Identification of therapeutic targets in coronary artery disease: from patient to mice and back

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CHAPTER 4

CC Chemokine ligand-18 (CCL18/PARC) enhances atherogenesis and induces T-cell accumulation in atherosclerotic plaques of ApoE-/- mice

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ABSTRACT

Background: Although serum CCL18 (PARC) levels were shown to be elevated in unstable angina pectoris and abundant expression of the chemokine CCL18 has been observed in human atherosclerotic plaques, a causal role of CCL18 in atherogenesis remains to be established. In this study we aimed to map the effects of focal and systemic CCL18 overexpression on plaque progression and stability.

Methods and Results: CCL18 mRNA expression was 4-fold up-regulated in autopsy derived early versus advanced lesions. Administration of synthetic CCL18 for two weeks to atherosclerotic, Western type diet fed ApoE-/- mice was seen to promote plaque progression (N=14, $P<0.05$) but led to decreased plaque macrophage content ($P<0.05$). CCL18 increased plasma IL-6 levels by >100 fold ($P<0.01$), without any effects on CCL2 and CCL3, indicative of a specific CCL18 induced pro-inflammatory effect. To dissect out peripheric from plaque directed effects of CCL18 treatment, CCL18 was over-expressed in pre-existing atherosclerotic plaques in Western type diet fed apoE-/- mice by adenoviral gene transfer (N=12). Two weeks following gene transfer, CCL18 over-expressing plaques did not show any differences in plaque size nor in collagen, macrophage or smooth muscle cell content as compared to Ad-empty controls. Interestingly, Ad-CCL18 treatment markedly increased plaque content of $CD3^+$ T-cells, which all were FoxP3 negative. In keeping, sCCL18 led to an impaired proliferative response in macrophages and to dose dependently induced Jurkat T-cell migration in a CCR3 independent manner.

Conclusions: CCL18 exacerbates plaque growth and acts as a potent atherosclerotic T-cell chemoattractant *in vivo*, making it an attractive target to influence plaque progression and stability.

INTRODUCTION

Inflammation and inflammatory responses play a pivotal role in atherosclerosis and cardiovascular disease [1]. Over the last decade, it has become increasingly clear that chemokines have a major part in these processes [2], influencing plaque progression and stability as well as cellular recruitment of inflammatory cells to sites of injury and ischemia [3, 4]. The causal involvement of various chemokines in cardiovascular disease is well documented, however for many candidate chemokines conclusive proof to support a causal association still is lacking.

This is certainly the case for CCL18, as only few studies have focused on this chemokine in relation to cardiovascular disease [5,6,7]. CCL18 or pulmonary and activation-regulated chemokine (PARC) is a 7.8 kDa chemokine with constitutively high expression in lung and human plasma. CCL18 is currently considered to be an orphan ligand, although it has been proposed to act as competitive inhibitor of CCL11 (eotaxin) binding to CC-chemokine receptor 3 (CCR3) [8]. Furthermore, CCL18 is expressed by a wide range of monocyte subsets to serve as a chemoattractant for naïve CD45RA T-cells. CCL18 was also reported to stimulate collagen production in fibroblasts [9, 10], and has a function in inflammatory T_H2-mediated pulmonary diseases [11].

We have recently shown that CCL18 is implicated in acute coronary syndromes, as elevated CCL18 levels were observed in patients presenting with unstable angina pectoris (UAP) [5]. UAP patients also displayed increased CCR3 protein levels on circulating mononuclear cells [5]. Additionally, CCL18 has been detected in atherosclerotic plaques, with abundant expression in areas of decreased stability [6, 7], and co-localizes with CD83+ dendritic cells [12]. As CCR3 is expressed by intimal vascular smooth muscle cells with a migratory phenotype [13], and as CCL18 was suggested to be responsible for the recruitment of T-cells to the atherosclerotic plaque [6], it is tempting to speculate that the CCL18/CCR3 axis might be implicated in atherosclerosis. Currently however, its precise role in plaque homeostasis is unknown, partly due to the fact that CCL18 lacks a murine counterpart.

To elucidate the exact role of CCL18 in atherogenesis and/or plaque stability, we set out to study plaque CCL18 expression at different stages of disease progression, and addressed effects of systemic as well plaque specific overexpression of CCL18 on (advanced) atherosclerotic lesion progression in ApoE-/- mice.

METHODS

Human carotid plaque micro-array and protein analysis

RNA was isolated from early (n=9) and advanced (n=8) stable carotid artery plaques collected at autopsy and stable (n=3) and ruptured (n=12) lesions from surgery. Plaques were staged by histological analysis of aligning slides (Virmani classification [14]). RNA was isolated by the Guanidine Thiocyanate (GTC)/CsCl gradient method [15] using a NucleoSpin RNA II (Macherey-

Nagel) kit. Samples were individually hybridized to HGU133 2.0 Plus arrays (Affymetrix, Santa Clara, California) according to the manufacturer's instructions.

Paraffin sections (4 µm in thickness) from human autopsy carotid arteries were stained using a primary rabbit anti-human CCL18 IgG Polyclonal Ab (Genway; 2 µg/mL), overnight at 4°C. As positive control, lung sections were stained for CCL18. Negative controls received no primary antibody. A rabbit IgG polyclonal Ab (Genway; 2 µg/mL) served as isotype control. CCL18 staining of the luminal endothelial layer, intima, media, adventitia as well as vasa vasora was scored by 1 independent observer in a blind manner. Few CCL18 positive cells (<10%) were scored as 1, moderate positivity (approximately 50%) as 2 and (almost) complete positivity (>50%) as 3.

