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ROLE OF INTERLEUKIN-18 IN VASCULAR INJURY: A NEW PHARMACOLOGICAL TARGET FOR THE PREVENTION OF RESTENOSIS

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ABSTRACT

Background: Studies in humans as well as in animal models suggest that interleukin-18 (IL-18) plays a crucial role in vascular pathologies. IL-18 is a strong predictor of cardiovascular death in angina and is involved in atherotic plaque destabilization. Higher IL-18 plasma levels are also associated with restenosis after coronary artery angioplasty performed in patients with acute myocardial infarction. We investigated the effective role of IL-18 in neointima formation in a rat model of vascular injury, known as balloon angioplasty.

Methods and Results: Endothelial denudation of the left carotid artery was performed by using a balloon embolectomy catheter. Increased expression of IL-18 and IL-18Rα/β mRNA was detectable in carotid arteries from day 2 to 14 after angioplasty. The active form of IL-18 was highly expressed in injured arteries. Strong immunoreactivity for IL-18 was detected in the medial smooth muscle cells at day 2 and 7 after balloon injury and in proliferating/migrating smooth muscle cells in neointima at day 14. Moreover, serum concentrations of IL-18 were

significantly higher among rats subjected to vascular injury. Rats treatment with neutralizing rabbit anti-rat IL-18 IgG significantly reduced by 27% (P<0.01) neointima formation. In addition, IL-18 neutralization reduced number of proliferating cells, inhibited IFN-γ, IL-6, IL-8 mRNA expression and nuclear factor-κB activation in injured arteries.

Conclusions: These results identify for the first time a critical role for IL-18 in neointima formation in a rat model of vascular injury and suggest a potential role for IL-18 neutralization in reduction of neointima development and progression.

1. INTRODUCTION

Vascular disease constitutes a major cause of death and disability in developed countries and will soon become a health threat worldwide. This trend motivates major efforts on multiple fronts to fight cardiovascular disease, with the goals of prevention as well as improved therapy. One prerequisite for success in this quest is increased understanding of the very dynamic environment represented by the vascular wall, where several cell types interact and undergo profound phenotypic modulation in development and in diseases such as restenosis.

Vascular smooth muscle cells (VSMCs), the major constituent of the normal vessel wall, play a pivotal role. In a traditional view, VSMCs were considered as differentiated, quiescent cells dedicated to vasomotor function. However, VSMCs are now considered to display multiple functions including regulation of extracellular matrix (ECM) composition as well as producers and targets for growth factors and pro-inflammatory cytokines, all important factors in the development of vascular diseases [1]. Activation of VSMCs results in the production of different cytokines,

which are involved in the regulation of several functions in vascular inflammation that include both innate and acquired immune responses [2,3,4].

Interleukin-18 (IL-18) is a nonglycosylated polypeptide member of the IL-1 superfamily [5]. Studies in humans as well as in animal models have suggested that this cytokine plays a crucial role in cardiovascular pathologies. Increased IL-18 expression has been reported in human atherosclerotic plaque [6]. Furthermore, animal models support the proatherogenic role of IL-18 [7] as well as the beneficial effect of inhibiting IL-18 on plaque progression and composition [8].

To date, however, the expression and function of IL-18 in neointima formation has not been investigated. This is of particular relevance, because it is well established that long-term failure of arterial stenting is due to neointimal formation [9].

1.1 Restenosis

An angioplasty is a safe and effective way to unblock coronary arteries. During this procedure, a catheter is inserted into the groin or arm of the patient and guided forward through the aorta and into the coronary arteries of the heart. There, blocked arteries can be opened with a balloon

positioned at the tip of the catheter (fig. 1). There are, however, limitations associated with angioplasty, one of which is called “restenosis” [1].

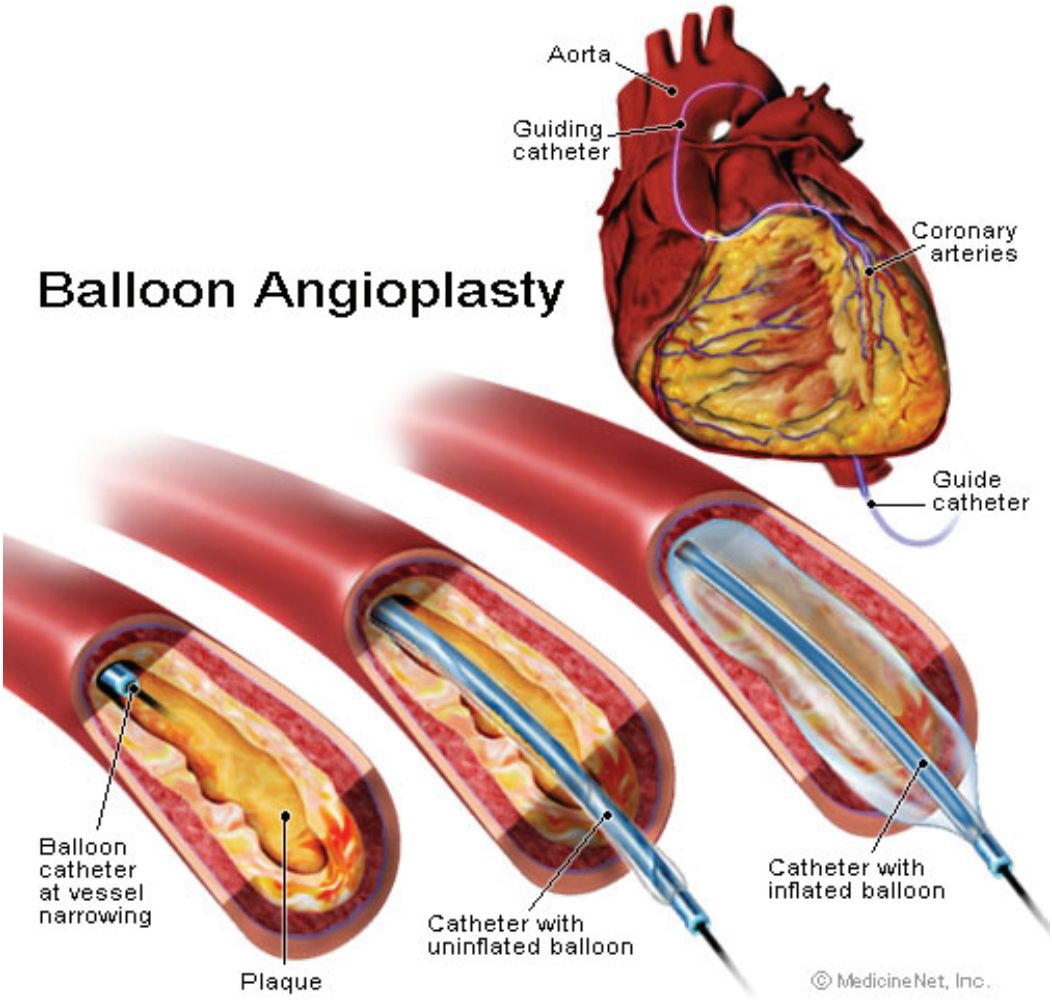

Figure 1. Balloon Angioplasty

Since the first reports of successful angioplasty of human coronary atherosclerotic lesions, restenosis has been encountered as a significant

limitation to the long-term efficacy of the procedure. In their 1979 landmark publication "Non-Operative dilatation of Coronary-Artery Stenosis," Gruntzig et al [10] reported that 6 of 32 patients undergoing successful initial angioplasty suffered restenosis, a rate of 19%.

Initially, angioplasty was performed only with balloon catheters, but technical advances have been made and improved patient outcome has been achieved with the placement of small metallic spring-like devices called "stents" at the site of the blockage. The implanted stent serves as a scaffold that keeps the artery open. Angioplasty and stenting techniques are widely used around the world and provide an alternative option to medical therapy and bypass surgery for improving blood flow to the heart muscle.

Restenosis occurs when the treated vessel becomes blocked again. It usually occurs within 6 months after the initial procedure [11]. Compared with balloon angioplasty alone, where the chance of restenosis is 40%, stents reduce the chance of restenosis to 25% [12]. Therefore, the majority of patients having angioplasty today are treated with stents. Restenosis can occur after the use of stents, and physicians refer to this as "in-stent restenosis".

1.1.1 Normal artery

Arteries are made up of three distinct layers: tunica intima, tunica media and tunica externa (fig. 2). Tunica intima forms the innermost layer (luminal surface) lined by endothelial cells. Tunica media is the thickest layer consisting of elastic fibers and smooth muscle cells. A thin layer of elastin separates the tunica intima and tunica media. Tunica externa consists of elastic connective collagen fibers.

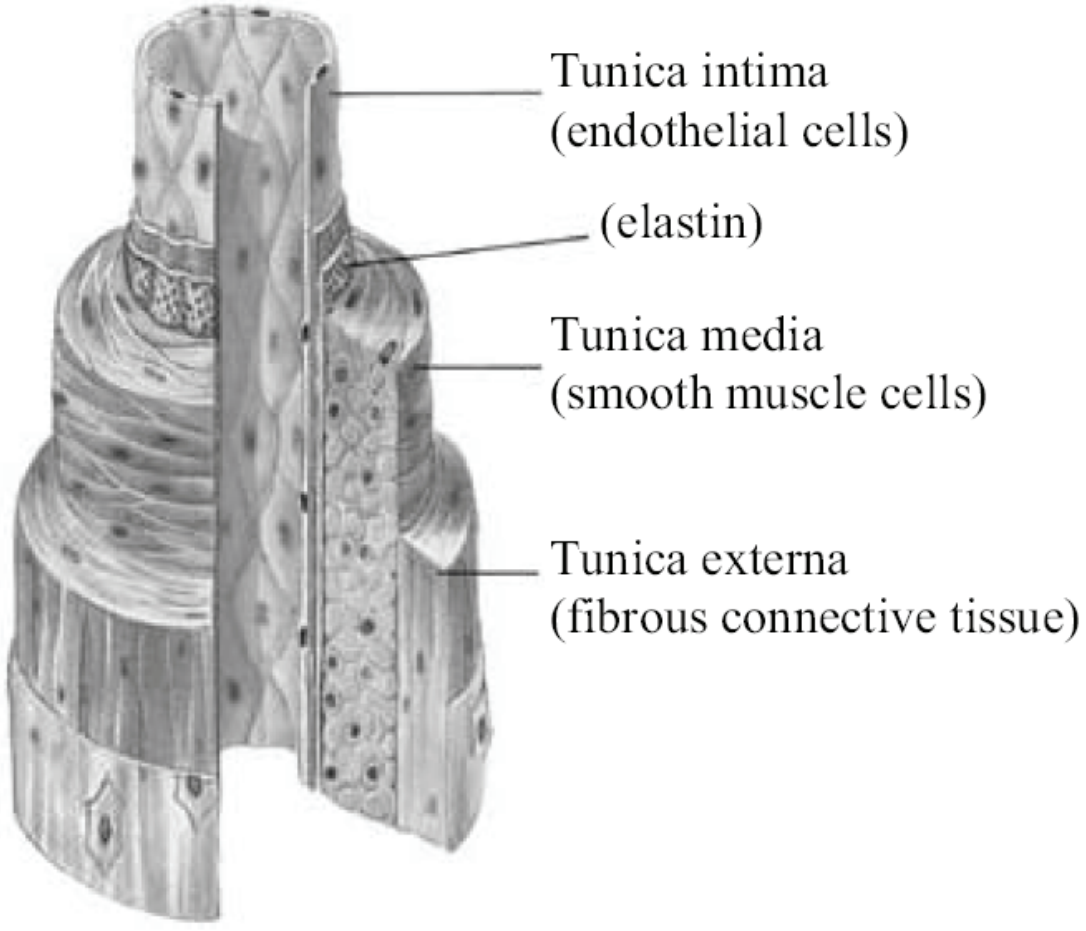

Figure 2. Structure of an artery.
The structure of arteries is briefly described consisting of three distinct layers of tunica containing endothelial cells, smooth muscle cells and connective tissue.

1.1.2 Pathogenesis of Restenosis

Restenosis is a hyperplastic, pathologic reaction involving SMC migration and proliferation, ECM formation and remodelling of the arterial wall leading finally to reocclusion of the enlarged artery (fig. 3) [13]. Similarly to early, fibrous atheromas, human restenotic lesions consist mainly of fibrocellular tissue [14].

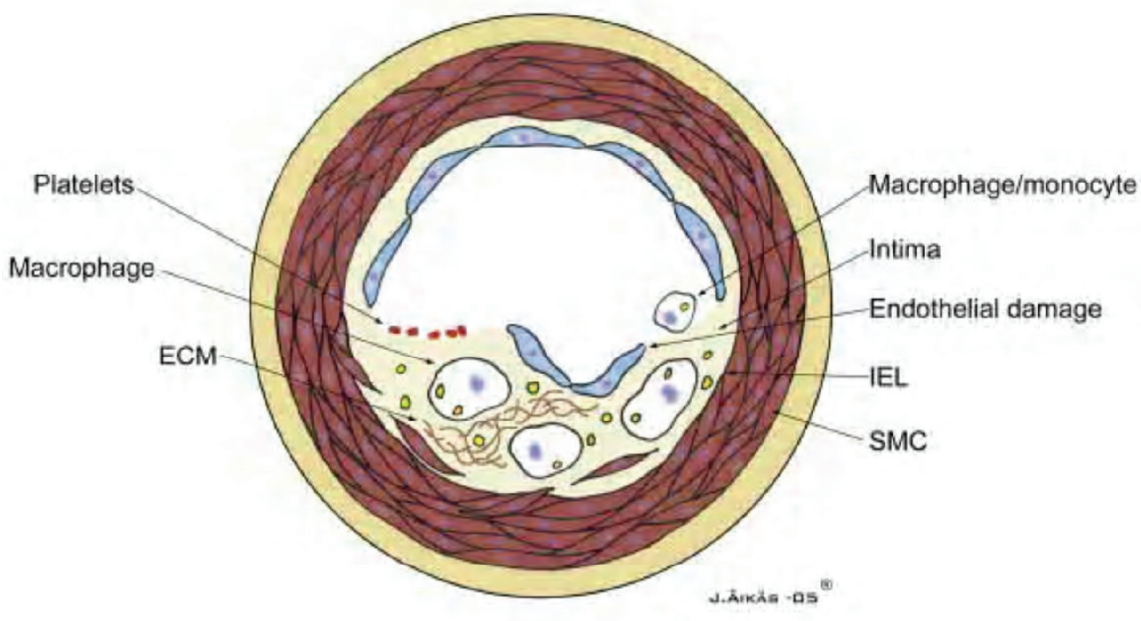

Figure 3. Pathogenesis of restenosis.
The balloon denudation damage the endothelia. Neointima proliferation can be seen before the thrombus is absorbed. SMCs start to migrate toward the intima-layer from the media-layer. Also, the cell infiltration from the damaged endothelia initiates the inflammation process. Internal elastic lamina (IEL), smooth muscle cell (SMC), extracellular matrix (ECM).

