

Original Article

Smooth muscle homeostasis in human atherosclerotic plaques through interleukin 15 signalling

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Abstract: Interleukin (IL)-15 is a cytokine that has a broad tissue distribution and is important in maintaining homeostasis of cells and stability of tissues. When IL-15 is also expressed by vascular smooth muscle cells (SMC), which are the dominant type of cells in most atherosclerotic plaques, it could be important in maintaining plaque tissue integrity and hence resistance of plaques towards development of clinically relevant complications such as plaque rupture and thrombosis. In this study, IL-15 and IL-15R α in vitro expression by coronary artery SMC was investigated using RT-PCR and FACS analysis. Immunohistochemistry was used to study in situ expression of IL-15 and IL-15R by SMC of human carotid artery atherosclerotic plaques. Multiplex ligand-dependent probe amplification (MLPA) was used to investigate the mRNA expression of 40 pro- and anti-inflammatory genes after stimulating coronary SMC with IL-15. We found that atherosclerotic SMC express both IL-15 and its receptor IL-15R, and IFN- γ and TNF- α enhance IL-15R expression in cultured SMC. MLPA studies on SMC revealed enhanced expression of PDGF beta mRNA after IL15 stimulation. In conclusion, our data suggest that IL-15 may contribute to atherosclerotic plaque integrity by stimulation of smooth muscle cells, probably in a PDGF dependent fashion.

Keywords: Atherosclerosis, smooth muscle cells, cytokines, plaque stability

Introduction

Interleukin (IL)-15 is a pleiotropic cytokine that plays an important role in the development and homeostasis of cells of both the innate and the adaptive immune system [1, 2], and is therefore considered of importance in the pathogenesis of atherosclerosis [3-5]. IL-15 signals through a receptor complex consisting of a private receptor 15R α [6, 7], the IL-2/15R β -chain and the common gamma chain (γ_c) [8, 9]. IL-15 as well as its receptor have a broad tissue distribution, indicating important functions for this cytokine also on non-immune cells. For example, IL-15 and IL-15R α protein expression have been detected on, among others, tubular epithelial cells from the kidney [10, 11], and on synoviocytes from patients with rheumatoid arthritis [12, 13]. It has been shown previously that IL-15 is abundantly expressed in atherosclerotic plaque macrophages [3, 4], which may therefore be important inducers of IL-15 functions in athero-

sclerosis. However, not all atherosclerotic plaques are inflammatory lesions, and many plaques in humans contain fibrous tissue, calcifications and smooth muscle cells (SMC) as their dominant tissue components. In fact, SMC are the most prevailing cell type in many plaques [14, 15] and are considered important for the tissue integrity and stability of the plaque structure. Neither the expression of IL15, nor its receptor IL15R have been detected in SMC of plaques, but experimental animal studies have revealed recently that IL-15 is expressed by SMC of the arterial duct [16], and in a cuff induced model of neointima formation [17]. Given the prominent presence of SMC in plaques and their function in maintaining plaque tissue integrity, we designed a study to evaluate IL-15 and IL-15 receptor expression by cultured SMC atherosclerotic plaque SMC. In addition, we investigated the effects of this cytokine on the expression pattern of pro- and anti-inflammatory by cultured SMC using Multiplex ligation-

dependent probe amplification (MPLA).

Materials and methods

Cell cultures

Primary human coronary artery smooth muscle cells (CaSMC) were obtained from Lonza (Breda, the Netherlands) and cultured in DMEM supplemented with 10% fetal calf serum, antibiotics (penicillin/streptomycin) and L-Glutamine (all from Life Technologies, Breda, and the Netherlands). Sub confluent monolayers of third to fifth passage CaSMC were used in all experiments. For cytokine stimulation experiments CaSMC were cultured with the following cytokines for indicated time points: TNF- α (20ng/ml), IFN- γ (100units/ml) and IL-15 (10/50ng/ml, all the cytokines were from Strathmann, Hamburg, Germany)

RNA isolation and cDNA synthesis

RNA was extracted from cells using Trizol reagent (Invitrogen, Breda, the Netherlands) according to the manufacturer's instructions. cDNA was synthesized from 2.5 μ g of total RNA using Pdn6 as a template and M-MLV reverse transcriptase (RT) (Invitrogen).