CCL18 peptide synthesis

CCL18 peptide (codon 21 (Ala) to codon 89 (Ala); sCCL18) was synthesized by standard solid phase Fmoc chemistry as described before [16]. Purity and identity of the peptide was established by LC-MS (Molecular weight 7,855 Da) [16].

CCL18 adenovirus

Adenoviral vectors expressing human CCL18 under a CMV promoter or an empty transcript, both carrying the gene encoding Green Fluorescent Protein (Ad-CCL18-GFP and Ad-empty-GFP), were generated, produced and titered as described previously (kindly provided by dr. Sergei Atamas, University of Maryland, Baltimore, USA) [17]. Virus stocks (titer: $1x10^{10}$ pfu/ml) were aliquoted and stored at -80°C until further use. Adenovirus batches were essentially free of replication competent virus as judged by PCR using GGGTGGAGTTTGTGACGTG as forward and TCGTGAAGGGTAGGTGGTTC as reverse primers.

Cellular response on CCL18

Primary murine vascular smooth muscle cells (VSMCs [18]) and Mouse H5V endothelial cells, RAW 264.7 murine macrophage as well as Jurkat T-cell line were used to examine the mitogenic response and gene expression patterns after incubation with sCCL18. In brief, cells were plated in 24-well plates and grown to 70% confluence in DMEM (VSMCs, H5V and RAW264.7 cells) or RPMI-1640 (Jurkat cells) supplemented with 10% fetal calf serum (FCS), 100 µg/ml streptomycin, 100U/ml penicillin and 2 mM L-glutamine. Afterwards, cells were serum starved in DMEM/RPMI-1640 + 1%FCS for 10 hours and subsequently incubated for 16 hours with 25 ng/ml of sCCL18, together with ^{3}H-thymidine (925 GBq/mmol; Amersham, Uppsala, Sweden). The next day, ^{3}H-thymidine incorporation was measured by a Packard 1500 liquid scintillation analyzer (PerkinElmer) and cells were harvested in parallel for RNA isolation. Guanidium thiocyanate-phenol was used to extract total RNA from cells and samples were subjected to DNAse I treatment (Promega, Madison, WS) after which cDNA was generated using RevertAid M-MuLV reverse transcriptase (Fermentas, Burlington, Canada) according to manufacturer's protocol. Semi-quantitative gene expression analysis of IL-6, CCR3, CCR5 and CD-69 was performed by

the SYBR-Green method on a FAST 7500 real time RT-PCR apparatus (Applied Biosystems, Foster City, CA) using the primer pairs depicted in table 1.

Table 1. Primer sequences used for real time-PCR analysis.

Gene	Forward	Reverse
IL-6	GAAGAATTTCTAAAAGTCACT TTGAGATCTAC	CACAGTGAGGAATGTCCACAAAC
CCR3	TGCAGGTGACTGAGGTGATTG	CGGAACCTCTCACCAACAAAG
CCR5	GACTGTCAGCAGGAAGTGAGCAT	CTTGACGCCAGCTGAGCAA
CD69	AAAGCACGAGCGATCCAGTTA	AGAAAATAATTCGTTCCTCACCAACTA
HPRT	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG
Cyclophilin	CCATTTCAAGAAGCAGCGTTT	ATTTTGTCTTAACTGGTGGT

Migration assay

Jurkat T-cells were stimulated with increasing concentrations (1-100 nM) of sCCL18 in a 96-well Neuroprobe plate (pore size 5 μm, Gaithersburg, MD). Chemotactic fMLP (1 nM, Sigma) was used as positive control, chemotaxis buffer (RPMI-1640, 25 mM HEPES and 0.1% BSA) as negative control. After 4 hours of incubation, pictures of the wells were taken and the migrated cells were counted manually.

In vivo kinetics of sCCL18

sCCL18 was radiolabeled with ^{125}I (GE Healthcare, Diegem, Belgium) according to the iodogen method [19]. To examine its *in vivo* kinetics after repeated administration, ^{125}I-sCCL18 was administered once daily to C57Bl6 mice (Charles River, Maastricht, the Netherlands) by intravenous injection (doses of 100 ng, 500 ng and 1 μg) or intra-peritoneal administration (doses of 5 and 10 μg); at 1, 4 and 24h after each injection blood samples were taken and counted for radioactivity. Mice were sacrificed at 72 h following the first injection, main organs and tissues were excised and ^{125}I accumulation was counted by a Wizard 1470 automatic gamma counter (PerkinElmer, Waltham, MA).

Systemic sCCL18 *in vivo* experiments

Effects of systemic administration of sCCL18 on (advanced) atherosclerosis were studied in female 12-week old ApoE-/- mice, fed with a Western-type diet containing 0.25% cholesterol and 15% cocoa butter (Special Diets Services, Witham, Essex, UK). Diet and water were provided ad libitum. After 6 weeks of western-type diet, mice (N=7) received an i.p. bolus injection of 5 μg sCCL18 in 500 μl of PBS/0.5%BSA, followed by two-daily injections of 2.5 μg sCCL18 for two weeks. As a control, mice were injected with 500 μl of PBS/0.5%BSA. Plasma cholesterol levels were monitored colorimetrically using enzymatic procedures (Roche Diagnostics, Mannheim, Germany). After two weeks, control and sCCL18 treated mice were sacrificed, blood was drawn for FACS analysis and differential blood cell count on an automated Sysmex XT-2000iV Veterinary Haematology analyzer (Sysmex corporation, Kobe, Japan) after which the arterial bed was

flushed for ten minutes with PBS and formaldehyde and heart, aorta and spleen were taken out for histological analysis.