The pathologic reaction of the arterial wall can be divided into four phases: (1) a mechanical phase (early elastic recoil in response to the mechanical dilatation of the vessel), (2) a thrombotic phase, (3) a proliferative phase (neointima formation by proliferation of SMC) and (4) a remodelling phase (differentiation of SMC to a synthetic phenotype and ECM deposition).

Table 1 summarizes the molecular mechanism involved in the development of restenosis and its regulators. The relative contribution of each of these depends on the type of injury.

Table 1. Molecular mechanisms of restenosis and their regulators.

PHASE	MOLECULAR MECHANISM	REGULATORS
Mechanical	Elastic recoil	No molecular regulation
Thrombogenic	Adherence and activation of platelets	Cytokines, VEGF, NO, thrombin, blood flow
	Recruitment of inflammatory cells: expression of adhesion molecules (P-selectin, ICAM) and chemotactic factors (IL-8, MCP-1)	Cytokines (IL-1, IL-6, TNF-α), growth factors (PDGF, thrombin)
Proliferative	SMC migration and proliferation: production of MMPs and growth factors (PDGF, TGFβ, IGF, FGF, VEGF, thrombin, ATII)	Cytokines (IL-1, IL-6, TNF-α, IFN-γ), NO
Remodeling	Remodeling (MMPs) and ECM deposition	Cytokines, growth factors (PDGF, TGFβ, IGF, VEGF)

Within minutes following balloon deflation, the artery undergoes elastic recoil due to contraction of the elastic fibers of the inner and external lamina, causing up to a 40% lumen loss. A thrombotic response triggered by endothelial denudation, and medial dissection due to the mechanical injury of the angioplasty procedure, lead to platelet adherence and aggregation on the exposed subendothelial surface. Neointimal formation is complicated process involving the recruitment of inflammatory cells to the site of injury, the migration of vascular SMC from the media to the intima, and the proliferation of these cells. Growth factors and cytokines are the major stimuli for proliferation of SMCs after the injury [15]. The earliest step in the process of in-stent restenosis, before SMC proliferation, is platelet deposition and aggregation [16]. Platelets release multiple growth and migratory-promoting factors in addition to those released from injured vascular cells and surrounding extracellular matrix such as thrombin, platelet-derived growth factor (PDGF), interleukin (IL)-1, insulin-like growth factor-1 (IGF-1), fibroblast growth factor-2 (FGF-2), vascular endothelial cell growth factor (VEGF), and others [17-19]. This complex interplay of growth factors then regulate SMC migration and proliferation through cell surface receptors and intracellular

signaling molecules inducing early response genes necessary for cells to leave their quiescent state and enter the cell cycle [20]. The dynamic process of SMC migration involves changes in matrix synthesis such as degradation and organization [21]. Matrix metalloproteinases, effectors of extracellular matrix degradation [22] are upregulated after injury [21] and the degradation of the extracellular matrix allows SMCs to migrate to the intima.

In this maladaptative response of the artery to injury, the inflammation plays a pivotal role. Angioplasty causes adhesion of inflammatory cells at the injury site and their migration into the artery wall and the insertion of a foreign body, such as a stent, further increases the inflammatory response [23,24]. Local inflammation and arterial injury after stent deployment and adjunctive balloon angioplasty augments neointimal growth of cells [25,26]. Nuclear factor-κB (NF-κB) is a transactivator of a diverse group of genes whose activation has been strongly associated with the cellular response to inflammation. Landry et al., identified that the activation of NF-κB is linked to the inflammatory response associated with neointima formation after vascular injury [27].

1.1.3 Vascular smooth muscle cells

Vascular smooth muscle cells are among the most plastic of all cells in their ability to respond to different stimuli. Specifically, VSMC may proliferate (hyperplasia with an increase in cell number), hypertrophy (an increase in cell size without change in DNA content), endoreduplicate (an increase in DNA content and usually size), and undergo apoptosis. Among the mechanisms utilized by VSMC to mediate these varying cellular responses are autocrine and paracrine growth pathways. An autocrine growth mechanism is one in which the individual cell, in response to a growth factor, synthesizes and/or secretes a substance that stimulates that same cell type to undergo a growth response. A paracrine growth mechanism is one in which the individual cells responding to the growth factor synthesize and/or secrete a substance that stimulates neighboring cells of another cell type. In many situations, autocrine and paracrine growth mechanisms occur simultaneously [28].

The concept of VSMC auto/paracrine growth was first proposed in the late 1970s as a result of work in the laboratories of Gospodarowicz et al. [29], Harker and Ross [30], Castellot and co-workers [31], and Chamley-Campbell et al. [32]. Dzau [33] and Nilsson et al. [34] were the first to use the term *autocrine growth* to describe increased expression of VSMC growth factors by

VSMC. It has now become clear that almost all VSMC growth factors elicit auto/paracrine growth pathways. However, recent data indicate that many other stimuli that modulate VSMC function including extracellular matrix, biomechanical forces, reactive oxygen species (ROS), lipids, and other proteins alter VSMC growth by inducing auto/paracrine growth mechanisms.

Although many investigators assume that smooth muscle cells in the vessel wall are morphologically similar, it has become clear that they are phenotypically and functionally heterogeneous, which has obvious consequences for responses to various growth factors. A basic question is whether this is due to differences in origin or to spatiotemporal heterogeneity in expression of differentiation markers due to local environmental and hormonal factors. Both developmental and environmental factors influence VSMC heterogeneity.

It is important to note that while the medial layer of the vessel is highly enriched in VSMC, other cell types may coexist in this layer. This has important implications since migration and growth of medial cells to form a neointima is an important pathological process in atherosclerosis and restenosis. By implication, not all cells that are present in the neointima may be VSMC. For example, Frid et al. [35] were able to isolate at least four

phenotypically unique cell subpopulations from the inner, middle, and outer compartments of the arterial media. Differences in cell phenotype were demonstrated by morphological appearance and by differential expression of muscle-specific proteins. The isolated cell subpopulations exhibited markedly different growth capabilities. Two SMC subpopulations grew slowly in 10% serum and were quiescent in plasma-based medium. The other two cell subpopulations, exhibiting nonmuscle characteristics, grew rapidly in 10% serum and proliferated in plasma-based medium. These differences in growth were subsequently related to production of autocrine growth factors [36]. Similar VSMC heterogeneity was observed for human VSMC [37]. Two morphological phenotypes of VSMC are usually defined, namely, the epithelioid and the spindle-shaped cell [38]. Functionally these phenotypes have been suggested to correlate with the synthetic and contractile cell types, respectively [32]. Contractile VSMC express high levels of contractile proteins including myosin and low levels of α-actin. In contrast, synthetic VSMC express high levels of α-actin, extracellular matrix proteins, and low levels of myosin. In general, the spindle-shaped, contractile VSMC are not proliferating or migrating, whereas the epitheliod, synthetic VSMC have entered the cell cycle and are proliferating.

Based on techniques used for cell isolation and growth, there may be enrichment of particular subpopulations of VSMC that may explain some of the different results that have been reported for in vitro studies of autocrine growth mechanisms. With the identification of genes whose expression is specific for VSMC (thereby enabling localization in situ by mRNA or protein detection), it has become clear that upon development of intimal thickening (e.g., during atherosclerosis, restenosis, or closure of the ductus arteriosus), there is re-expression of fetal genes. These findings suggest that there is significant plasticity in VSMC function. There may also be embryonic cells ("progenitors") left from development [39] similar to those isolated from fetal animals. For example, Majesky et al. [40] have shown that proliferating smooth muscle cells isolated from the aorta express unique cytochrome *P*-450 enzymes that are typical of embryonic smooth muscle cells. Also, the myofibroblast has been proposed to transdifferentiate into an endothelial-like cell as well as into synthetic phenotype VMSC during intimal thickening [41]. Finally, there is increasing evidence that differentiated cells can transdifferentiate in other cell types [42].

Heterogeneity within the vessel wall may be related to alterations in the local environment. Variations in the hemodynamic environments may modify local gradients in substances (e.g., increased residence time of

lipids) or local metabolic requirements (e.g., increased energy metabolism or altered cytoskeleton arrangements) [43]. The normal blood flow pattern may be described as pulsatile and laminar. This ensures that fluid shear stress (the dragging frictional force of blood on the vessel luminal surface) is maintained within the narrow range of 10–20 dyn/cm^2. When the blood flow pattern is no longer laminar, it may be described as turbulent, and as a consequence pulsatility may be lost resulting in oscillatory flow patterns. Intimal proliferation occurs most commonly in these areas of turbulent and oscillatory flow such as the human carotid bulb. One explanation for intimal proliferation at these sites is related to alterations in EC-derived factors; specifically, there may be a decrease in factors that inhibit VSMC growth and an increase in factors that stimulate VSMC growth [44]. Variations in physical forces at a particular site as a consequence of vessel architecture and flow pattern may modulate VSMC function [45]. While fluid shear stress is likely to be the major force that influences EC function, mechanical strain may be more important for VSMC. Changes in mechanical strain have been shown to induce many VSMC growth factors including PDGF, bFGF, IGF-I, and TGF-β [46-49]. In addition, mechanical strain may make VSMC more sensitive to the mitogenic actions of other factors [50].

Hyperplasia refers to an increase in VSMC cell number associated with DNA synthesis. Entry of VSMC into the cell cycle and proliferation appears to be governed by many of the same mechanisms common to all cells. Hyperplasia is an important component of hypertension as shown by a significant increase in smooth muscle cell proliferation rate and the number of cell layers in the media of vessels from animals with chronic hypertension [51]. It should be noted that hyperplasia is characteristic of intermediate and large arterioles, whereas small vessels undergo remodeling. Hyperplasia also occurs in many other vascular diseases including atherosclerosis, restenosis, and the response to vascular injury. Hyperplasia is a slow process in chronic human hypertension. In summary, VSMC proliferation is a common response to mechanical stress and injury.

Perhaps the best studied situation in which hyperplasia of VSMC occurs is after injury to the blood vessel. While the rat carotid balloon injury model has been investigated extensively for many years [52], the pattern of events that leads to vessel repair and intimal thickening appears similar in other species (pig, mouse, nonhuman primate, and human) and other arteries (aorta, iliac, femoral, and brachial). Many candidate molecules that regulate VSMC growth have been studied in the rat carotid injury model by use of pharmacological and gene therapy approaches.

Results suggest important roles for the renin-angiotensin system, catecholamines, ET-1, natriuretic peptides, thrombin, PDGF, TGF-β and other activins [53], fibroblast growth factor (FGF), and oxidative stress among other stimuli [54]. Recent results provide further support for these molecules as regulators of VSMC growth after injury as well as IL-1 (17.5 kDa) and IL-6 (20.5 kDa) [55,56]. Both IL-1 and IL-6 have been reported to have autocrine growth effects on VSMC. Cellular effects of interleukins are also regulated by levels of endogenous inhibitors of the IL-1 receptor and by processing of the IL-1 precursor to mature hormone. The growth effects of the interleukins are somewhat controversial because other investigators have observed that IL-1 inhibited VSMC growth [57]. However, cell lines constitutively expressing IL-1α precursor demonstrated metabolism to the mature peptide and increased growth [58]. Levels of IL-1 are regulated primarily by inflammatory cytokines such as TNF-α, which induces IL-1 mRNA in human endothelial cells and VSMC [59]. IL-1 can also induce its own expression [58] and is upregulated by TGF-β and by hypoxia [60]. Recently, the mechanism by which IL-1β is produced by VSMC has been elucidated [61]. VSMC express the IL-1β precursor upon stimulation and the IL-1β-converting enzyme (ICE) constitutively, but do not produce mature IL-1β or express ICE activity.

IL-1 has been reported to stimulate expression of PDGF-A chain [56], bFGF [62], and IL-1 itself, while IL-6 induces PDGF-A chain [55]. Other autocrine factors induced by IL-1 and IL-6 remain to be identified. In vivo, both IL-1 and IL-6 would be anticipated to show increased expression in atherosclerosis and in injured vessels. The strongest data for a role of IL-1 in neointima formation is the differential but concomitant expression of IL-1 system components after balloon angioplasty [63]. Cytokines such as IL-1 and TNF-α have been proposed as primary mediators of the inflammatory component of atherosclerosis [59] and can regulate the production of MCP-1, a potential signal for directed migration of monocytes into the intima. Cytokines can also regulate genes that encode other growth factors and cytokines themselves. TNF-α can induce IL-1 mRNA in human endothelial cells and VSMC. IL-1 and TNF-α can augment the production by vascular cells of macrophage-colony stimulating factor, which may promote growth and activation of mononuclear phagocytes. Because these activated macrophages are powerful producers of ROS, this process may generate additional VSMC autocrine growth mechanisms. Another autocrine mechanism for IL-6 has been proposed that involves the release of 60-kDa heat shock protein (HSP60) from apoptotic VSMC [28]. More recently, it has been reported that another pro-inflammatory cytokine, intelukine-18, is

mitogenic for aortic SMC and induces the release of a chemokine, CXCL16, upregulated in vascular injury [64].