Reverse transcriptase PCR

cDNA was amplified by PCR in a reaction mixture (25 μ l) containing Taq-buffer (Invitrogen), 1.5mM MgCl₂ (2.0mM for IL-15 PCR), 0.2mM dNTPs, 5pmol of each 5' and 3' primer and 0.25U Taq DNA polymerase (1.0 for IL-15 PCR).

The following primers and annealing temperatures were used: IL-15 sense 5'-GTATTGTAGGAGGCATCGTGG-3', antisense 5'-GGTCATGTGATCCAAGGTCTG-3'; β -actin sense 5'-ACCCAACACTGTGCCATCTA-3', antisense 5'-TAGAAGCATTGCGGTGGACGA-3'; IL-15R α sense 5'-CCTCAAGTCAAGGCAAACCTC-3', antisense 5'-GACTTCTGAGAGGCCTGGTG-3'; IL-2/15R β 5'-ACGTCCAGAAGTGGCTCTCT-3', antisense 5'-AGAAGTAACCTGGTTGGTGAAGC-3'; γ_c sense 5'-CTCCAGAGAACCTAACACTTC-3', antisense 5'-GATCCTCTAGGTTCTTCAGG-3'.

All PCR runs had 40 cycles and each cycle consisted of denaturation at 95°C for 1min, annealing at pre-determined temperatures for 1min, and elongation at 72°C for 2min preceded by initial denaturation at 95° for 5min, and a final

extension step at 72°C for 5min. Aliquots of PCR products were electrophoresed on a 1.0% agarose gel and visualized by ethidium bromide staining. A mock PCR (without cDNA) was included to exclude contamination in all experiments

Real-time PCR

Realtime RT-PCR was performed with the Lightcycler 2.0 system and the LightCycler Fast-Start DNA MasterPLUS SYBR Green I kit (both Roche, Almere, the Netherlands) according to the manufacturers instructions.

The following primers and annealing temperatures were used: IL-15 sense 5'-TTGAAGATCTTATTCAATCTATGC-3', antisense 5'-TGTTACATTCCCATTAGAAGAC-3'; IL-15R α sense 5'-CTCAAGTCAA GGCAAACCTC-3', antisense 5'-ACCTCTTCTCAGTCGTCTTT-3'; PDGF- β protein sense 5'-AGACCCCGGAGAGGAAGAT-3', antisense 5'-CGTTGGTGCGGTCTATGAG-3'. Analysis of real-time PCR data was performed with LinRegPCR software[18]. The data obtained are the result of two independent experiments. IL-15R α levels are expressed relative to the level of a housekeeping gene TATA box binding protein (TBP).

Multiplex ligation-dependent probe amplification

RNA from IL-15 stimulated (50ng/ml, 24 hours) and control SMC was isolated and analyzed by multiplex ligand-dependent probe amplification (MLPA)[19] using the SALSA MLPA R009 Inflammation Probe mix (MRC-Holland, Amsterdam, the Netherlands) as described previously[20]. The SALSA MLPA R009 inflammation mRNA probemix contains several probes specific for mRNAs that are strongly induced by treatment of blood in vivo or in vitro by lipopolysaccharide (LPS). The R009 probemix contains several different probes among which are probes for pro- and anti-inflammatory cytokines, chemokines, their receptors and nuclear factor κ B (NF- κ B) pathway components. Expression levels of mRNA were normalized to three house keeping genes (CDKN1A, PARN and B2M) included in the probe mix.

FACS analysis

Adherent CaSMC were harvested by gentle scraping with a rubber policeman. CaSMC were

incubated with 10% normal human serum (Sanquin, Amsterdam, the Netherlands) and subsequently stained with primary mouse antibodies directed against α -smooth muscle actin (clone 1A4, DAKO, Glostrup, Denmark) and IL-15 (clone mAb247, R&D) and polyclonal rabbit antibody against IL-15R α -chain (clone HO-107, Santa Cruz, CA, USA). Cells stained with primary antibodies were subsequently incubated with phycoerythrin (PE) conjugated goat anti-mouse immunoglobulins (GaM-PE; SBA, Birmingham, Al, USA) or fluorescein isothiocyanate (FITC) conjugated swine anti-rabbit immunoglobulins (SwaR-FITC; DAKO). Labelled cells were analyzed with a FACScalibur (Becton-Dickinson, Erembodegem, Belgium).