Focal overexpression of CCL18 in pre-existing plaques

Female ApoE-/- mice (N=24), aged 12 weeks, were obtained from our own breeding stock and were put on a Western type diet. After two weeks, mice received constrictive collars around both carotid arteries as previously described [20]. Briefly, semi-constrictive silastic collars (Dow Corning, Midland, MI) were placed bilaterally at both common carotids to induce the formation of complex fibroatheromatous lesions proximal to the collar within 4 weeks. The collars were removed and the internal carotid artery as well as the common carotid proximal to the plaque were clamped and the adenovirus suspension (20 µl of Ad-CCL18-GFP or Ad-empty-GFP at a non-inflammatory titer of 1.0×10^{10} pfu/ml) was instilled via the left common carotid artery and left to incubate for 10 minutes. This virus load was earlier shown to be well tolerated and not accompanied by virus related cytotoxicity. Two weeks after local incubation, the mice were sacrificed and before harvesting, the arterial bed was flushed for ten minutes with PBS and formaldehyde. Fixated carotid arteries were embedded in OCT compound (Sakura Finetek, Zoeterwoude, the Netherlands), snap frozen in liquid nitrogen and stored at -20°C until transverse, serial cryosections were prepared (5µm thick) on a Leica cryostat. All animal work was approved by the regulatory authority of Leiden University and performed in compliance with the Dutch government guidelines.

Histological analysis

Cryosections of aortic root sections and *en face* pinned aortas were stained for 15 minutes in 0.5% Oil-red-O (Sigma Diagnostics) to visualize lipid accumulation. The plaque size was calculated from the average size of ten sections per heart. Carotid artery sections were stained with hematoxylin (Sigma Diagnostics) and eosin (Merck Diagnostica) for morphometric analysis. Vascular smooth muscle cells (VSMCs), macrophages and collagen were stained as previously described [21]. T-cells were detected with CD3 (SP7, 1:150; Neomarkers, Fremont, CA) and FoxP3 antibodies (FJK-16S, 1:50; eBioscience, San Diego, CA). TUNEL staining was performed with In Situ Cell Detection Kit (Roche Diagnostics). Macrophage-, smooth muscle cell- and collagen-positive areas, the number of intimal $CD3^+$ T-cells as well as the number of TUNEL+ cells were determined by computer-assisted color-gated analysis using LeicaQwin software (Leica Imaging Systems, Cambridge, UK), and related to the total plaque area. Splenic cryosections (5µm) were double stained for T-cells (rabbit monoclonal CD3 Ab; SP7 clone, 1:100, Neomarkers, Fremont, CA) and macrophages (rat monoclonal F4/80 Ab; 1:200, BMA Biomedicals, Augst, Switzerland). Goat anti rat Cy3 and goat anti rabbit Alexa488 (1:100, Molecular Probes Inc., Eugene, OR) were used as secondary Ab's and nuclei were visualized by DAPI staining (Serva Feinbiochemica, Heidelberg, Germany). Slides were analyzed using a Bio-Rad Radiance 2100 MP confocal laser scanning system equipped with a Nikon Eclipse TE2000-U inverted fluorescence microscope.

ELISA

Mouse serum derived from the systemic sCCL18 experiment was used to determine circulating levels of IL-6, CCL2 (eBioscience) and CCL3 (Biosource, Nivelles, Belgium) according to manufacturer's protocol.

Flow Cytometry

Peritoneal leukocytes, derived from ascites, as well as blood samples from sCCL18 and control treated animals were obtained one hour after the final sCCL18 injection. Erythrocytes were removed by hypotonic lysis with NH_4Cl (155mM NH_4CL in 10mM Tris/HCL, pH 7.2). Leukocytes were stained with anti-CD8 (clone 53-6.7; 0.25 µl per sample; eBiosciences, San Diego, CA), anti-CD4 (GK1.5), anti-F4-80 (BM-8), anti-Gr-1 (1A8) and anti-CCR3 (83/03; BD Biosciences, San Jose, CA) antibodies, and analysed by fluorescence activated flow cytometry (FACSCalibur) using CELLQuest software (BD Biosciences); 20,000 cells were counted for each sample.