Despite this long history, the exact origin of the cell type that leads to formation of the neointima (dedifferentiated VSMC, VSMC progenitor cell, or myofibroblast) remains unknown.

1.1.4 Prevention and treatment

The biological processes in pathogenesis of restenosis suggest a number of targets for pharmacological intervention. These therapies can be divided into categories based on mechanisms of action: anti-thrombotic, anti-inflammatory, anti-mitotic and pro-mitotic agents for targeting of unwanted SMC proliferation or desiderable endothelial cell re-growth, respectively. Traditional pharmacological agents including antiplatelet agents, anticoagulants, angiotensin-converting enzyme inhibitors, calcium channel blockers and lipid-lowering agents have failed to reduce restenosis rates in clinical studies [65] mainly because the concentrations required for effective action at the site of injury have not been achieved.

Probucol is the first pharmacological agent showing to reduce coronary restenosis after angioplasty and the mechanism of preventing restenosis appears to be independent of its lipid-lowering effect [66]. The

positive results obtained with probucol suggest that restenosis process is associated with oxidative stress. Reactive oxygen species are produced after angioplasty and the generation of reactive oxygen species and oxidation of lipids impairs endothelial function. Oxidative stress exerts toxic effects on VSMC which leads to the activation of inflammatory reactions [67].

While restenosis can often be easily treated non surgically with the use of balloons, atherectomy devices, and the use of intracoronary radiation therapy [68], the prevention of restenosis remains a highly desirable goal. Recent technology has created a method of "coating" stents: using a coronary stent for local delivery of drugs combines scaffolding with targeted drug action. The initial research and clinical trials have been concentrated on sirolimus (rapamycin), a macrolide antibiotic with immunosuppressive and anti-mitotic properties [69]. Stents are coated with polymer containing low dose sirolimus then a layer of drug-free polymer, which serves as a barrier to diffusion. While stents may almost eliminate elastic recoil and negative remodelling, they can induce a more pronounced vascular response than angioplasty alone [70]. Also, after stent implantation restenosis occurs because of the formation of soft scar tissue in the center of the stent, which blocks coronary blood flow. With the increased usage of

the stents, there are reports of problems, such as late stent malapposition, subacute and late thromboses and aneurysm formations due to the toxicity associated with this method of treatment. In addition, the long term effects of stents are still unknown.

Gamma or beta radiation (brachytherapy) has been proposed as potential way of reducing restenosis, especially in-stent restenosis, because it is well known that low-dose radiation is highly effective and safe for preventing keloids and treating benign vascular malformations [71]. Also, low-dose radiation can delay normal would healing and impair SMC function. A number of clinical trials have been completed examining the use of intravascular radiation to prevent restenosis; the most positive results have come from treatment of in-stent restenosis [72,73]. The suggested beneficial effect of brachytherapy is the inhibition of SMC proliferation and the favourable arterial remodelling. Although brachytherapy is technically simple, it posses several difficulties concerning safety issues and side effects. A well documented consequence of brachytherapy is the aneurismal dilatation of the arterial wall and subacute and late stent thrombosis [71].

It is most important to note that all of these exciting developments only allow us to buy time for an individual but it is too far to consider these "the beginning of the end of restenosis".

1.2 Interleukin-18

Interleukin (IL) 18 was first described as an interferon (IFN) γ-inducing factor1 which circulated during endotoxaemia in mice preconditioned with an infection of *Propionibacterium acnes*. Because of its ability to induce IFN-γ, IL-18 is by default a member of the T cell helper type I (Th1)-inducing family of cytokines (IFN-γ, IL-2, IL-12, IL-15). However, because antibodies to IL-18 also reduced the hepatotoxicity of endotoxaemia, IL-18 was considered to possess other biological properties beyond that of inducing IFN-γ. It has become clear that IL-18 is a proinflammatory cytokine and that its mechanism of action can be independent of its ability to induce IFN-γ [74].

IL-18 is related to IL-1β more than any other cytokine. The similarities between IL-1β and IL-18 exist at several levels. Firstly, the IL-18 precursor form (pro-IL-18), like pro-IL-1β, does not contain a signal peptide, and pro-IL-18 requires cleavage to an active cytokine by the IL-1β

converting enzyme (ICE, caspase-1). IL-1β and IL-18 are structurally related because both cytokines are primarily all β-pleated sheet folded molecules [75]. This structural relationship is significant because there are very few all-β sheet molecules among the different cytokines.

At the receptor level, the activity of IL-18 is through a heterodimeric complex, the IL-18 receptor (IL-18R) complex. The IL-18R binding chain is termed IL-18Rα. IL-18Rα is a member of the IL-1 receptor family, previously identified as the IL-1R related protein (IL-1Rrp). A signalling chain (IL-18Rβ), also termed accessory protein-like (AcPL), is related to the IL-1R accessory protein. Although similar to the IL-1 receptor accessory protein in that the IL-18Rβ does not itself bind its ligand in solution, the IL-18Rβ chain is part of the IL-18 receptor complex. After binding of IL18 to the IL-18Rα, the IL-18R AcPL binds to form a high affinity heterodimeric complex with the ligand. The high affinity IL-18R complex recruits the IL-1 receptor activating kinase (IRAK), resulting in phosphorylation of nuclear NF-κB-inducing kinase (NIK) with subsequent translocation of NF-κB to the nucleus. Initially identified as part of the IL-1R signalling events, IRAK is recruited to the IL-1R complex after exposure to IL-1. In cells possessing both the IL-18Rα and β chains, nuclear translocation of NF-κB is seen after incubation with IL18, and this

property helps to explain the pleotropic nature of IL-18. In IL-18 deficient mice, production of IFN-γ and cytotoxic T cells is markedly diminished despite ample amounts of IL-12. A similar finding exists in mice deficient in ICE. The role of IL-12 in IFN-γ production therefore seems to require IL-18 [74].

Cells known to express IL-18 include monocytes/macrophages, dendritic cells, Kupffer cells, keratinocytes, glucocorticoid-secreting adrenal cortex cells, and osteoblasts [76].

Because of its ability to stimulate IFN-γ release by both natural killer (NK) cells and T lymphocytes, IL-18 is considered to be a key cytokine in both innate and acquired immunity [77]. IL-18 has been shown to be capable of directly inducing expression of proinflammatory cytokines such as tumor necrosis factor (TNF-α) and IL-1β in mature Th1 cells, macrophages, and natural killer cells [77-79], to up-regulate production of both CC and CXC chemokines [80], to stimulates gene expression and synthesis of Fas ligand [81], to enhance expression of costimulatory molecules such as CD40L and CD86 [82], and to induce tissue damage through the induction of cell-mediated cytotoxicity [5] (fig. 4).

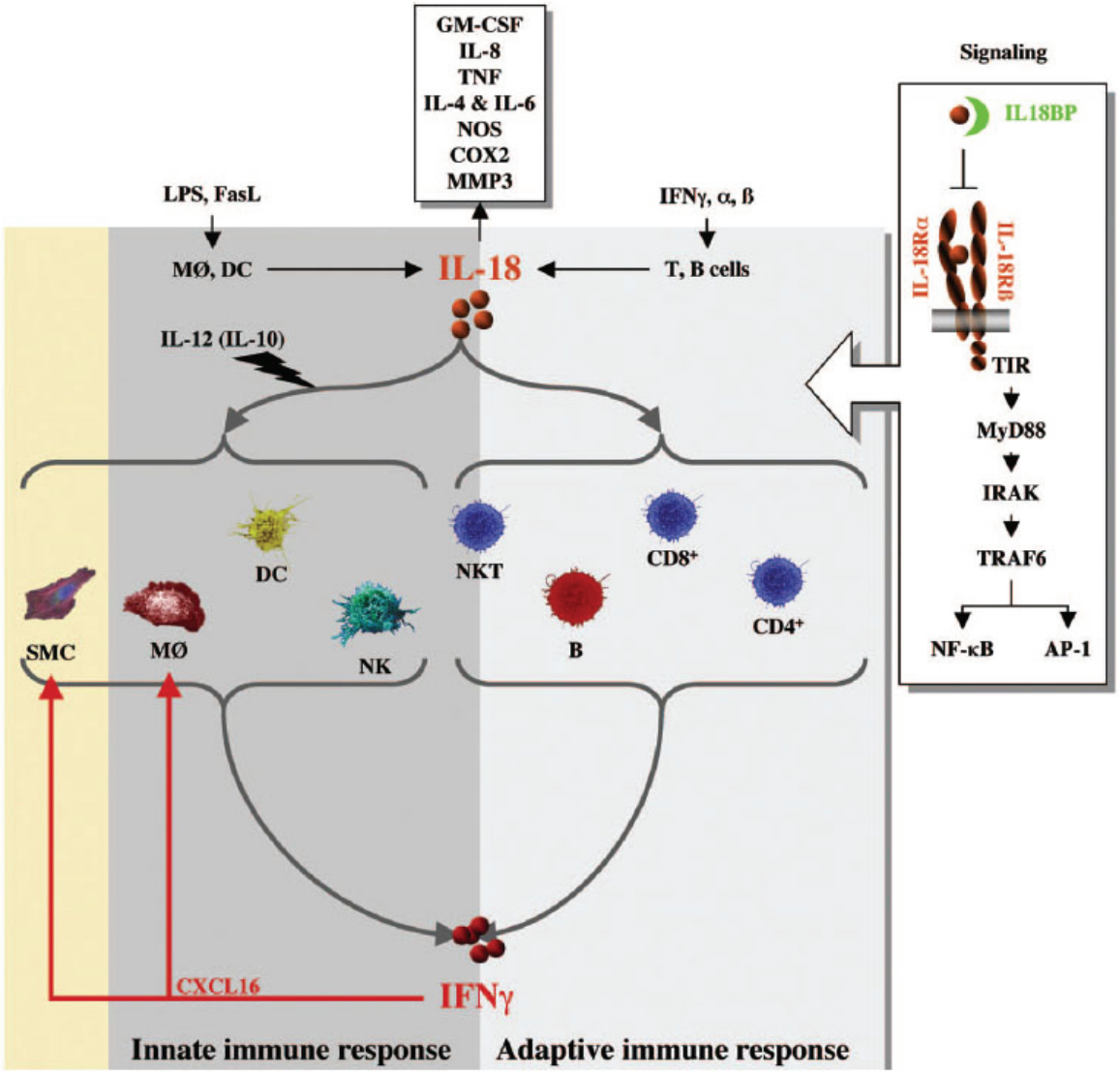

Figure 4. The inflammatory orchestra conducted by IL-18.
IL-18 is produced by several cells from the innate (MØ and DC) and adaptive (T, B cells) immune systems, on stimulation by lipopolysaccharide (LPS), FasL or interferons. IL-18 signals recruits MyD88 and leads to the activation of the NF-κB and AP-1 transcription factors (right inset). IL-18 signaling drives (top inset) the transcription of a set of cytokines, chemokines, growth factors and enzymes.
MØ indicates macrophages; DC, dendritic cells; NOS, NO synthase; COX2, cyclooxygenase 2; MMP3, stromelysin; TIR, Toll/IL-1R domain; IL-18BP, IL-18 binding protein; IRAK, IL-1 receptor–associated kinase; TRAF6, TNF receptor-associated factor 6; IL-18Rα,β, α and β chains of IL-18 receptor.
[from Caligiuri et al., Arterioscler Thromb Vasc Biol. 2005; 25: 55-657]

The biological activity of interleukins is partially regulated by anti-cytokine antibodies, soluble cytokine receptors, and cytokine-binding proteins, the elaboration of which is frequently controlled by the interleukin concerned [83,84]. IL-18 binding protein (IL-18BP) is a secreted protein that binds IL-18 and neutralizes its biological activities. IL-18BP is induced by IFN-γ in various cells, suggesting that it serves as a negative feedback inhibitor of the Th1 immune response [85]. Numerous recent *in vivo* studies using both IL-18-gene-targeted mice and neutralising agents such as anti-IL-18 antibody or IL-18 binding protein, implicate IL-18 in components of host defence and in responses in autoimmune models of disease [86-88], increasing interest in it as a therapeutic target [5].

1.2.1 Interleukin-18 and cardiovascular disease

Binding to the IL-18 receptor results in enhanced secretion of many cytokines and proteins involved in vascular pathologies, among which IL-6, IL-8, intercellular adhesion molecule-1 (ICAM-1), and various matrix metalloproteinases (MMPs) [89]. IL-18 and its receptor are expressed in human atheroma-associated endothelial cells, vascular smooth muscle cells (SMCs), and macrophages [89]. IL-18 binding to IL-18R activates the DNA-

binding activity of transcriptional factors, such as (NF-κB) and activator protein 1 (AP-1) in VSMCs [81,90,91].

Studies in humans as well as in animal models have suggested that this cytokine plays a crucial role in vascular pathologies. Increased IL-18 expression has been reported in human atherosclerotic plaque [6], mediating IFN-γ release locally [89]. Furthermore, animal models support the proatherogenic role of IL-18 [7]. Mallat et al. [8], using a mouse model of human-like atherosclerosis, establish an unsuspected and crucial role for IL-18 signaling pathway in atherosclerotic plaque development, progression, and stability. While preventing early lesion formation in the thoracic aorta, inhibition of IL-18 signaling by IL-18BP supplementation also profoundly affected advanced lesion composition in the aortic sinus, inducing a switch toward a stable plaque phenotype.

