methodological approaches, namely i) detection of TCR Vα24-Jα18 with the 6B11 mAb; ii) detection of Vα24 and Vβ11 co-expressing T cells freshly isolated from lesions, and iii) expansion and functional characterization of iNKT cell lines from plaques. We observed an intraplaque infiltration of iNKT cells and a significant reduction of iNKT cells in circulating blood in symptomatic patients. This relative accumulation in plaques could be caused by homing and retention following local activation and/or proliferation upon antigen recognition.

The presence of iNKT cells in lesions has been inferred in mouse atherosclerosis models by molecular investigations and not cellular isolation. In a pioneering study on ApoE-deficient mice under high cholesterol diet, the presence of iNKT cells was suggested by RT-PCR studies [13, 14, 32]. However, to date iNKT cells have neither been isolated from plaques nor functionally characterized. In one study, $CD3^+CD161^+$ cells were histologically detected in carotid specimens and appeared with a frequency of 0.3-2% among plaque infiltrating T cells [30]. CD161 is expressed by a variety of T lymphocytes and therefore is not a specific marker for iNKT cells. In a second study, all $CD3^+$ cells expanded from aortic aneurysms expressed the CD161 marker [33], suggesting an abnormal proliferation of this cell type *in vitro*. Since the expression of semi-invariant TCR Vα24/Vβ11 was not investigated the presence of iNKT cells was not confirmed. We isolated plaque-infiltrating iNKT cells, which were $Vα24^+Vβ11^+$. They also bound αGalCer-loaded CD1d dimers, providing clear evidence that they are classical iNKT cells, and were efficiently activated by αGalCer-loaded CD1d-expressing APC to release IL-4, IFN-γ, GM-CSF and TNF-α. Thus, in

atherosclerotic plaques there is accumulation of cells expressing phenotypic and functional features of *bona fide* iNKT cells.

Importantly, the iNKT cell lines isolated from plaques all showed an extremely low threshold of activation when stimulated with αGalCer. The same low threshold was found only in a minor fraction of iNKT cell clones isolated from peripheral blood. These findings might suggest that due to antigen recognition and expansion in plaques, there is a preferential accumulation of iNKT cells expressing TCR with high responsiveness to αGalCer *in vitro*. Whether high reactivity to αGalCer reflects high reactivity to lipids accumulating within plaques is unknown since endogenous self-lipid antigens stimulating iNKT cells remain poorly characterized. An additional and non-mutually exclusive possibility is that plaque-derived iNKT cells lack NK inhibitory receptors and therefore are activated by very low doses of antigen. Intriguingly, in a model of atherosclerosis in ApoE-deficient mice iNKT cells with low expression of the inhibitory Ly49 receptors showed proatherogenic activity which was more pronounced than that of Ly49-positive iNKT cells [34]. The high reactivity of plaque-derived human iNKT cells deserves further investigations, since it remains unclear whether the entire plaque infiltrating iNKT cell population shows this unusual behavior.

How iNKT cell activation exerts proatherogenic effects remains an open issue. One potential mechanism relates to inflammation, which in human atherosclerosis is characteristically progressive. Since human iNKT cells isolated from plaques do release proinflammatory cytokines, their chronic *in situ* activation by lipid antigens might lead to lesion progression. This hypothesis is in line with many studies conducted in mice. Injection of αGalCer increases size and number of plaques in a mouse atherosclerosis model [15]. This experimental iNKT cell activation elicits massive release of Th1 and Th2 cytokines and elevation in plasma levels of IL-6 and monocyte chemoattractant protein 1, which have been proposed to enhance local inflammation [11, 15]. In humans atherosclerosis is a slowly progressive disease and there is as yet no evidence of massive inflammation in the arterial wall in early lesions. Instead a chronic inflammatory reaction may apply, probably together with other disease-promoting mechanisms.

A second pathogenic mechanism concerns neovascularization. The significant association of $CD1d^+$ cells with neovascularization in plaques suggests that iNKT cells may be involved in angiogenic processes. Our findings revealed that iNKT cell activation by antigen has proangiogenic effects as shown by enhanced microvascular sprout formation in an *in vitro* assay of angiogenesis. This effect was associated with EC migration as demonstrated by enhanced EC motility in both wound-healing and transmigration Boyden chamber assays.

Amongst the multiple cytokines that were produced by activated iNKT cells, IL-8 was the most promising candidate to further investigate. IL-8 was detected previously in the supernatant from lipid-stimulated blood-derived iNKT cells [35]. We found that plaque-derived iNKT cells produce IL-8 as shown by intracellular staining. Further, the enhanced EC migration was dependent on release of IL-8 from iNKT cells since the migration response was abrogated by IL-8-blockade or IL-8 immunodepletion. The participation of IL-8 in atherosclerotic lesion progression is suggested by several studies [36]. IL-8 has been detected in atheromatous tissue [28, 37, 38] and can be induced in monocytes by oxidized LDL and cholesterol [37, 39]. Functionally, IL-8 contributes to intimal macrophage accumulation [40], to endothelial adhesiveness for monocytes [41], has mitogenic and chemoattractant effects on smooth muscle cells [42] and may also facilitate plaque recruitment of $CD8^+$ effector T cells with high cytotoxic potential [43]. IL-8 has been proposed as an important mediator of angiogenesis in CV lesions contributing to plaque growth [28]. It is tempting to speculate that iNKT cells, when chronically activated by lipid antigens in the arterial wall, exert both promigratory and proinflammatory functions which become important for plaque neovascularization and destabilization. These functions might be shared with resident monocytes and other T cells recognizing specific antigens in plaques.

In conclusion, our studies have revealed $CD1d^+$ cells in advanced, vascularized atherosclerotic lesions from patients with active disease. We have identified the presence of iNKT cells within plaques, isolated plaque iNKT cells and demonstrated their high sensitivity to antigen stimulation and their proinflammatory and proangiogenic potential *in vitro*. By

these mechanisms, iNKT cells might participate in plaque growth and destabilization. Gathering evidence suggests that atherosclerosis is an autoimmune disease treatable with immunotherapeutic approaches [44]. Our observations invoke iNKT cells and CD1d-expressing cells as additional potential candidate targets for immunopreventative interventions.

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Materials and methods

Patients and arterial tissues

All investigations with human subjects and tissues were approved by the regional ethical review board and performed in accordance with institutional guidelines. The arterial tissue microarrays have been described previously [5]. In brief, 0.5 cm long arterial ring segments were obtained systematically during autopsy from 36 deceased patients who were treated for a broad variety of medical conditions at the Department of General Medicine of an academic medical center (Cantonal Hospital Bruderholz). The arterial rings were removed always at the same anatomical site regardless of the local lesion severity: 2 cm before the bifurcation for the left common carotid, 2 cm after branching from the aorta for the left renal and 2 cm after the aortic bifurcation for the left iliac artery. The deceased patients entering this study were not selected but were by intention prospectively included in order to circumvent any relevant selection bias. Clinical characteristics are given in Table 1. Fifteen of these patients were known to have symptomatic, active atherosclerosis and to have suffered from CV events, defined as as myocardial infarction, angina pectoris with signs of myocardial ischemia, cerebrovascular ischemic stroke, transient ischemic attack, peripheral arterial occlusive disease, symptomatic aortic aneurysm, or any arterial revascularization procedure to treat atherosclerosis [5].

PBMC were obtained from a second cohort of 269 in-patients hospitalized for any reason and who, with written informed consent, participated in a cross-sectional observational study of atherosclerosis [45]. Twenty-eight of this second cohort had previous CV events in more than 1 organ system; among these SA patients, the 10 oldest individuals (median age: 78, range 76-83 years) were selected for the analysis of the number of circulating iNKT cells (Supporting Information Table 1). From this second cohort, 110 of 269 patients never reported any CV events in the past; among them, the 10 oldest subjects (median age: 79, range 78-83 years) were selected as an age-matched ASA group. The age of these patients matches that of SA and ASA patients from whom tissue microarrays were

generated. As an additional control group, 10 healthy young individuals free of any clinical evidence for atherosclerosis (median age 29, range 26-33 years) were included. PBMC from whole blood were isolated and processed as described [46]. Multicolor FACS was used to characterize iNKT cells in total PBMC as described in Supporting Information.

Analysis of iNKT cells in arterial tissue

Fresh-frozen, OCT embedded arterial rings obtained at autopsy from 13 symptomatic patients with advanced grade atherosclerotic plaques (AHA grade IV, V or VI) were variously used for the identification of TCR Vα24$^+$ or TCR Vβ11$^+$ T cells (n = 5) and of TCR Vα24-Jα18$^+$ T cells (n = 8) as detailed in Supporting Information.

Collagenase-assisted release of lymphocytes from fresh arterial tissue obtained from SA patients with advanced lesions undergoing thrombendarterectomy was performed as previously described [47] with some modifications (Supporting Information). After staining with anti-TCR Vα24-FITC and anti-TCR Vβ11-biotin/streptavidin-Cy5 cells were collected by cytospin and analyzed for TCR Vα24$^+$Vβ11$^+$ cells by confocal microscopy. In some experiments the released lymphocytes were resuspended in complete RPMI-1640 medium (Supporting Information), split, seeded into individual wells of a 96-well plate and subjected to 2 rounds of restimulation with DC obtained as described [48] plus 100 ng/ml αGalCer (kind gift of Kirin Breweries) and addition of anti-MHC class I and anti-MHC class II mAb (W6/32 and L243, both from ATCC) to avoid activation of MHC-restricted alloreactive T cells. The expanded plaque tissue-derived cells were assessed for the presence of iNKT cells by multicolor FACS (Supporting Information and figure legends) and antigen presentation assays.

***In vitro* study procedures**

Materials and methods for these studies are fully detailed and referenced in the Supporting Information. The following human cell lines and clones were used: MOLT-4 expressing negligible CD1d (ATCC CRL 1582), C1R-hCD1d and HeLa-hCD1d [49] as antigen

presenting cell (APC) lines, human microvascular endothelial cell line HMEC-1 (EC), iNKT cell clones from PBMC of healthy donors and iNKT cell lines from plaques. To generate conditioned medium (CM), iNKT cells were cultured with APC pulsed with αGalCer or vehicle, and supernatant was harvested after 48h. Cytokines in CM were quantified by sandwich ELISA, or by Multiplex analysis (BioRad; Human17-Plex Panel 171-A11171) in selected experiments. Assays testing angiogenic effects of CM on EC included the EC-spheroid model of *in vitro* angiogenesis, proliferation, Boyden-chamber transmigration and videomicroscopy of wound closure.

Statistical analysis

Patient data were compared between groups using the Mann-Whitney U test. Data from *in vitro* experiments were compared using the unpaired Student's 2-tailed t test and are given as mean±SD. All analyses were performed using GraphPad Prism software (version 5.03). Differences were considered significant at $P<0.05$.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

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FIGURE LEGENDS

Figure 1. APC in atherosclerotic lesions. The number of $CD68^+$ macrophages and $CD1d^+$ cells per intima area were determined with arterial tissue microarrays [5]. For each arterial sector, the intima area was morphometrically measured and the $CD1d^+$ and $CD68^+$ cells in the intima counted (expressed as cells/mm^2). (A) Macrophage and $CD1d^+$ cell counts in arterial sectors affected by atherosclerotic lesions of increasing severity according to the AHA classification. (B) Quantitative analysis of macrophage and $CD1d^+$ cell counts in arterial sectors according to disease activity (*i.e.* whether patients suffered CV events during their lifetime or not and said to have asymptomatic (ASA) or symptomatic atherosclerosis (SA)). Neovessels were detected as vWF-positive microvessels in the arterial intima [5] and plaques were scored positive (filled boxes) or negative (open boxes) with respect to this anatomical sign. Data in A and B are presented as box plots with median, interquartile range and 5-95 percentiles. n.s., not significant *P<0.5, **P<0.01, ***P<0.001, Mann-Whitney U test. §, compares all ASA with all SA.

Figure 2. iNKT cells in atherosclerotic lesions. (A) Lesional T cells *in situ*. Cryosections of intima of arterial rings from 5 patients with advanced atherosclerotic lesions were examined by confocal microscopy for the presence of T cells co-expressing CD3 and TCR Vβ11 (upper panels) or CD3 and TCR Vα24 chains (lower panels). Quantitative evaluation of cells positive for CD3/TCR Vα24 or CD3/TCR Vβ11 is given in Table 2. (B) Confocal analysis of iNKT cells freshly isolated from atherosclerotic plaques. Collagenase-released cells from fresh arterial tissue biopsies were collected by cytospin, co-stained with anti-TCR Vα24 and anti-TCR Vβ11 and analyzed by confocal microscopy. The data are representative of 4 independent experiments. All scale bars represent 2 μm.

Figure 3. Identification of iNKT cells in atherosclerotic lesions. Panels from left to right show the staining of iNKT cells with anti-TCR Vα24-Jα18, of $CD1d^+$ cells, iNKT cells (in red) merged to $CD1d^+$ cells (in green), and of nuclei (Hoechst). Scale bars: 10 μm. Stainings were performed on 8 patient tissue specimens and 2 representative stainings are shown. UPN, unique patient number. Boxed regions/arrows indicate co-localization of the iNKT TCR with CD1d (UPN 259) and iNKT TCR and CD1d polarization towards each other (UPN 189).

Figure 4. iNKT cells from atherosclerotic plaque tissue. (A) A representative (of 6 total) bulk T-cell lines isolated from plaques and expanded after stimulation with αGalCer. T cells were stained with anti-CD3, anti-CD4, anti-TCR Vα24 and anti-TCR Vβ11 and with αGalCer-loaded CD1d dimers. The FACS gating strategy is depicted in Supporting Information Fig. 7. Left panel shows density plot after gating on $CD3^+$ cells. The right panel shows density plot after gating on $CD3^+Vα24^+Vβ11^+$ cells. (B) Cytokine release from one representative plaque-derived iNKT cell line after *in vitro* stimulation with αGalCer. Empty circles show cytokine release in the presence of the maximum dose of αGalCer and absence of CD1d-expressing APC. Results are expressed as mean ± SD of triplicate determinations. One representative experiment out of 3 is shown. Similar results were obtained with the other 5 cell lines in at least 2 experiments. (C) Potency of αGalCer on 66 iNKT cell clones established from PBMC (open symbols) or with 6 iNKT cell lines isolated from plaque tissue (closed symbols). ED50 here defines the αGalCer dose inducing half-maximal IFN-γ release. Each point represents the ED50 value of one titration experiment, and for each group the median and interquartile range is given. ***P>0.001, unpaired Student's t-test.

Figure 5. Circulating iNKT cells are reduced in atherosclerosis patients. Distribution of iNKT cells in PBMC from healthy young donors (N) and from age-matched patients with asymptomatic atherosclerosis (ASA) or patients with symptomatic atherosclerosis (SA). iNKT cells were detected by FACS with αGalCer-loaded CD1d dimers (A and B) or with anti-TCR Vα24 and anti-TCR Vβ11 (C). The FACS gating strategy is depicted in Supporting

Information Fig. 7. In order to have a statistically quantifiable number of iNKT cells acquisition of at least 5 million $CD3^+$ cells was performed. Data are reported as percentage after gating on $CD3^+$ cells (A) or as percentage of total PBMC (B and C). Box plots with median, interquartile range and 5-95 percentiles are presented. * P<0.5, **P<0.01, ***P<0.001, Mann-Whitney U test.

Figure 6. Antigen activation of iNKT cells increases sprout outgrowth from EC spheroids. Conditioned media derived from iNKT cell cultures stimulated without (open bars) or with αGalCer (filled bars) were examined using the EC-spheroid model of *in vitro* angiogenesis. (A) Representative images of spheroids 24h after exposure to conditioned media. Spheroids were morphometrically analysed for total sprout number (B) and total sprout length (C). Bars undermarked "no APC" indicate the response to medium from iNKT cells cultured alone. Data are mean±SD from 6 experiments, each performed in triplicate. *P<0.05, **P<0.01, Student's t-test. A second iNKT cell clone elicited similar proangiogenic effects (Supporting Information Fig. 3).

Figure 7. Antigen activation of iNKT cells promotes EC migration. Confluent monolayers of EC were scrape-wounded and the subsequent rate of wound closure monitored over a time period of 12h by time lapse videomicroscopy. Acquired images were processed and analyzed using CellR software. (A) Representative images illustrating EC migration in the presence of CM+ or CM-. White lines indicate the location of the wound front and arrows indicate migration path length. (B) Quantitative analysis of the rate of EC migration from the initial wound front into the wound area (path length versus time). The data are representative of at least 30 experiments, each one performed in duplicate and values are given as averaged path length measurements ± SD from triple fixed observation fields/well. CM+ from different iNKT clones similarly enhanced motility (Supporting Information Fig. 5). (C) EC transmigration toward CM+ and CM- in Boyden chamber chemotaxis assay was quantified after a 6h incubation. Data are reported as mean±SD from 3 experiments each performed in

duplicate. ***, P < 0.001, Student's t-test. The CM used in the illustrated experiments were obtained using HeLa cells as APC. In other experiments C1R cells were used as APC with comparable results (data not shown).

Figure 8. Antigen activated iNKT cells produce IL-8 which promotes EC migration. (A) Intracellular IL-8 analyzed by FACS in plaque-derived lines either resting (APC alone) or activated with APC + αGalCer or with PMA + ionophore. Cells were stained intracellularly with anti-IL-8 and anti-CD3 mAb. Percentages of $CD3^+$ cells producing IL-8 are indicated. The FACS gating strategy is depicted in Supporting Information Fig. 7. (B) Effects of inclusion of anti-IL-8 blocking mAb (upper panels) and of IL-8 immunodepletion (lower panels) of CM on EC migration examined by wound assay. Upper graphs: assays were performed in the absence (open circles) and presence of neutralizing anti-IL-8 mAb or isotype control IgG (closed or open triangles, respectively). Lower graphs: assays were performed with untreated CM (open circles), or CM subjected to immunodepletion protocols using neutralizing anti-IL-8 mAb or isotype control IgG (closed or open squares, respectively). Data shown are the average path length measurements ± SD from triple fixed observation fields/well of duplicate samples. Similar results were obtained in three experiments using CM from different iNKT cell clones. The CM used in the illustrated experiments were obtained using HeLa cells as APC. In other experiments C1R cells were used as APC with comparable results (data not shown).

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Table 1. Clinical characteristics of the 36 patients.

	no cardiovascular events (*n* = 21)	cardiovascular events (*n* = 15)	P value
Cardiovascular risk factors			
Diabetes mellitus - no. (%)	1 (5)	7 (47)	0.004
Body mass index (kg/m^2) [a)]	23 ± 6	26 ± 5	0.06
Hypercholesterolemia - no. (%)	2 (10)	4 (27)	0.17
Arterial hypertension - no. (%)	4 (19)	6 (40)	0.17
Smoking - no. (%)	3 (14)	4 (27)	0.35
Male sex - no. (%)	13 (48)	11 (50)	0.90
Age (years) [a)]	74 ± 14	79 ± 9	0.12
History of cardiovascular disease			
Coronary heart disease [c)] - no. (%)	0 (0)	15 (100)	
Cerebrovascular disease [d)] - no. (%)	0 (0)	5 (33)	
Arterial occlusive disease [e)] - no. (%)	0 (0)	6 (40)	
Autopsy (hours after death) [a)]	24 ± 12	24 ± 13	0.94
Infection at death - no. (%) [b)]	9 (43)	8 (53)	0.53

[a)] mean ± standard deviation. [b)] Infection at death was defined by the presence of two or more of the following criteria: body temperature >38°C, C-reactive protein >50mg/L, neutrophils (band forms) >10%, positive blood cultures. [c)] myocardial infarction, angina pectoris with myocardial ischemia, revascularization, [d)] cerebrovascular ischemic stroke, transient ischemic attack, revascularization, [e)] symptomatic peripheral arterial occlusive disease, symptomatic aortic aneurysm, revascularization.

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Table 2. Lesional T cells *in situ: c*onfocal estimation of numbers of cells positive for CD3 and TCR Vα24 or TCR Vβ11.

Patient	AHA plaque type	TCR Vα24+/total CD3+ cells (%)	TCR Vβ11+/total CD3+ cells (%)
1	IV	5/163 (3.1)	8/143 (5.6)
2	IV	5/274 (1.8)	3/255 (1.2)
3	V	5/180 (2.8)	6/192 (3.1)
4	V	2/152 (1.3)	2/121 (1.6)
5	VI	8/153 (5.2)	7/185 (3.8)
		(2.8 ± 1.5)	(3.1 ± 1.8)

Values in parentheses express numbers of $V\alpha24^+/CD3^+$ or $V\beta11^+/CD3^+$ cells as a percentage of total $CD3^+$ cells and are given for each individual patient biopsy and as the mean ± SD for all 5 biopsies examined.