Immunohistochemistry

Atherosclerotic carotid artery endarterectomy specimens were obtained from 5 patients who were admitted to the hospital for the treatment of carotid artery stenosis. The use of the tissue samples was approved by the institutional medical ethical committee, and all data were analyzed anonymously. All specimens were instantly frozen in liquid nitrogen and stored at -80°C . Serial sections were cut at $5\mu\text{m}$ and fixed with acetone prior to staining. Haematoxylin and Eosin stains were used for histomorphological observations, and immunohistochemical analysis was performed on adjacent sections using double staining techniques. The following primary antibodies were used: anti-IL-15 (Diaclone, Besançon, France), anti-IL-15R (AF247, R&D, Oxon, UK) and anti- α -smooth muscle actin (clone 1A4, DAKO, reactive with vascular SMC). Before labelling with primary antibodies, endogenous peroxidase activity was blocked with 0.03% hydrogen peroxide and 1% sodium azide in TBS for 20 minutes. For double staining of SMC with IL-15 and with IL-15R, sections were first incubated with the unlabeled IL-15 or IL-15R antibody, followed by incubation with Envision goat anti-mouse-HRP. Peroxidase activity was developed with 3-amino-9-ethylcarbazole. After a blocking step with normal mouse serum, sections were incubated with anti- α -smooth muscle actin followed by streptavidin-HRP, and immunoreactivity was visualized with diaminobenzidine tetrahydrochloride. For confirmation of immune double staining of cells the sections were analyzed using a Nuance spectral imaging system (CRi, Woburn, MA) a computer assisted optical technique which distracts colours on the

basis of their spectral characteristics [21].

Results

Expression of IL-15 and IL-15 receptor by human coronary artery smooth muscle cells in vitro

To study whether smooth muscle cells are able to express IL-15 and its receptor IL-15R α *in vitro*, we performed RT-PCR and FACS analysis on cultured human CaSMC. Transcripts of IL-15 mRNA were detected in CaSMC (**Figure 1A**). FACS analysis showed IL-15 protein expression by cultured CaSMC (**Figure 1B**). IL-15R α mRNA as well as IL-15R α protein was expressed by cultured CaSMC (**Figure 1A and B**). In addition, both IL-2/15R β and γ_c mRNA transcripts were expressed by CaSMC *in vitro* (**Figure 1A**), indicating that CaSMC express a complete IL-15 receptor complex.

The effect of TNF- α and IFN- γ on mRNA expression of IL-15R α by cultured CaSMC was studied by real-time PCR on cDNA of CaSMC. Both IFN- γ and TNF- α enhanced the expression of IL-15R α mRNA in a time-dependent fashion (**Figure 1C**).

IL-15 and IL-15R α are expressed by smooth muscle cells in human atherosclerotic plaques

Samples of atherosclerotic plaque tissue contained multiple SMC rich areas in all 5 cases, which was confirmed by immunostaining. Because it was difficult to appreciate reliably the presence of double stained cells in light microscopical sections (in either the SMC/IL-15 or SMC/IL15R combinations), we analysed all sections with the use of spectral imaging software, which clearly identified the expression of IL-15 and also its receptor IL-15R on a subpopulation of SMC in all 5 atherosclerotic plaque samples. See also **Figure 2 and 3**.

IL-15 induces platelet derived growth factor- β expression in SMC

In this study the expression of 40 inflammatory genes (see **Table 1**) in IL-15 stimulated SMC was investigated using the SALSA MLPA R009 Inflammation Probe mix. Only PDGF- β showed a significant increase in expression upon 24 hours stimulation with IL-15 (**Figure 4A**). Of the other 39 studied genes only tissue factor showed a weak expression while all others were