After myocardial infarction (MI) in mouse, increased production of cardiac IL-18 mRNA and pro-IL-18, as well as circulating IL-18 occurs [92]. Increased levels of circulating IL-18 have been demonstrated in patients with acute coronary syndromes [93-95], and congestive heart failure [96,97]. Further, an epidemiologic study [98] had suggested that IL-18 can predict cardiovascular death in patients with stable and unstable angina. Interestingly, Yamagami et al. [99] found that the elevated serum IL-18 levels

are associated with increased carotid intima-media thickness as evaluated by B-mode ultrasound inpatients without histories of cardiovascular accidents. Also, the association was independent of traditional atherosclerotic risk factors, IL-6 and hs-CRP levels. In this study, IL-18 were higher in hypertensive patients and smokers than in those who were not, and had significant carrelations with traditional atherosclerotic risk factors such as sex, body mass index, diabetes and dyslipidemia. These findings are in line with those Ferrucci et al.[100], who showed associations of higher IL-18 levels with such risk factors. Also in accordance with previous studies [98,101,102], IL-18 levels had modest correlation with other inflammatory markers. Nevertheless, studies that examined the associations of IL-18 levels with atherosclerotic risk factors and other inflammatory markers are limited, requiring further studies to clarify their linkages. Although elevated IL-18 levels can predict the development of cardiovascular disease [98,101], their association with carotid intima-media thickness remains to be examined. Moreover, Kawasaki et al. [103] showed that higher IL-18 plasma levels were associated with restenosis after emergency coronary angioplasty performed in patients with acute myocardial infarction.

To date, however, the expression and function of IL-18 in neointima formation has not been investigated. This is of particular relevance, because it is well established that long-term failure of arterial stenting is due to neointimal formation, whereas a combination of arterial remodelling and proliferation of SMCs is responsible for restenosis following balloon angioplasty in humans [9]. In addition, VSMCs proliferation is a hallmark of restenosis and recent studies have provided strong evidence for an important role of IL-18 on SMCs proliferation and migration in vitro [91, 104].

1.3 Animal models for restenosis research

Animal models are important in understanding the arterial response to coronary injury following interventions and are essential for testing new treatment modalities to prevent restenosis. The ideal experimental model for assessing restenosis preventive strategies should reliably predict the outcome of clinical trials. However, differences in the severity of injury and substantial interspecies differences in the healing response and metabolism make conclusions about humans based on the animal data tenuous [105].

The healthy vessel in an experimental animal differs fundamentally from the diseased atherosclerotic coronary artery of the typical human. Experimental models do not display the features of complex atheroma, such as calcification, central necrosis, ulceration, thrombus formation, and plaque haemorrhage. It is unknown whether these characteristics of human plaque have an impact on the development of restenosis [106].

Although neointimal formation through smooth muscle cell migration, proliferation, and matrix synthesis is the unifying response to injury in each species, the generated neointima volume following injury differs immensely across species. Each animal model is valuable for its specific characteristics. The limits and strengths of each model should be used to maximum advantage before examining specific therapies in humans. It is crucial to describe precisely the mechanism of neointima formation specific for each species to improve the reliability of animal data. Although the molecular biology of smooth muscle cell proliferation is best understood in the rat carotid artery model (balloon angioplasty) [105].

The rat common carotid artery angioplasty model involves inducing fibroproliferative lesions within a long unbranched segment of artery using a 2F Fogarty catheter [107]. After denudation of the endothelium, a

hyperplastic neointimal response to injury is induced following repeated withdrawal of an inflated balloon catheter. Because this model was one of the first described and is not as technically challenging, it rapidly became extensively used. Classically, the response to injury in this model is referred to as the "three-wave paradigm", whereby endothelial denudation produces medial smooth muscle cell proliferation (peaking 3 days after injury), smooth muscle cell migration (from the media to the subendothelial/intimal border 4 days following injury), and finally, intimal proliferation coincidental with matrix synthesis (resulting in neointima formation, peaking 1 to 2 weeks after injury) [52,108].

Rats have been used extensively for restenosis research and have also figured prominently in the study of the contributions of thrombosis and hyperplasia to luminal narrowing following arterial injury [109]. Some of the reasons for the frequent use of rat models in restenosis research include, a) low cost, b) ready availability, c) reduced ethical concern compared to large animals and d) small size that limits the quantities of new agents required for in vivo screening. These characteristics have permitted rapid evaluation of new agents in sufficiently large populations to perform meaningful statistical analyses. In addition to these practical indications for

their use, small animal models have the added advantage of well-defined genetic characterization.

Despite the favorable characteristics of rat models, the predictive value of the data obtained from the study has been very limited. Greater than 40 large-scale clinical trials, that included thousands of patients, failed to establish significant effectiveness of multiple pharmacological agents in the prevention of restenosis following human angioplasty [110], even though most of the agents evaluated had been found to reduce luminal narrowing following arterial injury in rat models.

Any enthusiasm for more advanced testing of molecules should be tempered with the following caveats: (a) balloon angioplasty of the rat carotid artery is a model of restenosis far from perfect, and any effects observed may not predict outcome in more complex settings of vascular narrowing; (b) this model offers a way to better describe the mechanism of neointima formation.

1.4 Specific aims

It is not feasible to artificially regulate IL-18 in humans to determine its association with restenotic diseases, therefore, dissection of the role of this cytokine in lesion development will be dependent on animal models.

The aims of the present study were, first, to evaluate the expression of IL-18 and its related receptor, distribution and specific localization of IL-18 active form in rat carotid arteries subjected to vascular injury. Second, we assessed the relationship between IL-18 neutralizing and neointima formation in damaged arteries.

2. METHODS

2.1 Animals

Male Wistar rats (Harlan Italy, S. Pietro al Natisone, Udine, Italy) weighing 250g were used for the present study. Animals were housed in propylene cages with food and water ad libitum. The light cycle was automatically controlled (on at 7:00 AM and off at 7:00 PM), and the room temperature was thermostatically controlled to 22 ± 1°C. Before the experiments, the animals were housed in these conditions for 4 or 5 days to become acclimatized. Animal care was in accordance with Italian and European regulations on protection of animals used for experimental and other scientific purposes.

2.2 Balloon angioplasty

Animals were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (5 mg/kg) (Sigma, Milan, Italy). Endothelial denudation of the left carotid artery was performed by using a balloon embolectomy catheter (2F, Fogarty; SEDA, Trezzano s/Naviglio,

Milan, Italy). In brief, a catheter was introduced through the external branch of the carotid, advanced into the aortic arch, and inflated at 2 atmospheres with a calibrated inflation device (Indeflator Plus 20, Advanced Cardiovascular System, Inc). The vessel was damaged by passing inflated balloon catheter back and forth through the lumen three times. This sequence produced complete endothelial denudation of the left common carotid. Naive animals were used as control. Some animals were subjected to anesthesia and surgical procedure without balloon injury (sham-operated rats). Rats were euthanized 0, 4 and 24h and 2, 7 and 14 days after vascular injury, and carotid arteries were removed and processed as described below.

2.3 Anti-IL-18 treatment

To neutralize endogenous IL-18, rats were subjected to balloon angioplasty followed by i.p. injection of 3 mg of purified rabbit anti–rat IL-18 IgG, prepared by PRIMM (Milan, Italy). Subsequent injections were at days 4, 8 and 12. Control rats received normal rabbit IgG. The biological activity of the antibody was tested in vitro. A dose of 200 μg of anti-IL-18 antibody was shown to completely block IFN-γ inducing activity of 50 ng of IL-18 in rat spleen cells stimulated with Con A.

2.4 Morphology

At the time of the final experiments, the animals were anesthetized with an intraperitoneal injection of ketamine 100 mg/kg and xylazine 5 mg/kg, and the carotid arteries were fixed by perfusion at 120 mm Hg with 100 mL of PBS (pH 7.2) followed by 80 mL of prepared PBS containing 4% paraformaldehyde through a large cannula placed in the left ventricle. The carotid arteries were removed. Paraffin-embedded sections were cut (6 μm thick) from the approximate midportion of the artery and stained with hematoxylin and eosin to demarcate cell types, three sections were stained with aldehyde fuchsin and counterstained with van Gieson's solution to demarcate the internal elastic lamina (IEL). Ten sections from each carotid artery were reviewed and scored under blind conditions. Both the circumference and the cross-sectional area of external elastic lamina (EEL), internal elastic lamina (IEL), lumen, media and neointima were measured carefully, by using an image analysis system (Qwin Lite 2.2, Leica, Milan, Italy).

2.5 Total RNA isolation

Total RNA was isolated from the carotid arteries using TRIzol (Invitrogen, Milan, Italy). The carotid arteries (n=3 per group) were frozen

in liquid nitrogen, pooled and crushed into powder in a mortar with a pestle, transferred to a microcentrifuge tube and immediately suspended in TRIzol. The tissue suspensions were centrifuged at 7500*g* for 10 min. The supernatant was transferred to a new microcentrifuge tube and homogenized by passing 5-10 times through 20 gauge needle fitted onto a 3 ml syringe. 0.2 ml of chloroform was then added and the tube was shaken for 15s, followed by centrifugation at 12.000 g for 15 min. The aqueous phase was transferred to a new microcentrifuge tube, and the total RNA was precipitated using 0.5 ml of isopropyl alcohol. RNA was allowed to precipitate at room temperature for 10 min and centrifuged at 12.000 *g* for 10 min. The supernatant was removed, and the RNA pellet was washed with 1 ml of 70% ethanol followed by centrifugation at 7,500*g* for 5 min. The RNA pellet was air-dried for 5 min, resuspended in diethyl pyrocarbonate (DEPC)-treated water and then heated at 55°C for 15 min. The final amount of RNA was determined by absorbance at 260 nm.

2.6 RT-PCR of IL-18, IL-18Rα/β, IFN-γ, IL-6 and IL-8

Total RNA was reverse-trascribed into complementary DNA and then amplified by PCR using Taq polymerase (Promega, Madison, WI) according to manufacturer's instructions. Parallel amplification of rat

housekeeping gene β-Actin was performed as internal control. The primers were as follows: IL-18: sense: 5'-TGCAATACCAGAAGAAGGC-3', antisense: 5'-CCCCATTTTCATCCTTCC-3' [111]; IL-18Rα: sense 5'-CCAACGAAGAAGCCACAGACA-3', antisense: 5'-CTCAGGATGACACTCTCTCAG-3'; IL-18Rβ: sense 5'-CCTATCTGATGTCCAGTGGT-3', antisense: 5'-GGGGGCTCCTAATTCTGGG-3' [112], IFN-γ: sense 5'-GAAAGCCTAGAAAGTCTGAAGAAC-3', antisense: 5'-GCACCGACTCCTTTTCCGCTTCCT-3'; IL-6: sense: 5'-ATACCACCCACAACAGACCAGT-3', antisense: 5'-GATGAGTTGGATGGTCTTGGT-3'; IL-8: sense 5'-GAAGATAGATTGCACCGATG-3', antisense: 5'-CATAGCCTCTCACACATTCC-3'; β-Actin: sense 5'-ATGAAGATCCTGACCGCGCGT-3', antisense: 5'-AACGCAGCTCAGTAACAGTCCG-3'. The amplified fragments were 536bp, 270bp, 164bp, 366bp, 467bp, 365bp, and 584bp, respectively. The PCR reaction was performed under the following conditions: a first cycle of denaturation at 94°C for 1 min 40 sec, then 30 (IL-6, IL-8 and β-Actin) or 36 (all others) cycles of denaturation at 94°C for 40 sec, annealing at 50°C (IL-18) or 56°C (IL-8 and β-Actin) or 58°C (IL-18Rα, IL-18Rβ and

IFN-γ) or 60°C (IL-6) for 40 sec, extension at 72°C for 1 min and 1 additional cycle of extension at 72°C for 8 min. The PCR products were run on a 2% agarose gel and visualized by ethidium bromide staining. Subsequently, the relative bands were quantified by densitometric scanning of the pictures with with GS-700 Imaging Densitometer (Bio-Rad, Milan, Italy) and a computer program (Molecular Analyst, IBM, Milan, Italy). mRNA expression levels were normalized to expression levels of housekeeping gene β-actin and expressed as densitometric arbitrary units.

2.7 Preparation of cytosolic and nuclear extracts

All the extraction procedures were performed on ice with ice-cold reagents [113]. Briefly, carotid arteries crushed into powder as described above were resuspended in adequate volume of hypotonic lysis buffer, and chilled on ice for 5 min. Then, the homogenates were vigorously shaken for 15 sec in the presence of 100 µl of 5% Nonidet P-40, and incubated on ice for 15 min. The nuclear fraction was precipitated by centrifugation at 1500 g for 10 min and the supernatant, containing the cytosolic fraction, was removed and stored at -80°C. The nuclear pellet was resuspended in adequate volume of high salt extraction buffer and incubated with shaking at 4°C for 30 min. The nuclear extract was then centrifuged for 15 min at

13,000 g and supernatant was aliquoted and stored at -80°C. Protein concentration was determined by the Bio-Rad protein assay kit (Bio-Rad, Milan, Italy).

2.8 Western blot analysis

The level of IL-18 was evaluated in cytosolic extracts by immunoblot analysis. β-Actin immunoblot analysis was performed to ensure equal sample loading. Equivalent amounts of protein (60 μg) from each sample were electrophoresed in an 12% discontinuous polyacrylamide minigel. The proteins were transferred onto nitrocellulose membranes, according to the manufacturer's instructions (Bio-Rad). The membranes were saturated by incubation with 10% non-fat dry milk in PBS-0.1% Triton X-100 (ICN Biomedicals Inc.) for three hours at room temperature and then incubated with anti-IL-18 goat antibody (0.2 μg/ml) (R&D System) or anti-β-Actin (1:5000) (Sigma) mouse antibody over night at 4°C. The membranes were washed three times with 0.1% Tween 20 in PBS and then incubated with anti-goat or anti-mouse (1:1000) immunoglobulins coupled to peroxidase (DakoCytomation) for 1 hour at room temperature. The immune complexes were visualized by the enhanced chemiluminescence method (Amersham, Cologno Monzese, Italy). Subsequently, the relative intensities of the bands

were quantified by densitometric scanning of the X-ray films with GS-700 Imaging Densitometer (Bio-Rad) and a computer program (Molecular Analyst, IBM, Milan, Italy). IL-18 protein levels were expressed as arbitrary densitometric units.