Statistical analysis

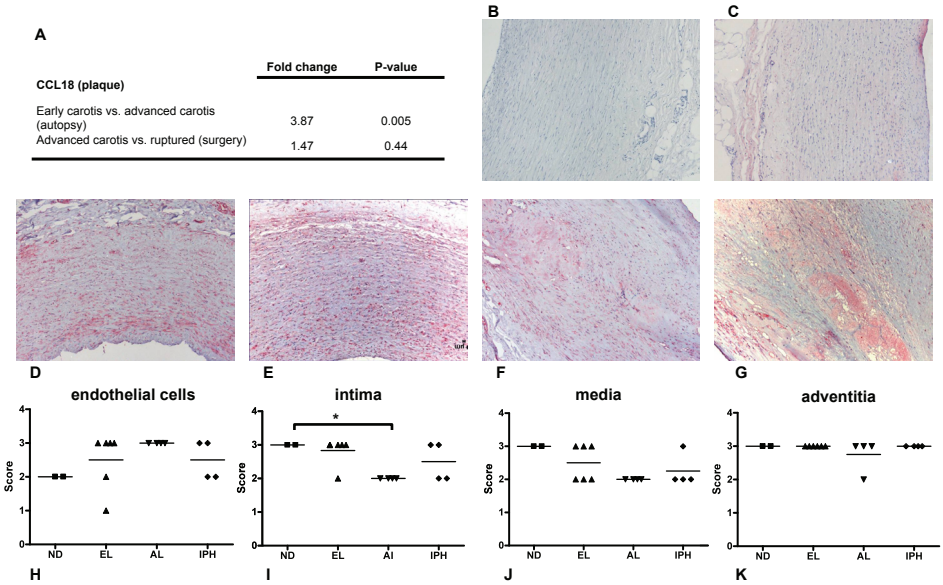
To determine microarray derived fold changes between groups, the error model of Rosetta Resolver (Rosetta Biosoftware, Seattle, Washington) was used. Analysis between groups was performed with Student's t-test or Mann-Whitney test, in case of a skewed distribution. Human CCL18 plaque expression between groups was analysed with Kruskal-Wallis testing. All values are expressed as mean ± SEM when appropriate. Analysis were performed with GraphPad Prism 4 (GraphPad software, San Diego, CA), a P-value of <0.05 was considered significant.

RESULTS

Microarray and protein expression

Human autopsy and endarterectomy derived carotid artery plaques were classified for progression stage according to the guidelines of Virmani and coworkers [14]. Advanced fibroatheroma displayed a 3.87 fold up-regulation of CCL18 mRNA expression as compared to early lesions ($P<0.005$, figure 1A). No significant differences in CCL18 expression were observed between advanced vs. ruptured endarterectomy derived plaques. These data suggest that CCL18 expression increases with lesion progression at least until the advanced plaque stage, which might affect leukocyte influx into the plaque. Immunohistochemistry analysis confirmed the abundant expression of CCL18 protein in both normal and atherosclerotic human lesions, at all stages of plaque progression (figure 1B-F), although IHC did not allow us to draw quantitative conclusions on protein expression. CCL18 was expressed by luminal endothelial and adventitial cells regardless of plaque progression stage. CCL18 expression by intimal and medial VSMCs tended to decrease during plaque progression ($P<0.05$ for intimal and $P=0.11$ for medial cells) (figure 1H-K).

Figure 1.



Micro-array analysis of mRNA obtained from human carotid plaque specimen derived at autopsy reveals a significant up-regulated CCL18 expression in advanced versus early plaques (A). Negative control (B) and isotype IgG antibody staining (C) showing no cross-reaction. CCL18 staining is present throughout the vessel wall in a non-diseased vessel (ND) (D), within early lesions (EL) (E), within advanced lesions (AL) (F) and in intra-plaque haemorrhage (IPH) (G). Scoring for $CCL18^+$ cells shows no difference in endothelial cells upon plaque progression (H), a significant down-regulation of CCL18 within intimal cells in advanced lesions as compared to early lesions (I) and a non-significant decrease in $CCL18^+$ medial cells in advanced versus early lesions (J). No differences are observed in $CCL18^+$ adventitial cells (K). Magnification 100x. * = $P<0.05$. For color figure see page: 220

Effect of sCCL18 *in vitro*

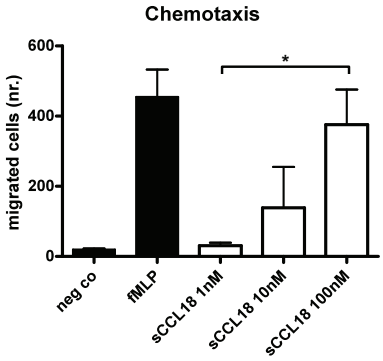
To verify the functionality of the sCCL18 peptide [16], we examined the effects of sCCL18 on cellular chemotaxis of Jurkat T-cells. sCCL18 induced a clear dose-dependent increase in migration, which confirms the specific T-cell chemo-attractive properties of CCL18 *in vitro* (figure 2). Next we studied mitogenic effects of CCL18 on the major cellular constituents of the atherosclerotic plaque. Incubation of murine RAW264.7 macrophages, H5V cells and primary VSMCs with 25 ng/ml of sCCL18 did not affect H5V endothelial cell proliferation, but decreased that of RAW264.7 cell ($P<0.01$) and tended to do so on VSMCs ($P=0.12$, figure 3A).