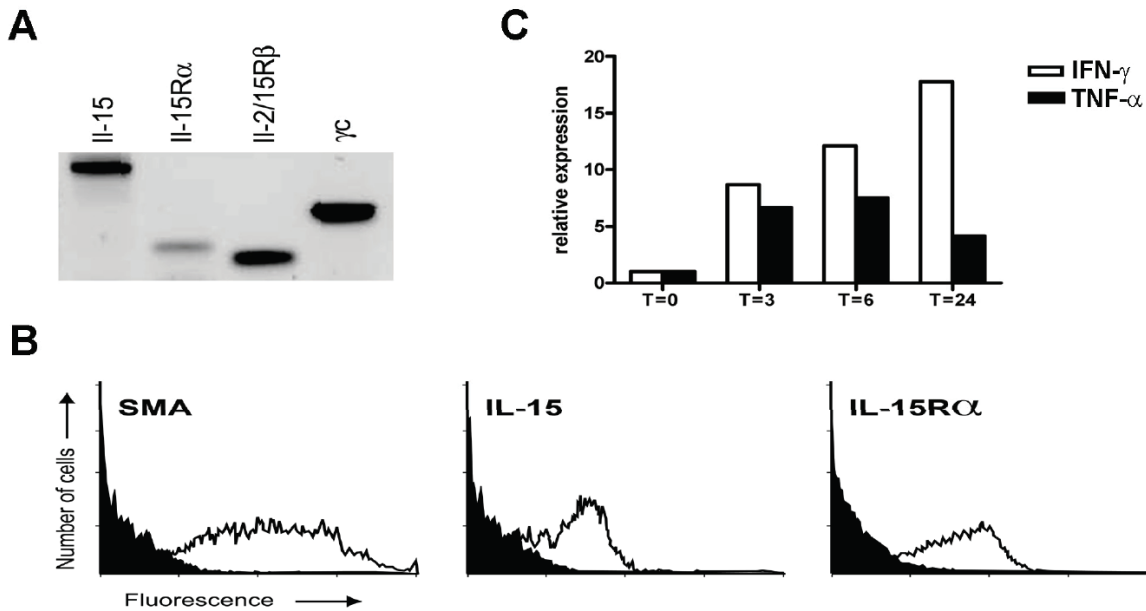


Figure 1. A. rtPCR of IL-15, IL-15R α , IL-2/15R β and the γ C in cultured CaSMC. B. FACS analysis of CaSMC stained with smooth muscle actin (SMA, open histograms), IL-15 and IL-15R α . Control isotype matched antibodies GaM-PE and SwaR-FITC are shown as closed histograms (C) relative expression of IL-15R α mRNA after stimulation with IFN- γ or TNF- α for 0, 3, 6 and 24 hours.