2.9 IL-18 immunohistochemistry

Carotid arteries were snap frozen in liquid nitrogen in OCT embedding medium (Tissue Tek, Sakura Finetek Europe, The Netherlands) and stored at -80°C. Cross sections were cut (6 μM) from the approximate midportion of the artery and used for IL-18 detection by immunohistochemistry (IHC). For staining, sections were incubated in acetone for 10 min, air dried and rehydrated with PBS before incubation in 0.3% H_2O_2 50% methanol in PBS for 15 min. Protein block serum free (DakoCytomation, Milan, Italy) was added for 30 min. To detect IL-18, sections were stained with 15 μg/ml anti-rat IL-18 Ab (goat IgG, R&D Systems, Minneapolis, MN) in PBS overnight, before being washed in PBS. Sections incubated with an isotype-matched control antibody were used as negative control. Subsequently, sections were incubated with 1/400 polyclonal rabbit anti-goat immunoglobulins/biotinylated (DakoCytomation) before washing. Streptavidin-HRP (LSAB kit, DakoCytomation) was added for 15 min

before washing as before. Enzymatic activity was detected with 3,3'-diaminobenzidine substrate (DakoCytomation) before washing in dH_2O. Hematoxylin was used to counterstain before rinsing in H_2O. Sections were subsequently exposed to 70%, 80%, 95%, then 100% ethanol for dehydration before clearing in xylene (Kaltek, Padova, Italy) and immediate mounting in Entellan (Merck, Darmstadt, Germany).

2.10 IL-18 and anti-α-smooth muscle actin immunofluorescence

Carotid arteries were snap frozen in liquid nitrogen in OCT embedding medium (Tissue Tek, Sakura Finetek Europe, The Netherlands) and stored at -80°C. Ten cross sections were cut (6 μM) from the approximate midportion of the artery and used for IL-18 and anti-α-smooth muscle actin (anti-α-SMA) detection by immunofluorescence. For staining, sections were fixed in acetone for 10 min, air dried and rehydrated with PBS before incubation in Protein Block serum free (DakoCytomation, Milan, Italy) for 30 min. To detect IL-18, sections were stained with 15 μg/ml anti-rat IL-18 Ab (goat IgG, R&D Systems, Minneapolis, MN) diluted in 1% blocking reagent (Perkin Elmer, Milan, Italy)/0.3% Triton X-100 (MP Biomedicals, Verona, Italy) in PBS overnight, before being washed in TNT wash buffer (Tris-HCl pH 7.5, 0.15 M NaCl, and 0.05% Tween 20; Sigma). Sections

incubated with an isotype-matched control antibody were used as negative control. Subsequently, sections were incubated with 1/75 Texas Red-donkey anti-goat IgG (Jackson ImmunoResearch Laboratories, United Kingdom) for 30 min, before washing. Monoclonal anti-α-smooth muscle actin FITC conjugate (1/250, clone 1A4, Sigma) was added in blocking buffer for 1 h before washing as before. Dapi was used for identification of nuclei. Images were taken by an AxioCam HRc video-camera (Zeiss, Arese, Milan, Italy) connected to an Axioskop fluorescence microscope (Zeiss), using the AxioVision 3.1 software.

Neointimal α-SMA positive cells number was determined by counting all nucleated cells with FITC fluorescence present on a carotid section. For each group studied carotid arteries obtained from 5 different rats per group were analyzed. Six sections from each carotid artery and 10 fields per section were reviewed and scored under blind conditions.

2.11 Proliferating Cell Nuclear Antigen Analysis

Proliferating cell nuclear antigen (PCNA) analysis was used to quantify the proliferative activity of cells at the balloon injury sites. After deparaffinization and rehydration the section were washed in a solution of 0.3% H_2O_2 in 50% methanol/PBS for 15 min. Sections were then washed

five times for 1 minute in PBS. Before incubation protein block serum free (DakoCytomation, Milan, Italy) was added for 30 min. To detect PCNA, sections were incubated with anti-PCNA (Signet Laboratories, Inc., Dedham, MA) antibody used overnight in 1:200 dilution. Subsequently, sections were washed in PBS and incubated with 1/400 polyclonal rabbit anti-goat immunoglobulins/biotinylated (DakoCytomation) before washing. Streptavidin-HRP (LSAB kit, DakoCytomation) was added for 15 min before washing as before. Enzymatic activity was detected with 3,3'-diaminobenzidine substrate (DakoCytomation) before washing in dH_2O. Hematoxylin was used to counterstain before rinsing in H_2O. Sections were subsequently dehydratated before clearing in xylene (Kaltek, Padova, Italy) and immediate mounting in Entellan (Merck, Darmstadt, Germany).

For each group studied carotid arteries obtained from 5 different rats were analyzed. Six sections from each carotid artery and 10 fields per section were reviewed and scored under blind conditions. Number of PCNA-positive cells counted in media and neointima 7 days following angioplasty was calculated.

2.12 Electrophoretic Mobility Shift Assay

Electrophoretic Mobility Shift Assay (EMSA) studies were performed on nuclear extract obtained as previously described. Briefly, double-stranded NF-κB consensus oligonucleotide probe (5' AGC TTC AGA GGG GAC TTT CCG AGA GG 3' [114]) was end-labelled with [^{32}P]γ-ATP. Nuclear extract (10 μg protein from each sample) was incubated for 20 min with radiolabelled oligonucleotides (2.5-5.0x10^4 cpm) in 20 μl reaction buffer containing 2 μg poly dI-dC, 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 μg/μl bovine serum albumin, 10% (v/v) glycerol. Nuclear protein-oligonucleotide complexes were resolved by electrophoresis on a 5% non-denaturing polyacrylamide gel in 0.5 x Tris-borate/EDTA at 150 v for 2 h at 4°C. The gels were dried and autoradiographed with intensifying screen at -80°C for 24 hours.

2.13 Enzyme-linked immunosorbent assay (ELISA)

Serum IL-18 levels (2-7-14 days after angioplasty) were measured by ELISA. Briefly, 96-well plates (Maxisorb, Nunc-Immuno, Roskilde, Denmark) were coated with anti-rat IL-18 Ab (1 μg/ml in 0.1 M NaH_2CO_3; R&D Systems) overnight at 4°C, blocked and serial dilution of sera were added. Bound IL-18 was detected with 1/20000 polyclonal rabbit anti-goat

immunoglobulins/biotinylated (DakoCytomation), followed by 1/200 streptavidin-peroxidase polymer (Sigma) and developed with tetramethylbenzidine substrate (Sigma). Plates were read at 630 nm. The results expressed as pg/ml.

2.14 Statistical Analysis

Results are expressed as mean ± SEM of n samples for in vivo experiments and mean ± S.E.M. of multiple experiments for molecular biology. Student *t* tests were used to compare 2 groups, or ANOVA with the Dunnett's post tests for multiple groups using Prism software (Graph Pad, San Diego, CA). The level of statistical significance was 0.05 per test.

3. RESULTS

3.1 Time course of neointima formation

Balloon angioplasty led to a time-dependent increase in neointima formation (fig. 5). In normal rats (n=5) there was no neointimal formation. In rats subjected to angioplasty the neointimal area was not detectable at day 1 (n=10), while it was 0.013 ± 0.001 mm^2 (n=10) at day 7 and 0.213 ± 0.010 mm^2 (n=10) at day 14. In sham-operated rats (n=5) not subjected to vascular injury, there was no neointimal formation at all time points analyzed. Medial area (0.145 ± 0.008 mm^2 in Sham group) was not affected by vascular injury at all time points analyzed .

3.2 Expression of IL-18, IL-18Rα/β, IFN-γ, IL-6 and IL-8 mRNA in rat carotid arteries after balloon angioplasty

To determine whether IL-18 and IL-18Rα/β mRNA were expressed in rat carotid arteries after balloon angioplasty, RT-PCR was performed (fig. 6). Both IL-18 and IL-18Rα/β mRNA were detected in carotid arteries subjected to vascular injury (L, lesion), whereas their expression was very

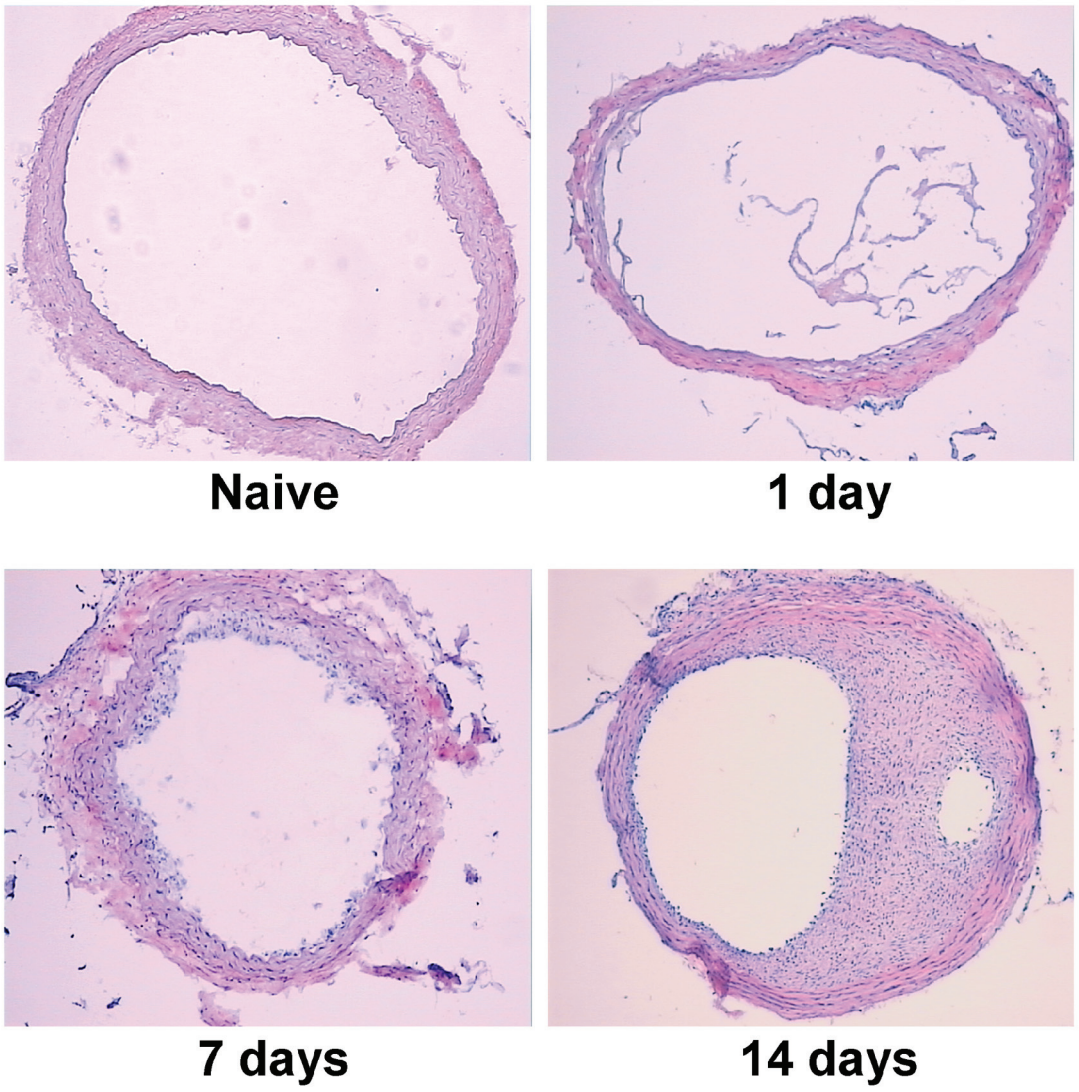

Figure 5. Time course of neointima formation.

low in controlateral (CL) carotid arteries at all time points analyzed (day 2, 7, and 14 after angioplasty) (fig. 6). Little expression was detected in normal arteries. The expression of both IL-18 and IL-18Rα/β mRNA was

found to reach a peak level at 7 days and diminished 14 days following angioplasty (fig. 6).