CCL18 treated RAW264.7 cells showed decreased transcript levels of IL-6 and chemokine receptors CCR3 and CCR5 (figure 3B).

sCCL18 in vivo kinetics

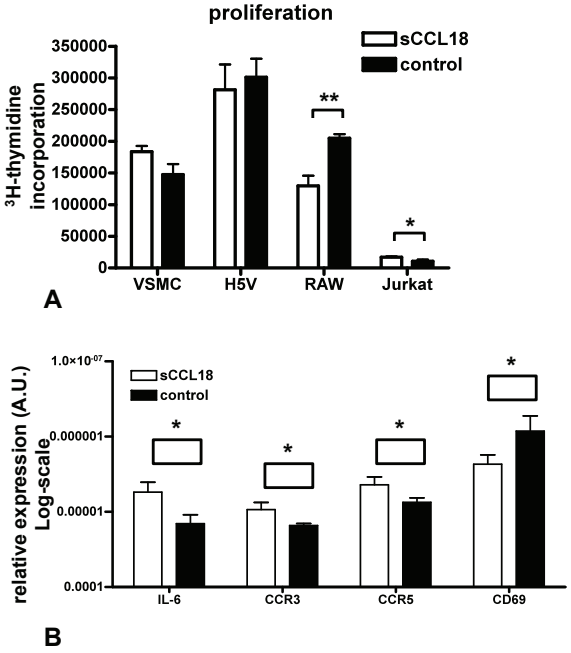
The kinetics of sCCL18 *in vivo* in mice was assessed by administering ^{125}I labelled sCCL18 and monitoring plasma levels as well as the uptake by various organs in time. After testing various concentrations, administered by intraperitoneal (i.p.) as well intravenous injection (data not shown), we concluded that an i.p. starting bolus of 5 µg ^{125}I sCCl18 followed by 2.5 µg ^{125}I

Figure 2.



sCCL18 induces a significant, concentration dependent increase in migration of Jurkat T-cells as evidenced by the increased number of migrated cells. fMLP = positive control, values are presented as mean ± SEM, * P<0.05, N=4 per group

Figure 3.

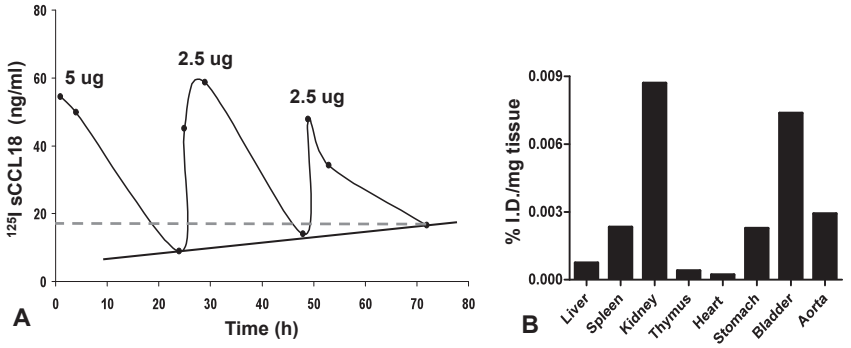


sCCL18 incubation (25 ng/ml) induces a decreased proliferative response in RAW264.7 cells, but an increase in Jurkat T-cell proliferation. No significant differences are observed in VSMC and H5V endothelial cell proliferation (A). Comparative expression analysis of mRNA from RAW264.7 cells treated for 16h with sCCL18 or PBS shows a significant decreased mRNA expression of IL-6, CCR3 and CCR5, and an increased expression of CD69 in sCCL18 treated cells (B). Values are presented as mean ± SD, * P<0.05, ** P<0.001

sCCL18 i.p. afterwards was the most efficient dosing strategy to achieve physiological mean plasma ^{125}I sCCL18 levels of approximately 25-30 ng/ml (figure 4A). Regarding organ distribution, ^{125}I sCCL18 accumulated in kidney and bladder after 72 hours, reflective of efficient renal

^{125}I sCCL18 excretion (figure 4B). ^{125}I sCCL18 was furthermore present in spleen and, interestingly, aorta.

Figure 4.



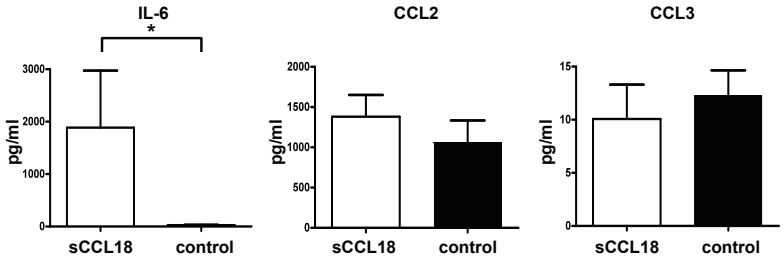
In vivo kinetics of ^{125}I labelled sCCL18 after repeated intraperitoneal injections. Minimum serum levels gradually increase to approximately 17 ng/ml after three repetitive daily injections (dotted line). Daily injections result in an average CCL18 serum level of 25-30 ng/ml (A). Injected ^{125}I sCCL18 accumulates in kidneys and bladder, but also, to a lesser degree, in heart, spleen and aorta (B). I.D. = injected dose

Effects of systemic CCL18 treatment

Western type diet fed ApoE-/- mice were treated with a starting dose of 5 µg sCCL18 i.p. and subsequent injections of 2.5 µg every other day for two weeks. sCCL18 administration did not affect plasma cholesterol levels (sCCL18 1076 vs. control 1182 mg/dl, P=0.50)), but had considerable impact on the plasma cytokine profile. As Wimmer *et al.* previously reported that CCL18 induced IL-6, CCL2 and CCL3 secretion by monocytes in vitro [22], we particularly focussed on this set of cytokines. Circulating levels of IL-6 were sharply elevated in mice receiving sCCL18 (1,885 vs. 27 ng/ml for the PBS controls)(figure 5A). No significant differences in CCL3 and CCL2 levels were observed (figure 5B,C).