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Figure 1

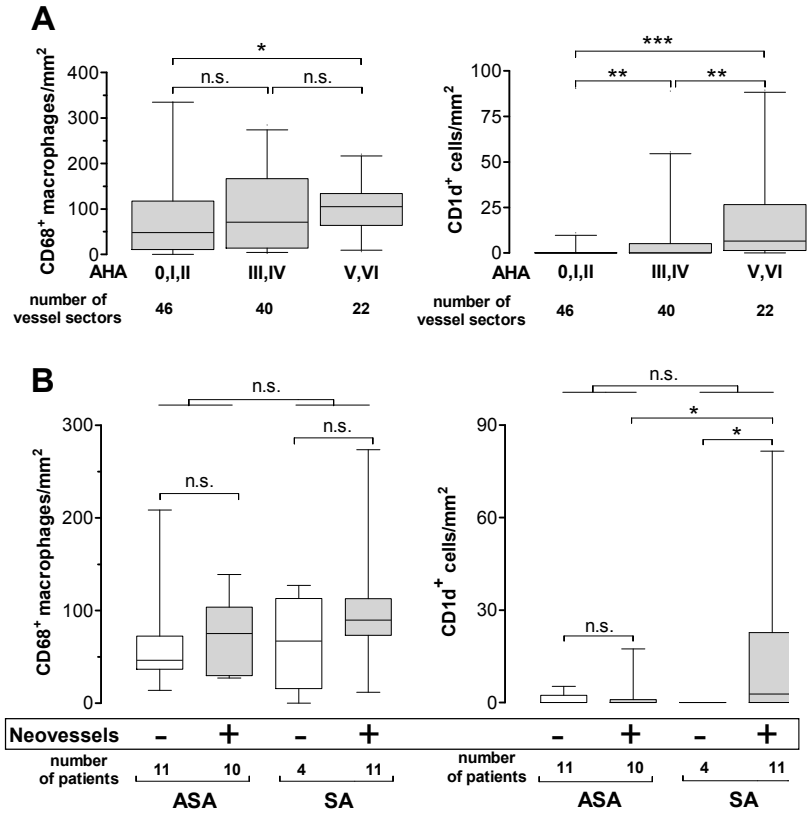

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Figure 2

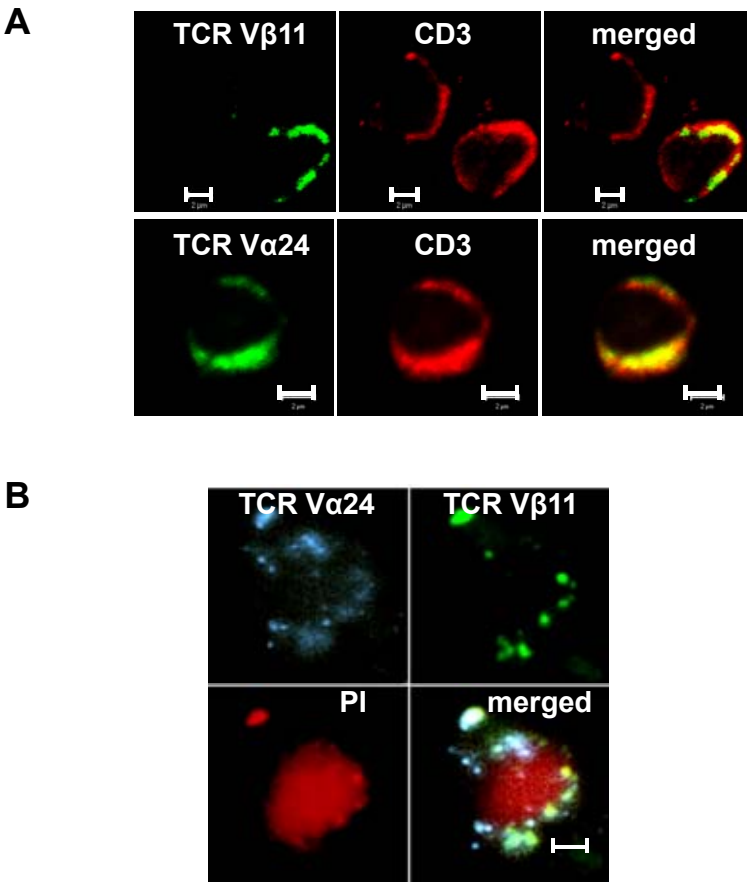

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Figure 3

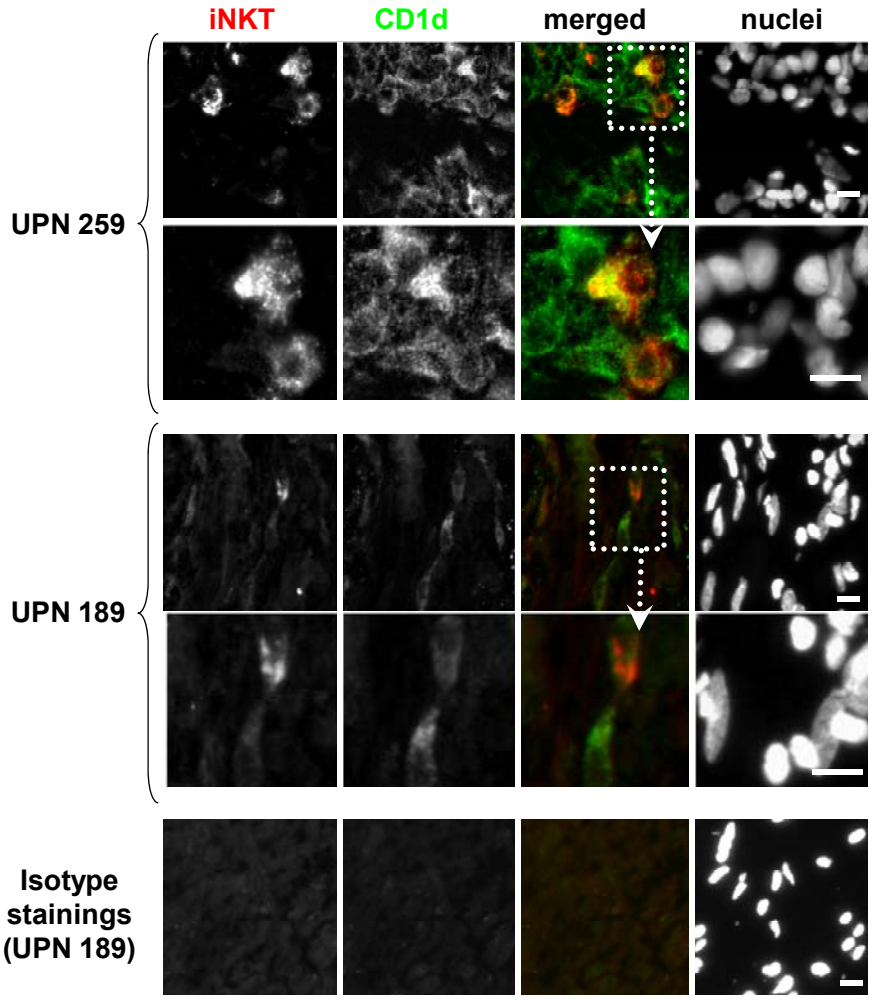

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Figure 4

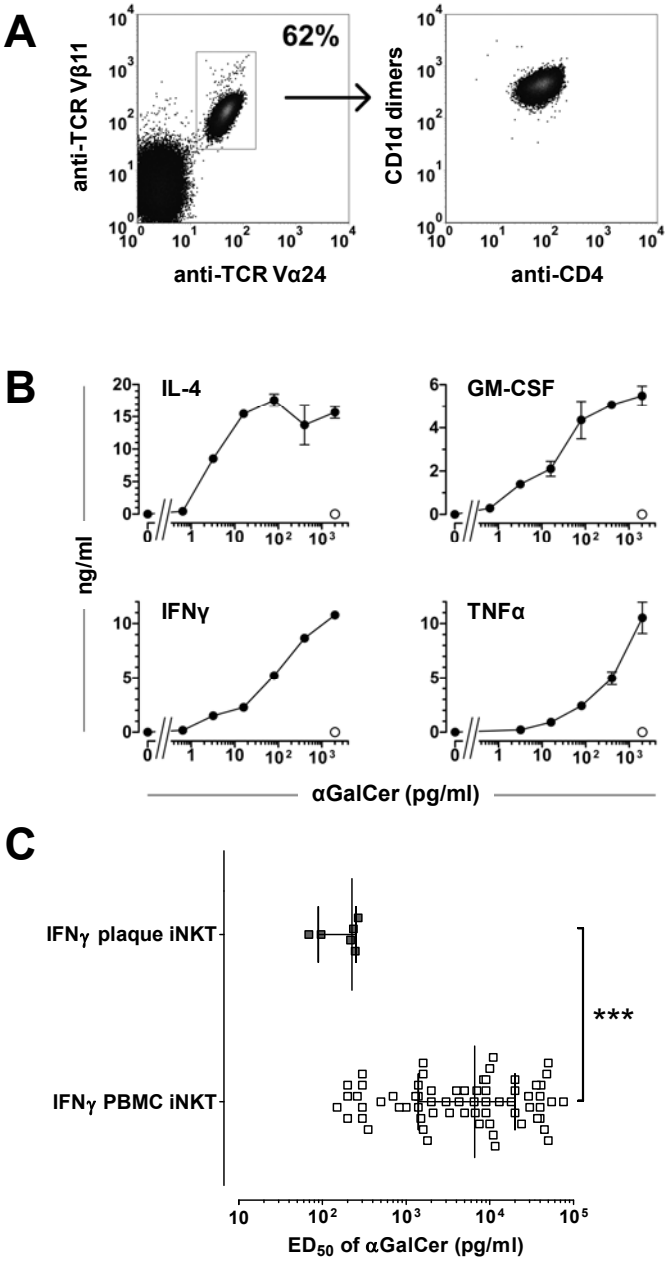

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Figure 5

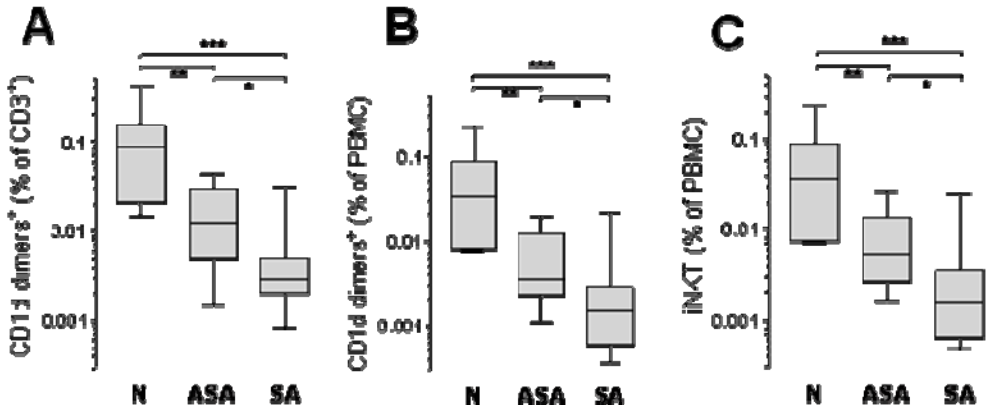

Figure 6

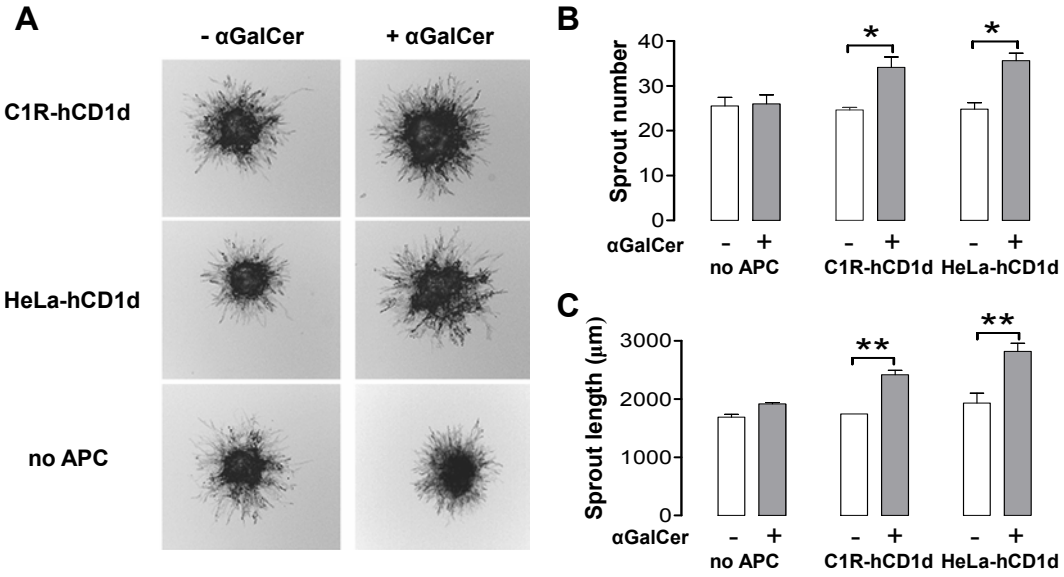

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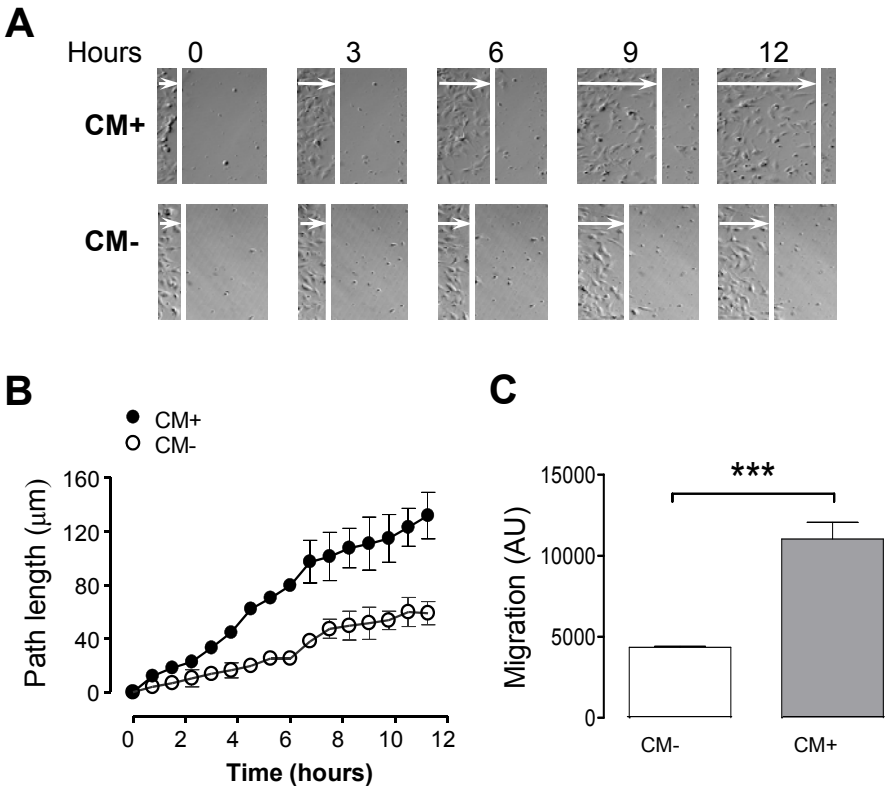
Figure 7



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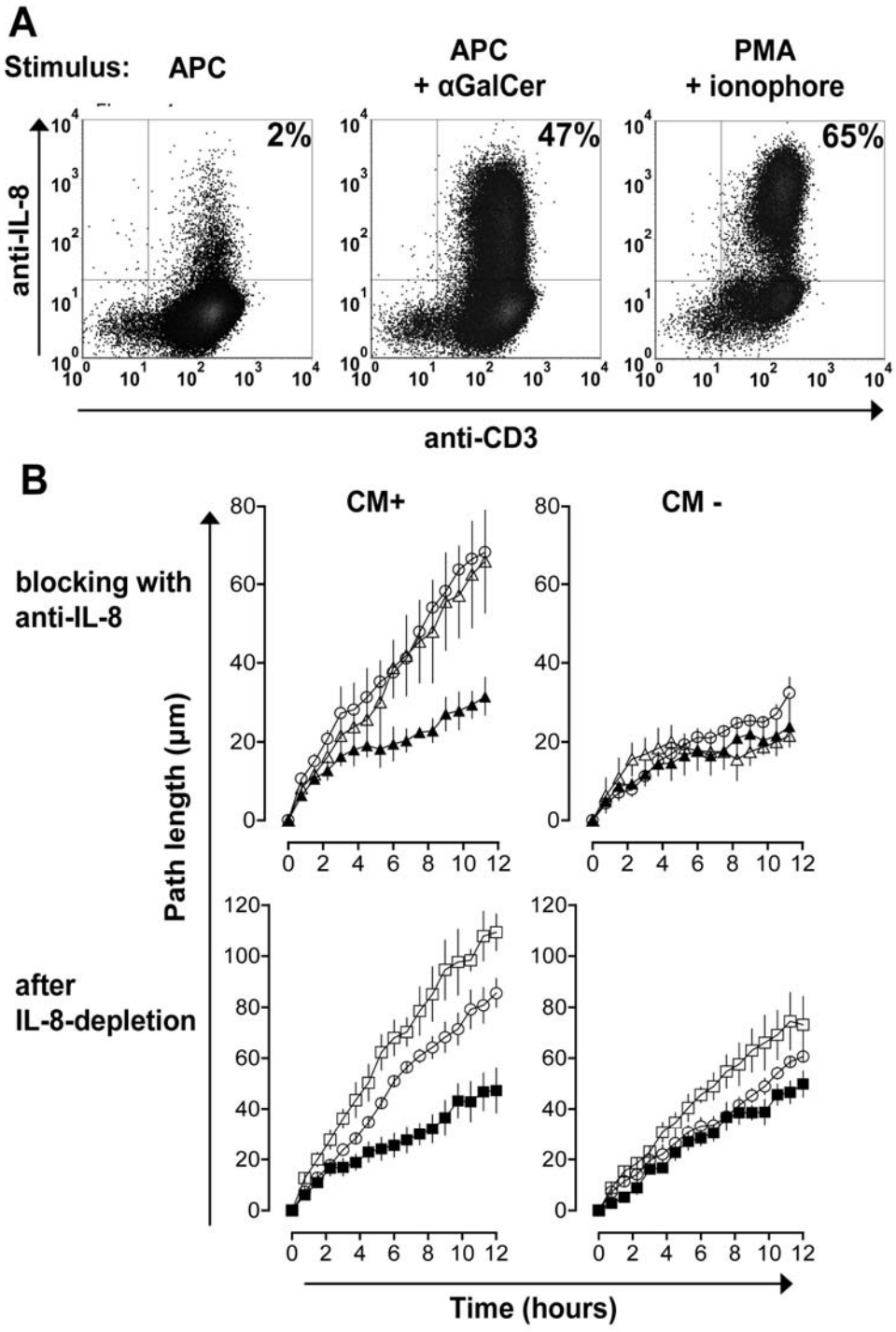

Invariant natural killer T cells: linking inflammation and neovascularization in human atherosclerosis

(*Kyriakakis, Cavallari, Andert, Philippova, Koella, Bochkov, Erne, Wilson, Mori, Biedermann, Resink, De Libero*)

SUPPORTING INFORMATION

METHODS

Arterial tissue arrays and immunohistochemistry

From each patient 3 autoptic arterial ring segments were taken, 1 each from the left common carotid, the left renal and the left common iliac artery. In each case the most affected ring sector was typed histopathologically according to the AHA consensus report [1], punched out, incorporated into tissue array paraffin blocks and processed for immunohistochemical staining [2]. The arterial sectors (3x36=108) were quantitatively analyzed for $CD1d^+$ cells and $CD68^+$ macrophages in the arterial intima (assessed as cells/mm^2), and ectopic plaque neovascularization in the arterial intima was by staining for vWF. For the immunohistochemical analysis, mAb for CD1d (MCA982, Serotec), CD68 (M-0876, Dako) and polyclonal antiserum against von Willebrand Factor (vWF; M-0616, Dako) were used. Antibody was detected by a peroxidase-conjugated secondary antibody (Dako Envision), diaminobenzidine (Vector Laboratories) as a chromogenic substrate, and hemalaun as counterstain. The CD1d staining data are shown in Supporting Information Fig. S1. For each arterial sector, the intima area was morphometrically quantified and the numbers of $CD1d^+$ and $CD68^+$ cells in the intima were counted. Ectopic neovascularization was defined by the presence of any microvessels in the arterial intima [2]. The time between death and autopsy had no effect on the staining intensity of any of the epitopes analyzed (data not shown)

Confocal analysis of iNKT cells in atherosclerotic plaque tissue

Primary antibodies used were mouse anti-TCR Vα24 (clone C15), anti-TCR Vβ11 (clone C21 [3]), rat anti-CD3 (MCA1477, Serotec), mouse anti-TCR Vα24-Jα18 (clone 6B11, BD) and mouse anti-CD1d (clone 51.1, eBioscience). Mouse and rat IgG were used as negative control antibodies (Serotec). Secondary antibodies used were biotin-conjugated donkey anti-mouse IgG followed by streptavidin-Cy2/-Cy5, Cy3-conjugated donkey anti-rat IgG (Jackson ImmunoResearch), goat anti-mouse IgG1 Alexa-555 and goat anti-mouse IgG2b Alexa-647 (Invitrogen). Sections were counterstained for nuclei with Hoechst 33342 or DAPI. Sections were

analyzed on an Olympus BX61 fluorescence microscope and on a Zeiss LSM-510 or LSM-710 confocal laser scanning microscope. Colocalization of the TCR Vα24 chain and the TCR Vβ11 chain with CD3 was determined after image merging with Zeiss Image Browser (version 3.2.0). All $CD3^+$ cells per intima were detected, counted and checked for double positivity. Images of tissue sections stained with anti-TCR Vα24-Jα18 and anti-CD1d were loaded into ImageJ, visualized using HiLo LUT for background correction (thresholding), and further processed to montages.

Collagenase-assisted isolation of lymphocytes from fresh arterial tissue

This was performed according to previously described protocols [4] with some modifications. Fresh arterial tissue biopsies obtained during thrombendarterectomy were used. Only patients with symptomatic disease undergo this clinical procedure. Due to local ethical restrictions bioptic tissue was primarily reserved for diagnostic purposes and only a small part was available to us for cell isolation. Excised arterial tissue was kept in PBS at room temperature until initiation of cell isolation procedures. The biopsy was transferred to a *Petri* dish containing cold PBS, cells were gently scraped off from the inside of the artery with a scalpel and retained. Then the artery was cut into small pieces, and after extensive washing transferred to a Falcon tube for digestion. Digestion to access tissue penetrating lymphocytes was performed in RPMI-1640 medium containing 5% HS (AB human serum Blutspendezentrum beider Basel, Basel Switzerland), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 µM non-essential aminoacids, 100 µg/ml kanamycin (all Invitrogen), and 100 U/ml recombinant IL-2 (defined as complete RPMI-1640 medium) plus 20 µg/ml gentamycin (Gibco), ciproxin (HBayerH), and 2.5 µg/ml fungizone (HGibcoH) including 400 U/ml collagenase Type IV (Sigma) and 500 U/ml benzonase nuclease (HSigmaH). Scraped and collagenase-freed cells were pooled and separated from debris by Ficoll density gradient centrifugation.

Multicolor FACS analysis of iNKT cells isolated from human atherosclerotic plaque tissue

This was performed with the following reagents: anti-TCR Vα24-Alexa488, anti-TCR Vβ11-biotin followed by streptavidin-allophycocyanin, anti-CD3-Pacific blue or -Cascade yellow (Dako), anti-CD4-Pacific blue, anti-CD8-PE-Cy5.5 (Caltag), and α-GalCer-loaded human CD1d dimers (produced in our laboratory, Cavallari M. et al., manuscript in preparation) followed by goat anti-mouse IgG2b-PE. Cells were analyzed on a CYAN ADP flow cytometer (Dako), and events were gated to exclude nonviable cells on the basis of light scatter and propidium iodide (PI) incorporation as well as on pulse-width of the forward scatter signal to include only single living cells.

Multicolor FACS analysis of iNKT cells in total PBMC

PBMC were separated from total blood cells with a ficoll gradient and iNKT cells were detected by Multicolor FACS analysis using αGalCer-loaded CD1d dimers, anti-TCR Vα24 and anti-TCR Vβ11 mAb (as described above) together with anti-CD3ε mAbs (BD). In order to have a statistically quantifiable number of iNKT cells acquisition of at least 5 million $CD3^+$ cells was performed. Living cells were gated as above.

Cell culture

The APC lines MOLT-4 (ATCC CRL 1582), which expresses only negligible CD1d, and human CD1d-transfected C1R and HeLa (C1R-hCD1d and HeLa-hCD1d, respectively) [5] were maintained in RPMI-1640 medium containing 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 µM non-essential aminoacids, and 100 µg/ml kanamycin.

Isolation of iNKT cell clones from PBMC of healthy donors has been described before [6]. Mainly, four different clones were used in this study: iNKT1, iNKT2, iNKT3, iNKT4. iNKT cells were maintained in RPMI-1640 medium containing 5% HS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 µM non-essential aminoacids, 100 µg/ml kanamycin, and 100 U/ml recombinant IL-2.

Human microvascular endothelial cell line HMEC-1 (EC) [7] was maintained in endothelial cell growth medium (ECGM) consisting of basal endothelial cell medium and endothelial cell growth supplement mix (PromoCell GmbH; C-22210) with addition of FCS to a final concentration of 10% 1 d before passaging into experimental culture dishes. For all experimental protocols, unless otherwise stated, EC were cultured in ECGM supplemented with FCS to a final concentration of 4%.

Generation of conditioned medium (CM)

ECGM with 4% FCS was used in all activation assays to generate conditioned medium (CM). APC were plated at 2.5 x 10^4/well in 96-well plates and incubated during the whole assay at 37°C with vehicle or 100 ng/ml αGalCer. After 1 h iNKT cells (0.5-1 x 10^5/well) were added. Cell culture supernatants (CM- and CM+ indicate absence and presence of αGalCer during incubations, respectively) were harvested after 48 h, filtered (0.22 µm) and stored in aliquots at -70°C. Unless otherwise specified for all *in vitro* assays on endothelial cell behavior CM was mixed 1:1 with fresh ECGM supplemented with FCS to a final concentration of 4%.

Antigen titration assay and ED_{50} calculation

APC were plated at 2.5 x 10^4/well in 96-well plates and incubated during the whole assay at 37°C with vehicle or αGalCer at the indicated concentrations. After 1 h iNKT cells (0.5-1 x 10^5/well) were added. Supernatants were harvested after 24-48 h and released cytokines were

measured by ELISA. The ED_{50} was calculated using a response curve mathematical fitting as the dose giving half maximal activation.

Measurement of cytokines

IL-4, TNFα, GM-CSF and IFNγ were quantified in cell culture supernatants by sandwich ELISA with the following mAb: 8D4-8 and MP4-25D2 (anti-human IL-4, BD), MAb1 and MAb11 (anti-human TNFα, BD), 6804 and 3209 (anti-human GM-CSF, R&D), HB-8700 and γ69 (anti-human IFNγ, ATCC and [8]). Cytokine concentrations were expressed as mean pg/ml ± SD of triplicate samples by comparison to standards of human lymphokines. In some experiments CM were also analyzed for an array of cytokine/chemokine using the Bioplex Multiplex system (BioRad; Human17-Plex Panel 171-A11171) according to the manufacturers' instructions. Beads specific for the following cytokines/chemokines were used: IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, MCP-1, MIP-1β, TNFα, IL-1β, IFNγ, G-CSF and GM-CSF. Data obtained by Bioplex assay are given in Supporting Information Fig. S2.

IL-8 depletion of conditioned medium

CM- and CM+ were incubated overnight at 4°C with 40 µg/ml of either anti-CXCL8/IL-8 (clone 6217, R&D Systems) or isotype control IgG (Sigma-Aldrich) under rotation. The next day media were incubated for 2 h at 4°C and under rotation with 40 µl/ml of protein G Sepharose beads (GE Healthcare Bio-Sciences AB). Beads were pelleted by centrifugation (10,000 rpm for 30 min at 4°C) and the supernatants used to overlay EC in wound assays.

Spheroid assay of angiogenesis *in vitro*

EC spheroid assay in 3-D fibrin gels was performed as described previously [9]. EC spheroids were composed of 500 cells/spheroid and fibrin gels containing approximately 30 spheroids/gel were overlaid with either 500 µl ECGM, or with CM, with standard inclusion of 100 ng/ml VEGF and incubation under normoxic conditions for 24 h. For microscopic visualization of spheroids, in gel-fixation and staining with 0.5 µg/ml TRITC-conjugated phalloidin (Sigma-Aldrich) were performed. Outgrowth of capillary-like structures from spheroids was evaluated by morphometric analysis of the number and total length of sprouts per spheroid using AnalySIS software (Soft Imaging System GmbH). At least 20 spheroids from two parallel wells were analyzed for each experimental point.

Proliferation assay

Effects of CM on EC proliferation were evaluated by cell enumeration and BrdU incorporation assays. For cell enumeration, EC that had been growth arrested following 18 h culture in basal ECGM containing 0.1% FCS and 0.1% BSA were enzymatically detached and re-seeded at 2 x 10^3 cell/well into 96-well plates and cultured for 3 d in the presence of CM. Cell numbers were

determined after complete adherence (d 0) and thereafter daily by trypsinization and counting in a Coulter counter. For BrdU incorporation assay EC were seeded into 96-well dishes (2 x 10^3 cells/well), growth arrested for 18 h, then cultured with CM for 24 h followed by use of a BrdU colorimetric assay according to manufacturers' instructions (Roche Diagnostics). Measurements were made in triplicate wells for both cell enumeration and BrdU incorporation assays.

Wound assay and time-lapse videomicroscopy

Confluent monolayer cultures of EC were scrape-wounded (5 mm wide wound) and then normal growth medium substituted with CM. In some experiments CM included neutralizing anti-CXCL8/IL-8 (clone 6217, R&D Systems) or isotype control IgG (Sigma-Aldrich), or was depleted of IL-8 prior to wound assay. Wound closure was filmed at a rate of 1 frame/15 min, for at least 12 h using an Olympus IX-81 inverted time-lapse microscope equipped with a digital camera within a humidified incubation chamber with 5% CO_2 at 37°C (Olympus Optical). Acquired images were processed and analyzed using CellR software (Soft Imaging System GmbH). Each experiment contained parallel wells for every experimental condition. Three different fields of observation at the initial wound front (time 0) were randomly selected and fixed and the distance of cell migration into the wound area was measured every 15 min. Every 45 min a data point for path length was calculated as the average path length from three consecutive 15 min frames. Representative videos of the assay are shown in Supporting Information FigS5video1-CM-.avi and FigS5video1-CM+.avi.

Boyden chamber-transmigration assay

Migration assay was performed in a modified 96-well Boyden micro-chamber (MBA96, Neuro Probe) as described previously [9, 10]. Membranes (10 µm pore size) were precoated on the underside only with collagen I in PBS (50 µg/ml) and CM was placed into the lower wells of the chamber. Upper wells of the chamber contained 5 x 10^4 EC/well in DMEM containing 0.1% BSA. After a 6 h incubation period cells that had migrated through to the underside of the membrane were fixed and stained with Diff-Quik® Staining Kit (Dade Behring). The membrane was scanned with a desktop scanner, and the intensity of spots (given as AU) analyzed using Aida software.

Staining for intracellular IL-8

APC were first pulsed for 1 h with 100 ng/ml αGalCer or vehicle and then cocultured with iNKT cells isolated from plaques or from PBMC. In parallel lymphocytes and iNKT cells were directly stimulated with PMA/ionomycin (200 ng/ml and 2 µg/ml) as a positive activation control. After 20 min, Brefeldin A (10 µg/ml, Sigma-Aldrich) was added and incubation allowed to proceed for an additional 16 h. After washing, the cells were fixed with 2% PFA and permeabilized with 0.1% of saponin. Immunofluorescence analysis was performed using anti-IL-8 (clone 6217, R&D

Systems) followed by goat-anti-mouse IgG1 PE-labeled (SBA) and anti-CD3-biotin (clone S4.1; also known as 76D, Caltag) followed by streptavidin-APC (Caltag). Cells were analyzed on a CYAN ADP flow cytometer and data analyzed using Summit™ 4.3 software (Dako).

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SUPPORTING DATA

INDEX OF SUPPORTING DATA

Table 1 *Clinical data from the patients who donated blood.*

Figure 1 *shows staining controls for CD1d and a representative arterial tissue sector.*

Figure 2 *shows that IL-8 is amongst different cytokines released by antigen-activated human iNKT cell clones as determined by Bio-Plex cytokine assay.*

Figure 3 *shows stimulation of sprouting from EC spheroids following antigen-activation of different human iNKT cell clones and also illustrates the requirement for APC-presentation of αGalCer.*

Figure 4 *shows effects of CM on EC-proliferation.*

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Figure 7 *depicts FACS gating strategies.*

Videos *show EC motility in the presence of CM- (FigS5video1-CM-.avi) and CM+ (FigS5video2-CM+.avi).*

Table 1. Clinical data from the patients who donated blood

UPN	gender	age (yr)	a.h. [a)]	d.m. [b)]	h.c. [c)]	s. [d)]	f.h. [e)]
Patients with ≥ 2 cardiovascular events in the past (symptomatic arteriosclerosis, SA)							
773	f	76	no	no	yes	no	yes
602	m	76	yes	yes	yes	yes	no
756	m	76	no	no	no	yes	yes
655	f	77	yes	yes	yes	no	yes
749	m	77	no	yes	yes	yes	yes
664	m	79	yes	no	yes	yes	no
716	f	82	yes	yes	no	no	no
764	m	84	yes	no	no	yes	no
582	m	89	no	no	no	no	no
698	f	92	yes	no	no	no	no
Patients without cardiovascular events in the past (asymptomatic arteriosclerosis, ASA)							
622	f	74	no	no	no	no	no
581	f	77	yes	no	no	no	no
601	f	78	no	no	yes	no	yes
771	m	78	yes	no	no	yes	yes
651	m	79	yes	no	no	yes	no
624	f	79	yes	yes	no	no	yes
632	f	81	no	no	no	no	yes
596	m	84	no	no	no	yes	no
741	f	85	yes	no	no	no	no
574	m	88	yes	no	no	no	no

UPN, unique patient number. [a)] arterial hypertension. [b)] diabetes mellitus. [c)] hypercholesterolemia. [d)] smoking. [e)] positive family history for cardiovascular events. For detailed definition of cardiovascular events see reference [11].

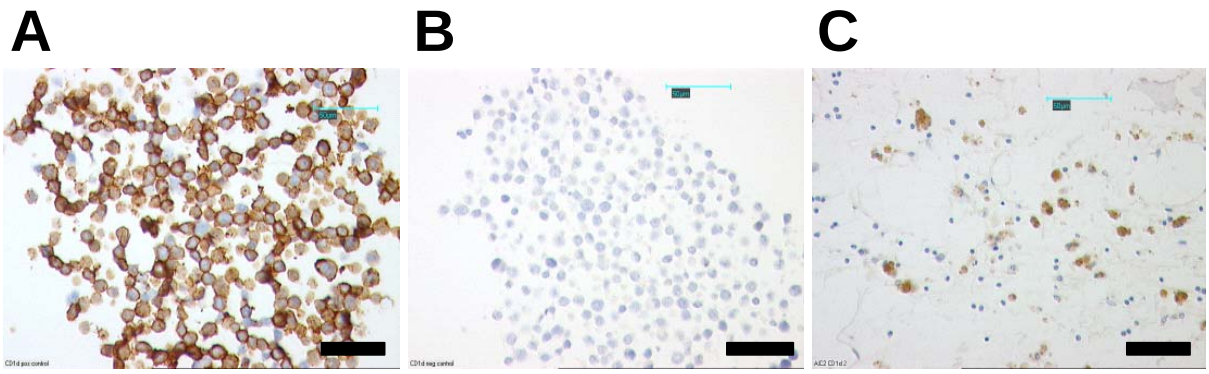

Figure 1. CD1d staining controls and representative arterial sector. mAb for CD1d (MCA982, Serotec) were used. (A) CD1d-transduced C1R cells. (B) Untransduced C1R cells. (C) Intramural CD1d positive cells in the common iliac artery. Scale bar, 50 μm.