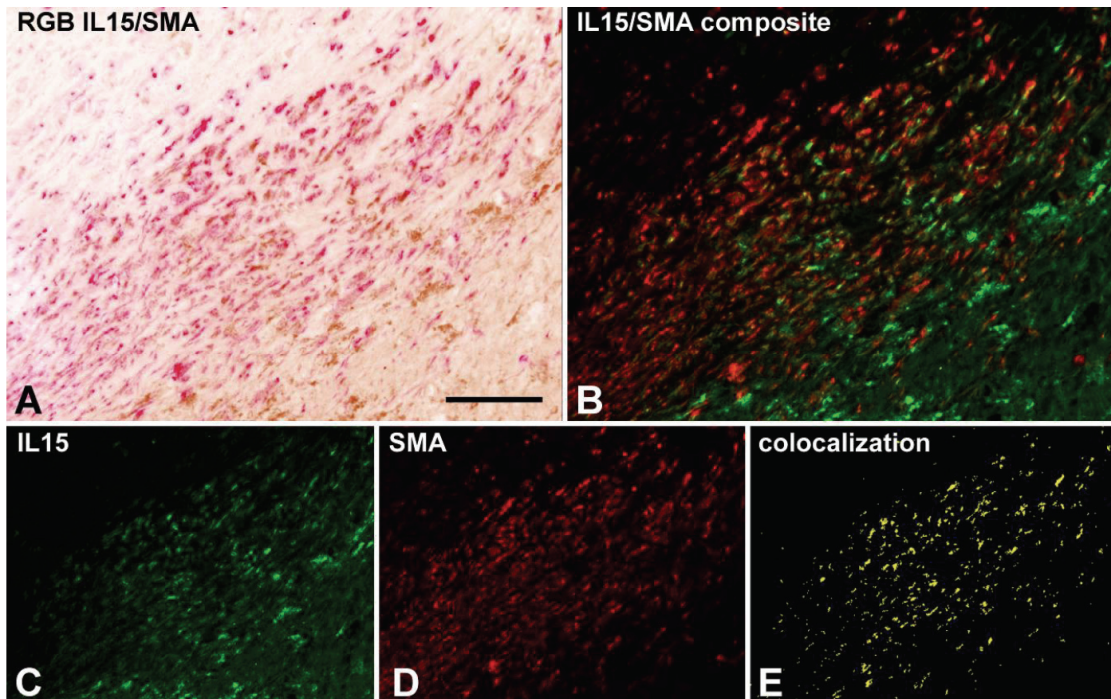


Figure 2. Spectral analysis of immunodouble stained section of an intimal atherosclerotic plaque using IL-15 and SMA antibody combination. A. Light microscopical image of the original immunostained tissue section showing IL-15 in red and SMA in brown. B. Spectral analysis of the same section showing IL-15 staining in green and SMC staining in red. C-E: same section showing only IL15+ cells

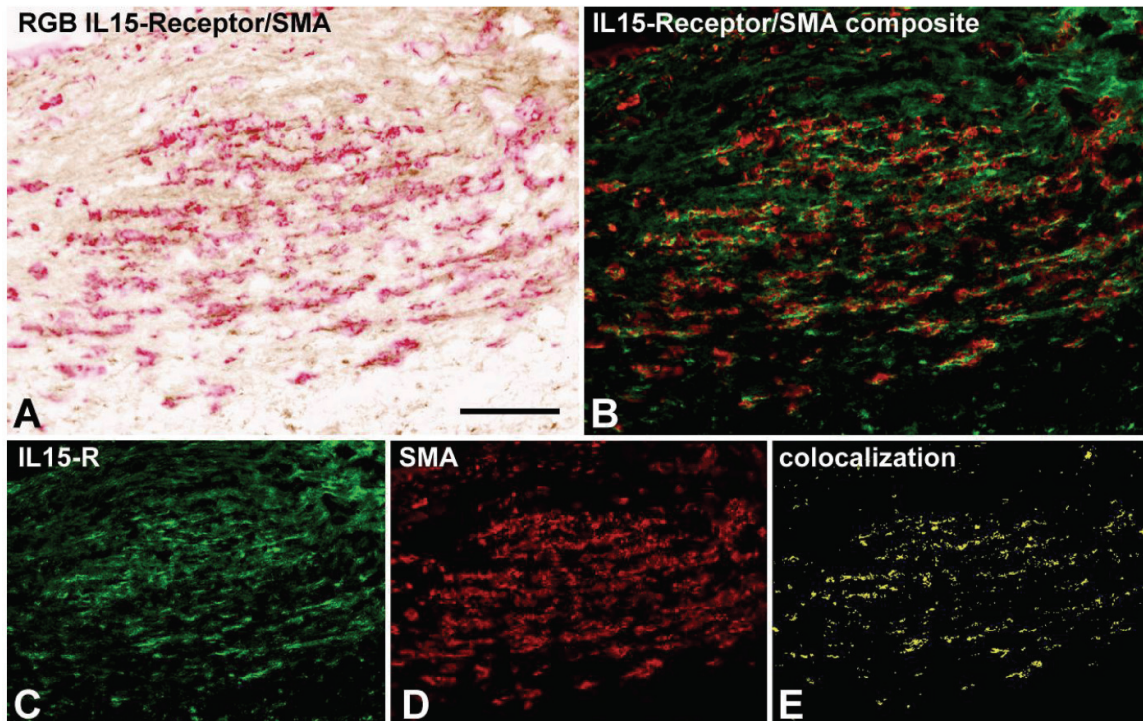


Figure 3. Spectral analysis of immunodouble stained section of an intimal atherosclerotic plaque using IL-15R and SMA antibody combination. **A.** Light microscopical image of the original immunostained tissue section showing IL-15R in red and SMA in brown. **B.** Spectral analysis of the same section showing IL-15 staining in green and SMC staining in red. **C-E:** same section showing only IL15R+ cells (**C**), only SMC (**D**), and only double stained cells (**E**). Bar = 0.1mm