CCL18 treatment for only 2 weeks during 8 weeks period of Western type diet feeding was seen to aggravate atherogenesis in the aortic arch(+45% ; sCCL18 $3.3x10^5$ +/- μm^2 vs. control $2.3x10^5$ +/-μm^2; P=0.03, figure 6A). Surprisingly *en face* analysis of total dissected aortas revealed no significant differences (plaque/area ratio: sCCL18 0.29 vs. control 0.24; P=0.37). Regarding

Figure 5.

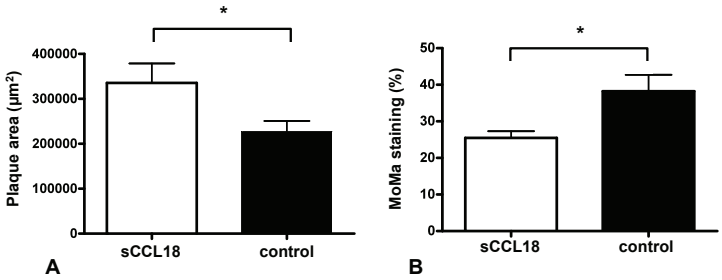


ELISA analysis performed in mouse serum of the systemic sCCL18 experiment. Levels of IL-6 are sharply increased after sCCL18 treatment compared to control (A). No significant differences are observed in CCL3 and CCL2 levels (B,C). Values are presented as mean ± SEM, * P<0.05

plaque composition, plaque T-cell content did not differ (data not shown) but the sCCL18 treatment resulted in a diminished plaque macrophage content (sCCL18 25% vs. control 38%; P=0.02, figure 6B). In keeping with earlier findings by Luzina et al. on a pro-fibrotic effect of CCL18 in lung, plaque collagen content tended to be enhanced in the sCCL18 treated group, although not significant (sCCL18 17% vs. control 12%; P=0.15) [17].

In view of the effect of CCL18 on plasma cytokine patterns, we assessed if systemic sCCL18

Figure 6.



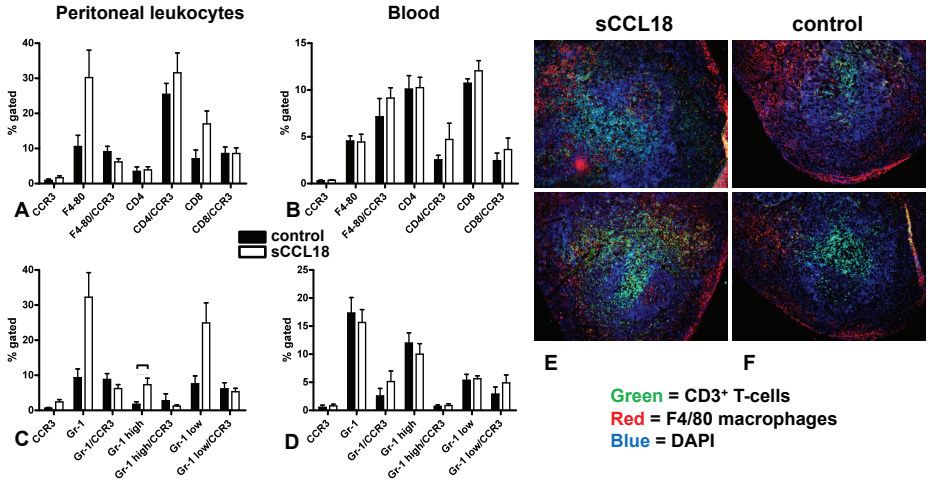
Morphometric analysis of aortic root sections. sCCL18 causes a significant increase in plaque size and a significant decrease in plaque macrophage content (A,B). No differences were observed in VSMC and collagen content. Values are presented as mean ± SEM, * P<0.05

administration had influenced blood leukocyte content and differentiation status. Differential cell count analysis did not reveal overt changes in major leukocyte subpopulations in sCCL18 treated mice (data not shown). More detailed analysis by flow cytometry confirmed no significant differences in distribution of granulocytes and T-cell sub-populations in blood (figure 7B,D). We did not observe any changes in peritoneal or circulating $CD4^+$ and $CD8^+$ T-cell numbers, suggesting that CCL18 does not influence T-cell release by the lymphoid system or the spleen. Interestingly total peritoneal $CCR3^+$ cell numbers were not changed as well after i.p. CCL18 administration, although we observed a non-significant trend in $CD4^+/CCR3^+$ cells in sCCL18 treated mice (figure 7A,C). Furthermore, $F4/80^+$, Gr-1^{high} and Gr-1^{low} monocytes as well as $CD8^+$ T-cell numbers were increased in the sCCL18 group, but only the Gr-1^{high} distribution reached statistical significance (P<0.05). Histological analysis of spleens revealed a striking difference in germinal centre architecture as well as in $CD3^+$ T-cell distribution of sCCL18 treated mice. T-cells were more dispersed and not confined to the germinal centres but present in the marginal zones, potentially reflecting enhanced T-cell mobilisation or interference with splenic T-cell/stroma interaction in response upon systemic CCL18 administration (figure 7E-F).