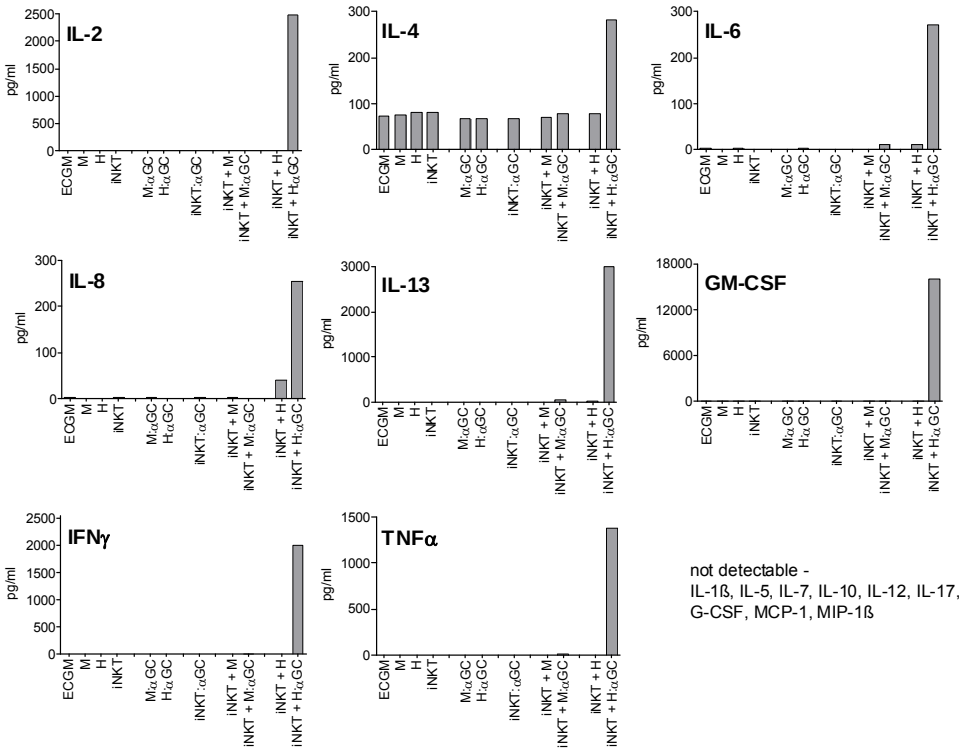

Figure 2. Antigen-activated human iNKT cells release different cytokines. CM from different activation conditions were analyzed for the presence of an array of cytokines by MultiplexPlex assay.

ECGM = normal EC growth medium;

M, H, iNKT = CM from APC (MOLT-4, HeLa-hCD1d) or iNKT cultured alone;

M/H:αGC = CM from cultures of αGalCer-pulsed APC (MOLT-4 or HeLa-hCD1d);

iNKT + αGC = CM from iNKT cultured in the presence of αGalCer;

iNKT + M/H = CM from cultures of iNKT and APC (MOLT-4 or HeLa-hCD1d);

iNKT + M/H:αGC = CM from cultures of iNKT and αGalCer-pulsed APC (MOLT-4 or HeLa-hCD1d).

Data are representative of 2 experiments performed on the same iNKT cell line.

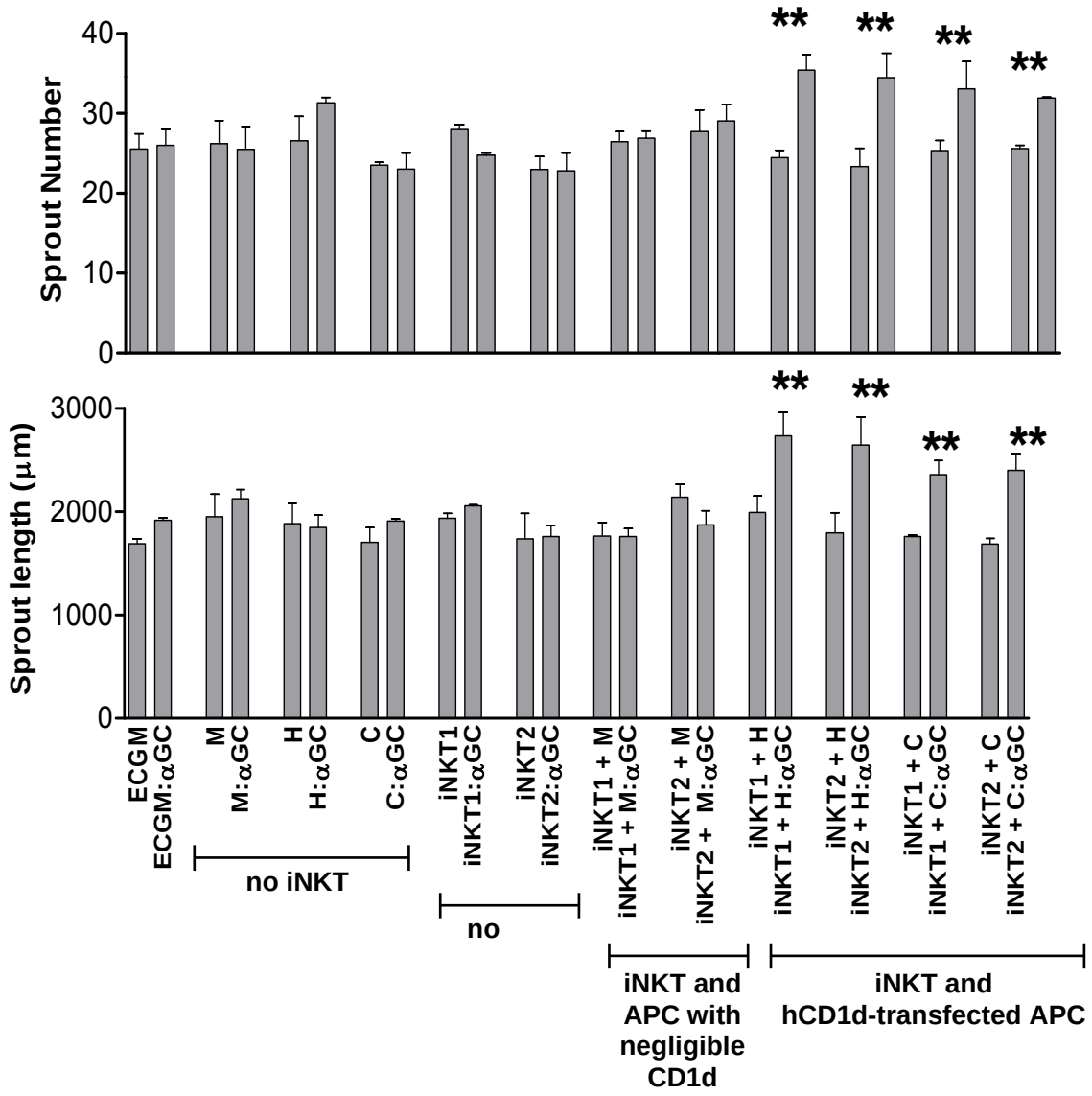

Figure 3. Only CM from antigen-activated iNKT cell clones stimulates sprouting from EC spheroids. The EC-spheroid *in vitro* angiogenesis assay was applied to test angiogenic potentials of CM collected from the following culture sets:

ECGM = normal EC growth medium alone or with inclusion of αGalCer (ECGM:αGC);

no iNKT = APC lines MOLT-4 (expressing negligible CD1d) or HeLa-hCD1d or C1R-hCD1d alone (M, H, C) or pulsed with αGalCer (M:αGC, H:αGC, C:αGC);

no APC = iNKT cell clones alone (iNKT1 and iNKT2) or with inclusion of αGalCer (iNKT1:αGC or iNKT2:αGC),

iNKT and APC expressing negligible CD1d = iNKT1 + M or + M:αGC;

iNKT and hCD1d-transfected APC = iNKT1/iNKT2 + H/C, or + H:αGC/C:αGC).

Normal endothelial cell growth medium (ECGM) served as the baseline assay control. Morphometric analysis of sprout number and total sprout length was performed after a 24 h assay period. Data (mean ± SD) are from at least 3 experiments. **, $P<0.001$, Student's t-test.

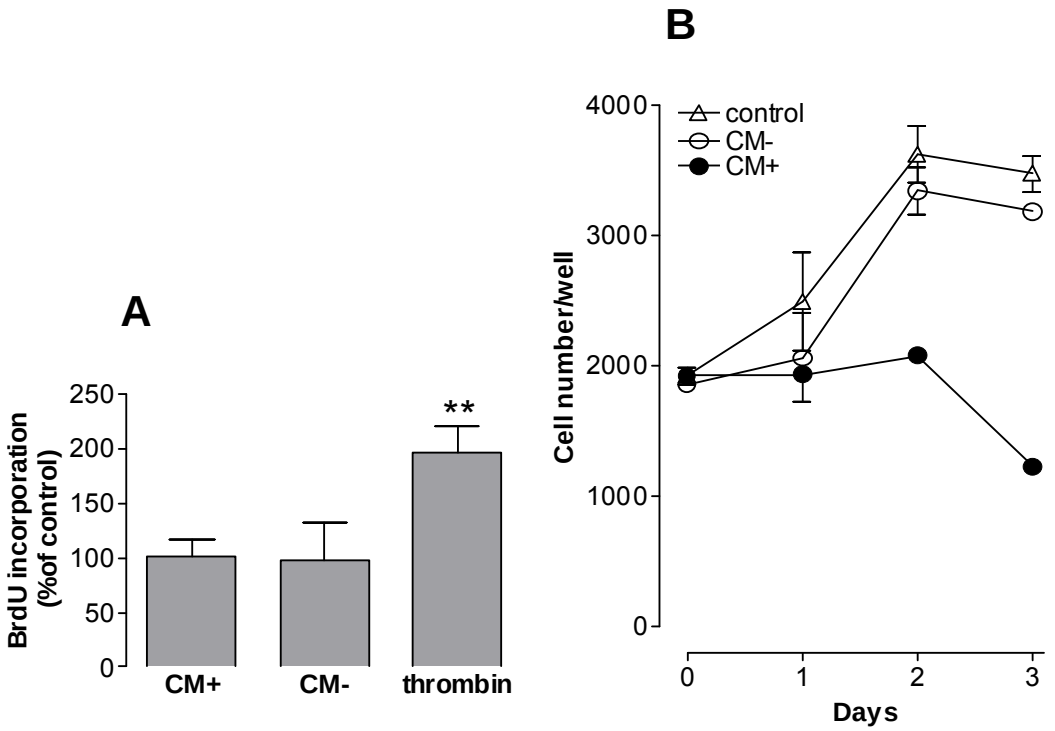

Figure 4. CM from antigen-activated iNKT cells does not stimulate proliferation. Effects of CM- and CM+ on EC proliferation were measured by BrdU incorporation (A) and cell enumeration (B) assays. EC cultured in normal ECGM served as baseline control and for BrdU assays thrombin (4U/ml) was included as a positive activation stimulus. For BrdU incorporation assay EC were seeded into 96-well dishes (2 x 10^3 cells/well), growth arrested for 18 h, then cultured with CM for 24 h followed by use of a BrdU colorimetric assay according to manufacturers' instructions (Roche Diagnostics). For cell enumeration, EC that had been growth arrested following 18 h culture in basal ECGM containing 0.1% FCS and 0.1% BSA were enzymatically detached and re-seeded at 2 x 10^3 cell/well into 96-well plates and cultured for 3 d in the presence of CM. Cell numbers were determined after complete adherence (d 0) and thereafter daily by trypsinization and counting in a Coulter counter. Measurements were made in triplicate wells for both cell enumeration and BrdU incorporation assays. Data are mean ± SD from 3 separate experiments using iNKT cell clone iNKT1. **, $P < 0.001$, Student's t-test. Experiments in which CM from other iNKT cell clones was tested yielded similar results. Whereas EC start to proliferate after 24 h under control conditions or in the presence of CM-, and at similar rates, there was no proliferation in the presence of CM+ and cell death began to occur after 48 h. The latter is most likely due to the fact that CM+ contains high levels of IFNγ and TNFα, which can induce apoptosis in EC (Molostvov, G., A. Morris, P. Rose, and S. Basu. 2002. Modulation of Bcl-2 family proteins in primary endothelial cells during apoptosis. *Pathophysiol Haemost Thromb*. 32:85-91).

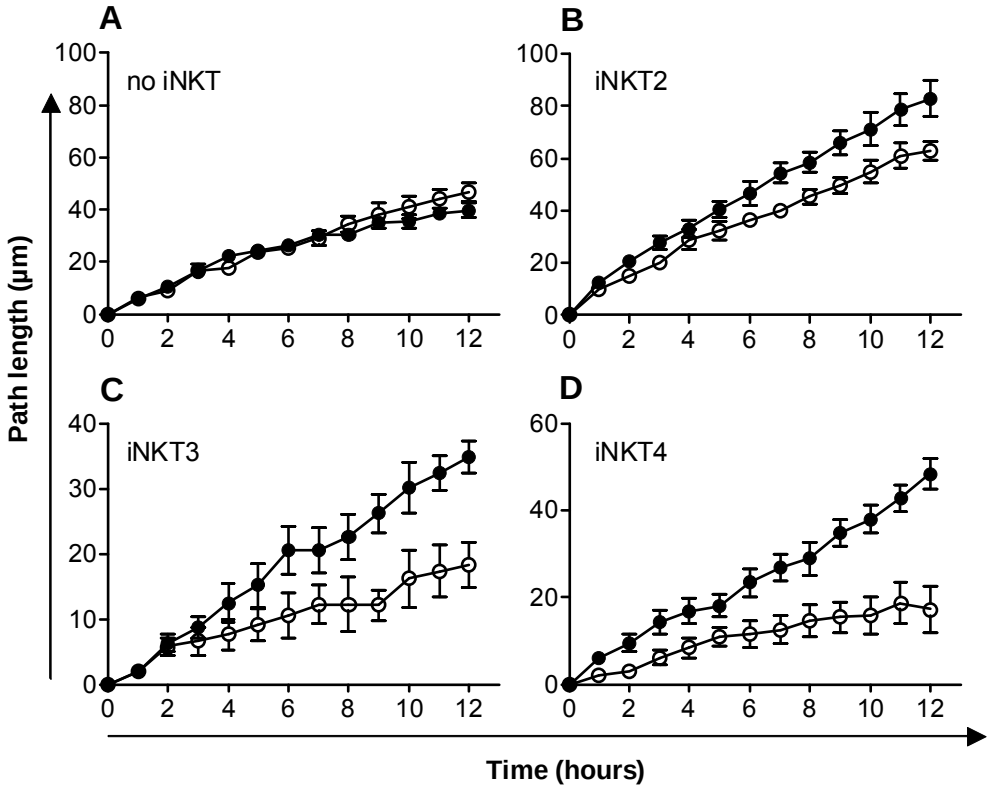

Figure 5. CM from antigen-activated iNKT cell clones stimulates endothelial cell motility. Wounded EC monolayers were incubated with CM from cultures of APC (HeLa-hCD1d) ± αGalCer (A) or cultures of three different iNKT clones cocultured with APC ± αGalCer (B-D). Closed and open symbols indicate the presence and absence of αGalCer, respectively. Motility of EC was monitored over a 12 h period by time lapse videomicroscopy (see FigS5video1-CM-.avi and FigS5video2-CM+.avi for representative recordings) and the rate of EC migration from the initial wound front into the wound area (path length versus time) was measured by morphometric analysis of acquired images. Data (averaged ± SD) shown here are from a single experiment in which the different CM were tested in parallel and in duplicate wells. The data are representative of at least 30 other motility experiments in which different CM were tested individually or in parallel.

FigS5video1-CM-.avi. Wounded EC monolayers were incubated with CM from non-activated iNKT cells (iNKT1 cocultured with HeLa-hCD1d) and motility of EC monitored over a 12 h period by time lapse videomicroscopy.

FigS5video2-CM+.avi. Wounded EC monolayers were incubated with CM collected from activated iNKT cells (iNKT1 cocultured with αGalCer-pulsed HeLa-hCD1d) and motility of EC monitored over a 12 h period by time lapse videomicroscopy.

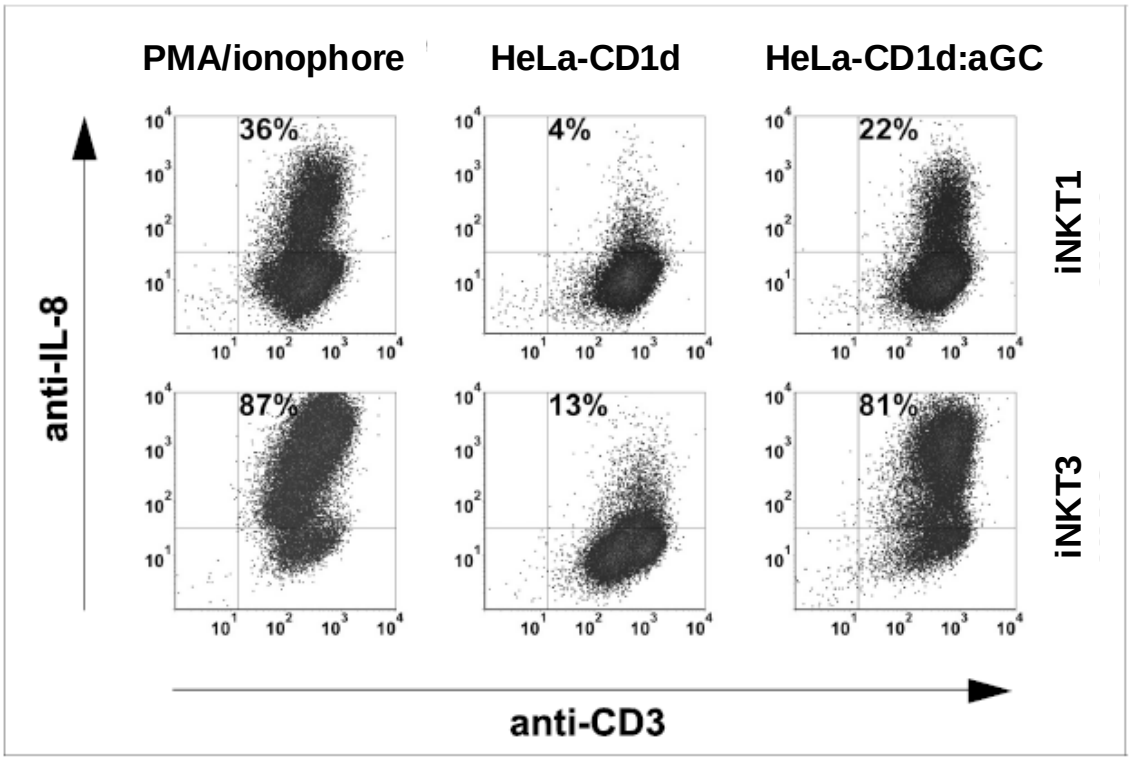

Figure 6. Antigen-activated iNKT cells express IL-8. Human iNKT1 and iNKT3 cell clones were activated with PMA/ionophore or APC + αGalCer (HeLa-hCD1d:αGC) or were non-activated (HeLa-hCD1d). Intracellular IL-8 was detected by FACS after staining fixed and permeabilized cells with anti-CD3 and anti-IL8 mAb. Similar results were obtained with two other iNKT cell clones.

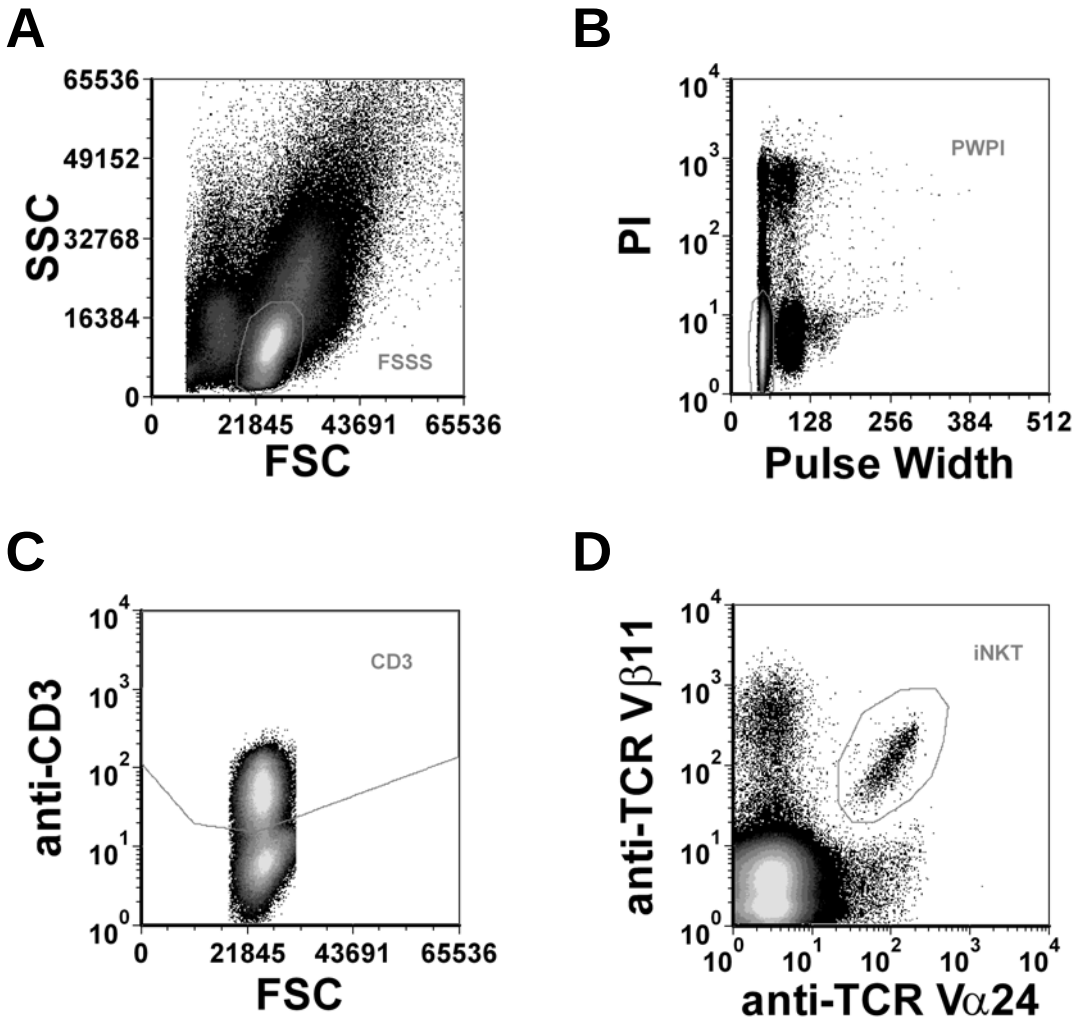

Figure 7. FACS gating strategies. (A) Cells were gated for lymphocytes according to forward scatter (FSC) and side scatter (SSC) properties (FSSS gate). This gating was used in Fig. 8A and Supporting Information Fig. 6. (B) Dead cells (propidium iodide, PI) and multipletts (Pulse Width, PW) were excluded from FSSS cells by further gating on the PWPI gate. This gating was used in Fig. 5B and 5C. (C) Single living lymphocytes (FSSS and PWPI gated) gated as being CD3-positive T cells (CD3 gate). This gating was used in Fig. 4A and Fig. 5A. (D) T cells were gated according to their expression of TCR Vα24 and TCR Vβ11 chains as iNKT cells if being double-positive (iNKT gate). This gating was used in Fig. 4A. After gating the cells were further analyzed for the markers of interest.

Project 2

Molecular mechanisms involved in response to iNKT cell activation during neovascularization

The results of this project have been drafted as a preliminary manuscript.

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(The manuscript is appended)

IL-8-mediated angiogenic responses of endothelial cells to lipid-antigen activation of iNKT cells depend on EGFR transactivation

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ABSTRACT

Invariant natural killer T (iNKT)[2] cells are a unique T cell subset contributing to both innate and acquired immunity. This T cell subset is CD1d-restricted and specific for glycolipids, including the marine sponge α-galactosylceramide (α-GalCer) and unidentified self-molecules. In advanced atherosclerotic plaques, focal collections of inflammatory cells correlate with areas of intraplaque neovascularisation. We recently found that iNKT cells can promote plaque neovascularization by enhancing endothelial cell (EC) migration and sprouting in an interleukin-8 (IL-8)-dependent manner. Here we investigate the participating signal transduction mechanisms. EC were treated with conditioned medium derived from iNKT cells activated (CM+) or not (CM-) by αGalCer. CM+-induced migration in 2D-wound assay and sprout outgrowth in a 3D-model of angiogenesis *in vitro* was abolished by epidermal growth factor receptor (EGFR) inhibitors cetuximab, gefitinib or lapatinib, as was CM+-induced acquisition of angiogenic morphology. Immunoblotting and reverse protein microarray (RPA) revealed that CM+ increased (vs. CM-) phosphorylation of several signalling effectors including focal adhesion kinase (FAK), Src, extracellular-signal-regulated kinase, c-Jun N-terminal kinase, p38-mitogen activated protein kinase, signal transducer and activator of transcription 1 and 3. EGFR-inhibition or IL-8-immunodepletion of CM+ abrogated activation of FAK and Src only. RPA also revealed CM+-induced phosphorylation of EGFR, which was blocked by EGFR-inhibition or IL-8 immunodepletion of CM+. Thus IL-8-dependent activation of angiogenic behaviour in EC in response to lipid-antigen activation of iNKT occurs *via* transactivation of EGFR. The data support EGFR as a non-traditional proangiogenic receptor driving inflammation-associated neovascularization in atherosclerotic disease.

Short title: EGFR transactivation in iNKT cell driven angiogenesis

Keywords: plaque neovascularization, inflammation, EGFR transactivation, EGFR pharmacological inhibition, motility, signal transduction

[2]**Abbreviations used:** APC, antigen presenting cell; αGalCer, α-galactosylceramide; iNKT cells, invariant natural killer T cells; CM, conditioned medium; EC, endothelial cells; ECGM, endothelial cell growth medium; EGFR, epidermal growth factor receptor; GSK3β, glycogen synthase kinase 3β; GPCRs, G-protein coupled receptors; HB-EGF, heparin-binding EGF-like growth factor; HCMV, human cytomegalovirus; HMEC-1, human microvascular endothelial cell line; IL-8, interleukin 8; MMP, matrix metalloproteinase; RPA, reverse protein microarray; FAK, focal adhesion kinase; Erk, extracellular-signal-regulated kinase; Jnk, c-Jun N-terminal kinase; p38MAPK, mitogen activated protein kinase; S6rp, S6 ribosomal protein, STAT, signal transducer and activator of transcription.

INTRODUCTION

Atherosclerosis is now generally accepted as an inflammatory disease, characterized by degenerative changes and extracellular accumulation of lipid and cholesterol. The evolving inflammatory reaction plays an important role in the initiation of atherosclerotic plaques and their destabilization, converting a chronic process into an acute disorder with ensuing thromboembolism [1]. The main risk factor for the throboembolic complications of atherosclerotic disease is the development of complex and unstable forms of atherosclerotic plaques that are prone to spontaneous erosion, fissure or rupture. Most clinical events result from unstable plaques which do not appear severe on angiography, and thus plaque stabilization may be a way to reduce morbidity and mortality.