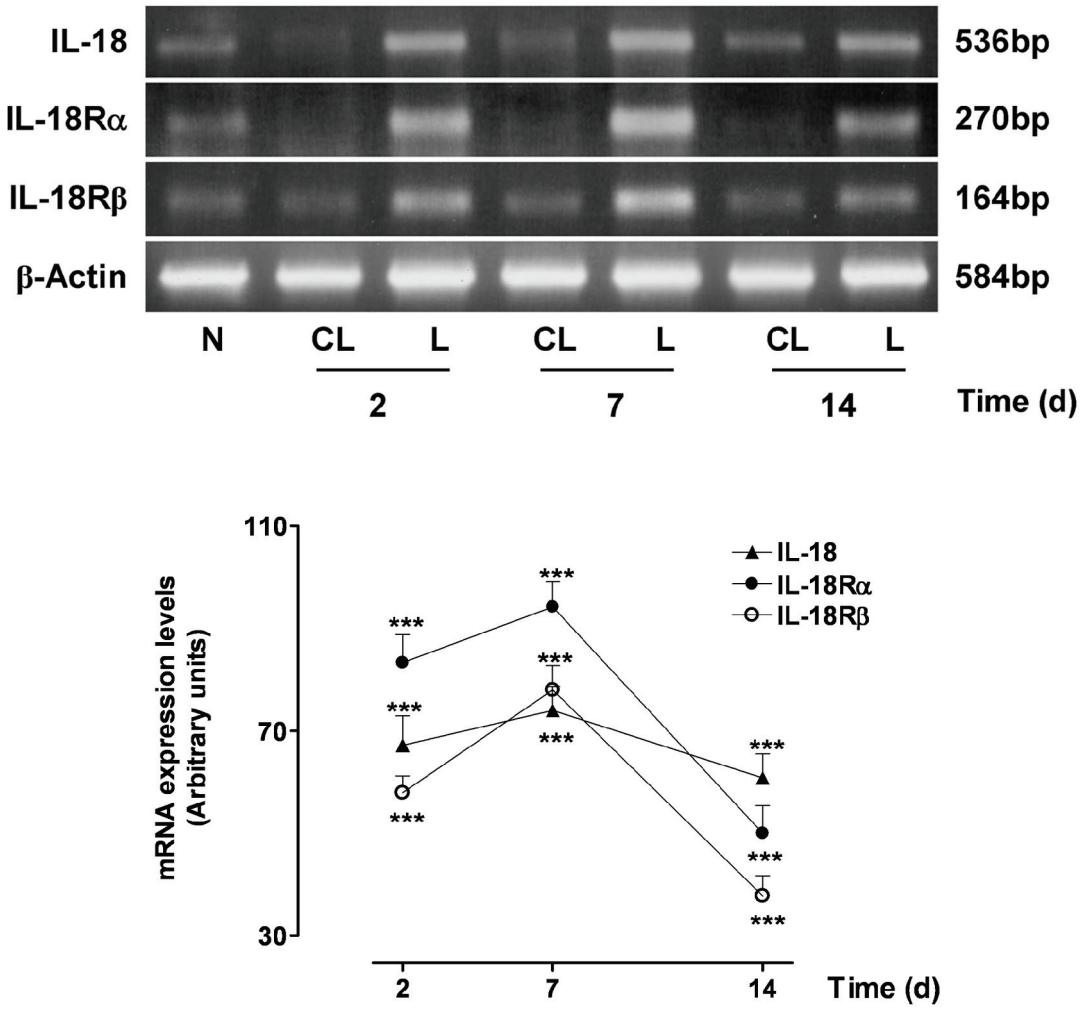

Figure 6. Expression of IL-18 and IL-18Rα/β mRNA in rat carotid arteries after balloon angioplasty.
(Upper panel) PCR amplification of reverse-transcribed mRNA was performed on total RNA isolated from pooled (n=3 per group) normal carotid arteries (N), controlateral arteries (CL) and injured carotid arteries (L, lesion) at different time point after angioplasty. Data shown are representative of three different experiments performed.
(Lower panel) Densitometric analysis of IL-18 mRNA expression, normalized to expression of the housekeeping gene β-Actin, was determined by computer program. The results are expressed as mean ± S.E.M. of n=3 experiments. ***P<0.001 vs normal carotid arteries.

A similar pattern of expression was evident for all cytokines/chemokines analyzed. IFN-γ and IL-8 mRNA were mostly evident at day 7. IL-6 mRNA expression reached a peak level at day 2 diminishing thereafter (fig. 7).

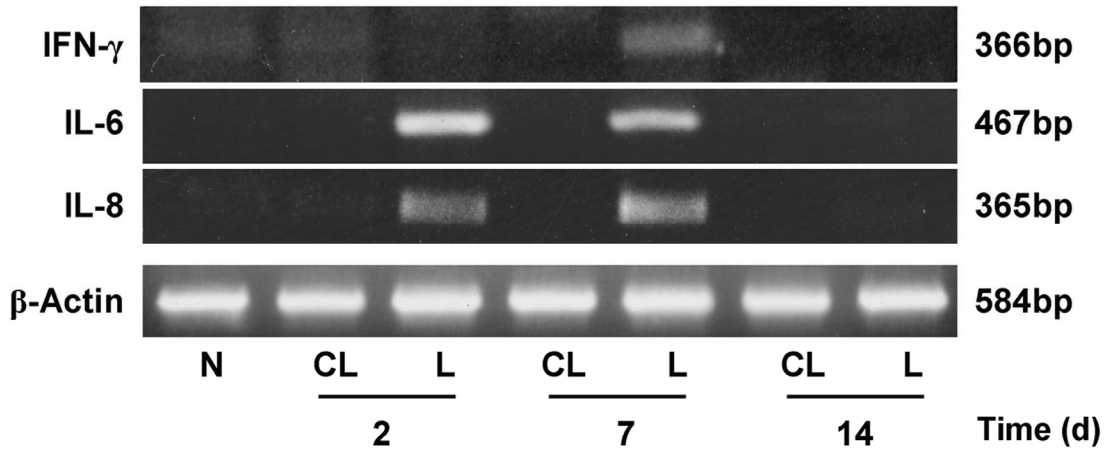

Figure 7. Expression of IFN-γ, IL-6 and IL-8 mRNA in rat carotid arteries after balloon angioplasty.
PCR amplification of reverse-transcribed mRNA was performed on total RNA isolated from pooled (n=3 per group) normal carotid arteries (N), controlateral arteries (CL) and injured carotid arteries (L, lesion) at different time point after angioplasty. Parallel amplification of rat housekeeping gene β-Actin was performed as internal control. Data shown are representative of three different experiments performed.

3.3 Time course of IL-18 protein expression in rat carotid arteries after balloon angioplasty

Western blot assays were performed on cytosolic protein extracts from carotid arteries. The active form of IL-18 was highly expressed in carotid

arteries subjected to angioplasty. No expression was detected in normal arteries (fig. 8). Densitometric analysis of IL-18 levels showed a peak of IL-18 expression intensity at day 7 (fig. 8), concomitantly with the beginning of neointima formation.

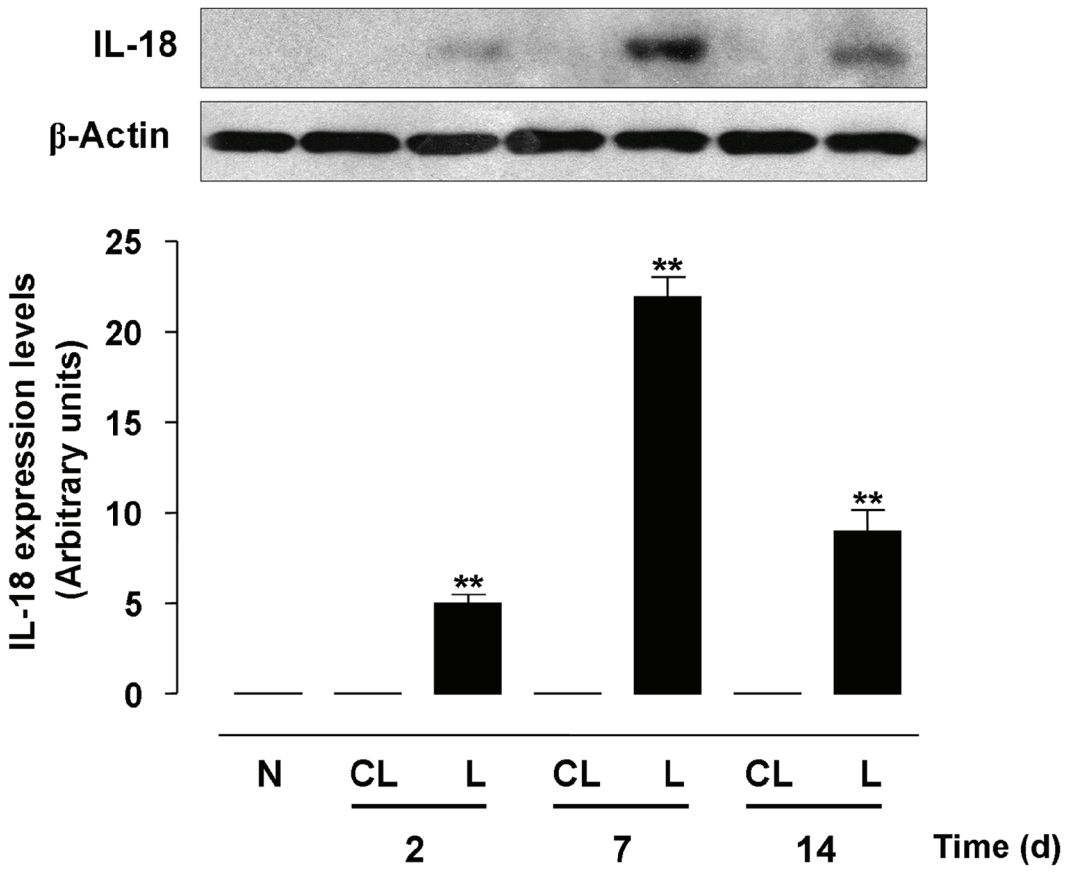

Figure 8. Time course of IL-18 protein expression in rat carotid arteries after balloon angioplasty.
(Upper panel) Western blot analysis was performed on cytosolic extracts of pooled (n=3 per group) carotid arteries at different time point after angioplasty. Equal loading was confirmed by β-Actin staining. Data shown are representative of three different experiments.
(Lower panel) Densitometric analysis of IL-18 protein expression levels, normalized to expression levels of the housekeeping gene β-Actin, was determined by computer program. The results are expressed as mean ± S.E.M. of n=3 experiments. ***P*<0.01 vs normal carotid arteries.

3.4 Localization of IL-18 in rat carotid arteries by immunohistochemistry

To determine the temporal expression, spatial distribution and cellular localization of IL-18, immunohistochemical studies were performed on carotid arteries subjected to vascular injury. Non-injured arterial tissue lacked immunoreactive IL-18 (not shown). In contrast, injured carotid arteries stained strongly for IL-18 (fig. 9).

Control IgG showed no signal (fig. 9, a). In preliminary data no IL-18 positive staining was detectable in injured vessel up to day 2 (0h-24h, data not shown). Whereas, strong immunoreactivity for IL-18 was detected in the medial SMCs at day 2 after balloon injury (fig. 9, b). At day 7 IL-18 was also expressed in some medial cells and in occasional neointimal cells (fig.9, c). At day 14 IL-18 was observed only in neointima (fig. 9, d).

3.5 Cellular localization of IL-18 and α-SMA in rat carotid arteries

A clear immunoreactivity for IL-18 was detected in the medial α-actin positive SMCs at day 2 after balloon injury (fig. 10). Intriguingly, at day 7 medial SMCs started to loose α-actin staining and to increase IL-18 expression (may be a consequence of changes in phenotype), in addition IL-18 was detected in neointimal cells (fig. 10).

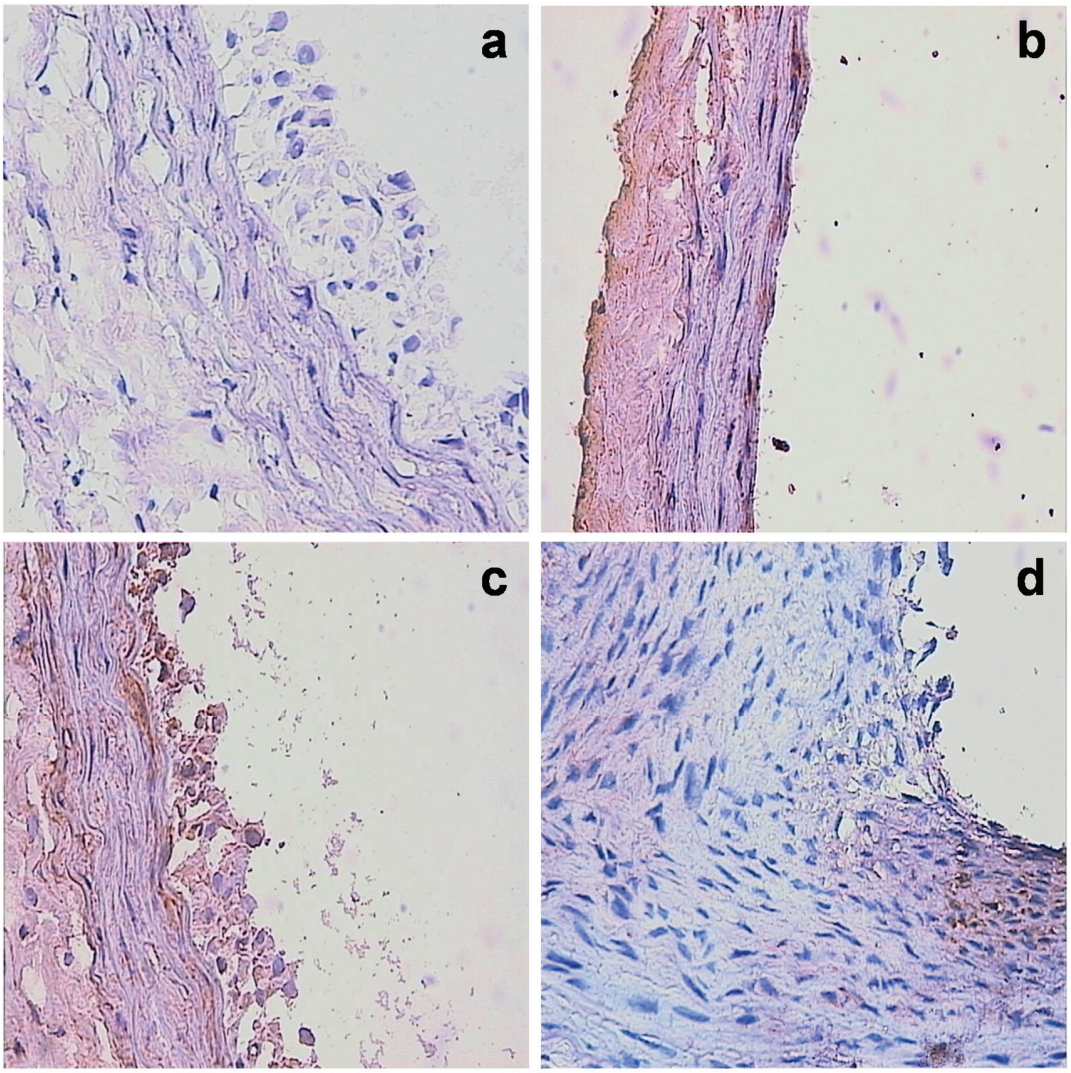

Figure 9. Localization of IL-18 in rat carotid arteries.
Results illustrated are from a single experiment and are representative of three separate experiments. (a): Negative Control; (b): 2 days after angioplasty; (c): 7 days after angioplasty; (d): 14 days after angioplasty. Original magnification: 400x.