Effects of local CCL18 expression

Finally, given the overt effects of CCL18 in the periphery and as systemically elevated CCL18 levels may have blunted chemokine receptor mediated chemotactic responses or elicited a peripheric inflammatory response, we set out to study effects of focal over-expression of CCL18 in pre-existing plaques on plaque stability. Adenoviral infection of carotid artery plaques was

Figure 7.



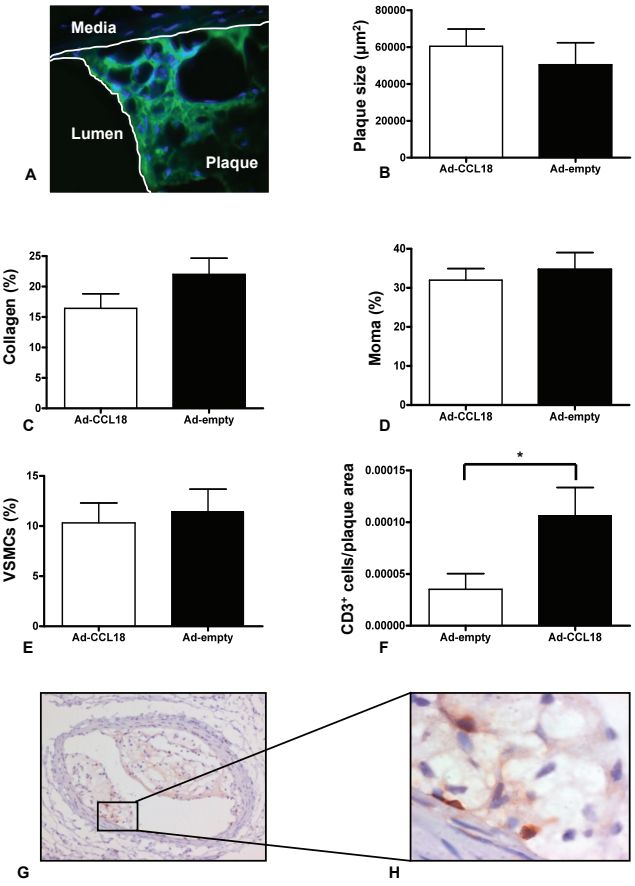
Flow cytometric analysis of peritoneal and blood cells. In general, sCCL18 does not induce major differences in macrophage and T-cell distribution, but causes an increase in peritoneal Gr-1^{high} positive cells (A-D). Representative pictures of T-cell (green) and F4-80 macrophage (red) distribution within the germinal centres of the spleen. Nuclei are stained blue with DAPI. sCCL18 treated mice show a perturbed germinal centre architecture in that T-cells seem to be more scattered within these germinal centres and germinal centres are more diffusely confined (E,F). Values are presented as mean ± SEM, * $P<0.05$. Magnification 100x. For color figure see page: 221

successful as judged from the pronounced GFP expression in endothelial cells as well as in the central atheroma of the plaque (figure 8A). CCL18 over-expression for 2 weeks did not affect plaque progression (figure 8B). Regarding plaque composition, Ad-CCL18-GFP treatment did not induce any notable differences in VMSC, macrophage as well as collagen content of the plaque (figure 8C-E). Furthermore, the number of internal elastic lamina ruptures per plaque did not differ between treatment groups, indicating that the plaque elastolytic activity remained unchanged, nor were there any differences in necrotic core size and TUNEL positive cells (data not shown). In keeping with the reported T-cell recruiting capacity of CCL18 we indeed observed a sharply increased $CD3^+$ T-cell content of Ad-CCL18-GFP transduced plaques ($P=0.028$)(figure 8F-H). The T-cell enrichment mainly involved non-regulatory T-cells, as only very few FoxP3+ cells were observed in the intima. Therefore, a two-week focal over-expression of CCL18 in advanced plaques clearly increased the T-cell content, but did not noticeably affect plaque stability.

DISCUSSION

CCL18 expression has been detected in human atherosclerotic plaques [6, 7], and its serum levels shown to be up-regulated during acute cardiovascular syndromes [5]. However, direct experimental evidence on a causal role in cardiovascular disease is still lacking. Here, we not only provide additional gene and protein expression data of CCL18 in human plaques, we are

Figure 8.



Local overexpression of CCL18 in the carotid plaque was successful as abundant GFP expression is present within the plaque (A, 400x magnification). However, two weeks after incubation, this does not result in differences in plaque size, macrophage, collagen content and VSMC content (B-E). Interestingly, T-cells are present in relatively large amounts in Ad-CCL18-GFP incubated plaques (F), as demonstrated in panel G (100x) and H (1000x). Values are presented as mean ± SEM, * P=0.028. For color figure see page: 222

also the first to describe the impact of systemic as well as local human CCL18 expression on atherogenesis and plaque stability in a mouse model of atherosclerosis.

CCL18 gene expression was markedly up-regulated in autopsy derived advanced atherosclerotic plaques. These results are in line with those of Papaspyridonos et al., who observed a 4-fold increase in CCL18 gene expression in unstable regions versus stable regions in atherosclerotic plaques of the same patient [7]. However, they did not use the Virmani plaque classification but rather a surface morphology scoring system, hampering a valid comparison. Our results can either point to enhanced presence of $CCL18^+$ macrophages as recently suggested by Hagg *et al.* [23], or to intrinsically enhanced expression of CCL18 in the whole atherosclerotic plaque during disease progression. The latter finding seems more likely, given the abundant

CCL18 protein expression throughout the entire atherosclerotic plaque, including the medial area. Li *et al.* recently reported the co-localization of CCL18 with $CD83^+$ cells in atherosclerotic plaques, but CCL18 expression was confined to the plaque surface [12]. In any case our results suggest that CCL18 is not only expressed throughout the atherosclerotic neo-intima, but also, in a more constitutive manner in the vascular wall.