Progressive atherosclerotic disease and the development of acute lesion instability are linked with plaque angiogenesis (reviewed in [2-6]). In early lesions local hypoxic conditions within the developing and expanding intima induce vasa vasorum angiogenesis and medial infiltration, which prevents cellular death and contributes to plaque growth and stabilization. However, ectopic neovessels are detrimental to plaque integrity because they are immature and fragile and promote development of an unstable haemorrhagic rupture-prone environment. Intraplaque haemorrhage autoamplifies both the angiogenic process and the intraplaque inflammatory response, and thereby exacerbates plaque vulnerability. In more advanced plaques, focal collections of inflammatory cells correlate with areas of intraplaque neovascularization and haemorrhage, suggesting that release of growth factors and cytokines by macrophages and leukocytes may have a key role in modulating the neovascularization process.

Although neovascularization and inflammation are recognized as closely linked processes, specific inflammatory cell protagonists and angiogenic mediators underlying intraplaque neovascularization remain poorly understood. Invariant natural killer T (iNKT) cells are a unique T cell subset contributing to both innate and acquired immunity. This peculiar T cell subset is CD1d-restricted and specific for glycolipids, including unidentified self-molecules and the marine sponge α-galactosylceramide (α-GalCer) [7]. We recently identified iNKT cells as novel cellular candidates promoting plaque neovascularization and destabilization in human atherosclerosis [8]. We demonstrated a significant association of $CD1d^+$ cells with neovascularization in plaques as well as intraplaque infiltration of iNKT cells in advanced human atherosclerotic lesions [8]. Activation of plaque-derived iNKT cell lines by CD1d-expressing antigen presenting cells (APC) presenting α-GalCer enhanced endothelial cell (EC) migration *in vitro* and promoted sprout outgrowth in an EC-spheroid model of *in vitro* angiogenesis. Proangiogenic activity was ascribed to interleukin-8 (IL-8) released by the activated iNKT cells.

The notion of IL-8 as plaque destabilizing and/or angiogenic factor in human atherosclerosis is not new. In 1996 Wang et al, disclosed IL-8 expression primarily in the macrophage-abundant zones of the plaque by *in situ* hybridization and immunohistochemical staining of human coronary atheromata [9]. IL-8 was shown to downregulate expression of tissue inhibitor of metalloproteinase-1 expression in cholesterol-laden human macrophages, thereby favouring local extracellular matrix degradation and plaque instability [10]. Simonini et al., reported that IL-8 was expressed at higher levels in homogenates of coronary artherectomy tissue than in homogenates of internal mammary artery

tissue; however, localization of IL-8 (immunohistochemistry and *in situ* RT-PCR) was exclusive to coronary atherectomy tissue sections and correlated with the presence of factor VIII–related antigen [11]. Corneal *in vivo* angiogenesis induced by coronary atherectomy homogenates was completely inhibited by anti-IL-8 antibodies, revealing the relevance of atheroma IL-8 to angiogenesis [11].

However, signal transduction pathways mediating angiogenic properties of IL-8 remain poorly defined. IL-8 has been reported to affect different aspects of angiogenic behaviour of EC and by different signal transduction mechanisms. Li et al., reported a direct stimulatory effect of IL-8 on proliferation, survival and matrix metalloproteinases (MMP) [12]. Schraufstatter et al., demonstrated that IL-8 induces a haptotactic response and also Rac-dependent cytoskeletal reorganization leading to cell retraction and gap formation between adjacent cells [13]. Schraufstatter et al., subsequently reported that IL-8-mediated migration depended on cathepsin B activity and also involved epidermal growth factor receptor (EGFR) [14]. While we have demonstrated that lipid-antigen activation of iNKT cells increases the migration capacity of EC in an IL-8 dependent manner, associated signalling pathways involved were not studied. Here we report that stimulatory effects of activated iNKT cells on EC migration, sprouting and actin reorganization are driven through EGFR with selective downstream activation of focal adhesion kinase (FAK) and Src.

EXPERIMENTAL

Lipid antigen activation of iNKT cells and generation of conditioned medium

The generation of conditioned medium (CM) following activation of CD1d-restricted iNKT cells (clones isolated from PBMC of healthy donors [15] by CD1d-expressing APC (human CD1d-transfected HeLa [16]) presenting α-GalCer lipid antigen was carried out as detailed previously [8]. Briefly, APC were suspended in EC growth medium (ECGM) consisting of basal endothelial cell medium and endothelial cell growth supplement mix (C-22210; PromoCell GmbH; Heidelberg, Germany) with addition of FCS to a final concentration of 4% FCS (ECGM-4% FCS), plated at 2.5 x 10^4/well in 96-well plates and preincubated for 1 h at 37°C with vehicle or 50-100 ng/ml α-GalCer. The iNKT cells (0.5-1 x 10^5/well) were added and culture supernatants (CM- and CM+ indicate absence and presence of α-GalCer during APC preincubation, respectively) were harvested after 48 h, filtered (0.22 μm) and stored in aliquots at -70°C. For some experiments the CM were subjected to IL-8 immunodepletion protocols using anti-CXCL8/IL-8 (clone 6217, R&D Systems Europe Ltd., Abingdon, UK) or isotype control IgG (Sigma-Aldrich, Buchs, Switzerland) as detailed before [8].

Endothelial cell culture

Human microvascular endothelial cell line HMEC-1 [17] were normally maintained in ECGM-10% FCS. Seeding densities for experiments were as follows: 2 x 10^4 cells/well into 24-well plates or onto 0.1% gelatin-coated glass cover slips inserted into 24-well plates for morphology analysis or confocal

microscopy respectively, 8 x 10^4 cells/well into 24-well plates for wound assay, 2 x 10^5 cells/well into 6-well plates for immunoblotting and 1 x 10^4 cells/well into 96-well plates for reverse phase protein array (RPA). Each experiment contained duplicate or triplicate wells for every experimental condition. Culture medium was removed after overnight adherence, cell layers were rinsed with PBS and stimulation protocols were initiated by addition of either ECGM-4% FCS (control) or CM mixed 1:1 with ECGM-4% FCS. When used, the EGFR tyrosine kinase inhibitors gefitinib (Iressa®, Astra Zeneca AG, Zug, Switzerland) and lapatinib (Tykerb®, GlaxoSmithKline, London, UK), or the neutralizing anti-EGFR monoclonal antibody cetuximab (Erbitux®, Merck KGaA, Darmstadt, Germany) and isotype control IgG (Sigma-Aldrich) were added to cultures 1 h prior to and again at the start of experiments.

Wound assay and time-lapse videomicroscopy

Real time analysis of cell migration in wound assay was performed as detailed before [8]. Briefly, closure of scrape-wounded cultures of HMEC-1 was filmed over 12 h using an Olympus IX-81 inverted time-lapse videomicroscope (Olympus Optical Co., Geneva, Switzerland). Acquired images were processed and analyzed using CellR software (Soft Imaging System GmbH, Muenster Germany). Three different fields of observation at the initial wound front (time 0) were randomly selected and fixed and the distance of cell migration into the wound area was measured every 15 min. Every 45 min a data point for path length was calculated as the average path length from three consecutive 15 min frames.

Spheroid assay of angiogenesis *in vitro*

The EC spheroid assay of angiogenesis *in vitro* in 3-D fibrin gels has been detailed previously [18]. After 24 h of experimental incubation spheroids were fixed in-gel and stained with 0.5 µg/ml TRITC-conjugated phalloidin (Sigma-Aldrich). Outgrowth of capillary-like structures from spheroids (at least 20 for each experimental point) was evaluated by morphometric analysis of the total length of sprouts per spheroid using AnalySIS software (Soft Imaging System GmbH) [18].

Immunofluoresence microscopy

After 24 h of experimental incubation HMEC-1 were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS and stained with 0.5 µg/ml TRITC-conjugated phalloidin (Sigma-Aldrich) followed by counterstaining of nuclei with Hoechst (Molecular Probes, Leiden, Netherlands) [19]. After extensive washes with PBS samples were mounted in Mowiol 4.88 reagent (Calbiochem, Darmstadt, Germany) and analysed using an LSM-710 Laser Scanning Microscope (Zeiss, Feldbach, Switzerland).

Immunoblotting

Whole cell lysis and immunoblotting procedures have been detailed [20, 21]. The following primary antibodies were used: rabbit anti-phospho$^{thr202/tyr204}$-Erk (extracellular-signal-regulated kinase), rabbit anti-phospho^{ser473}-Akt, phospho^{ser9}-GSK3β (glycogen synthase kinase 3β) rabbit anti-phospho$^{Thr180/Tyr182}$-p38MAPK (mitogen activated protein kinase) (Cell Signalling, Bioconcept, Allschwil, Switzerland) phospho^{tyr397}-FAK (BD, Allschwil Switzerland), goat anti-GAPDH (Abcam, Cambridge, UK). Secondary HRP-conjugated goat anti-mouse IgG, anti-rabbit IgG (Southern Biotechnology, BioReba AG, Reinach, Switzerland) or donkey anti-goat IgG (Santa Cruz, Heidelberg, Germany) together with Amersham ECL (Amersham Biosciences, Little Chalfont, UK) were used for detection of immunoreactive proteins. Scanned images of autoradiograms were analyzed using AIDA Image software.

Reverse protein microarray

All experimental and analytical protocols for the platform of planar-waveguide reverse (phase) protein microarray (RPA) as developed by Zeptosens (www.zeptosens.com) have been precisely detailed previously [22, 23] as have the sources and validation of the phospho-specific antibodies used (phospho^{tyr1173}-EGFR, phospho$^{thr202/tyr204}$-Erk, phospho$^{Thr180/Tyr182}$-p38MAPK, phospho^{Tyr701}-STAT1 (signal transducer and activator of transcription 1), phospho^{tyr705}-STAT3, phospho$^{Thr183/Tyr185}$-Jnk (c-Jun N-terminal kinase), p-Akt^{ser473}, phospho$^{ser235\&ser236}$-S6rp (S6 ribosomal protein) phospho$^{Tyr576/577}$-FAK, phospho^{Tyr416}-Src) [23].

***In situ* zymography**

Gelatinolytic activity of the metalloproteinases MMP-2 (72kDa gelatinase A/type IV collagenase) and MMP-9 (92 kDa gelatinase B/type IV collagenase) was measured by *in situ* zymography. Sample supernatants collected from EC treated with CM were loaded on 10% polyacrylamide gels containing gelatin (1 mg/ml). The gel was washed in 2.5% (v/v) Triton X-100 followed by repeated washes in distilled water, and then incubated overnight at 37°C in a gelatin renaturation buffer followed by staining with 2% Coomassie Blue. Proteolysis was detected as white zones in a dark field.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software 5.0 (GraphPad Software, http://www.graphpad.com) using Student's *t* test or one-way ANOVA followed by Tukey's multiple comparison tests with $P<0.05$ taken as statistically significant.

RESULTS

Angiogenic behavioural responses induced in EC in response to lipid antigen activation of iNKT cells are sensitive to EGFR inhibition

We have demonstrated that activation of iNKT cells by APC presenting the lipid antigen α-GalCer can elicit angiogenesis *in vitro* and identified IL-8 released by iNKT cells as the chemokine involved in endothelial cell migration and sprouting; the migration response of HMEC-1 to culture supernatants derived from α-GalCer-activated iNKT cells could be completely abrogated by inclusion of anti-IL-8 blocking antibodies or using culture supernatants which had been subjected to immunodepletion of IL-8 prior to assay [8]. Here, and since IL-8 has previously been shown to transactivate EGFR in HMEC-1 [14], we investigate a possible involvement of EGFR in the angiogenic response of HMEC-1 to lipid antigen activation of iNKT cells.

Conditioned media were collected from iNKT cell cultures either stimulated with α-GalCer (CM+) or not (CM-), applied to scrape-wounded HMEC-1 monolayers or fibrin-embedded HMEC-1 spheroids without (control) or with inclusion of either anti-EGFR blocking monoclonal antibodies (cetuximab) or EGFR tyrosine kinase inhibitors (gefitinib or lapatinib). Figure 1 recapitulates our previous findings that migration of HMEC-1 into the wound area is increased in the presence of CM+ (vs. CM-) and demonstrates that each of the EGFR inhibitory treatments markedly attenuated the enhanced migration response to CM+ (Figure 1 B, D, F). The migration of HMEC-1 exposed to supernatants from non-activated iNKT (CM-) was minimally affected by the treatments (Figure 1 A, C, E). Figure 2 recapitulates our previous findings of a stimulatory effect of CM+ (vs. CM-) on sprout outgrowth in the EC-spheroid model of angiogenesis *in vitro* and demonstrates complete abrogation of this effect in the presence of EGFR tyrosine kinase inhibitor gefitinib. Sprout outgrowth in the presence of CM- was not affected by gefitinib. We did not examine the effects of lapatinib or cetuximab in this experimental model.

We next examined the influence of iNKT cell activation on cell morphology following culture of HMEC-1 in the presence of CM+ or CM- for 24 h. Phase contrast microscopy revealed that CM+ treated cells were more elongated and exhibited more frequent and pronounced lamellipodial extensions, characteristics of the motile phenotype (Figure 3A). Accordingly, evaluation of actin cytoskeletal organization by TRITC-phalloidin staining showed that stress fibers were more prominent in CM+ treated HMEC-1 (Figure 3A). Morphometric analysis of cell lengths showed that average cell length for CM+ treated HMEC-1 cells was markedly greater than that for CM- treated ones (≈ 80 μm and ≈ 50 μm, respectively). As shown by presentation of the data as a frequency distribution of cell length, HMEC-1 treated with CM+ exhibited a high frequency (≈ 67.4%) of cells with lengths of ≥ 61 μm, whereas the majority (≈ 70.7%) of HMEC-1 treated with CM- exhibited lengths of ≤ 60 μm (Figure 3B). Average cell length in the presence of either gefitinib or lapatinib remained at ≈ 50 μm for CM-treated HMEC-1 but decreased to ≈ 60 μm for CM+ treated HMEC-1, the latter reflecting a decrease in the frequency (from ≈ 67.4% to 40%) of cells ≥ 61 μm in length (Figure 3B). Abrogating effects of EGFR tyrosine kinase inhibition on the CM+-induced acquisition of a motile phenotype are consistent with the abrogation of CM+-enhanced migration and sprout outgrowth. The differential effects of CM- and CM+ on EC behaviour were not associated with differences in metalloproteinases MMP-2 or MMP-9

as determined by *in situ* gelatin zymography of culture supernatants collected from HMEC-1 after 2, 6 and 24 h incubation with CM- or CM+ (Supplementary Figure S1).

Lipid antigen stimulation of iNKT cells activates multiple signal transduction effectors: unique sensitivity of Src and FAK to inhibitors of EGFR or IL-8 neutralization.

In order to reveal the signalling pathways that might be triggered in HMEC-1 upon activation of iNKT cells, we investigated the activation status of a variety of effector molecules known to be involved in regulating endothelial cell migration. Whole cell lysates from HMEC-1 under control culture medium conditions (ECGM-4% FCS) or treated with CM- or CM+ for 15 min were subjected to immunoblot analysis for phosphorylated Akt, GSK3β, p38MAPK, Erk and FAK. Compared to control culture conditions, CM- and CM+ similarly increased phosphorylation of Akt and GSK3β phosphorylation, whereas only CM+ increased the phosphorylatin of FAK, p38MAPK and Erk (Figure 4A). The presence of lapatinib or gefitinib attenuated the CM+-induced increase in phosphorylation of FAK (Figure 4B).

Next we utilized the highly sensitive Zeptosens chip-based reverse protein array (RPA) platform [22, 23] which allows the generation of high-fidelity data for protein expression profiling of low levels or transient states of protein abundance. This method revealed an increased level of EGFR phosphorylation in HMEC-1 treated with CM+ or with IL-8, but not with CM- (Figure 5A). IL-8-immunodepletion of CM completely abrogated the phosphorylation of EGFR induced by CM+ (Figure 5A).

RPA was further applied to validate the findings obtained by immunoblot analysis and to identify additional candidate signalling effectors. Elevated phosphorylation of FAK, p38MAPK and Erk in CM+ treated HMEC-1 could be confirmed and the RPA revealed that also levels of phosphorylated Jnk, STAT1, STAT3, Src and S6rp were higher in CM+ treated HMEC-1 than in CM- treated ones (Figure 5B). Either inclusion of gefitinib or IL-8-immunodepletion of CM fully attenuated the CM+-induced increase in FAK and Src phosphorylation (Figure 5C) whereas the phosphorylation of Akt, Jnk, STAT1, STAT3, p38MAPK and S6rp were not affected (Supplementary Figure S2).

DISCUSSION

Antiangiogenic strategies have already shown promising results in cancer disorders [24] and in other conditions such as macular degeneration [25] and rheumatoid arthritis [26], where angiogenesis plays a crucial role in disease progression. The accumulating evidence linking ectopic neovascularization and immature microvessels with atherosclerosis, reinforced inflammation, and plaque destabilization has spurred considerable interest in the concept of antiangiogenic therapy for atherosclerotic disease [3-5, 27]. This poses a challenge since a multitude of cellular receptors and signalling components can contribute to angiogenesis. The design of treatments that target plaque angiogenesis requires a detailed knowledge of the critical participating cellular and molecular regulatory pathways.

In previous work we identified iNKT cells as a novel culprit leukocyte population involved in plaque destabilization. Plaque-derived iNKT promptly released a spectrum of proinflammatory cytokines and chemokines upon lipid antigen activation and the culture supernatants from activated iNKT cells induced migratory and tubulogenic responses in EC that specifically depended upon IL-8 released by iNKT cells [8]. In this study we provide evidence that these IL-8-mediated functional proangiogenic activities are driven through EGFR. Firstly, in functional analyses CM+-induced EC migration and angiogenic sprout formation were completely abolished by EGFR function blocking antibodies or EGFR tyrosine kinase inhibitors [8]. Secondly, in morphological studies EGFR inhibition impeded CM+-induced acquisition of the elongated, motile phenotype. Thirdly, in signal transduction analysis either IL-8-immunodepletion or EGFR inhibition completely abolished CM+-induced phosphorylation of EGFR, Src and FAK.

VEGFR-1 and VEGFR-2 are key cellular receptors on EC that trigger angiogenesis [28]. However, there are numerous other receptors that may participate in plaque angiogenesis including hepatocyte growth factor receptor, fibroblast growth factor receptor, platelet-derived growth factor receptor, Tie1/Tie2 as well as G-protein coupled receptors (GPCRs) for ligands such as thrombin, bradykinin, angiotensin II, endothelin-1 lysophosphatidic acid and IL-8 [29]. GPCRs are capable of mediating some of their biological effects through the transactivation of the EGFR [30]. Our cumulative findings (this study and [8]) that CM+-stimulated migration, EGFR phosphorylation and activation of FAK and Src could be completely abolished by either IL-8 or EGFR inhibition are consistent with involvement of EGFR transactivation. Previous studies have demonstrated that the migration response of EC to exogenous IL-8 depends upon activation of the IL-8 receptor CXCR2 [13, 31] and involves EGFR transactivation through cathepsin B-mediated cleavage of EC-expressed transmembrane pro-heparin-binding EGF-like growth factor (pro-HB-EGF) to soluble HB-EGF [14], a cognate member of the EGFR family of ligands [32]. HB-EGF directly induces EC migration *in vitro* and angiogenesis both *in vitro* and *in vivo* [33]. Although we did not directly address whether HB-EGF is indeed the molecule transactivating EGFR in our experimental model, our observed inhibition of CM+-induced migration by cetuximab supports a requirement of EGFR ligand-induced EGFR transactivation rather than an alternate transactivation *via* non-receptor tyrosine kinases (e.g. proline-rich tyrosine kinase 2 and Src) activated downstream of IL-8 receptor ligation [34-36]. Expression of HB-EGF is upregulated in macrophage-rich areas of human atherosclerotic plaques [37, 38]. Specific associations between the presence of HB-EGF and iNKT cell-associated plaque neovascularization have not been investigated. However, the complete attenuation of CM+-induced Src and FAK activation (this study) and *in vitro* angiogenesis [8] by IL-8 neutralizing mAbs would exclude that HB-EGF derives from lipid antigen activated iNKT cells.

EGFR-mediated signalling has been linked to pathogenic angiogenesis in tumor growth [28, 39, 40] and now is a major pharmacological target for inhibiting tumor growth [39-41]. Accumulating data suggest that EGFR signalling may also participate in vascular disease [42]. Numerous studies have demonstrated that EGFR ligands promote dedifferentiation and proliferation of vascular smooth muscle cells and on chemotaxis and proliferation of monocyte-derived macrophages, processes which actively drive atherosclerosis [42]. Much less is known about EGFR-dependent control of EC angiogenic behaviour and signalling in atherosclerosis. The association of human cytomegalovirus (HCMV) infection with atherosclerosis, transplant vascular sclerosis and coronary restenosis was recently attributed to the ability of HCMV to trigger angiogenesis via specific binding of HCMV to EGFR and β1 and β3 integrins and the downstream activation of the phosphatidylinositol 3 kinase and

MAPK pathways in EC [43]. Schraufstatter *et al*., demonstrated that the EC migration induced through IL-8-mediated EGFR transactivation involved activation of small GTPase Rac [13]. Although our study did not directly examine small GTPase activation, the CM+-induced alterations in EC cytoskeletal organization (strong stress fibres) and morphology (more frequent and pronounced lamellipodial extensions) suggest activation of at least Rac [44]. In signal transduction analysis we found that a broad spectrum of signalling pathways (Erk, Jnk, p38MAPK, STAT1, STAT3, Src/FAK) were activated to a greater extent in CM+ treated EC, which was not surprising given that lipid-antigen activation of iNKT cells induces the release of a broad spectrum of chemokines and cytokines (including tumor necrosis factor α, interferon γ, granulocyte-macrophage colony-stimulating factor, interleukins -2, -4, -13 and -8) [8]. However, only Src and FAK activation were sensitive to inhibition by either IL-8-immunodepletion or gefitinib, suggesting an IL-8/EGFR transactivation mechanism that is rather selective with respect to subsequent intracellular signal pathway activation in EC. The complete attenuation of CM+-induced EGFR phosphorylation by IL-8 immunodepletion confirms that IL-8 derived from lipid antigen activated iNKT cells mediates EGFR transactivation in EC.

The non-receptor tyrosine kinases Src and FAK are key regulators of cell migration and their activities are regulated through alterations in phosphorylation at mutiple sites [45]. EGFR, via SRC-3Δ4 as a bridging protein [46], is known to induce the autophosphorylation of FAK at tyrosine 397 which creates a binding site for Src through its SH2 homology domain. Resultant activation of Src through autophosphorylation on tyrosine 416 contributes to maximal FAK activation by phosphorylation of FAK at several sites including tyrosine 576 and tyrosine 577 [47]. Our immunoblot and RPA data showing phosphorylation at these sites are consistent with the abovementioned EGFR-induced cascade of FAK/Src activation.

In conclusion, our study supports EGFR as a non-traditional proangiogenic receptor driving inflammation-associated neovascularization in atherosclerotic disease. Figure 6 presents a model for events involved in the angiogenic response of EC to lipid antigen activation of iNKT cells, and different modalities for inhibiting this response. The model, depicting IL-8-mediated transactivation of EGFR, incorporates also the observations of Schraufstatter *et al*., with respect to Rac activation [13] cathepsin B generation of EGFR ligand HB-EGF [14]. While the model presents HB-EGF as an autocrine ligand for EC, this ligand can be elaborated by several other cell types (monocytes, smooth muscle cells, T-cells) resident and activated within atherosclerotic lesions *in vivo* [42], thereby facilitating paracrine activation of EGFR on EC and exacerbation of angiogenesis. Thus EGFR inhibition represents a novel therapeutic modality for control of neovascularisation within developing atherosclerotic plaques.

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FIGURE LEGENDS

Figure 1. The enhanced migration response of endothelial cells to conditioned medium from activated iNKT cells is sensitive to EGFR inhibition.

Confluent monolayers of EC were scrape-wounded and cultured in the presence of conditioned culture media derived from non-activated (CM-, **A, C, E**) or α-GalCer-activated (CM+, **B, D, F**) iNKT cells, and without (**A-F** -open circles) or with inclusion of 1 μg/ml anti-EGFR blocking mAbs (**A, B** -closed triangles), 1 μg/ml isotype control IgG (**A, B** -open triangles), 0.5 μM gefitinib (**C, D** -closed circles) or 0.1 μM lapatinib (**E, F** - closed squares). Wound closure was monitored over a time period of 12 h by time lapse videomicroscopy. The rate of EC migration from the initial wound front into the wound area (path length versus time) was determined by analysis of acquired images using CellR software. The data are representative of 1 of 3 independent experiments, each performed in duplicate and values are given as averaged path length measurements ± SD from triple fixed observation fields/well.

Figure 2. Increased sprout outgrowth induced by conditioned medium from antigen activated iNKT cells is attenuated upon EGFR inhibition.

EC-spheroids were incubated for 24 h in the presence of conditioned media derived from iNKT cell cultures stimulated without (CM-, white bars) or with (CM+, grey bars) αGalCer, and without (control) or with inclusion of gefitinib (0.5 μM). Representative images of whole spheroids at the end of the 24 h culture period are shown (scale bar = 200 μm). The total length of sprouts growing out from any given spheroid was morphometrically analyzed; at least 40 spheroids from duplicate wells per condition were analyzed. Results are mean ± SD for 3 independent experiments and express total sprout length relative to that under control (no inhibitor) CM- conditions. ***P<0.001.

Figure 3. Cytoskeletal rearrangement and phenotype alterations induced by conditioned medium from antigen activated iNKT cells is sensitive to pharmacological inhibition of EGFR.

(**A**) HMEC-1 at ≈ 70% confluency were cultured with CM- or CM+ for 24 h and cell morphology was examined by phase contrast microscopy (upper panels, at 10x and 20x magnification) and confocal microscopy (lower panels, at 40x and 60x magnification) after staining for actin with TRITC-labeled phalloidin. (**B**) Culture with CM- (white bars) or CM+ (grey bars) was performed without (control) or with inclusion of gefitinib (0.5 μM) or lapatinib (0.1 μM). Cell lengths were measured using AnalySIS software. At least 100 cells from each of at least three fields in duplicate wells per condition were analyzed. The histogram presents a relative frequency distribution analysis of data acquired and is representative of 1 of 3 independent experiments.

Figure 4. Effect of antigen activated iNKT cells on some signalling effectors involved in EC migration and angiogenesis; immunoblot analysis.

(**A**) Whole cell lysates (10-15 μg per lane) from HMEC-1 incubated for 15 min with control medium (ECGM-4% FCS; Ctrl), CM- or CM+ were immunoblotted for phospho (p)-FAK, p-Erk, p-p38MAPK, p-Akt, p-GSK3β and GAPDH (as internal control). Qualitatively similar data were obtained in 4 independent experiments, each using CM derived from a different iNKT cell clone. (**B**) HMEC-1 were incubated for 15 min with CM- or CM+ without or with inclusion of gefitinib (0.5 μM, gef) or lapatinib (0.1 μM, lap) and whole cell lysates immunoblotted for p-FAK, p-Erk, p-p38MAPK and GAPDH. Phosphorylation levels in CM+ treated HEMC-1 (filled bars) are expressed relative to that in HMEC-1 treated with CM- (open bars), and results are mean ± SD for 3 independent experiments. Blots from one experiment are presented.

Figure 5. Analysis of signalling responses of HMEC-1 to antigen activation of iNKT cells using reverse protein array.

EC were variously incubated for 15 min as depicted within the figure panels **A, B** and **C** with control medium (ECGM-4% FCS; Ctrl) without or with inclusion of IL-8 (10ng/ml), CM+ or CM- without or with inclusion of gefitinib (0.5 μM), CM+ or CM- subjected to immunodepletion protocols using anti-IL-8 mAb or non-immune (n.i.) IgG. Whole cell lysates were analysed by reverse protein array for the phosphorylation status of the indicated molecules, expressed relative to the status in EC incubated with control medium (Ctrl). Data in **B** are the average of 2 experiments. Results in **A** and **C** are mean ± SD from 3 independent experiments. **P<0.01, ***P<0.001. (a) compares native CM- or n.i. IgG-immunoprecipitated CM- with native CM+ or n.i. IgG-immunoprecipitated CM+, respectively (b) compares anti-IL-8 mAb immunoprecipitated CM+ with native CM+ or n.i. IgG-immunoprecipitated CM+.

Figure 6. A simple model for events involved in the angiogenic behavioural response of endothelial cells to lipid antigen activation of iNKT cells, and different modalities for inhibiting this response.