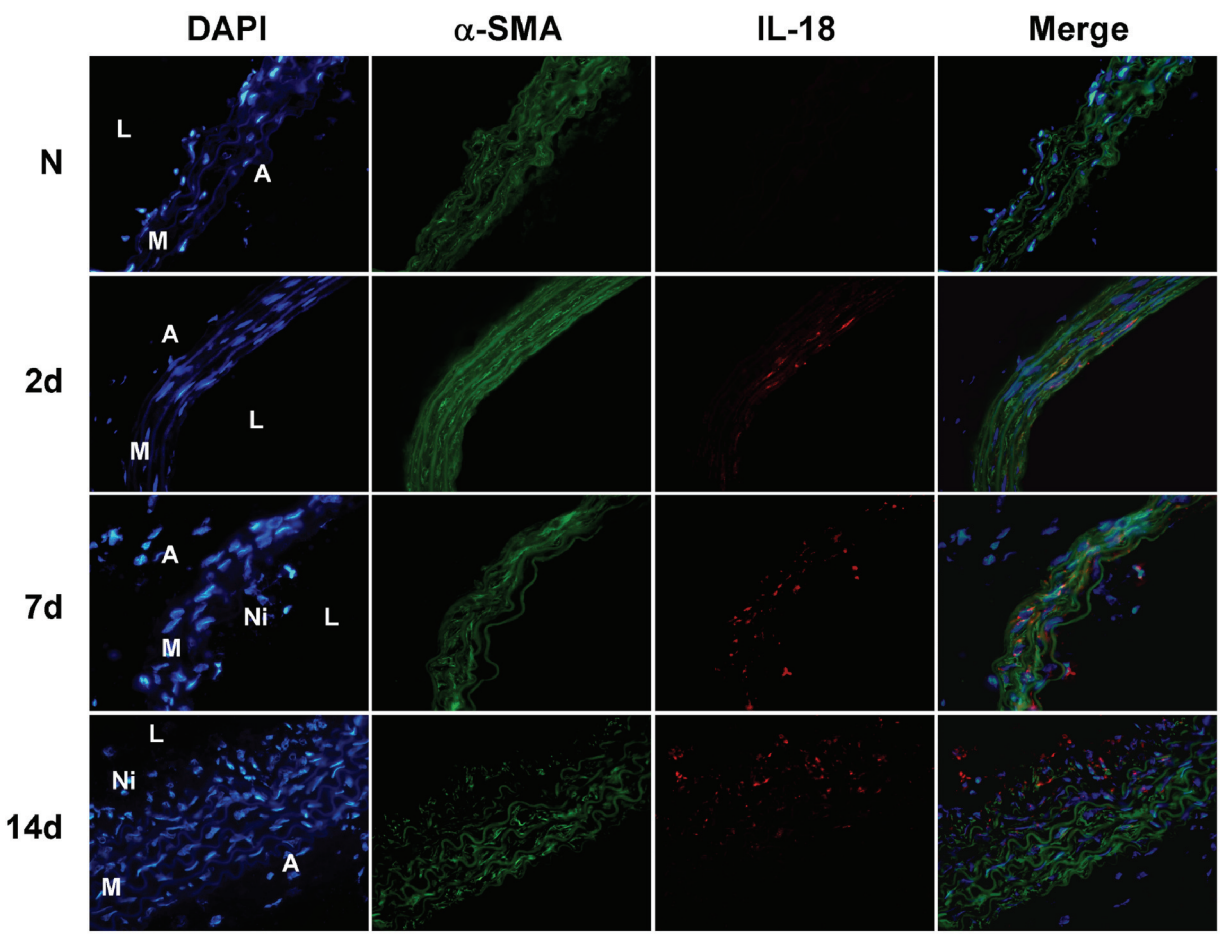

Figure 10. Cellular localization of IL-18 and α-SMA in rat carotid arteries.
Immunofluorescence visualization of α-smooth muscle actin (α-SMA, green) and IL-18 (red) in rat carotid arteries 2, 7, and 14 days following balloon angioplasty. Serial sections were stained with Dapi (blue) to locate nuclei. Results illustrated are from a single experiment and are representative of three separate experiments. (N): Normal carotid artery; (A): Adventitia, (L): Lumen, (M): Media, (Ni): Neointima. Original magnification: x 400.

Immunoreactivity for IL-18 was mostly observed in neointima at day 14 (fig. 10), also at this stage co-localization of IL-18 and α-actin was evident in some cells (fig. 10). SMCs in the neointima, although stained with the anti-α-actin antibody, typically showed weaker signal than contractile-state cells. No IL-18 expression was seen in the adventitia at all time points analyzed. These results suggest that IL-18 is mainly expressed in proliferating-migrating SMCs cells highly contributing to neointima formation.

3.6 IL-18 serum levels

Serum IL-18 levels were measured by ELISA to determine whether balloon angioplasty could affect circulating levels of IL-18 (fig. 11). Serum concentration of IL-18 was higher in rats subjected to vascular injury than among naïve rats. IL-18 was slightly increased in serum at day 2 after angioplasty, reached to a maximal level at day 7 (113 ± 18 pg/ml $P<0.01$, n=10) and diminished at day 14 (83 ± 6.5 pg/ml $P<0.05$, n=10) compared to naive rats (40 ± 7 pg/ml, n=10) (fig. 11). Serum concentrations of IL-18 in sham-operated rats were similar to naive rats 2, 7 and 14 days following surgical procedure (data not shown).

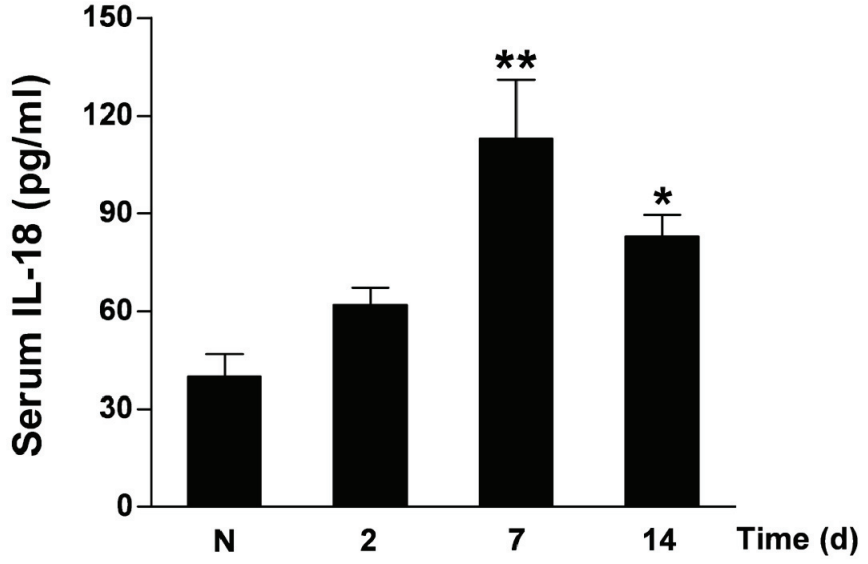

Figure 11. IL-18 serum levels
Serum IL-18 concentration in rats 2, 7 and 14 days following angioplasty. N: naive rats. Data are expressed as mean (pg/ml) ± S.E.M. of 10 rats per group. *P<0.05, **P<0.01 vs naive group.

3.7 Neutralization of IL-18 inhibits neointima formation

Finally, we tested the hypothesis that endogenous IL-18 affects neointima development and progression by using neutralizing rabbit anti–rat IL-18 IgG. Several group of rats (n=5) were treated with neutralizing anti-rat IL-18 IgG or normal rabbit IgG (Control) beginning at the time of angioplasty, the antibody injections were repeated at day 4, 8 and 12. A

remarkable increase in the number of PCNA positive cells was demonstrated in the media and intima 7 days after injury in control rats, which was much higher than the number of PCNA-positive cells in anti-IL-18 IgG treated group at the same time (fig. 12).

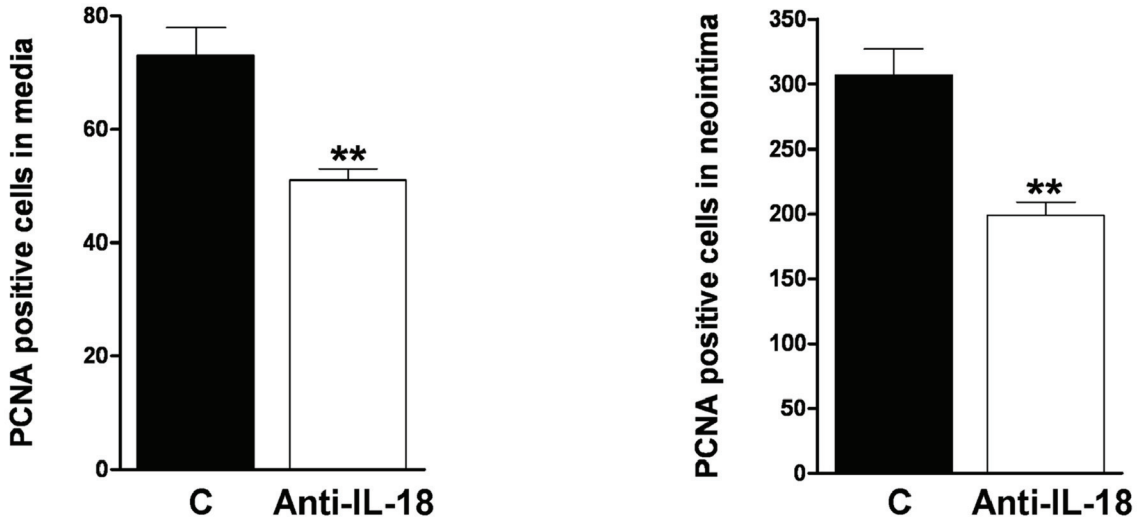

Figure 12. PCNA positive cells
Injured carotid arteries (n=5 per group) were harvested at day 7 after angioplasty. Extent of proliferative cells was determined by counting PCNA-positive cells in intima and media (10 fields per section). ***P*<0.01 vs Control group (C).

Intriguingly, IL-18 neutralization caused a significant inhibition of neointima formation by 27% (*P*<0.01) at day 14, compared to control group (fig. 13), concomitantly, anti-IL-18 IgG treated rats exhibited significantly (*P*<0.01) diminished neointimal content of α-SMA positive

cells (fig. 14), clearly suggesting a potential role of IL-18 in balloon induced SMCs proliferation.

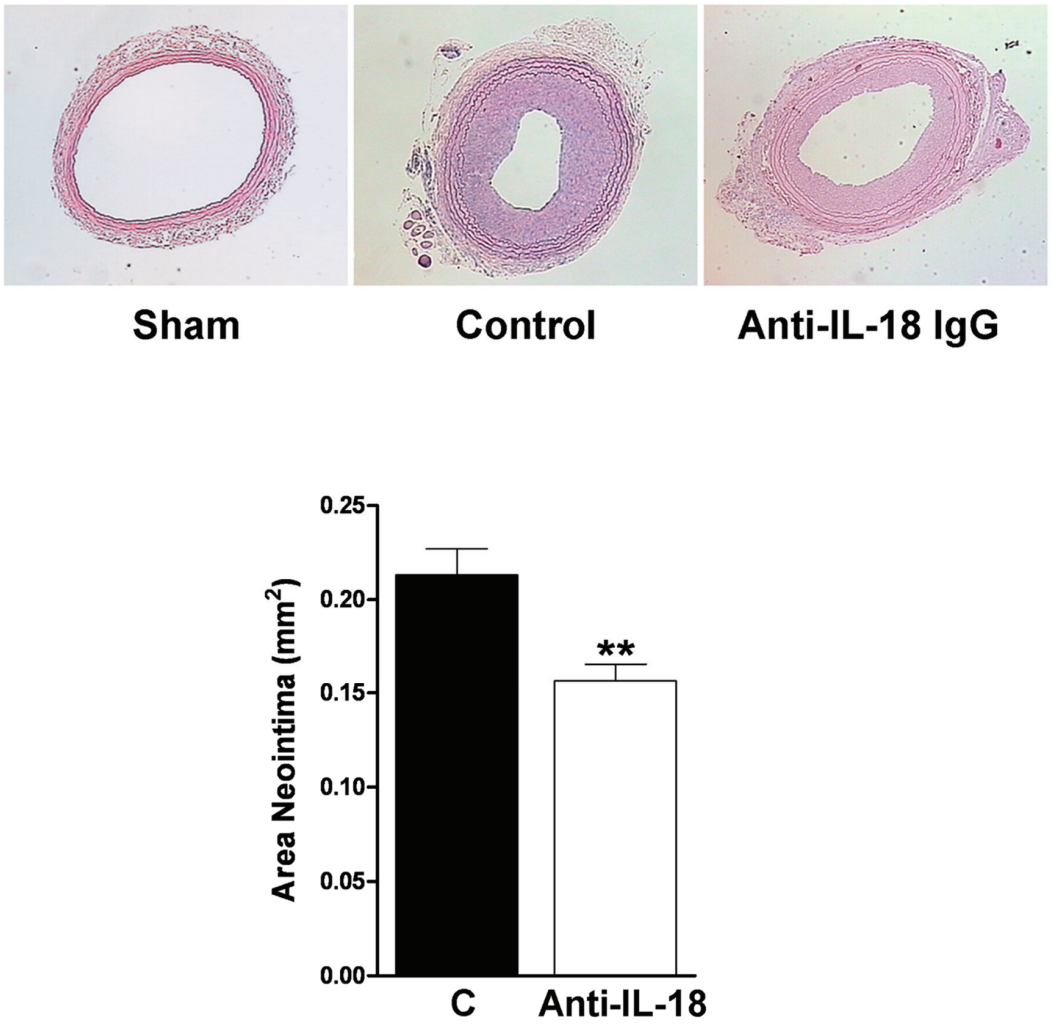

Figure 13. Effect of IL-18 neutralization on neointima formation
Photomicrographs showing the effect of IL-18 neutralization on neointima formation in rat carotid arteries after balloon angioplasty at day 14. Original magnification: x 50. Effect of IL-18 neutralization (Anti-IL-18 IgG, n=5) on neointimal areas of rat injured carotid arteries. Control animals (n=5) were treated with normal rabbit IgG following angioplasty as described in methods section. Results are expressed as mean ± S.E.M. $^{**}P<0.01$ vs Control group (C).