Human CCL18 levels in healthy control subjects are reported to be approximately 25 ng/ml, reaching levels >100 ng/ml in patients with acute lymphoid leukaemia [24], while *in vitro* reports used concentrations ranging from 0.1 to 1000 ng/ml [9]. Therefore, we consider average CCL18 serum levels of 18 ng/ml with peak values of up to 100 ng/ml achieved after a two-daily injection regimen into mice to be physiologically relevant.

sCCL18 not only effected a dose-dependent migration of human T-cells in vitro, which is in concordance with earlier observations [17, 25], but also decreased RAW264.7 macrophage proliferation. Gene-expression analysis revealed a down-regulation of CCR3 and CCR5 during proliferation. CCL18 is currently considered an orphan ligand, but has shown to be a non-competitive inhibitor of eotaxin binding to CCR3 [9]. Next to quenching the proliferative response, CCL18 also induced macrophage activation as CD69 expression was seen to be up-regulated upon sCCL18 stimulation. Further proof for the pro-inflammatory role of CCL18 was obtained from the *in vivo* studies as we detected markedly raised levels of IL-6 but not CCL2 and CCL3 in sCCL18 treated mice. Therefore, systemic administration of sCCL18 seems to induce a pro-inflammatory IL-6 response which might act in concert with CCL18. IL-6 is an acute phase cytokine which is mainly secreted by macrophages and Th2 type T-cells [26]. IL-6 has been implicated both as a pro-atherogenic as well as an anti-atherogenic cytokine [26]. It remains to be determined whether the profound rise in IL-6 levels is a direct consequence or secondary to CCL18 treatment. Previously, TGF-β up-regulation was seen to be induced in CCL18 treated T-cells in vitro, which together with the induced IL-6 levels raises the intriguing premise that CCL18 favors Th17 polarization and in this way influences the atherogenic process.

As demonstrated by Hagg et al. CCL18 expression between lipid-loaded and naive macrophages is essentially similar, suggesting that CCL18 expression in macrophages is not dependent on lipid accumulation [23]. In particular, the decreased proliferative response of sCCL18 exposed RAW264.7 cells may partly have hampered macrophage expansion in atherosclerotic plaques, reducing plaque macrophage content in the aortic root as indeed observed from sCCL18 treated mice. Systemic chemokine gradients which signal via the CCR3 receptor and potentially other chemokine receptors as well may hypothetically been disrupted by the sCCL18 presence, enhancing plaque monocyte infiltration. Furthermore, total plaque area was reduced in sCCL18 treated mice at the aortic root level, but not in *en face* specimens of the aorta, suggesting that pro-atherogenic responses of CCL18 may be stage or site specific. In contrast to focal expression, two week systemic sCCL18 expression did not influence plaque T-cell presence. Apparently, local CCL18 gradients rather than alterations in systemic T-cell mobility seems to be instrumental in T-cell chemo-attraction towards the atherosclerotic plaque. In humans, CCL18 mainly attracts naive CD45RA+ T-cells [25]. In line with previously

published observations, CCL18 did not induce plaque Foxp3+ regulatory T-cell content, both in Ad-CCL18-GFP as well as Ad-empty-GFP treated mice [10]. Furthermore, Luzina *et al.* observed minimally activated infiltrates of T lymphocytes (relatively low numbers of $CD25^+$ and $CD69^+$ T-cells) in a CCL18 induced model of pulmonary fibrosis [17]. Collectively, these data suggest that CCL18 acts as naive T-cell chemo-attractant in atherosclerosis, but requires the presence of other pro-inflammatory signals for T-cell activation in the plaque.

A few limitations of our studies need to be addressed: since CCL18 has no murine counterpart and as chemokine/chemokine receptor interactions may differ from species to species, we should be cautious with extrapolating effects of CCL18 in a murine context to the human situation. However, as administration of human CCL18 in both our mouse models as well in vitro mouse cell cultures led to increased atherosclerotic T-cell presence and cellular proliferation and migration, a functional equivalent of CCL18 is likely to exist in mice. Furthermore, we cannot exclude that neutralizing or cross-reactive antibodies will be generated in response to sCCL18 treatment.

To conclude, CCL18 expression is present during all stages of plaque progression. Systemic treatment of atherosclerotic ApoE-/- mice to physiological levels was seen to promote plaque progression possibly by eliciting a profound rise in IL-6 levels. Plaque specific CCL18 expression by adenovirus is associated with enhanced T-cell presence thereby augmenting plaque progression and destabilisation. Whether this is a direct effect precipitated by CCL18 and whether it is reflective of CCL18 effects in human disease remains to be determined. Given the correlation of CCL18 serum levels with future atherosclerosis related cardiovascular disease, it will be important to address the underlying pathways and the effects of CCL18 antagonism on plaque initiation, progression and stability. Our results suggest that murine models seem feasible for this purpose.

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