Activation of CD1d-restricted iNKT cells through the presentation of lipid antigen α-GalCer to iNKT cells by CD1d-expressing APC induces release of IL-8 from iNKT cells which induces angiogenic behaviour in EC. Angiogenic responses of EC can be fully attenuated by either IL-8 neutralizing antibodies or IL-8 immunodepletion [8], anti-EGFR blocking monoclonal antibodies (cetuximab) or EGFR tyrosine kinase inhibitors (gefitinib or lapatinib), implicating a mechanism of IL-8 mediated EGFR transactivation. This is supported by blockade of EGFR phosphorylation following IL-8 immunodepletion of the secretome released by iNKT cells. IL-8 (CRCX2) mediated EGFR transactivation has been proposed to depend on cathepsin B induced cleavage of pro-HB-EGF to HB-EGF [14], a cognate ligand for EGFR [32]. FAK and Src are major downstream migration signal transduction effectors which can target many proteins, including Rac [13], that participate in the dynamic regulation of cortical actin organization and cell locomotion.

Figure 1

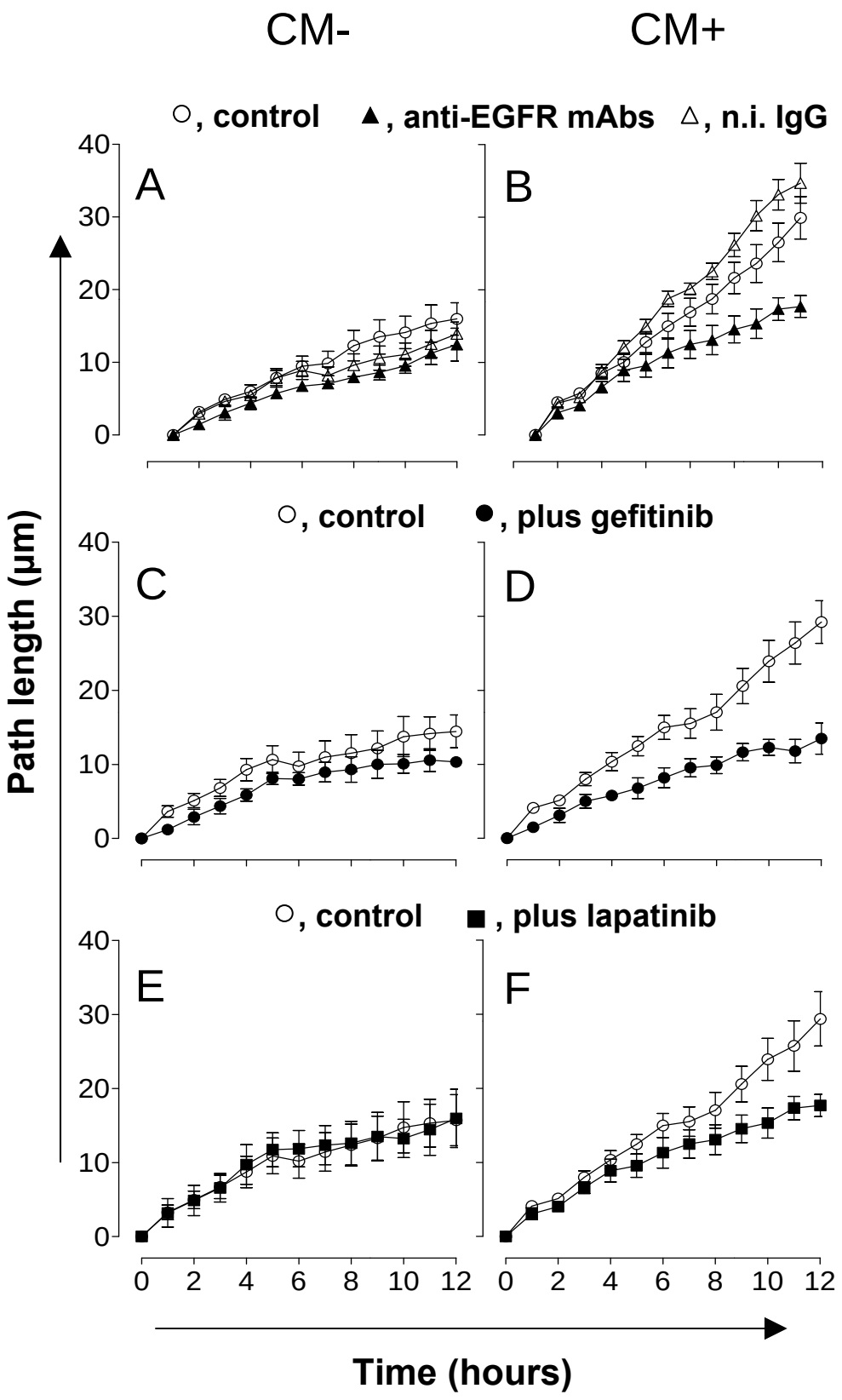

CM-
CM+
○, control
▲, anti-EGFR mAbs
△, n.i. IgG
○, control
●, plus gefitinib
○, control
■, plus lapatinib
A
B
C
D
E
F
Path length (μm)
Time (hours)
0
10
20
30
40
0
2
4
6
8
10
12

Figure 2

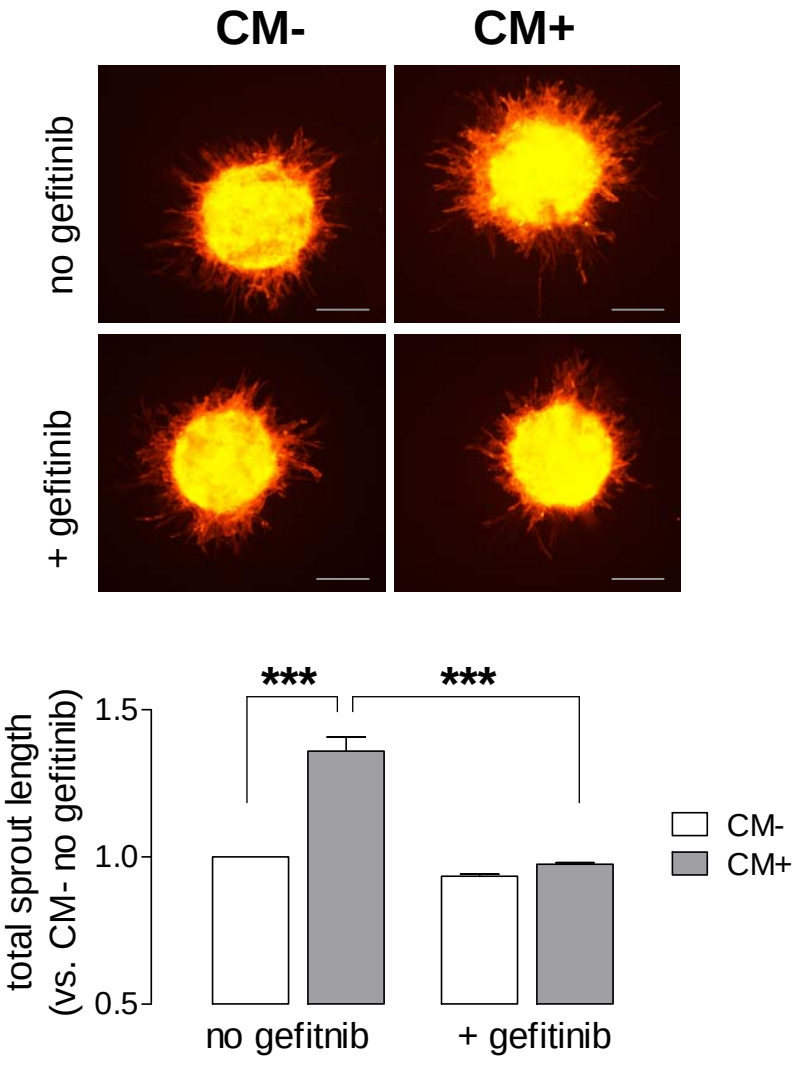

CM-
CM+
no gefitinib
+ gefitinib
***
***
total sprout length
(vs. CM- no gefitinib)
1.5
1.0
0.5
no gefitnib
+ gefitinib
CM-
CM+

Figure 3

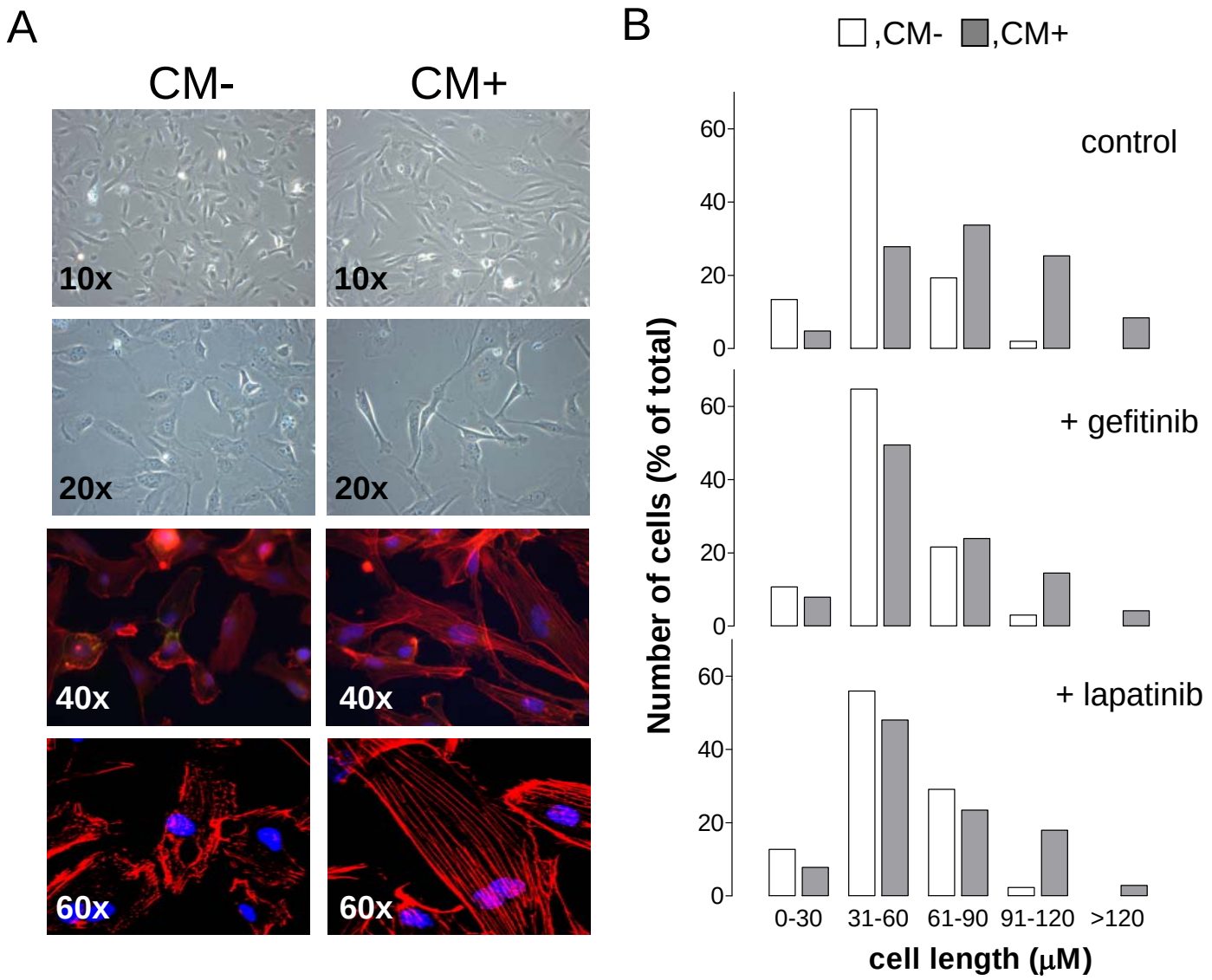

A
CM-
CM+
10x
10x
20x
20x
40x
40x
60x
60x
B
,CM-
,CM+
control
+ gefitinib
+ lapatinib
Number of cells (% of total)
0
20
40
60
0-30
31-60
61-90
91-120
>120
cell length (μM)

Figure 4

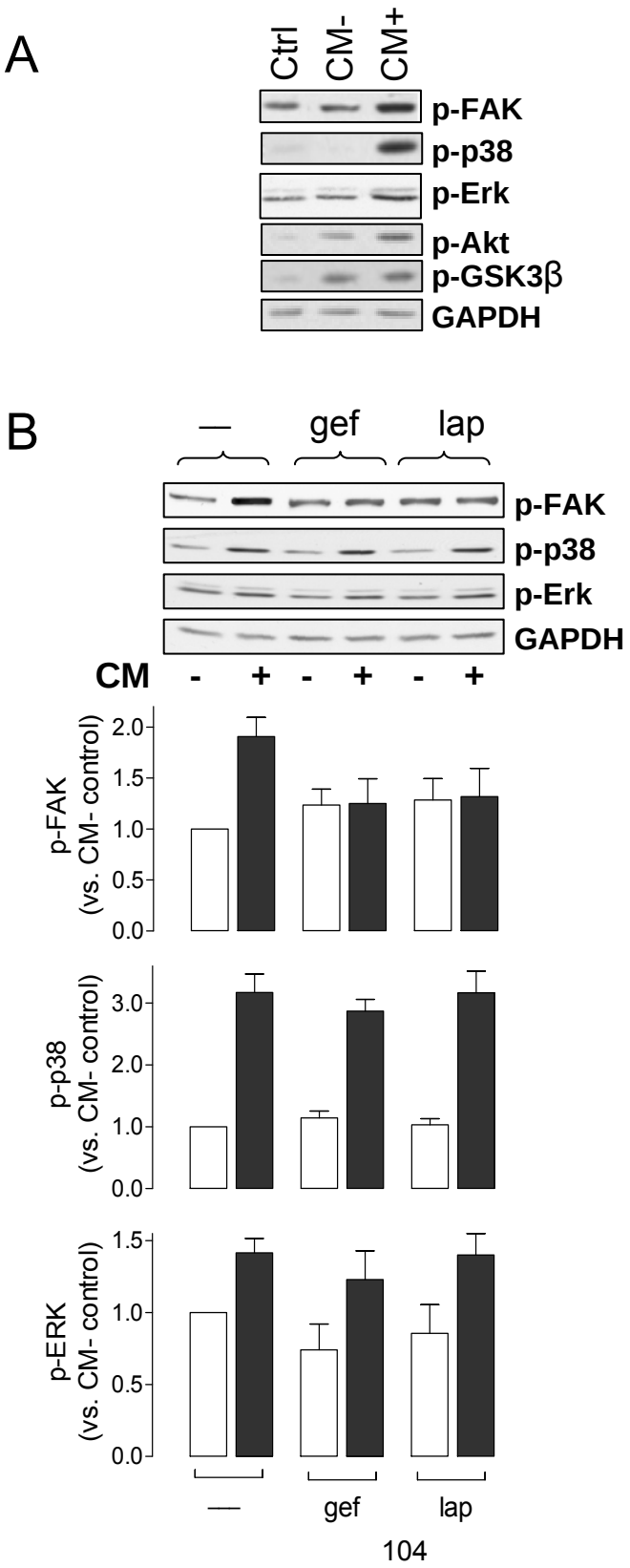

A
Ctrl
CM-
CM+
p-FAK
p-p38
p-Erk
p-Akt
p-GSK3β
GAPDH
B
—
gef
lap
p-FAK
p-p38
p-Erk
GAPDH
CM - + - + - +
p-FAK (vs. CM- control)
2.0
1.5
1.0
0.5
0.0
p-p38 (vs. CM- control)
3.0
2.0
1.0
0.0
p-ERK (vs. CM- control)
1.5
1.0
0.5
0.0
—
gef
lap

Figure 5

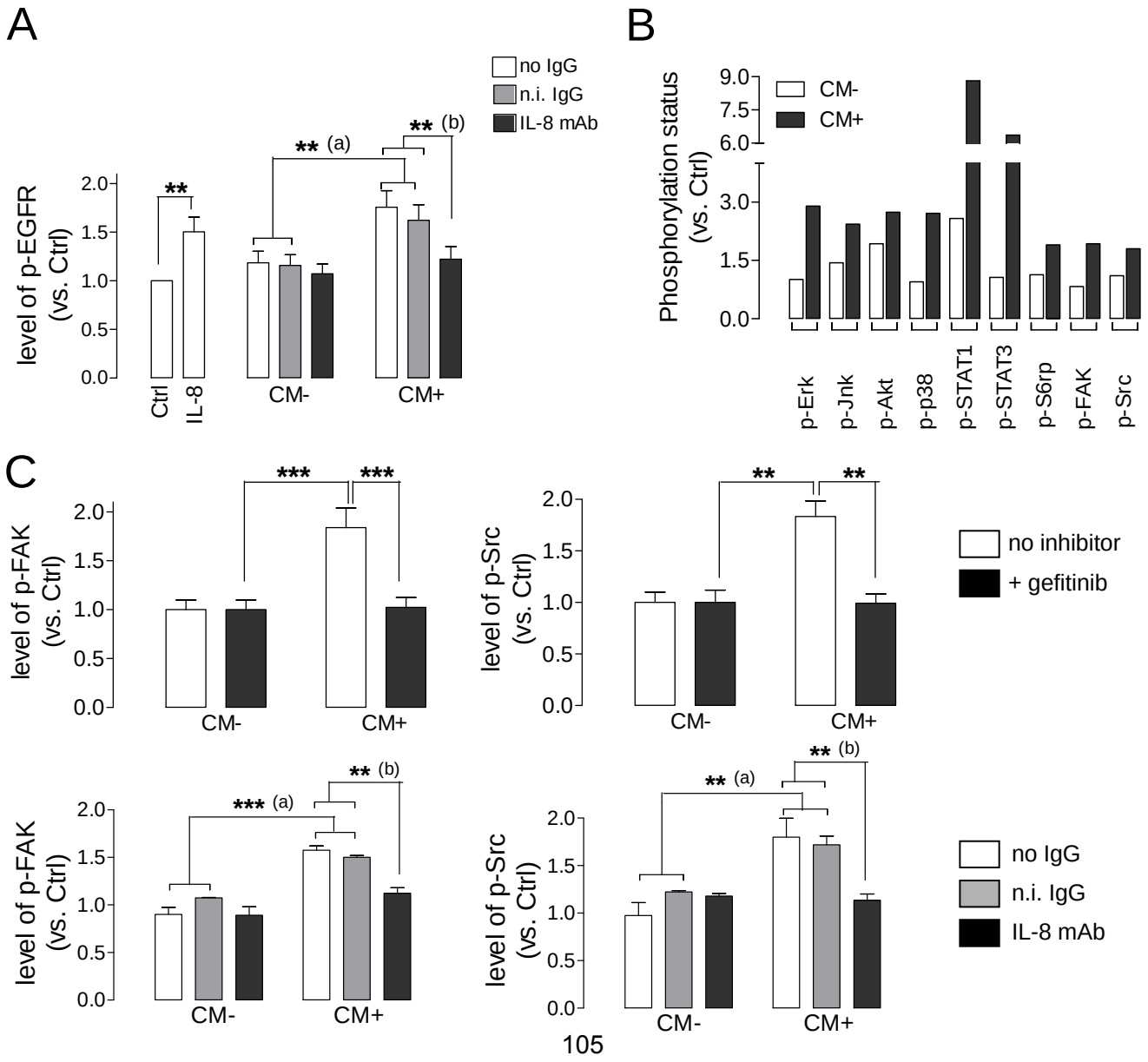
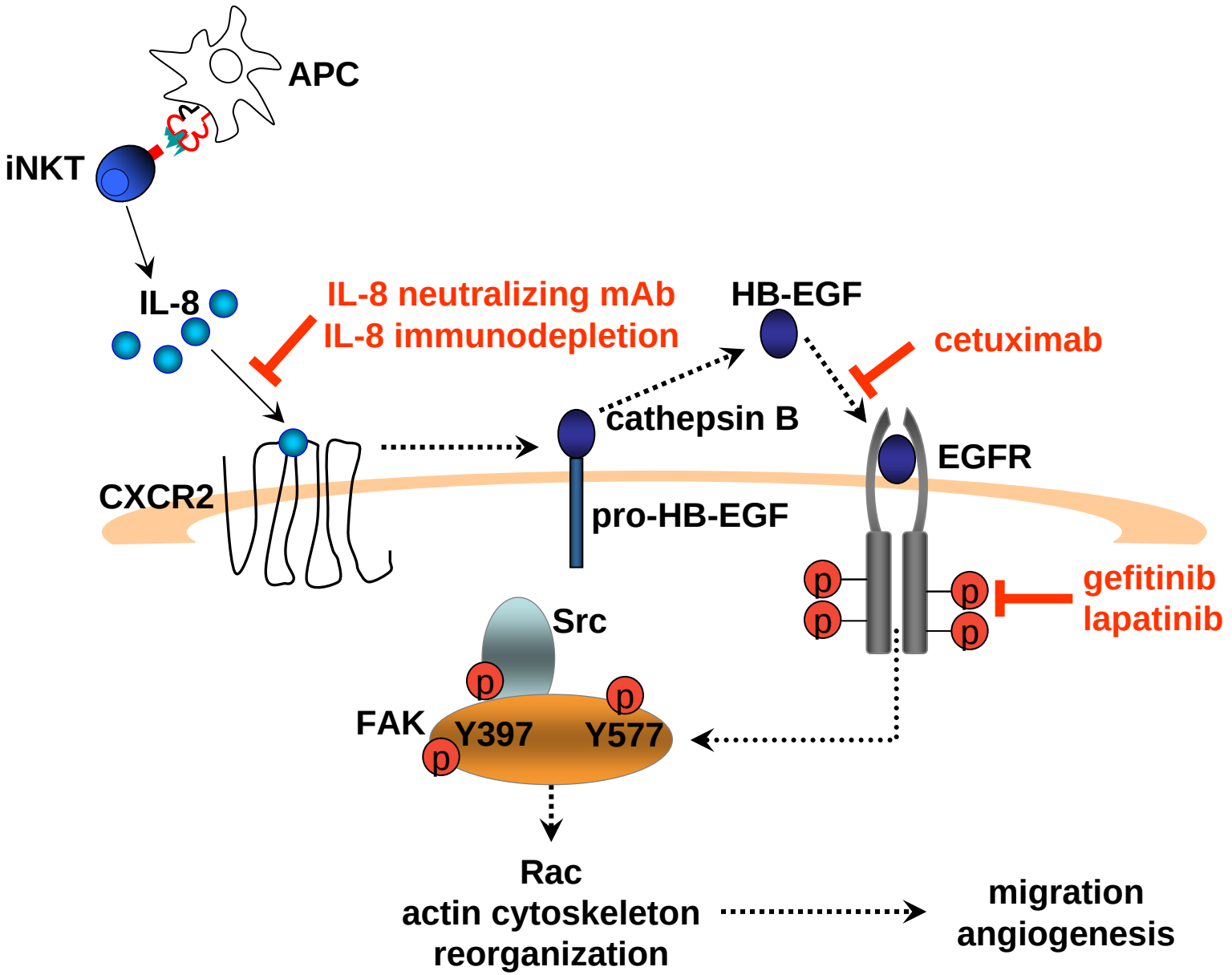

A
level of p-EGFR (vs. Ctrl)
2.0
1.5
1.0
0.5
0.0
**
** (a)
** (b)
no IgG
n.i. IgG
IL-8 mAb
Ctrl
IL-8
CM-
CM+
B
Phosphorylation status (vs. Ctrl)
9.0
7.5
6.0
3.0
1.5
0.0
CM-
CM+
p-Erk
p-Jnk
p-Akt
p-p38
p-STAT1
p-STAT3
p-S6rp
p-FAK
p-Src
C
level of p-FAK (vs. Ctrl)
***
***
level of p-Src (vs. Ctrl)
**
**
no inhibitor
+ gefitinib
*** (a)
** (b)
no IgG
n.i. IgG
IL-8 mAb

Figure 6


APC
iNKT
IL-8
IL-8 neutralizing mAb
IL-8 immunodepletion
HB-EGF
cetuximab
cathepsin B
EGFR
CXCR2
pro-HB-EGF
gefitinib
lapatinib
Src
FAK
Y397
Y577
Rac
actin cytoskeleton
reorganization
migration
angiogenesis

Supplementary data

IL-8-mediated angiogenic responses of endothelial cells to lipid-antigen activation of iNKT cells depend on EGFR transactivation

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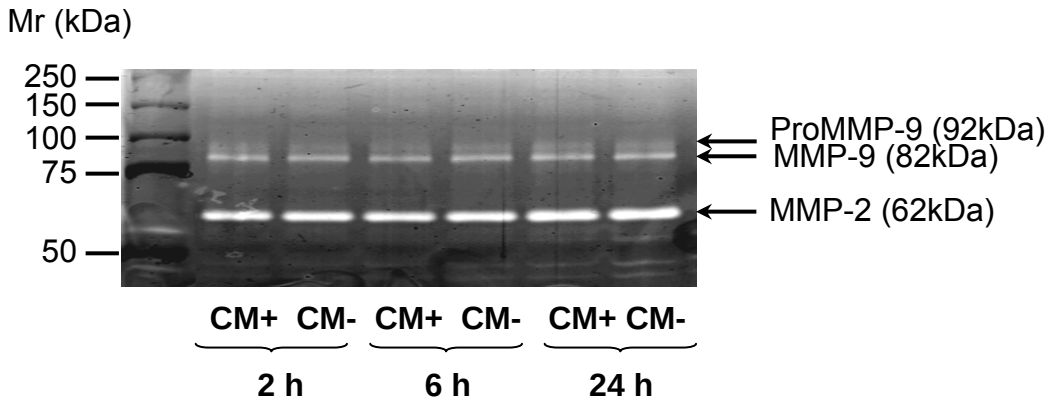

Figure S1. Conditioned medium from lipid antigen activated iNKT cells does not affect secretion of metalloproteinases by endothelial cells.

Human microvascular endothelial cell monolayers at 80% confluency were cultured for the indicated periods in the presence of conditioned culture media derived from non-activated (CM-) or α-GalCer-activated (CM+) iNKT cells. Gelatinolytic activity of the metalloproteinases MMP-2 and MMP-9 in HMEC-1 culture supernatants was measured by *in situ* zymography. The Coomassie blue-stained gel shown here is representative of at least 3 other independent experiments.