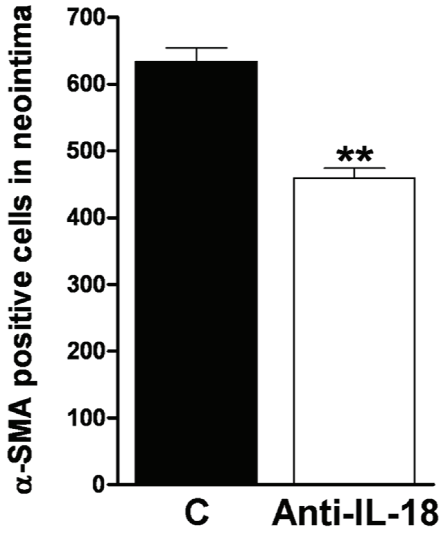

Figure 14. Neointimal α-SMA positive
Neointimal α-SMA positive cells number was determined 14 days following angioplasty as described in methods section. For each group studied carotid arteries obtained from 5 different rats were analyzed. Six sections from each carotid artery and 10 fields per section were reviewed and scored under blind conditions. ***P*<0.01 vs Control group (C).

3.8 Neutralization of IL-18 inhibits cytokines production and NF-κB activation

In order to provide mechanistic insights, the effect of IL-18 neutralization on several factors involved in neointima formation was investigated. Interestingly, IL-18 neutralization inhibits IFN-γ, IL-6 and chemokine IL-8 mRNA expression 7 days following angioplasty (fig. 15), in addition anti-IL-18 IgG treatment reduced balloon-induced NF-κB activation in injured arteries.

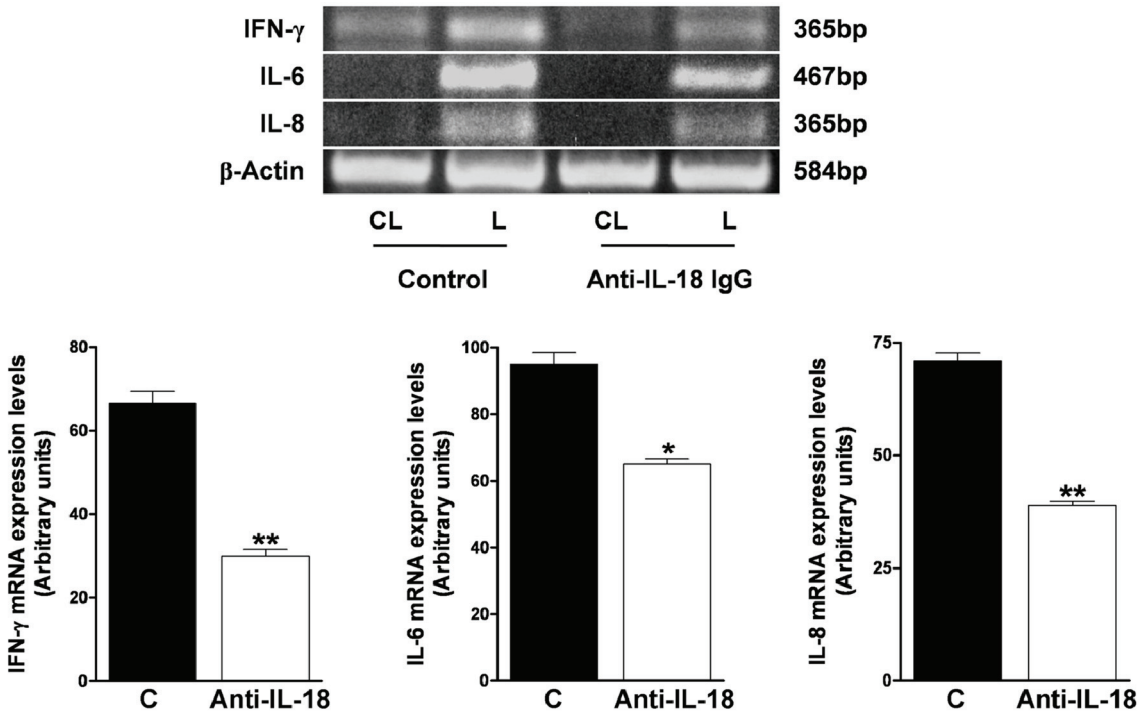

Figure 15. Effect of IL-18 neutralization on IFN-γ, IL-6 and IL-8 mRNA expression

Effect of IL-18 neutralization (Anti-IL-18 IgG) on IFN-γ, IL-6 and IL-8 mRNA expression in rat carotid arteries 7 days following balloon angioplasty. Control animals were treated with normal rabbit IgG following angioplasty as described in methods section. PCR amplification of reverse-transcribed mRNA was performed on total RNA isolated from pooled (n=3 per group) controlateral arteries (CL) and injured carotid arteries (L, lesion). Parallel amplification of rat housekeeping gene β-Actin was performed as internal control. Data shown are representative of two different experiments. In densitometric analysis IFN-γ, IL-6 and IL-8 mRNA expression levels were normalized to expression levels of housekeeping gene β-actin and expressed as densitometric arbitrary units. Results are expressed as mean ± S.E.M. of n=2 experiments. **P*<0.05, ***P*<0.01 vs Control group (C).

At day 14 a low level of NF-κB/DNA binding activity was detected in nuclear protein extracts from controlateral carotid arteries (CL) and from carotid arteries of sham-operated rats (n=5) (fig. 16). Conversely, a retarded band was clearly shown in injured carotid arteries (L, lesion) from control rats treated with normal rabbit IgG (n =5). Treatment of rats with neutralizing anti-rat IL-18 IgG (n=5), as described above, caused a marked inhibition of NF-κB activation in injured carotids (Fig. 16).

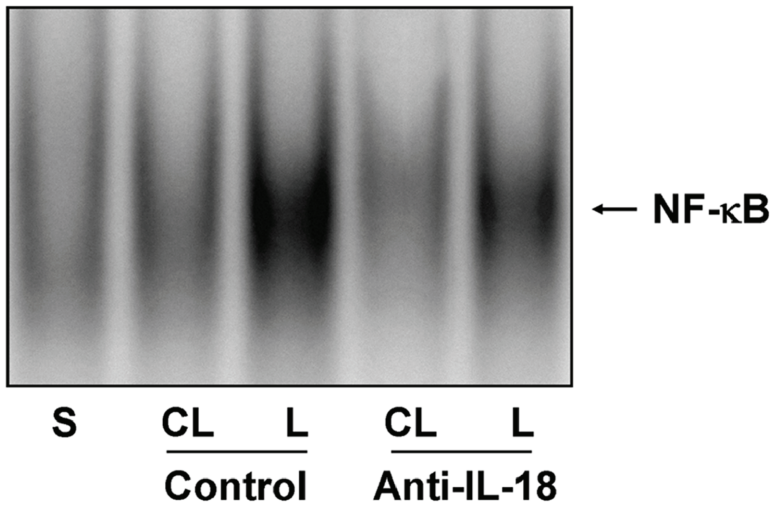

Figure 16. Effects of IL-18 neutralization on NF-κB/DNA binding activity
Effects of IL-18 neutralization on NF-κB/DNA binding activity in rat carotid arteries after balloon angioplasty. EMSA was performed on nuclear extracts of sham carotid arteries (S), controlateral (CL) and injured (L, lesion) carotid arteries from control rats treated with normal rabbit IgG or rats treated with neutralizing anti-rat IL-18 (Anti-IL-18 IgG), collected 14 days after angioplasty. Data shown are representative of three different experiments.

4. DISCUSSION

The present results identify, for the first time, a critical role for IL-18 in neointima formation in a rat model of vascular injury and suggest a potential role for IL-18 neutralization in reduction of neointima development and progression. In this study, we examined the temporal expression of IL-18 and IL-18Rα/β mRNA, and IL-18 active form in rat carotid artery after balloon angioplasty. We report increased expression of the pro-inflammatory cytokine IL-18 and of its signaling receptor, IL-18R (α/β-chain), in rat carotid arteries after vascular injury. Moreover, the levels of circulating IL-18 were found to be increased. IL-18 and IL-18Rα/β mRNA were equally expressed during the time course of neointima formation. A clear induction of IL-18 and IL-18Rα/β mRNA and active peptide was observed 2 days after balloon angioplasty and the elevated levels were sustained up to 14 days, with maximal expression evident at day 7 concomitant with the beginning of neointima formation.

Our in situ findings indicate that carotid arteries strongly express IL-18 after angioplasty, in contrast, non-injured arterial tissue did not contain

IL-18. It is interesting to note that expression of IL-18 is primarily located in SMCs that are actively involved in proliferation and migration, suggesting its potential role related to the neointimal formation [9]. SMCs IL-18 positive staining agrees with previous reports, demonstrating IL-18 expression by intimal SMCs in human atherosclerotic plaques [6].

Several cells in injured-arteries may express the IL-18 receptor, namely ECs, SMCs, macrophages and T lymphocytes, as demonstrated elsewhere [2]. Unfortunately, unavailability of appropriate antibodies hampered immunohistochemical analysis of IL-18Rα/β expression in situ. Low level of IL-18Rα/β mRNA in non-injured tissue, supported by RT-PCR data, suggested modest basal expression of the receptor on vascular cells a finding consistent with reports of constitutive expression of the IL-18 receptor on hematopoetic cell lines [115]. Interestingly, the combination of several cytokines found in neointima, namely IL-1β, TNF-α could promote the expression of both IL-18 receptor chains [89].

Increased serum levels of IL-18 in animals subjected to vascular injury is in keeping with an active role for this cytokine in the tissue pathogenesis and correlates well with epidemiological evidence showing higher IL-18 plasma levels associated with human restenosis [103,116].

To clearly elucidate the precise contribution of IL-18 involvement in development of vascular damage following balloon angioplasty we examined the effect of IL-18 neutralization on neointima formation. Animals treated with neutralizing IL-18 IgG exhibited a significant reduction in the size of neointima, concomitantly showing diminished neointimal content of α-SMA positive cells. Intriguingly, IL-18 neutralization diminished number of PCNA positive proliferating cells in the media and intima 7 days after injury, concomitantly with the beginning of neointima formation, clearly suggesting a potential role of IL-18 in balloon induced SMCs proliferation.

The precise molecular pathways responsible for the inhibitory effect of IL-18 neutralization on neointima formation in vivo remain to be elucidated. Several hypotheses can be put forward to explain these results. Recent studies have provided strong evidence for an important role of IL-18 on SMCs proliferation and migration in vitro. Sahar et al. [91] showed that IL-18 activates several key signaling pathways including MAPKs, transcription factors NF-κB and AP-1, and induces the expression of proinflammatory cytokines and chemokines such as IL-6, IL-8 and Monocyte Chemoattractant Protein-1 (MCP-1) in vascular SMCs. Activation of these signaling kinases may also be related to vascular SMCs

migration. Effects of IL-18 on cell survival and proliferation are cell type dependent. Unlike in endothelial cells [104], IL-18 in vitro failed to induce SMCs death. In fact, IL-18 induced SMCs proliferation in CXCL16-dependent manner [64].

Interestingly, we demonstrate, for the first time in vivo in a rat model of vascular injury, that IL-18 neutralization inhibits balloon induced cytokines (IFN-γ, IL-6) and chemokine (IL-8) mRNA expression, in carotid arteries 7 days following injury.

Furthermore, IL-18 neutralization reduced NF-κB activation, a transcription factor involved in neointimal development and progression [117,118]. A recent study suggests that angioplastic injury elicits an early, transient vascular NF-κB activation in media and a late, persistent activation in intima, critical in controlling intimal hyperplasia and the associated vascular inflammation [118].

The induced expression of IL-18 in neointima formation may involve additional functions. For example, IL-18 production may induce the expression of adhesion molecules [119,120], matrix metalloproteinases [121], as well as growth factors (e.g., GM-CSF), inducible nitric oxide synthase, or inducible cyclooxygenase [77], all factors regulated at transcriptional level by

NF-κB [122]. This could explain why at day 14 cytokine is decreasing while pathology is maximal.

There are some limitations for the current study. The rat balloon angioplasty model could be considered ideal to study the proliferation of smooth muscle cells in vivo, but is not a reliable experimental model of human angioplasty, because the injury is performed on a normal nonatheromatous arterial bed, this method doesn't take into account the contribution of vascular remodeling in human restenotic process, in addition, the carotid is not similar to other arterial beds (e.g. coronary arteries) with respect to its reaction to stimuli. Although it is not feasible to artificially regulate IL-18 in humans to determine its association with restenotic diseases, therefore, dissection of the role of this cytokine in lesion development is dependent on animal models.

In conclusion, these results identify, for the first time, a critical role for IL-18 in neointima formation in a rat model of vascular injury and suggest a potential role for IL-18 neutralization in reduction of neointima development and progression. The disease modifying activity of IL-18 neutralization in this model can offer a clue to better understand the role of IL-18 in human restenotic process.

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