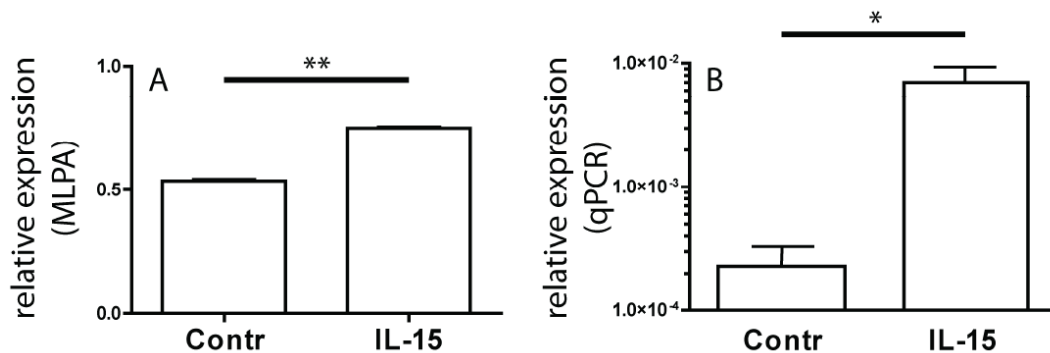


Figure 4. IL-15 induces PDGF- β mRNA expression in SMC. Enhanced expression of PDGF- β mRNA in SMC after IL-15 stimulation was first observed with MLPA (**A**) and confirmed by real time PCR (**B**). Asterisks indicate a significant difference ($p < 0.05$) between IL-15 stimulation and control. The data obtained are the result of two independent experiments.

negative. Results of the MLPA were confirmed with real time PCR: IL-15 stimulated SMC showed an almost 20 fold increase of PDGF- β mRNA as compared to control (**Figure 4B**).

Discussion

IL-15 is a pleiotropic cytokine involved in a range of biological activities maintaining cellular

Table 1. List of inflammatory genes analysed by MLPA

Cytokines	
Interleukin-1a (IL-1a)	
Interleukin-1b (IL-1b)	
Interleukin-1RA (IL-1RA)	
Interleukin-2 (IL-2)	
Interleukin-4 (IL-4)	
Interleukin-6 (IL-6)	
Interleukin-10 (IL-10)	
Interleukin-12A (subunit p35)	
Interleukin-12B (subunit p40)	
Interleukin-13 (IL-13)	
Interleukin-15 (IL-15)	
Interleukin-18 (IL-18)	
Interferon-gamma (IFN-g)	
Tumor necrosis Factor-a (TNF-a)	
Lymphotoxin (TNF-β)	
TNF Receptor 1 (TNFR1)	
Chemokines	
Interleukin-8 (IL-8)	
Monocyte chemotactic protein-1 (MCP-1)	
Monocyte chemotactic protein-2 (MCP-2)	
Macrophage inflammatory Protein-1 alpha (MIP1a)	
Macrophage inflammatory Protein-1 beta (MIP1b)	
Signal Transduction Factors	
Nuclear Factor Kappa-B (NF-kB) 1	
Nuclear Factor Kappa-B (NF-kB) 2	
I-Kappa-B alpha (IκBa)	
Others	
Platelet derived growth factor (PDGF)	
p21 (WAF1, CIP1, MDA6)	
Thrombospondin 1	
Tissue Factor (TF)	
MYC	
Macrophage migration inhibitory factor (MMIF)	
Beta-2 microglobulin	
Glutathione S Transferase (GST3)	
Homolog of Leukemia Viral BMI-1 Oncogene	
Phosphodiesterase 4B	
Protein-Tyrosine	
Phosphatase (PTB1B)	
Protein-Tyrosine phosphatase type 4A (PTP)	
Poly(A) specific ribonuclease	
Serine Proteinase inhibitor	
BMI-1 oncogene homolog	

homeostasis, and which has been studied extensively in cells of the immune system. However, the expression of its specific receptor IL-15Rα is widespread in tissues, which implicates that IL-15 has not only effect on cells of the immune system, but also on non-immune cells. Using RT-PCR and FACS analysis, we found expression of IL-15 mRNA and IL-15 protein in cultured coronary artery derived SMC (CaSMC). Using spectrally analysed immunohistochemical double stains applied on intimal plaque tissues, we also revealed that most, but not all, plaque SMC express IL-15. In a previous study using conventional immunohistochemical methods we