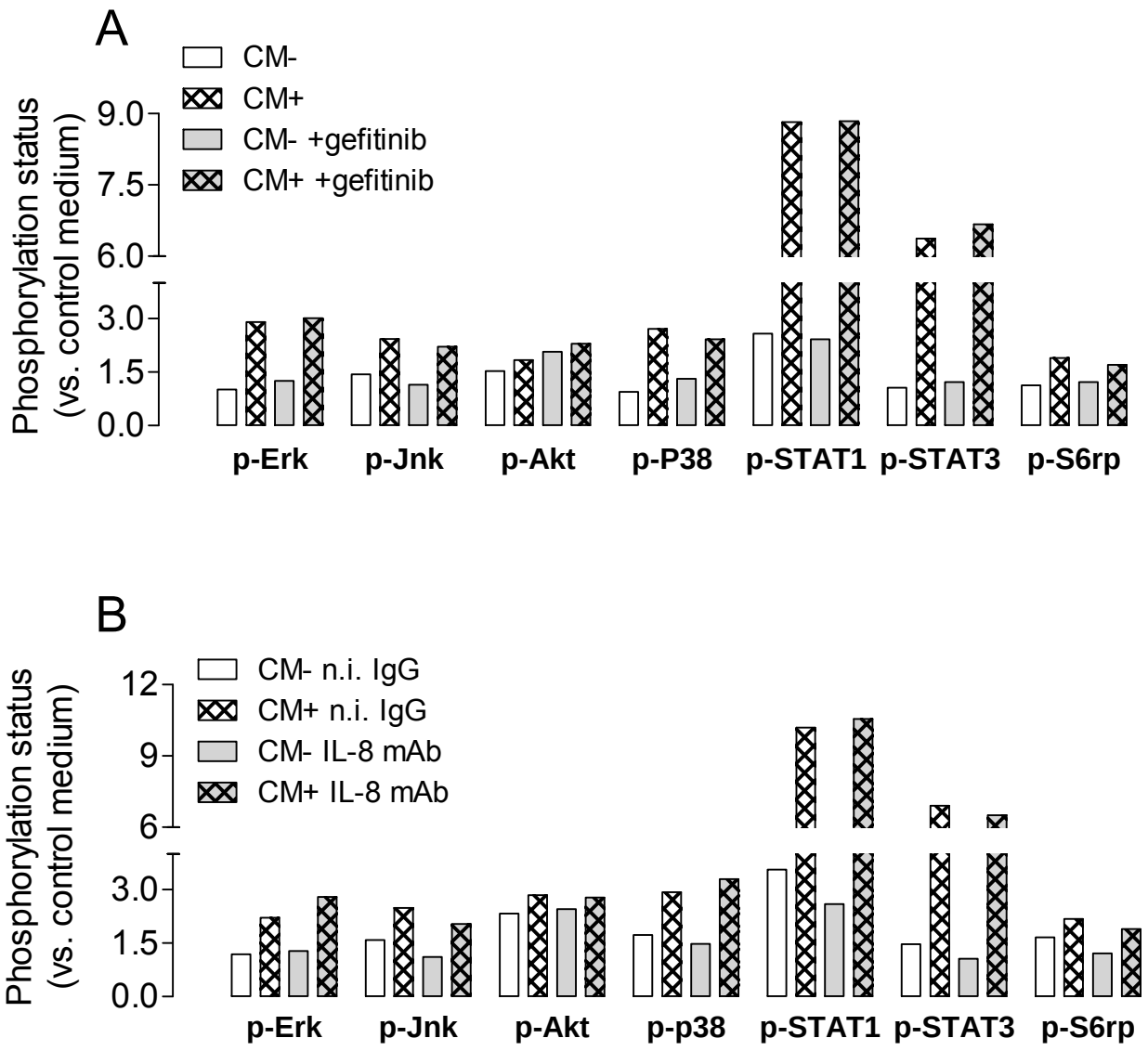

Figure S2. Lipid antigen activation of iNKT cells also activates numerous signal pathways in endothelial cells independently of endothelial cell EGFR tyrosine kinase activity and IL-8 released by the iNKT cells. Human microvascular endothelial cells were incubated for 15 min with CM- or CM+ without or with inclusion of gefitinib (0.5 μM) (**A**) **,** or with CM+ or CM- that had been subjected to immunodepletion protocols using anti-IL-8 mAb or non-immune (n.i.) IgG (**B**). Whole cell lysates were analysed by reverse protein array for the phosphorylation status of the indicated molecules, expressed relative to the status in EC incubated with control medium (ECGM-4% FCS). The data in **A** and **B** are the average of 2 experiments each performed in triplicate.

Project 3

The role of T-cadherin during endoplasmic reticulum stress

The results of this project have been published.

T-cadherin attenuates the PERK branch of the unfolded protein response and protects vascular endothelial cells from endoplasmic reticulum stress-induced apoptosis.

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T-cadherin attenuates the PERK branch of the unfolded protein response and protects vascular endothelial cells from endoplasmic reticulum stress-induced apoptosis

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ABSTRACT

Endoplasmic reticulum (ER) stress activated by perturbations in ER homeostasis induces the unfolded protein response (UPR) with chaperon Grp78 as the key activator of UPR signalling. The aim of UPR is to restore normal ER function; however prolonged or severe ER stress triggers apoptosis of damaged cells to ensure protection of the whole organism. Recent findings support an association of ER stress-induced apoptosis of vascular cells with cardiovascular pathologies. T-cadherin (T-cad), an atypical glycosylphosphatidylinositol-anchored member of the cadherin superfamily is upregulated in atherosclerotic lesions. Here we investigate the ability of T-cad to influence UPR signalling and endothelial cell (EC) survival during ER stress. EC were treated with a variety of ER stress-inducing compounds (thapsigargin, dithiothereitol, brefeldin A, tunicamycin, A23187 or homocysteine) and induction of ER stress validated by increases in levels of UPR signalling molecules Grp78 (glucose-regulated protein of 78 kDa), phospho-eIF2α (phosphorylated eukaryotic initiation factor 2α) and CHOP (C/EBP homologous protein). All compounds also increased T-cad mRNA and protein levels. Overexpression or silencing of T-cad in EC respectively attenuated or amplified the ER stress-induced increase in phospho-eIF2α, Grp78, CHOP and active caspases. Effects of T-cad-overexpression or T-cad-silencing on ER stress responses in EC were not affected by inclusion of either N-acetylcysteine (reactive oxygen species scavenger), LY294002 (phosphatidylinositol-3-kinase inhibitor) or SP6000125 (Jun N-terminal kinase inhibitor). The data suggest that upregulation of T-cad on EC during ER stress attenuates the activation of the proapoptotic PERK (PKR (double-stranded RNA-activated protein kinase)-like ER kinase) branch of the UPR cascade and thereby protects EC from ER stress-induced apoptosis.

1. Introduction

Endothelial dysfunction (ED) plays a key role in the pathogenesis of atherosclerosis [1]. ED is characterized by impairment of endothelium-dependent vasorelaxation and by a specific state of endothelial "activation" which manifests itself in increased endothelial cell (EC) production of proinflammatory and proliferative mediators, adhesion molecules and procoagulants. Thus injured EC significantly impair vascular function and trigger thrombogenic and atherogenic reaction cascades.

Important pathophysiological roles have been attributed to different types of stress in the development of ED and the progression of atherosclerosis. Luminal endothelium which is in direct contact with plasma and blood components is the first target of pathogens and damaging toxic compounds including risk factors for atherosclerosis. Acute and chronic oxidative stress associated with lipoprotein oxidation, activation of the inflammatory response and generation of

Abbreviations: apoE, apolipoprotein-E; ASK1, apoptosis signal-regulated kinase 1; ATF, activating transcription factor; BCL-2, B-cell leukemia/lymphoma-2; Bref.A., brefeldin A; CHOP, C/EBP homologous protein; DTT, dithiothreitol; EC, endothelial cells; ED, endothelial dysfunction; eIF2α, eukaryotic (translation) initiation factor 2 alpha; ER, endoplasmic reticulum; ERAD, ER associated degradation system; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Grp78, glucose-regulated protein of 78 kDa; GSK3β, glycogen synthase kinase 3 beta; Hcys, homocysteine; HUVEC, human umbilical vein endothelial cells; HMEC-1, human microvascular endothelial cell line; IRE1, inositol requiring kinase 1; JNK, Jun N-terminal kinase; LDLR, low density lipoprotein receptor; NAC, N-acetylcysteine; PERK, protein kinase RNA (PKR)-like ER kinase, phosphatidylinositol-3-kinase; ROS, reactive oxygen species; RT-PCR, real time PCR; T-cad, T-cadherin; Thapsi, thapsigargin; TRAF2, TNF receptor-associated factor 2; Tunica., tunicamycin; UPR, unfolded protein response; XBP-1, X-box binding protein-1.

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reactive oxygen species (ROS) is well recognized as an early event which plays an initiating role in cardiovascular disorders, while the ability of EC to resist stressors determines the progression and outcome of the disease [2,3]. Endoplasmic reticulum (ER) stress caused by perturbations in ER function including accumulation of misfolded proteins, changes in redox status or intraluminal calcium concentration, nutrient deprivation, altered glycosylation and pathogen infections contributes to pathogenesis of many human pathologies including neurodegenerative disorders, diabetes, obesity and cancer [4–10]. ER stress triggers the unfolded protein response (UPR), an adaptive signalling cascade aimed at restoring normal ER folding capacity and promoting cell survival, or, if ER stress is severe or prolonged, at promoting death of damaged cells to ensure protection of the whole organism [4–10].

UPR has recently been demonstrated to be involved in ED and related cardiovascular pathologies. Accumulation of free cholesterol in macrophages was shown to trigger ER stress and C/EBP-homologous protein (CHOP)-induced apoptosis [11]. Upregulation of ER chaperone glucose-regulated protein of 78 kDa (Grp78), apoptosis signal-regulated kinase 1 (ASK1) and other ER stress-related genes were detected in hypoxic cultured myocytes and in a murine model of myocardial infarction [12,13]. Both cytoprotective and apoptotic components of ER stress signalling are upregulated in myocardial ischemia and heart failure [14,15]. High levels of apoptosis and expression of Grp78 and pro-apoptotic transcription factor CHOP were found in smooth muscle cells and macrophages within the fibrous caps of thin-cap atheroma and ruptured plaques of both autoptic human coronary specimens and atherectomy specimens from patients with unstable angina pectoris [16]. Studies in apolipoprotein E knock-out (apoE$^{-/-}$) mice showed that UPR activation occurred at all stages of atherosclerosis, and evidence was provided to support the relevance of ER stress to macrophage apoptosis and enlargement of the necrotic core in advanced atherosclerotic plaques [17,18]. ER stress markers are also upregulated in atherosclerotic lesions of apoE$^{-/-}$ mice fed a hyperhomocysteinemic diet [19]. Homocysteine, a well known inducer of ER stress, induces apoptosis *in vitro* in a number of cell types including EC [20–23] and is believed to adversely affect stability and thrombogenicity of atherosclerotic lesions. In EC ER stress is activated by shear stress *in vitro* [24] and *in vivo* at atherosclerosis-susceptible arterial sites [25]. Pharmacological inducers of ER stress including tunicamycin, thapsigargin, Sin-1 and A23187 have variously been demonstrated to activate apoptosis in EC *via* induction of UPR-related proapoptotic factors CHOP and T-cell death-associated gene 51 [22,26]. Taken together, these findings support an association of ER stress and induction of UPR induced-apoptosis of EC, smooth muscle cells and macrophages with progression of atherosclerosis and development of plaque vulnerability.

T-cadherin (T-cad), an unusual glycosylphosphatidylinositol–anchored member of the cadherin superfamily of adhesion molecules, is upregulated in vascular cells under pathological conditions associated with abnormal vascular tissue remodeling and stress including atherosclerosis [27] and restenosis [28]. We have previously demonstrated that T-cad is involved in regulation of EC survival under conditions of oxidative stress. T-cad is markedly upregulated in EC by ROS, and its overexpression decreases oxidative stress-induced apoptosis [29]. Both overexpression and ligation of T-cad on the EC surface activate signalling pathways (phosphatidylinositol-3-kinase (PI3-kinase), Akt, glycogen synthase kinase 3 beta (GSK3β)) important for cell survival and proliferation [29,30]. There is considerable interplay between ER stress and oxidative stress. Oxidative stress, which occurs when the production of ROS overwhelms the antioxidant defenses, may cause alterations in ER homeostasis and activate UPR-related signalling, while conversely ER stress may result in accumulation of ROS and initiate expression and activation of oxidation-related signalling mediators [7]. Together these data suggest that T-cad upregulation represents a protective mechanism aiming at resisting stress conditions and might be involved in endothelial ER stress response. Moreover, our recent data on physical colocalization of T-cad and ER chaperon Grp78 in EC [31] further point to a possible role for T-cad in UPR signalling. In this study we investigate the ability of T-cad to influence UPR activation and EC survival during ER stress.

2. Experimental

2.1. Cell culture

Human umbilical vein EC (HUVEC), purchased from PromoCell GmbH (Heidelberg, Germany), were cultured on plates precoated with 0.1% gelatine in EC growth medium containing EC growth supplement (PromoCell). Human microvascular EC line (HMEC-1) was cultured in the same medium supplemented with 10% fetal calf serum (FCS). Cells were seeded either at 2×10^5 cells/well into 6-well plates (for immunoblotting) or at 2×10^4 cells/well into 96-well plates (for Homogenous Caspases ELISA) and allowed to adhere overnight. Culture medium was refreshed and cells were treated for selected intervals with a variety of ER stress-inducing agents including thapsigargin (500 nM), dithiothreitol (DTT, 1 mM), brefeldin A (5 μg/ml), tunicamycin (3 μg/ml), calcium ionophore A23187 (2 μM) and homocysteine (5 mM). Some experiments included ROS scavenger N-acetylcysteine (NAC, 15 mM), PI3-kinase inhibitor LY-294002 (10 μM) or Jun N-terminal kinase (JNK) inhibitor SP600125 (20 μM), which were added to cultures 1 h before stimulation protocols. All compounds were purchased from Sigma-Aldrich (Buchs, Switzerland) with the exception of SP600125 which was purchased from VWR/Merck (Dietikon, Switzerland). Agent vehicles at the appropriate final concentrations were tested in pilot studies and found not to affect any of the parameters measured in this study.

2.2. Overexpression and silencing of T-cad

Overexpression of T-cad in HMEC-1 was achieved by using Adeno-X Expression System (Clontech, Palo Alto, USA) as detailed previously [32]. Briefly, HMEC-1 in normal growth media were infected overnight with empty, LacZ or T-cad containing adenoviral particles at a final approximate concentration of 4 pfu/cell; expression of T-cad was monitored by immunoblotting (representative blots shown in Fig. 3). T-cad silencing in HMEC-1 was performed using MISSION® Lentiviral transduction system (Sigma-Aldrich Chemie, Buchs, Switzerland). Lentiviral particles expressing T-cad short hairpin RNA (product number TRCN0000055546) and non-target control transduction particles (product number SHC002V) were used at a multiplicity of infection of 4 lentiviral particles per cell according to manufacturer's recommendations. Infected cells were selected against puromycin for about 10 days. Efficiency of T-cad silencing was in the order of 80–90% as controlled by immunoblotting (representative blots shown in Fig. 4).

2.3. Homogenous caspases assay

Following exposure to ER stress-inducing agents caspase activity in treated cells was evaluated using Homogeneous Caspases ELISA that detects activated caspases 2, 3, 6, 7, 8, 9, and 10 (Roche Diagnostics GmbH, Mannheim, Germany) as described previously [29]. Substrate solution was added to wells without prior removal of any detached cells or medium change.

2.4. Immunoblotting

Immunoblotting was performed on whole cell lysates prepared by lysis with PBS containing 1% SDS and protease inhibitor cocktail

(Sigma), with inclusion of 1 mM orthovanadate and 5 mM NaF in samples analysed for protein phosphorylation status. In some experiments immunoblotting for CHOP protein was additionally performed on nuclear extracts prepared using the CHEMICON® Nuclear Extraction Kit according to the manufacturers' protocol (Millipore, Billerica MA, USA). Protein concentrations were determined using the Lowry method. Samples were loaded at 5–10 μg per lane and electrophoresed in 8% SDS-polyacrylamide gels under reducing conditions. The following antibodies were used for immunoblotting: goat anti-T-cad (R&D Systems Europe Ltd., Abingdon, UK), goat anti-GAPDH (Abcam, Cambridge, UK), mouse anti-Grp78 (BD Biosciences, Basel, Switzerland), rabbit anti-cleaved caspase 3 and rabbit anti-phospho-eIF2α (Cell Signalling, New England Biolabs, Frankfurt, Germany), rabbit anti-CHOP (sc7351, Santa Cruz from Lab Force AG, Nunningen, Switzerland), mouse anti-lamin A/C (LaZ-1) (kind gift of Prof. Harald Herrmann, Department of Molecular Genetics, German Cancer Research Center, Heidelberg, Germany). Secondary HRP-conjugated goat anti-mouse IgG or anti-rabbit IgG (Southern Biotechnology (BioReba AG, Reinach, Switzerland) or donkey anti-goat IgG (Santa Cruz) together with Amersham ECL (Amersham Biosciences, Little Chalfont, UK) were used for detection of immunoreactive proteins. Scanned images of autoradiograms were analyzed using AIDA Image or Scion (NIH) Image software.

2.5. RT-PCR

Isolation of RNA, reverse transcription, and real-time PCR analysis were performed as described previously [33]. The expression of target molecules was normalized to the expression of $ß_2$-microglobulin or to GAPDH. Primer sequences are available on request.

2.6. Statistical analysis

All experiments were performed on at least three separate occasions. Results are given as mean ± SD unless otherwise stated. Differences were variously determined with 1-way or 2-way repeated measures ANOVA with Tukey's or Bonferroni's multiple comparison test, respectively, using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). A P value of <0.05 was considered significant.

3. Results

3.1. Activation of the UPR and apoptosis in EC by ER stress inducers

In order to establish the experimental model we examined the kinetics of responses of HMEC-1 to a variety of pharmacological

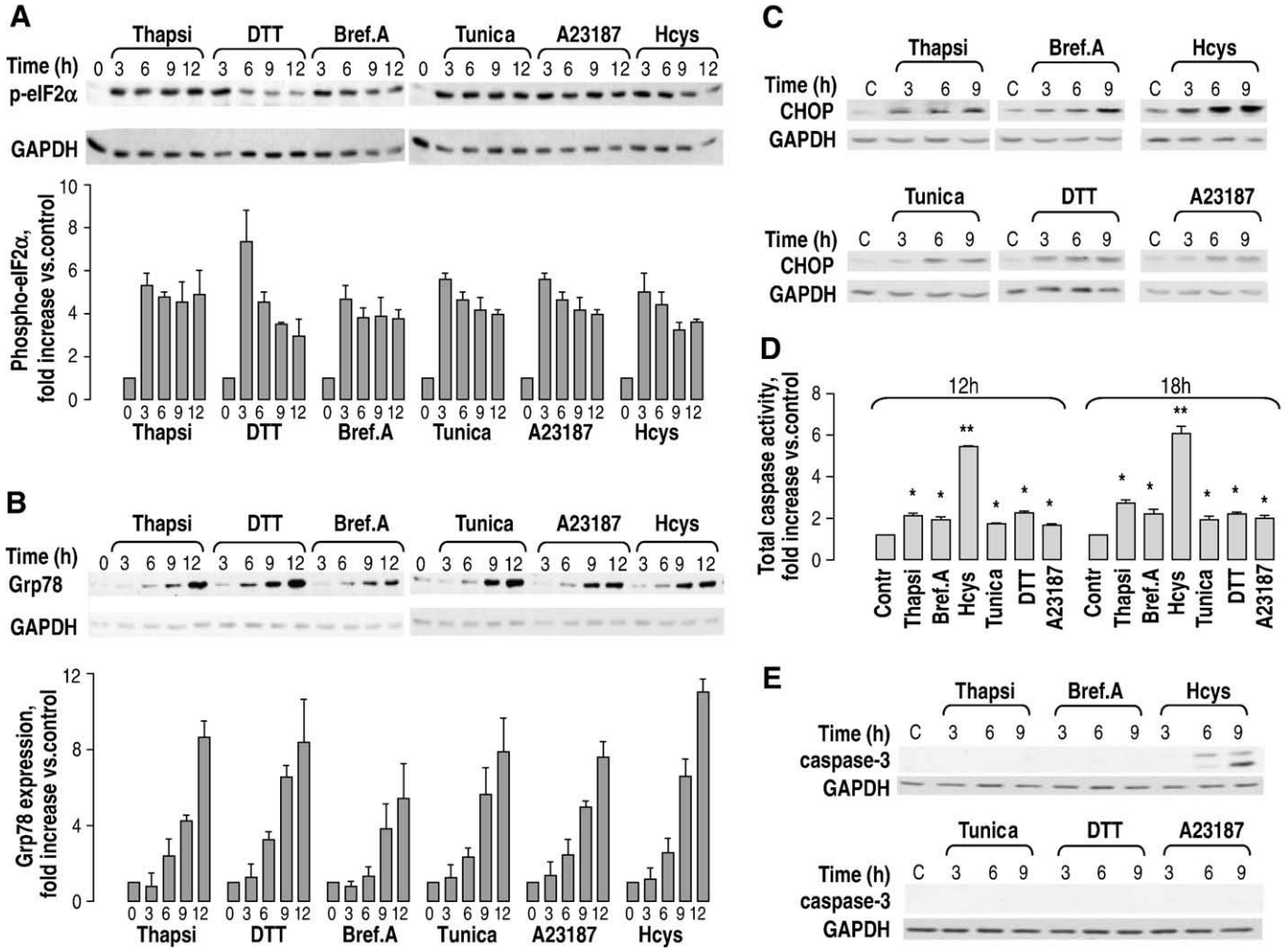

Fig. 1. Induction of ER stress in HMEC-1. HMEC-1 cultures were exposed for the indicated periods of time to the different ER stress inducers: thapsigargin (Thapsi), DTT, brefeldin A (Bref.A), tunicamycin (Tunica), A23187 and homocysteine (Hcys). Cells were processed for immunoblot analysis of phospho-eIF2α (A), Grp78 (B) or CHOP (C). Blots were probed with GAPDH to control for equivalence of protein loading. Changes in levels of phospho-eIF2α, Grp78 and CHOP are expressed relative to their respective levels in untreated cells (0 h). Total caspases activity in HMEC-1 cultures was measured using homogenous caspases assay kit (D). Data (mean ± SD, n = 3) are expressed relative to the baseline fluorimetic level in untreated control cells. Activity of caspase-3 in HMEC-1 lysates were analysed by immunobloting (E). The histograms show data (mean ± SD) obtained from 3 to 4 independent experiments. Blots are representative of 3–4 separate experiments.

compounds that are known to induce ER stress by different mechanisms, namely disturbance of calcium homeostasis (ER calcium pump inhibitor thapsigargin, calcium ionophore A23187), inhibition of N-glycosylation in the ER (tunicamycin), inhibition of ER/Golgi transport (brefeldin A) and reduction of disulfide bonds (dithiothreitol, homocysteine). Cells were treated with the compounds for up to 12 h. As read-outs for UPR activation we determined the phosphorylation status of the α-subunit of eukaryotic translational initiation factor 2 (eIF2α) as an index of translational attenuation and the level of Grp78 protein as an index of transcriptional induction of ER chaperone genes. All the chemicals tested induced an increase in phosphorylation of eIF2α within 3 h that remained generally steady during treatment for up to 12 h (Fig. 1A). Levels of Grp78 increased between 3 and 6 h of exposure to the chemicals and thereafter continued to rise steadily during treatment for up to 12 h (Fig. 1B). Similar responses were elicited in HUVEC (Supplemental Fig. S1).

Since excessive or prolonged ER stress is linked to the triggering of programmed cell death [4–10] the effects of the ER stressors on indices of apoptosis in HMEC-1 were investigated. CEBP homology protein (CHOP), a major ER stress-inducible pro-apoptotic transcription factor which operates as a downstream component of ER-stress pathways, at the convergence of the inositol-requiring kinase 1 (IRE1), protein kinase RNA (PKR)-like ER kinase (PERK) and activating transcription factor 6 (ATF6) [34] was induced by all the ER stressors (Fig. 1C). The specificity of the anti-CHOP antibodies used in this study (Sc-7351 from Santa Cruz) and our use of whole cell lysates for immunoblotting [35] were validated by comparison of immunoreactivity in whole cell lysates and nuclear extracts prepared in parallel following treatment of parental HMEC-1 with thapsigargin, brefeldin A or homocysteine (Supplemental Fig. S2). Analysis for caspases activity with the Homogeneous Caspases ELISA revealed a ~5-fold increase in cells treated with homocysteine and a lesser ~2-fold increase in cells treated with thapsigargin, brefeldin A, A23187, tunicamycin and DTT (Fig. 1D). Immunoblot analysis for active caspase-3 revealed its presence only in cells treated with homocysteine (Fig. 1E). This, taken together with the ~2–3-fold greater induction of caspases activity by homocysteine as compared with the other stressors (Fig. 1D), might reflect that, under our experimental conditions, homocysteine elicited the most potent proapoptotic response in EC.

3.2. Induction of the UPR in EC is associated with upregulation of T-cad

Next we investigated the effects of the ER stressors on induction of T-cad mRNA and protein. All ER stressors induced a 1.5 to 3-fold upregulation of T-cad transcripts within 2 h that remained elevated for up to at least 6 h (Fig. 2A). Likewise all compounds induced a 1.5 to 3-fold increase in T-cad protein expression within 3 h, which generally remained stable for up to at least 9 h (Fig. 2B). HUVEC responded similarly to conditions of ER stress (Supplemental Fig. S1).

3.3. T-cad expression modulates ER stress responses of EC

To determine whether T-cad plays a role in ER stress response in EC we examined the consequences of overexpression and silencing of T-cad on UPR activation. These gain-of-function and loss-of-function

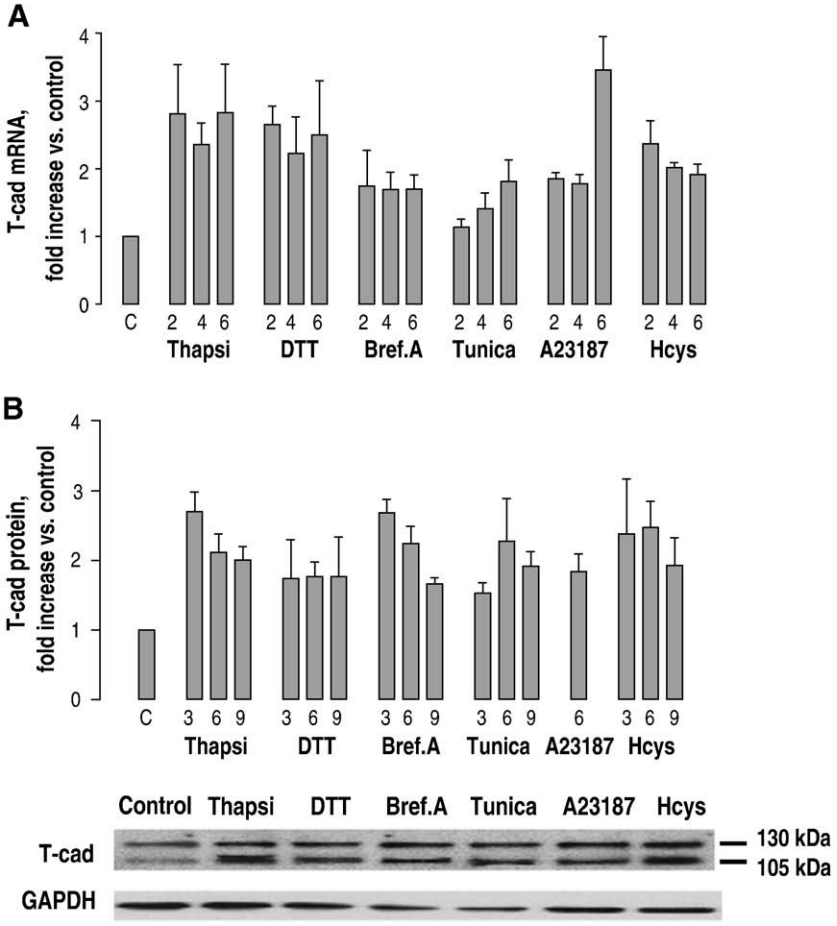

Fig. 2. Induction of T-cad expression in HMEC-1 in response to ER stress. HMEC-1 cultures were exposed for the indicated periods of time to the different ER stress inducers: thapsigargin (Thapsi), DTT, brefeldin A (Bref.A), tunicamycin (Tunica), A23187 and homocysteine (Hcys). Cells were processed for RT-PCR analysis of T-cad transcript levels (A) or for immunoblot analysis of T-cad protein (B). The immunoblot shows samples from cells treated for 6 h. Changes in T-cad mRNA and protein expression are expressed relative to their respective levels in untreated cells. The histograms present data (mean ± SD) obtained from 3 to 4 independent experiments.

experiments were performed using HMEC-1. Homocysteine, thapsigargin and brefeldin A were selected as the test ER-stress and UPR activation agents. Compared with the responses in control empty vector- or LacZ-transduced cells, T-cad overexpression significantly attenuated the elevation in levels of phospho-eIF2α, Grp78 and pro-apoptotic C/EBP homologous protein CHOP induced by the ER stressors (Fig. 3). In contrast, and compared with control shRNA-transduced cells, T-cad silencing significantly augmented the stimulatory effects of the ER stressors on phospho-eIF2α, Grp78 and CHOP levels (Fig. 4).

3.4. T-cad promotes EC survival during ER stress

In order to determine if modulation of UPR signalling by T-cad translates into a functional response and influences apoptosis rates during ER stress we studied effects of T-cad overexpression and silencing on caspase activation. T-cad overexpression blunted the induction of total caspases activity by thapsigargin, brefeldin A and homocysteine (Fig. 5A) and upregulation of cleaved caspase-3 level by homocysteine (Fig. 5B). In T-cad silenced cells these proapoptotic responses to the ER stressors were amplified (Fig. 5C, D).

3.5. T-cad effects on UPR signalling in EC do not depend on PI3-kinase pathway, JNK activity or protection against oxidative stress

We have previously demonstrated that T-cad-dependent activation of prosurvival signalling protects EC from oxidative stress. Since generation of ROS can be a cause of disturbances in ER function we investigated whether T-cad may prevent ER stress-induced apoptosis by acting not on UPR pathways directly but rather by limiting the impact of oxidative stress. As described above, upregulation of proapoptotic factor CHOP by thapsigargin and homocysteine was much more prominent in EC transduced with T-cad shRNA as compared to control shRNA-expressing cells. ROS scavenger N-acetylcysteine did not prevent the stress-induced increase in CHOP or Grp78 levels in T-cad-silenced cells (Fig. 6A), suggesting that the ability of T-cad to attenuate ER stress is distinct from its ability to protect EC from oxidative stress [29]. ER stress attenuation in T-cad overexpressing cells could not be prevented by PI3-kinase inhibitor LY-294002 suggesting that activation of PI3-kinase pathway by T-cad [29] does not contribute to protection of cells from excessive UPR (Fig. 6B). Inclusion of anthrapyrazolone SP600125 to inhibit JNK, which is a target of ASK1 and plays an important role in ER stress-

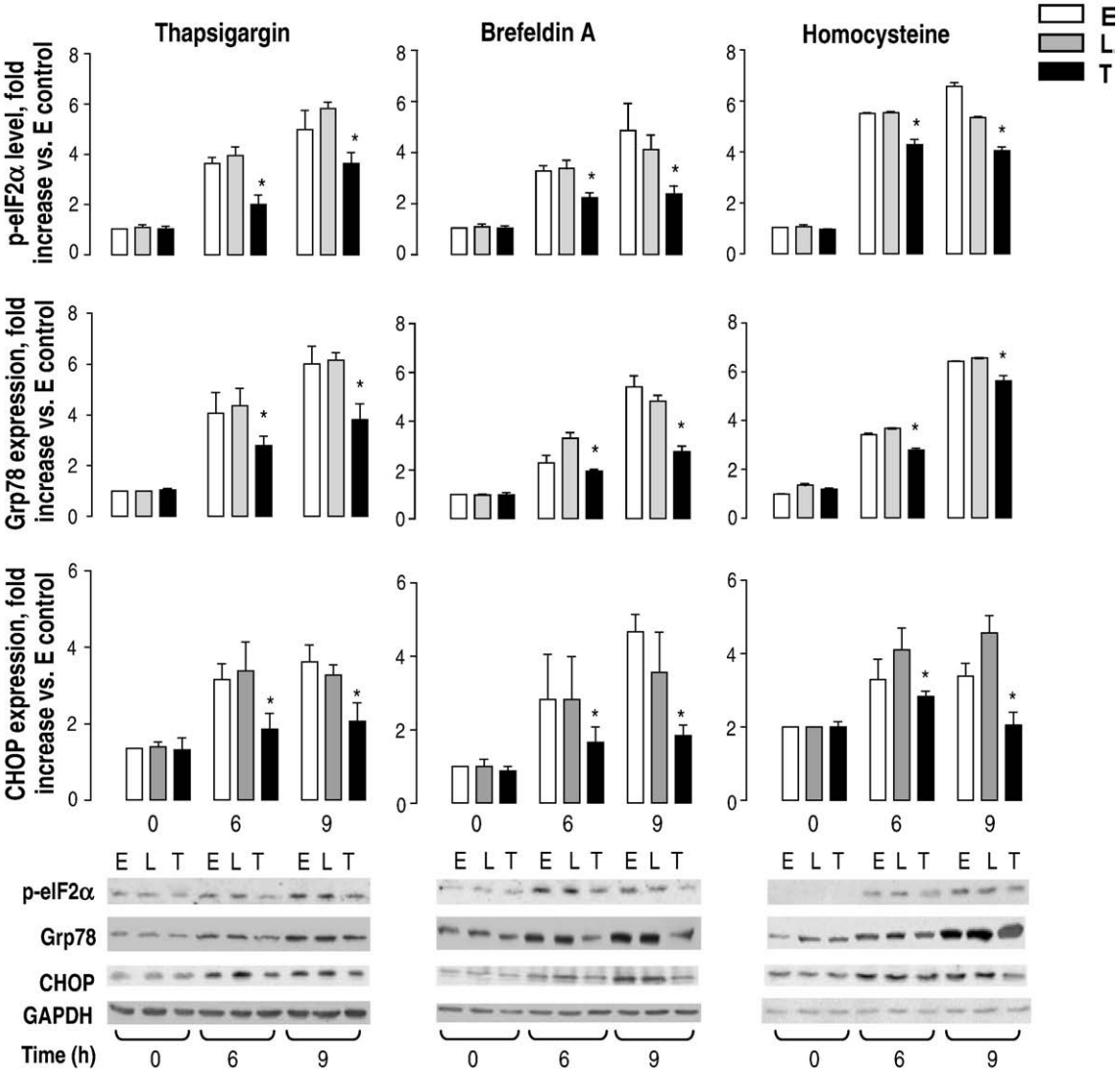

Fig. 3. T-cad overexpression attenuates ER stress response. Empty- (E, open bars), LacZ- (L, grey bars) and T-cad- (T, black bars) lentivector transduced HMEC-1 were treated with thapsigargin, brefeldin A or homocysteine for the indicated times. Whole cell lysates were prepared for immunoblot analysis of eIF2α phosphorylation and expression of Grp78 and CHOP proteins. Changes are expressed relative to levels in untreated E-transduced HMEC-1. The histograms present data (mean ± SD) obtained from 3 independent experiments. Representative blots, including the controls for T-cad overexpression, are shown. Asterisks indicate a significantly decreased response in T vs. E or L (P at least <0.05).

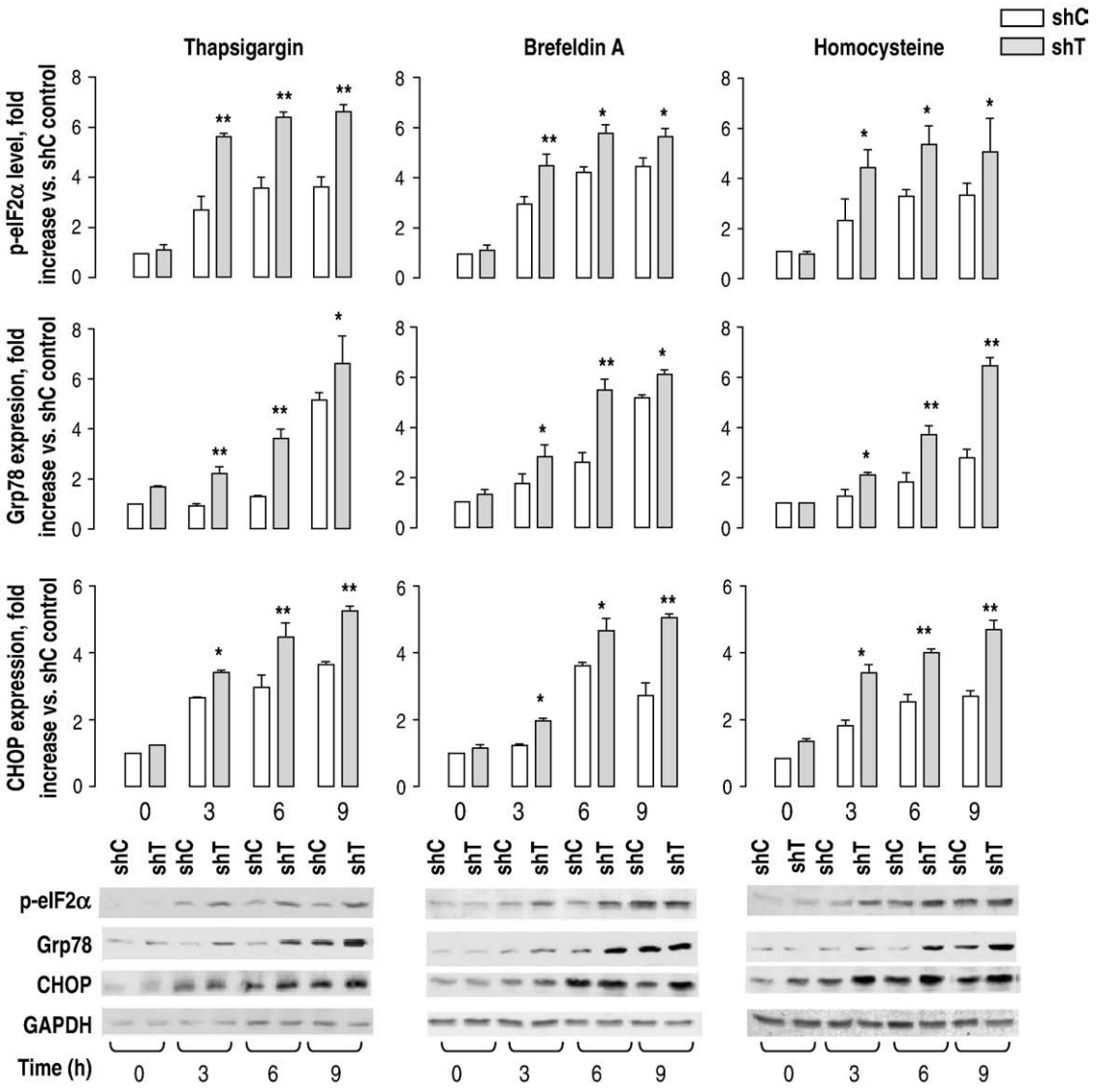

Fig. 4. Silencing of T-cad expression augments ER stress response. HMEC-1 transduced with lentiviral vectors expressing T-cad-specific (shT, grey bars) or control (shC, open bars) shRNAs were treated with thapsigargin, brefeldin A or homocysteine for the indicated times. Whole cell lysates were prepared for immunoblot analysis of T-cad, eIF2α phosphorylation and expression of Grp78 and CHOP proteins. Changes are expressed relative to levels in the control shRNA-transduced HMEC-1. The histograms present data (mean ± SD) obtained from 3 independent experiments. Representative blots, including the controls for T-cad silencing, are shown. Asterisks indicate a significantly increased response in shT vs. shC (*$P<0.05$, ** P at least <0.01).

induced apoptosis, also did not prevent the stress-induced increase in CHOP or Grp78 levels in T-cad-silenced cells (Fig. 6C).

4. Discussion

We have previously reported that upregulation of T-cad in EC occurs *in vivo* [27,28] and *in vitro* under conditions associated with oxidative stress [29] and functions as a protection mechanism promoting EC survival through activation of the PI3K/Akt/mTOR survival signal pathway and concomitant suppression of the p38 MAPK proapoptotic pathway [29]. The present study has demonstrated that upregulation of T-cad in EC also takes place during ER stress. T-cad overexpression and silencing studies collectively suggest that upregulation of T-cad attenuates ER stress by restricting activation of the proapoptotic PERK branch of the UPR cascade and thereby limits ER stress-induced apoptosis.

The cellular ER stress/UPR signalling cascade falls into several phases with different effector functions (reviewed in [4–9]). It is triggered by an accumulation of misfolded proteins in the ER which bind to ER chaperone Grp78, causing dissociation of Grp78 from the three major ER stress sensors PERK, ATF6 and IRE1 and a resultant launching of the UPR [4–9]. The first response aims at re-establishing homeostasis and normal ER function. PERK-dependent phosphorylation of eIF2α results in translational attenuation reducing the load of new protein synthesis on the ER. IRE1 induces expression of X-box binding protein XBP-1, while ATF6 is translocated to the Golgi and activated by proteolysis. XBP-1 and ATF6 act together with eIF2α-downstream target ATF4 as transcription factors activating expression of ER chaperones and components of ER associated degradation system (ERAD) eliminating misfolded proteins. In a later phase, immune and anti-apoptotic responses are activated *via* the NFκB pathway. When the adaptive mechanisms fail to compensate in the face of protracted or excessive ER stress apoptotic cell death is induced to protect the organism by eliminating the damaged cells. Several apoptosis pathways are known to be involved, the central role being played by the proapoptotic transcription factor CHOP which blocks expression of antiapoptotic protein Bcl-2. Transcriptional induction of CHOP mostly depends upon activation of PERK/eIF2α; however, IRE1 and ATF6 pathways also stimulate CHOP transcription, meaning that CHOP operates at the convergence of all UPR branches. ER stress-induced

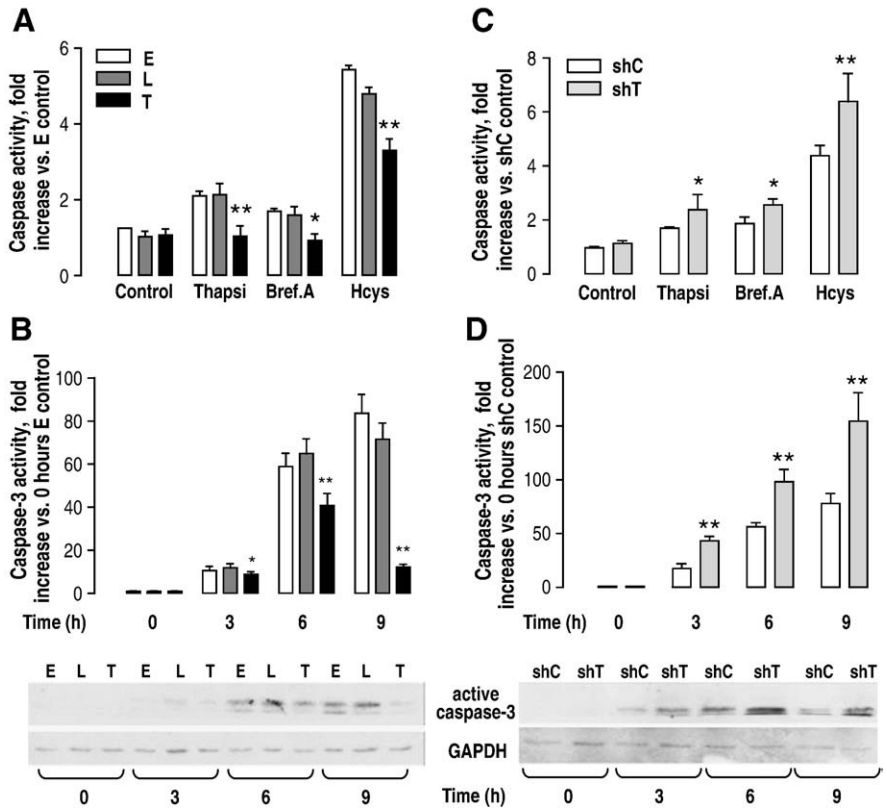

Fig. 5. Effects of T-cad overexpression and silencing on ER stress-induced apoptosis. Apoptosis was measured by homogenous total caspase activity assay (A, C) or by immunoblotting with anti-cleaved caspase-3 antibody (B, D) in empty- (E, open bars), LacZ- (L, grey bars) and T-cad- (T, black bars) lentivector transduced HMEC-1 (A, B) or in HMEC-1 transduced with lentiviral vectors expressing T-cad-specific (shT, grey bars) or control (shC, open bars) shRNAs (C, D) after treatment with thapsigargin (Thapsi), brefeldin A (Bref.A) or homocysteine (Hcys) for 15 h. Changes in caspase activity or level of caspase 3 are expressed relative to baseline levels (untreated) in respective control-transduced HMEC-1. The histograms present data (mean ± SD) obtained from 3 independent experiments. Asterisks in A and B indicate a significantly decreased response in T vs. E or L (*, $P<0.05$; ** P at least <0.01). Asterisks in C and D indicate a significantly increased response in shT vs. shC (*$P<0.05$, ** P at least <0.01).

apoptosis can also occur *via* IRE1-dependent activation of TRAF2/ASK1/JNK cascade and *via* Ca^{2+}-dependent activation of caspase-12.

Experimental evidence suggests that all stress sensors activate both protective and proapoptotic signalling. The final outcome of UPR will depend on a complex interplay of various factors such as balance between activity of different pathways and persistence of stress conditions, so any influence that can shift the phase of UPR between adaptation and apoptosis is likely to affect the cell fate decision between survival and death (reviewed in [4–9]). In terms of cardiovascular disease this phenomenon of "the double-edged sword" is illustrated by several studies demonstrating that UPR can be either beneficial or damaging for vascular and cardiac cells. Upregulation of Grp78 by endothelin-1 or salvianolic acid protects hypoxic cardiomyocytes and HUVEC from ER-stress [36]. Overexpression of Grp78 inhibits homocysteine-induced ER stress [37] associated with endothelial injury and increased risk of thrombosis. On the other hand, downregulation of UPR markers including Grp78 by darbepoetin and kaempferol exerts cardioprotective effect in autoimmune cardiomyopathy [5,38]. Increasing eIF2α phosphorylation status by pharmacological inhibition of its dephosphorylation may either protect cells [4] or potentiate ER stress-induced apoptosis caused by fatty acids [39]. Many studies suggest that prevention of UPR-induced apoptosis is an attractive target for minimizing tissue damage under stress conditions. In an experimental model of myocardial infarction $ASK1^{-/-}$ mice show reduced cardiomyocyte apoptosis rates and myocardial injury. Inactivation of CHOP gene exerted a survival benefit during diabetes [40] and prevented plaque growth, apoptosis and necrosis in $apoE^{-/-}$ and $LDLR^{-/-}$ mouse models of atherosclerosis [41].

In this study we have demonstrated that T-cad overexpression-dependent attenuation of UPR signalling is beneficial and results in the shift of the balance between UPR signalling branches in favour of prosurvival signalling, while T-cad silencing promotes apoptotic cell death. We have previously demonstrated that T-cad upregulation improves survival of EC during oxidative stress *via* concomitant activation of PI3-kinase/Akt signalling and inhibition of p38 MAPK signalling [29]. There is bi-directional cross-talk between oxidative and ER stress: protein folding and generation of ROS as a by-product of protein oxidation in the ER are closely linked events, and activation of the UPR upon exposure to oxidative stress is an adaptive mechanism to preserve cell function and survival [7]. Therefore, it was logical to consider that protective effects of T-cad against ER stress might be related to its ability to minimize ROS-induced damage [29]. However, we were unable to prevent the amplified ER stress-induced apoptosis of T-cad-silenced EC by treatment with ROS scavenger N-acetylcysteine. Inhibition of PI3-kinase did not eliminate the attenuating effects of T-cad overexpression on Grp78 and CHOP expression, supporting that T-cad limits ER stress in EC by mechanisms distinct from those (i.e. PI3-kinase/Akt signalling axis) mediating T-cad-dependent protection of EC from oxidative stress. Furthermore, JNK inhibitor failed to prevent the amplified ER stress-induced apoptosis of T-cad-silenced EC, indicating that anti-apoptotic effects of T-cad upregulation under conditions of ER stress are also unlikely to involve inactivation of ASK1 signalling (and by inference p38 MAPK [5,10]). On the other hand, we have clearly demonstrated T-cad-dependent modulation of phospho-eIF2α and CHOP which represent the proapoptotic PERK branch of the UPR cascade [4–10]. Thus, the protection afforded by T-cad against ER stress-induced apoptosis likely involves main ER stress pathways directly rather than modulation of related anti-stress signalling which converge with UPR.

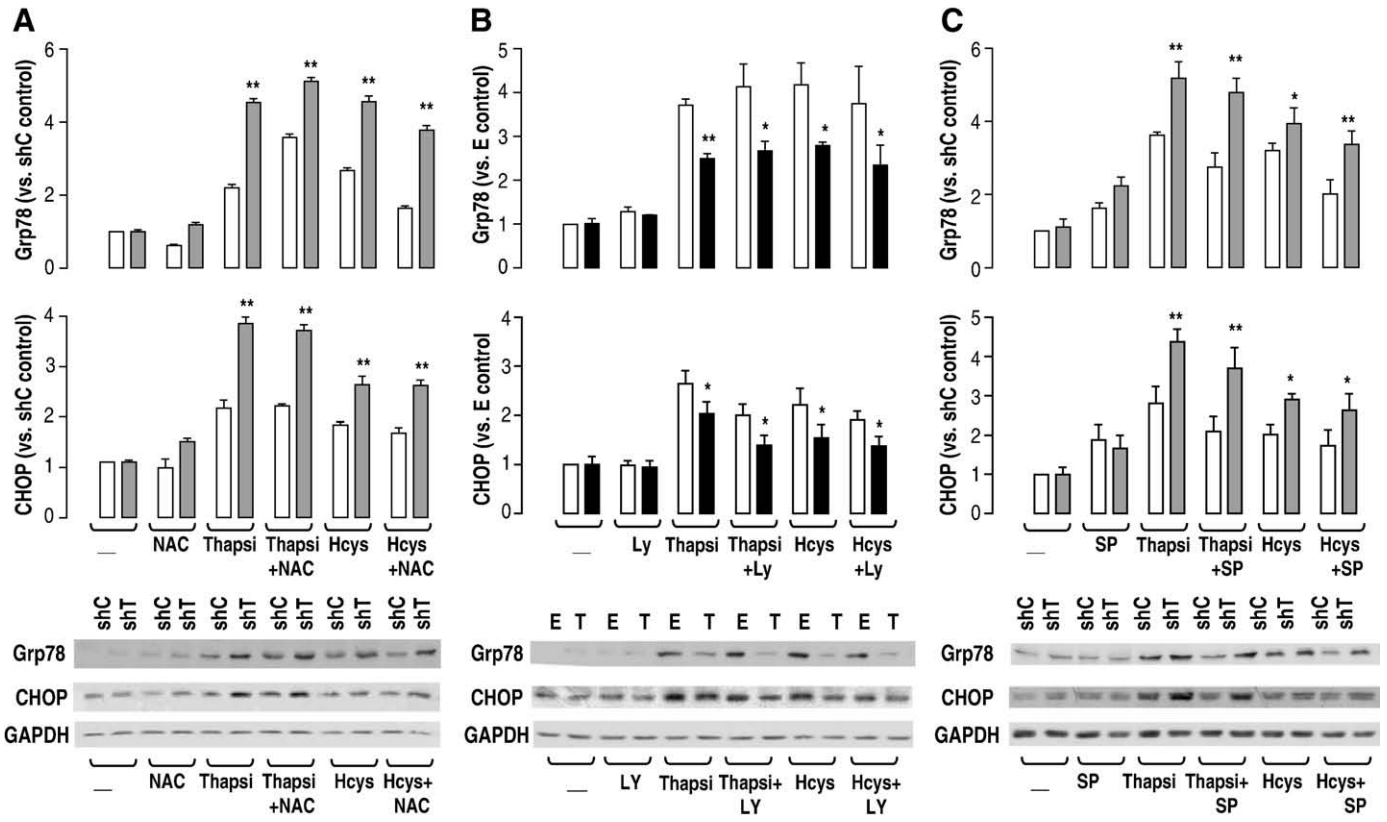

Fig. 6. T-cad dependent effects on ER stress response do not involve oxidative stress, PI3-kinase and JNK. HMEC-1 transduced with lentiviral vectors expressing T-cad-specific (shT, grey bars) or control (shC, open bars) shRNAs (A, C) or Empty- (E, open bars) and T-cad- (T, black bars) lentivector transduced HMEC-1 (B) were treated with thapsigargin (Thapsi) or homocysteine (Hcys) for 6 h in the absence or presence of ROS scavenger N-acetylcysteine (NAC; 15 mM), PI3-kinase inhibitor LY-294002 (LY; 10 μM) or JNK inhibitor SP6000125 (SP; 20 μM). Levels of Grp78 and CHOP protein were determined by immunoblot analysis of whole cell lysates. Changes are expressed relative to baseline levels (untreated) in the respective control-transduced HMEC-1. The histograms present data (mean ± SD) obtained from 3 independent experiments. Asterisks indicate significant differences between shC and shT or between E and T (*$P<0.05$, ** P at least <0.01).

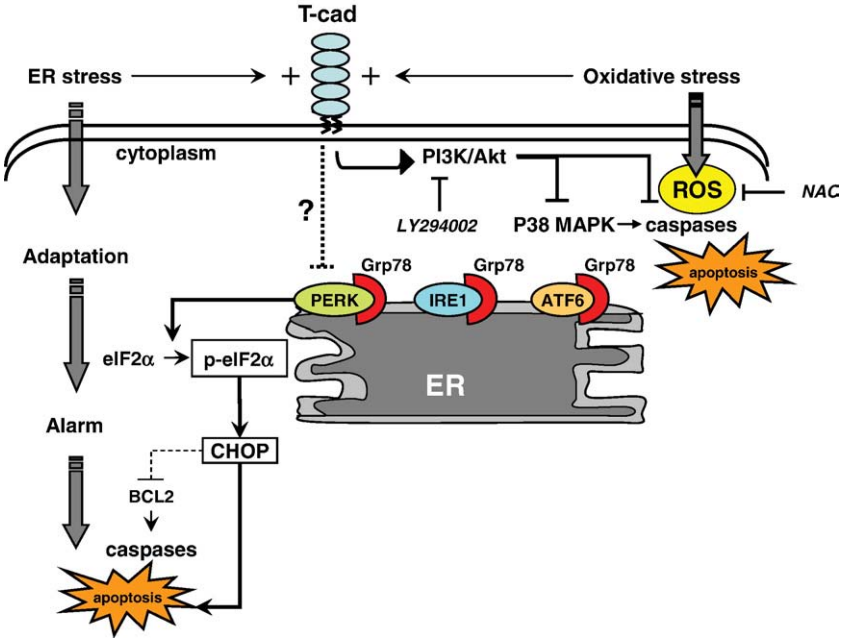

Fig. 7. Schematic diagram illustrating different mechanisms whereby upregulation of T-cad induced in response to cellular stress protects EC from apoptosis. In EC GPI-anchored T-cad is upregulated in response to ER stress and oxidative stress. The diagram shows the three major ER stress sensors (PERK, IRE1 and ATF6) which, for the sake of simplicity, are all depicted in association with Grp78. Our results suggest that T-cad protects against ER-stress-induced apoptosis by restricting the PERK arm of the UPR response, and in a manner that is insensitive to PI3-kinase inhibitor LY-294002 or ROS scavenger N-acetylcysteine. This is distinct from the PI3-kinase/Akt-dependent mechanism whereby T-cad protects EC from oxidative stress-induced apoptosis. How T-cad communicates signals to the ER stress machinery is not yet known.

The exact mechanisms linking T-cad to UPR mediators have yet to be elucidated. Of interest in view of the present data is our recent finding that T-cad associates with Grp78 in EC [31]. However, we detected colocalization not with ER-localized Grp78, which is a well-recognized trigger of UPR, but with its cell surface pool [31]. Surface Grp78 has been demonstrated to be present on the plasma membrane of several cell types including vascular endothelium [42,43]. Increased levels of surface Grp78 have been detected in different pathological conditions, for example on highly metastatic cancer cells [44], on endothelial and monocyte/macrophage-like cells in atherosclerotic lesions [42], and on procoagulant microparticles shed from the plasma membrane of activated EC [45]. When expressed on the cell surface, Grp78 is able to initiate various functional responses including inhibition of tissue factor procoagulant activity [42], mediation of signal transduction from activated α2-macroglobulin in peritoneal macrophages [44] and 1-LN prostate cancer cells [44,46,47], binding of dengue virus [48] and coxsackievirus [49], regulation of EC apoptosis induced by K5 kringle domain of plasminogen [43] and association with MHC class I molecules [50] and with Ro-52 antigen on mouse splenocytes presumably contributing to autoimmunity during rheumatoid arthritis [51]. There is also some evidence for participation of surface Grp78 in ER stress responses; in 1-LN cells ligation of activated α2-macroglobulin by cell-surface expressed Grp78 was associated with induction of UPR signalling [47]. Our study did not address the contribution of surface Grp78 to the protective effects of T-cad against adverse effects of ER stress. One might speculate that association of T-cad with surface Grp78 may either regulate downstream signalling or modulate localization or trafficking of Grp78 in the cell thus affecting intracellular levels of Grp78, which is the main sensor and trigger of the UPR.

5. Conclusions

The study reveals a novel regulatory mechanism for modulation of ER stress responses in the context of cardiovascular disease. We have demonstrated that upregulation of T-cad in EC occurs early following induction of ER stress, and that it functions to protect EC from adverse outcomes (e.g. apoptosis) caused by excess and chronic UPR. The mechanism of protection primarily involves an attenuation of signalling through the PERK arm of the UPR and is distinct from the mechanism whereby T-cad protects EC from oxidative stress-induced apoptosis [29] (Fig. 7). Upregulation of T-cad on the surface of vascular cells in atherosclerotic lesions may represent a protective mechanism for limiting tissue damage during cardiovascular disease progression.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cellsig.2010.04.008.