were not able to detect immunopositivity of IL-15 on plaque SMC, and thus far only Wuttge et al observed IL-15 positivity in SMC of experimental animals [3, 4]. However, in the present study we evaluated the IL-15 staining patterns with the use of spectral image analysis of immunodouble stains, which allows to detect reliably the topographic localization of lower levels of immunostaining in tissues and cells [22]. Moreover, the immunostaining results were confirmed by means of additional molecular and functional studies, which put the activity of IL-15 in plaques into new perspectives. IL-15 signals either via its designated receptor IL-15Rα alone [23, 24], or via a heterotrimeric receptor complex consisting of IL-15Rα, IL-2/15Rβ and γc(8). In this study we showed that SMC in human atherosclerotic plaques express IL15Ra *in vivo*, and human CaSMC express IL-15Rα, IL-2/15Rβ and γc, *in vitro*. Furthermore, we showed that IL-15Rα mRNA expression was up regulated in CaSMC *in vitro* after stimulating the cells with IFN-γ and TNF-α. This suggests that in a pro-inflammatory atherosclerotic microenvironment vascular SMC become more receptive for IL-15 signalling. Moreover, since apparently both IL-15 and its receptor are expressed by intimal SMC, our results reveal a potential autocrine pathway of IL-15 signalling by SMC in plaques. Initially, IL15 was believed to be operative only as a secreted cytokine, but there is now evidence that IL-15 may also exert biological effects in a cell membrane-associated form [25-27]. Via binding of IL-15- to its IL15Rα complex on the cell membrane, IL-15 may be *trans* presented to neighbouring (smooth muscle) cells [26, 27]. In addition, also reverse signalling may occur when IL-15 is presented by its receptor on the cell membrane, since experimental stimulation with recombinant soluble IL-15Rα or anti IL-15 antibodies has shown that membrane-bound IL-15 can mediate the activation of several intracellular signalling cascades [28]. Regrettably, we could not distinguish membrane bound IL-15 from intracellular IL-15 in our FACS analysis, since the cells were permeabilized during isolation and staining procedures.

In a further attempt to elucidate the effect of IL-15 on SMC, the mRNA expression of 40 genes was studied by means of a MLPA. Only the expression of the PDGF-β protein was found to be significantly up-regulated after IL-15 stimulation. PDGF-β expression by SMC in normal arter-

ies is low or absent but its expression is enhanced by SMC in atherosclerosis [29, 30]. *In vivo*, PDGF- β forms a homodimer (PDGF-BB), which signals via its receptor PDGFR- β . Several studies in experimental models of neointima formation have shown that PDGF-BB signalling occurs during SMC proliferation and migration from the media into the neointima[31]. Once IL-15 upregulates PDGF- β , it may contribute to the accumulation of SMC in the atherosclerotic plaque in a PDGF-BB dependent fashion. However, the situation may in fact be more complicated, since experimental studies in animals showed that IL-15 also inhibits the proliferation SMC in the arterial duct, and blockade of IL-15 increased neointimal formation in a model for cuff induced plaque formation [16, 17]. However, in these experiments the inhibiting effects in both studies were at most modest, and SMC were studied under proliferative conditions (arterial duct closure and intimal response to injury respectively). This clearly differs from the quiescent SMC that maintain a state of homeostasis in the plaque through supporting the tissue integrity, and which were subject to our study. IL-15 signalling may affect atherogenesis also along other pathways. First, PDGF-BB contributes to the formation of lipid laden (foamcell type) macrophages via up regulation of Lipoprotein Lipase (LPL) expression [32, 33], and second, IL-15 may have an effect on the activation of T cells in plaques [3]. These effects can also be exerted by IL15 secreting plaque macrophages³, but most lesions have a significant SMC mass, and numbers of SMC cells generally exceed by far the numbers of macrophages, as was also the case in the plaque samples that we studied[14, 15]. In conclusion, our data suggest that IL-15 and its receptor complex may contribute to atherosclerotic plaque integrity by modulating smooth muscle cells, likely in a PDGF dependent fashion.

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