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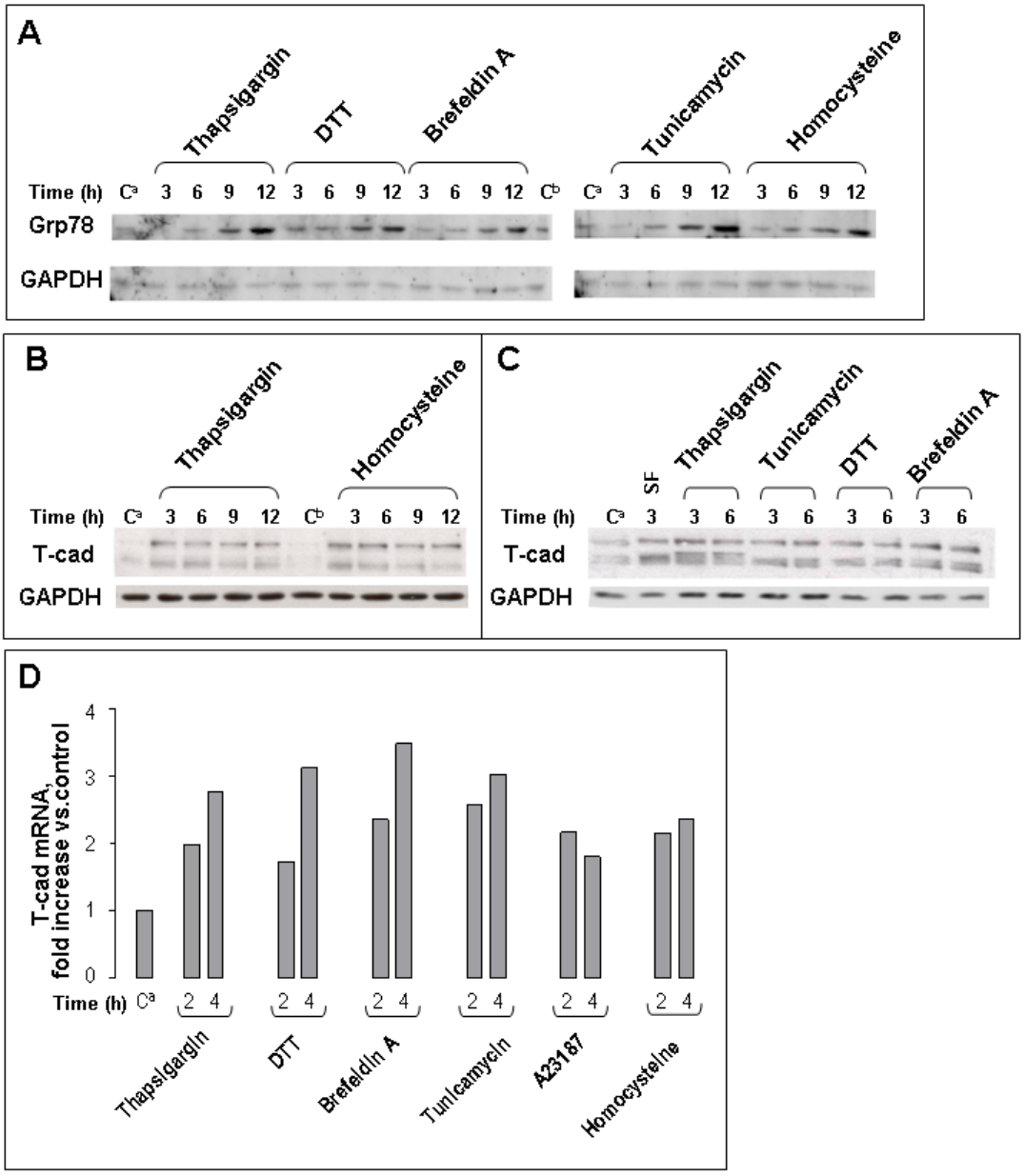
"T-cadherin attenuates the PERK branch of the unfolded protein response and protects vascular endothelial cells from endoplasmic reticulum stress-induced apoptosis"



Supplemental Figure S1. HUVEC were variously treated with thapsigargin (500 nM), DTT (1 mM), brefeldin A (5 µg/ml), tunicamycin (3 µg/ml), A23187 (2 µM), homocysteine (5 mM), or were incubated under serum-free conditions (SF). At the indicated time points HUVEC were processed for immunoblot analysis of Grp78 (A) or T-cad (B, C) with GAPDH as loading control (A-C), or for RT-PCR analysis of T-cad transcript levels (D). C^a and C^b specify control, untreated HUVEC processed at 0 h or 12 h, respectively. Data in A, B and C are from independently performed experiments. Data in D are given as the mean of triplicates in a single experiment.

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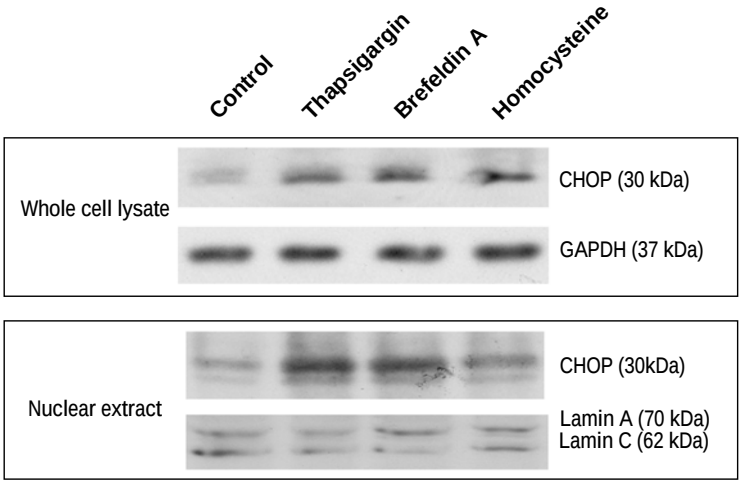


Supplemental Figure S2. HMEC-1 were incubated for 6 hours in the absence (control) or presence of thapsigargin (500 nM), brefeldin A (5 μg/ml) or homocysteine (5mM). Whole cell lysates (from cells in 6-well plates) or nuclear extracts (from cells in T-75 flasks) were subjected to immunoblot analysis for CHOP (using Santa Cruz monoclonal anti-CHOP antibodies; Sc-735). Blots were probed with anti-GAPDH or lamin A/C to control for equivalence of protein loading.

3. CONCLUSIONS AND FUTURE PERSPECTIVES

Endothelial activation and dysfunction play key roles in the pathogenesis of atherosclerosis. Inflammation and different types of stress may cause endothelial cells to become activated or to dysfunction. The present dissertation has addressed a number of issues related to atherosclerosis and endothelial dysfunction including:

A. The role of iNKT cells during the development of atherosclerosis and more specifically

i. whether iNKT cells infiltrate atherosclerotic plaques

ii. whether iNKT cell activation induces EC activation or/and dysfunction

B. The signalling mechanisms generated in EC in response to iNKT activation

i. which molecules expressed upon iNKT activation elicit EC activation

ii. which signalling cascade is responsible for EC migration and sprout outgrowth

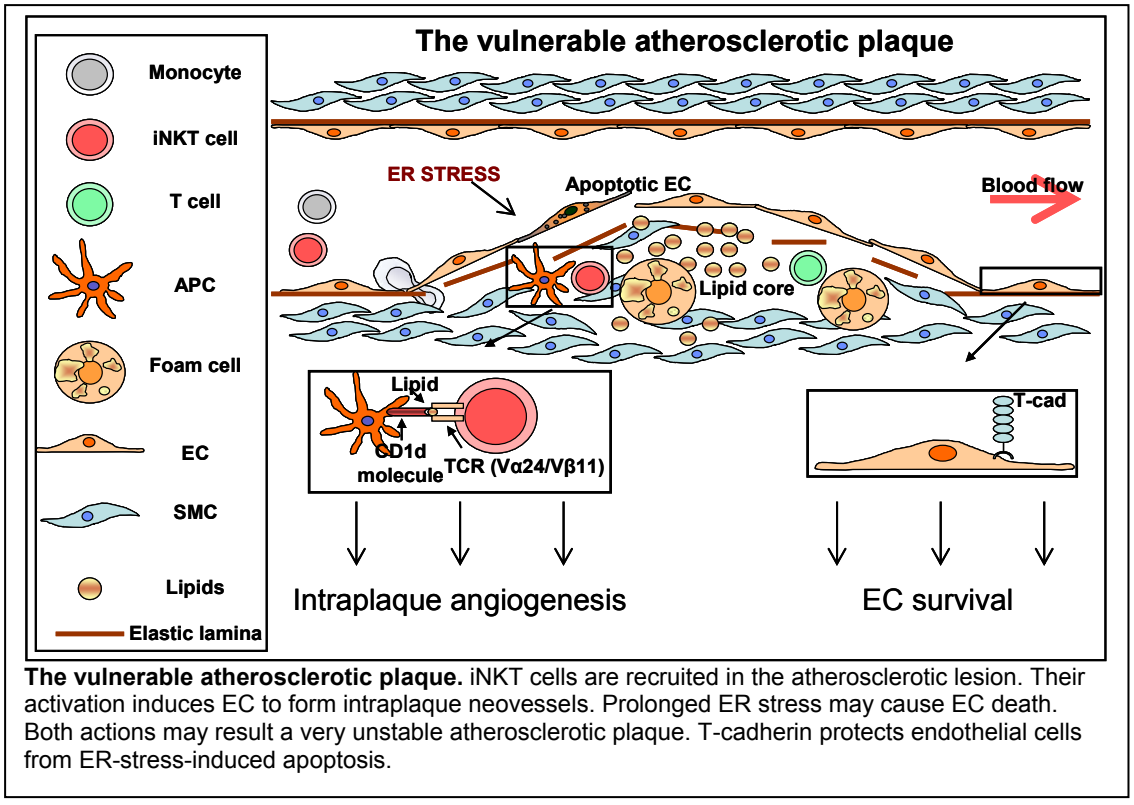
iii. whether pharmacological interventions may influence neovascularization

C. The role of T-cadherin during endoplasmic reticulum stress

i. whether T-cadherin is modulated under endoplasmic reticulum stress

ii. whether T-cadherin protects endothelial cells from apoptosis during ER stress

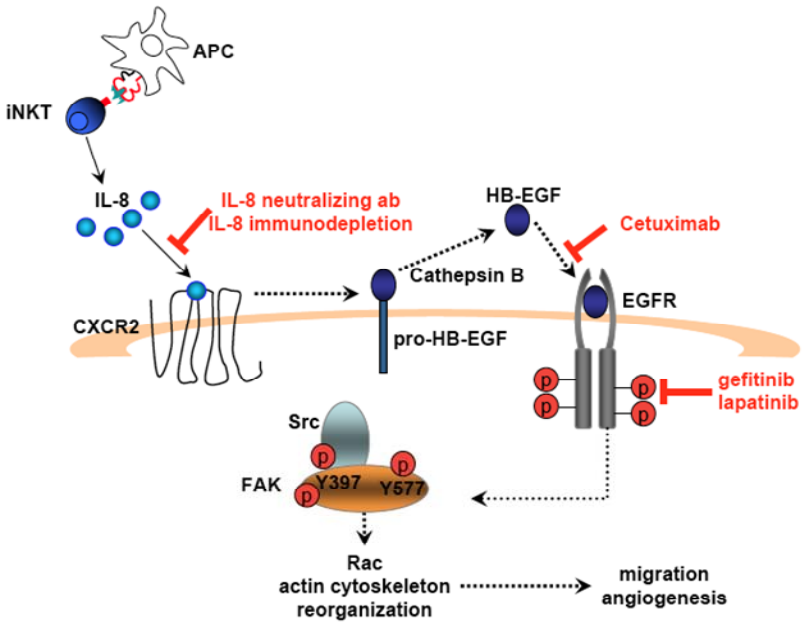
iii. which signalling pathways are involved for T-cadherin induced survival during ER stress



The vulnerable atherosclerotic plaque. iNKT cells are recruited in the atherosclerotic lesion. Their activation induces EC to form intraplaque neovessels. Prolonged ER stress may cause EC death. Both actions may result a very unstable atherosclerotic plaque. T-cadherin protects endothelial cells from ER-stress-induced apoptosis.

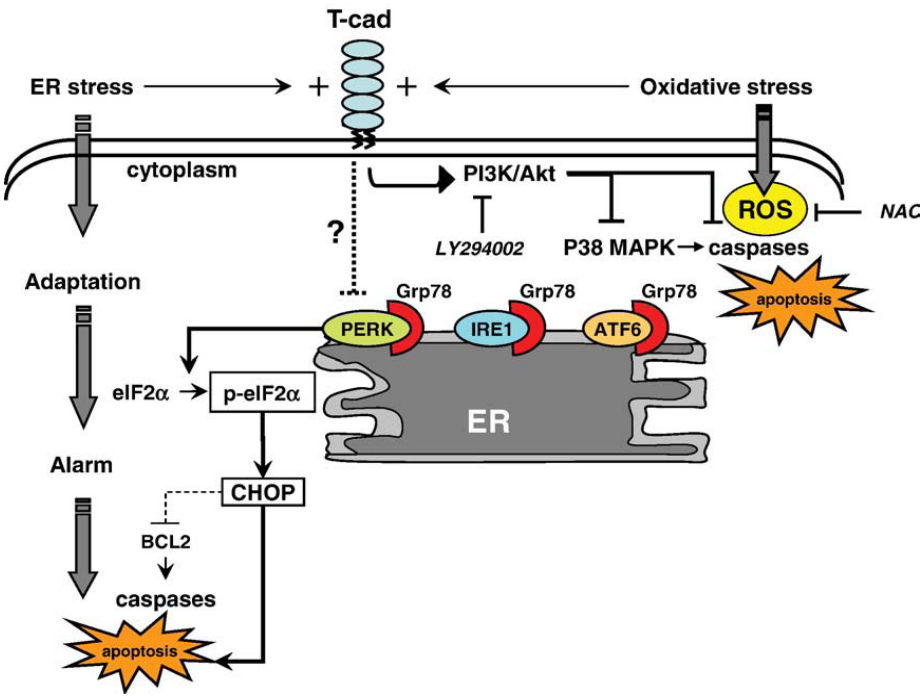
Key findings

In the present study we provide evidence for the presence of iNKT cells in atherosclerotic plaques and that plaque-isolated iNKT cells are more sensitive to antigen stimulation. In addition CD1d-expressing APCs are present in advanced, vascularized plaques from patients with active disease. Our *in vitro* data identified IL-8 as the main molecule, amongst the many cytokines and chemokines that are released upon activation of iNKT cells, mediating pro-migratory and pro-angiogenic responses in EC. A possible mechanism underlying intraplaque activation and angiogenic behaviour of EC in response to iNKT cell activation was then investigated. We were able to demonstrate that IL-8 released by antigen-loaded iNKT cells, activates the EGF receptor in EC. IL-8 is able to induce the activity of various matrix metalloproteinases, cathepsins or members of the ADAM proteases which cause shedding of EGFR ligands. We found that subsequent EGFR transactivation further activates FAK and Src, key regulators of cell migration and actin cytoskeleton re-organization. Thus the data support the notion that the angiogenic behaviour observed in EC in response to lipid-antigen activation of iNKT cells occurs via EGFR transactivation and therefore it represents a non-traditional proangiogenic receptor driving iNKT cell-associated neovascularization in atherosclerotic disease. The proposed hypothetical pathway is shown in the figure below.



Hypothetical mechanism involved in EC activation. Upon activation of iNKT cells by lipids, IL-8 is released among other chemical stimuli. EGF ligands are released and ECs are activated through EGFR, which in return transmits signals to the interior of the cell activating FAK and Src, important regulatory components for actin cytoskeleton reorganization and migration. EC obtain a more motile type of phenotype, migrate faster and eventually get organized to form sprouts.

The second study reveals a novel regulatory mechanism for modulation of ER stress responses in the context of cardiovascular disease. ER stress has attracted the attention of several studies the last years because of its association with cardiovascular diseases and the progression of atherosclerosis. We have demonstrated that upregulation of T-cad in EC occurs early following induction of ER stress, and that it functions to protect EC from adverse outcomes (e.g. apoptosis) caused by excess and chronic UPR. The mechanism of protection primarily involves an attenuation of signalling through the PERK arm of the UPR and is distinct from the mechanism whereby T-cad protects EC from oxidative stress-induced apoptosis. Upregulation of T-cad on the surface of vascular cells in atherosclerotic lesions may represent a protective mechanism for limiting tissue damage during cardiovascular disease progression. Below the schematic diagram illustrates the T-cadherin-associated mechanisms involved during ER stress and their distinction from the T-cadherin associated pathway operative during oxidative stress.



Signalling mechanism involved for the protection of vascular endothelial cells from endoplasmic reticulum stress-induced apoptosis by T-cad. The PERK branch of the UPR response is restricted by T-cad.

Future perspectives

Although we successfully elucidated the involvement of CD1d expressing APCs and CD1d-restricted iNKT cells in the progression of atherosclerosis and the importance of T-cadherin in EC survival during ER stress there are still a lot of issues to delineate.

The role of other stimuli released by iNKT cells while activated and their participation in signalling pathways.

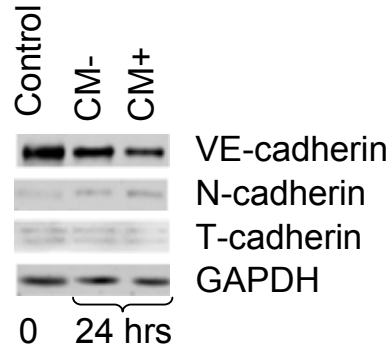
Analysis of an array of cytokines and chemokines using the Bioplex Multiplex system or by ELISA revealed that several cytokines and chemokines are found in the CM derived from activated iNKT cells apart from IL-8. It was found that IL-2, IL-4, IL-6, IL-13, INF-γ and TNF-α are present at quite high levels. All these molecules are either pro-inflammatory or/and potential angiogenic modulators. We already demonstrated that IL-8 is mainly responsible for the enhanced migration of EC and eventually for the sprout formation. However, we cannot rule out the possibility that there is an interplay or crosstalk between the other factors or that further signalling pathways are activated. Microarray technology could be a good approach for addressing the different pathways involved and the effect of the CM on EC under physiological conditions or under conditions of different disease stages.

Role of the VE-cadherin and N-cadherin on EC treated with CM.

Morphological responses also include changes of adherens junctions, the primary sites of mechanical coupling between ECs. VE-cadherin, which is restricted to the EC and is located at intercellular junctions, is responsible for controlling cell contacts. For example in epithelial cells E-cadherin is important for cell contact integrity. Delocalization of VE-cadherin from cell junctions, facilitates a mesencymal type of phenotype (Arciniegas et al., 2007). VE-cadherin downregulation is required for endothelial-to-mesenchymal transition and in analogy to epithelial-mesenchymal transition (EMT), N-cadherin upregulation serves as a marker of ongoing mesenchymal transition of EC. In the following table the most well studied markers for epithelial-mesenchymal transition markers are shown and can potentially serve as endothelial-mesenchymal transition markers.

Epithelial-mesenchymal transition markers (Lee et al., 2006)				
Increased proteins levels	**Decreased proteins levels**	**Increased protein activity**	**Nuclear protein accumulation**	**Functional markers**
N-cadherin	E-cadherin (VE-cadherin for endothelial cells)	ILK	β-catenin	Increased migration
Vimentin	Desmoplakin	GSK-3β	Smad-2/3	Increased invasion
Fibronectin	Cytokeratin	Rho	Snail1 and 2	Increased scattering
Snail1 and 2	Occludin		Twist	Elongation of cell shape
Sox10			NF-κβ	Resistance to anoikis
Integrin αvβ6				

EC in the presence of CM became elongated, resembling mesenchymal cells. Our data showed a downregulation of VE-cadherin in migrating cells and N-cadherin upregulation (Western blot analysis is shown below) suggesting a transition to a more mesenchymal phenotype when EC are exposed to CM+. T-cadherin was not altered under the specific experimental conditions. Endothelial-to-mesenchymal transition may contribute to neovascularisation and vascular remodelling.



Western blot analysis. VE-cadherin downregulation and concominant N-cadherin upregulation while treated with CM+ is shown. T-cad remained unaltered.

Further investigation would shed more light for the pathways involved.

Conditional knockdown of CD1d in atherosclerotic mice model.

We revealed the significance of CD1d and CD1d-restricted iNKT cells in respect to atherosclerosis and neovascularization in humans and *in* vitro. There are no known studies *in vivo* about the importance of iNKT cells on angiogenesis. Mice that are deficient in apolipoprotein E (apoE) develop atherosclerotic lesions resembling those observed in humans. The effect of iNKT cells in atherosclerosis has been studied in αGalCer treated apoE$^{-/-}$ and apoE$^{-/-}$CD1d$^{-/-}$ mice (Tupin et al., 2004). They demonstrated that activation of iNKT cells induces atherosclerosis. Aortic sections of those mouse models were made available to us, but the quality of the sections were not ideal for investigating any correlation, between neovascularization and progression of atherosclerosis.

The exact mechanism linking T-cadherin to UPR sensors has to be elucidated

T-cadherin colocalizes and associates functionally with surface expressed Grp78 (Philippova et al., 2008). Since dissociation of Grp78 from the UPR sensors is needed to initiate the UPR and recover protein homeostasis it would be of interest to examine whether T-cadherin may modulate localization and trafficking of Grp78, thus regulating the initiation and progression of UPR. One approach to address these questions is to use RNAi for directed silencing of Grp78. A second approach would be the use of pharmacological inhibition of Grp78, by which we can distinguish between surface and endoplasmic reticulum localization of Grp78. Both of these approaches would be applied to our gain- and loss-of function models.

Conditional knock-out and knock-in on T-cadherin in endothelium in mice.

The mouse is the most commonly used mammal for genetic manipulation. A conditional knock-out or knock-in mouse on T-cadherin in endothelium could be a valuable tool for investigating the T-cadherin effects *in vivo*. It is known that T-cadherin may get activated by homophilic ligation, but whether homophilic ligation occurs *in* vivo and what is the biological function remains elusive. A double knock-out mouse model for T-cadherin and apoE would be of great interest for investigating the pro-angiogenic and pro-survival properties of T-cadherin during atherosclerosis. Endothelial specific T-cadherin transgenic mice could be exploited to address issues regarding ER stress in human diseases. Mouse models for monitoring ER stress exist (Iwawaki et al., 2004) and can be used to cross it with the T-cadherin transgenic mouse, thus creating a powerful *in vivo* system.

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5. CURRICULUM VITAE

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Summary:

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Specialties:

- The study of cellular signalling pathways in mammalian systems in physiological and pathophysiological conditions (cardiovascular diseases, cancer, diabetes)
- The study of neovascularization *ex vivo* (IC imaging of organs from mouse models) and *in vitro* (cell cultures, 3D models, co-cultures)
- Experienced on synthesizing bioinorganic vanadium compounds as insulin mimetics. Synthetic, spectroscopic and structural studies.

Publications

1. Pfaff, D., Phillipova, M., **Kyriakakis, E.,** Joshi, M.B., Erne, P., Resink, T.J. T-cadherin in squamous cell carcinoma (SCC): The double-edged sword. (in preparation).

2. **Kyriakakis, E**., Cavallari, M., Fabbro, D., Mestan, J., Phillipova, M., De Libero, G., Erne, P., Resink, T.J. IL-8 mediated angiogenic responses of endothelial cells to lipid-antigen activation of iNKT cells depend on EGFR transactivation. **Biochem J** (submitted).

3. **Kyriakakis, E.,** Cavallari, M., Andert, J., Phillipova, M., Koella, C., Bochkov, V., Erne, P., Wilson, B.S., Mori, L., Biedermann, B.C., Resink, T.J., and De Libero, G. (2009).Invariant NKT cells: Linking inflammation and neovascularization in human atherosclerosis. **European J. Immunology.** 2010, DOI:10.1002/eji.201040619

4. Phillipova, M., Suter, Y., Toggweiler, S., Schoenenberger, A.W., Joshi, M.B., **Kyriakakis, E.**, Erne, P., Resink, T.J: T-cadherin is present on endothelial microparticles and is elevated in plasma in early atherosclerosis. **European Heart Journal.** 2010, DOI: ehq206 [pii] 10.1093/eurheartj/ehq206

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11. Kaliva, M., **Kyriakakis, E.,** Gabriel, C., Raptopoulou, C. P., Terzis, A., Tuchagues, J.P., Salifoglou, A. Synthesis, isolation, spectroscopic, and structural characterization of a new pH complex structural variant from the aqueous vanadium(V)-peroxo-citrate ternary system. **Inorg. Chim. Acta**, 2006, 359, 14, 4535-4548

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Conferences

Oral presentations

1. The protective effect of T-cadherin on vascular endothelial cells under endoplasmic reticulum stress. ***European Society of Cardiology****, Barcelona, Spain, August 29- September 2, 2009*

2. The role of T-cadherin during endoplasmic reticulum stress in endothelial cells.
14th Cardiovascular Biology and Clinical Implications Meeting*, Muntelier-Switzerland, 2-3 October 2008*

3. CD1d-restricted human NKT cell activation; a novel pro-angiogenic mechanism.
13th Cardiovascular Biology and Clinical Implications Meeting*, Muntelier-Switzerland, 4-5 October 2007*

4. iNKT cell dependent angiogenesis.
Annual Meeting of the Society for Microcirculation and Vascular Biology and 6th International Symposium on the Biology of Endothelial Cells*, Heidelberg, Germany, October 4-6, 2007*

5. Lipids in inflammation and atherosclerosis: Effects of CD1d-restricted human NKT cell activation on angiogenesis.
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Poster Presentations

1. T-cadherin prevents apoptosis in human vascular cells during endoplasmic reticulum stress.
Kyriakakis, E., Joshi, M.B., Phillipova, M., Bochkov, V., Erne, P., Resink, T.J. ***15th Cardiovascular Biology & Clinical Implications Meeting****, Muntelier, Switzerland. 1-2 October 2009*

2. Adhesion molecule T-cadherin is elevated in plasma at early stages of atherosclerosis and activates prosurvival signalling in endothelial cells.
Kyriakakis, E., Phillipova, M., Joshi, M.B., Phillipova, M., Erne, P., Resink, T.J. ***European Society of Cardiology****, Barcelona, Spain, August 29- September 2, 2009*

3. T-cadherin prevents apoptosis following endoplasmic reticulum stress in human endothelial cells.
Kyriakakis, E., Joshi, M.B., Phillipova, M., Bochkov, V., Erne, P., Resink, T.J. ***34th FEBS Congress****, Prague, Chech Republic, July 4-9, 2009*

4. iNKT cells and plaque vulnerability: the missing link
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5. Endoplasmic reticulum stress and the role of T-cadherin
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6. CD1d-restricted NKT cell activation: a novel mechanism for neovascularisation in atherosclerosis.
   Kyriakakis, E.., Phillipova, M., Cavallari, M., De Libero, G., Erne, P., Resink, T.J. ***Annual Meeting of the Swiss Cardiovascular and Reseach Training Network****, Bern-Switzerland April 24-25, 2008*

7. Oxidative stress-induced T-cad expression is regulated by thioredoxin TRX1
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8. Activation of CD1d-restricted human NKT cells stimulates angiogenesis *in vitro*.
   Kyriakakis, E.., Phillipova, M., Cavallari, M., De Libero, G., Erne, P., Resink, T.J. ***12th Cardiovascular Biology & Clinical Implications Meeting****, Muntelier, Switzerland, 5-6 October 2006,*

9. Interconversions between dinuclear vanadium (IV, V)-citrate complexes in aqueous solutions.
   Kyriakakis, E., Kaliva, M., Salifoglou, A. ***16th Pan-Hellenic Conference on Chemistry****, Heraklion, Greece 28-30 June 2002*

10. Closer look into interconnections between dinuclear vanadium (IV, V)-citrate complexes in aqueous solutions
    Kyriakakis, E., Kaliva, M., Salifoglou, A. ***8th Pan-Hellenic Conference of Postgraduate students on Chemistry****, Anogeia, Greece 28-30 June 2002*