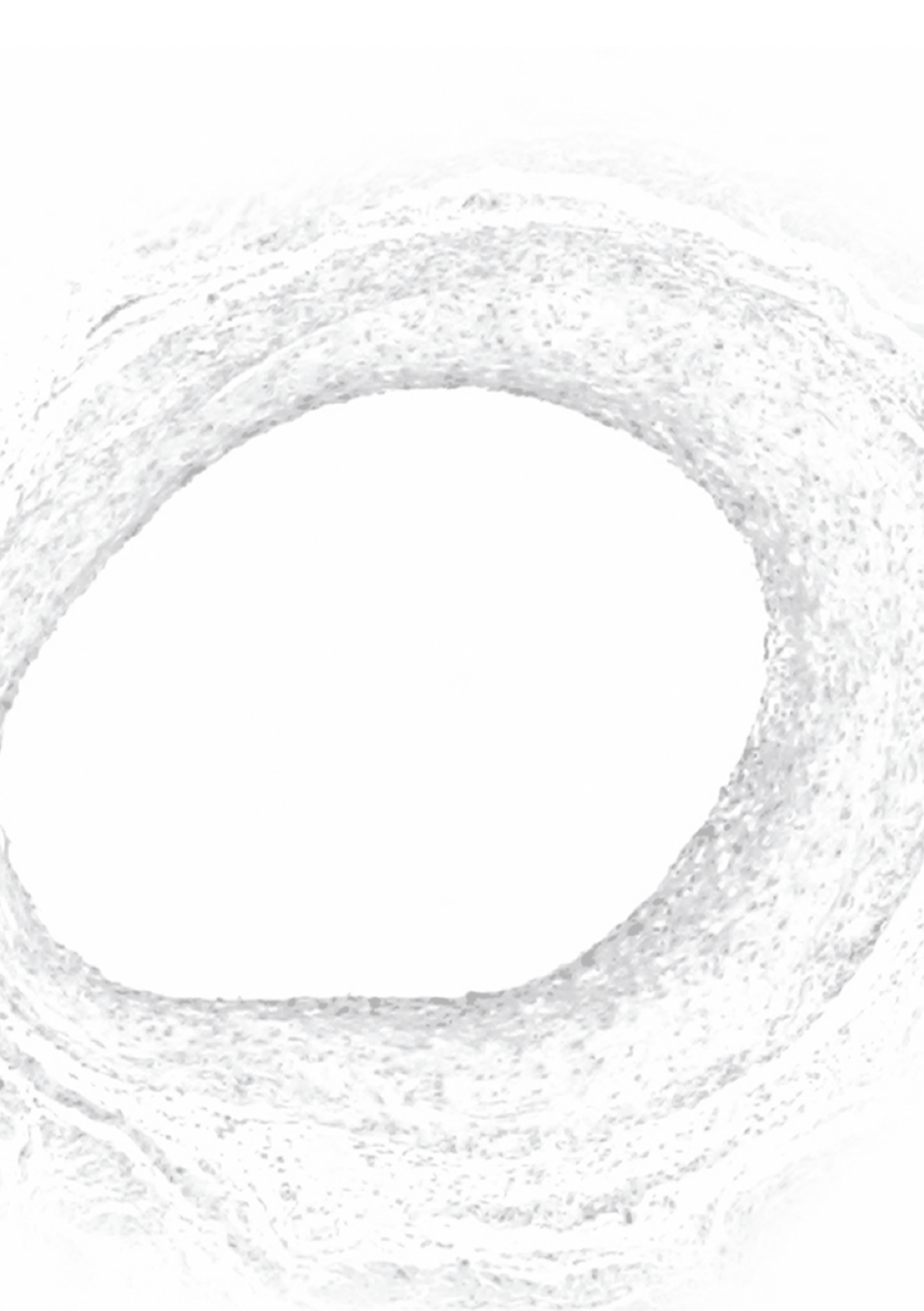
Inflammation in injury-induced vascular remodelling : functional involvement and therapeutical options

Schepers, A.



CHAPTER 3

The effect of interleukin-10 knock-out and overexpression on neointima formation in hypercholesterolemic ApoE3Leiden mice.

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ABSTRACT

Objective: Inflammatory factors are thought to play a regulatory role in restenosis. Interleukin-10 (IL10) is an important anti-inflammatory cytokine with anti-atherogenic potentials. The aim of this study was to assess the effects of IL10 modulation on cuff-induced neointima formation in hypercholesterolemic ApoE3Leiden mice.

Methods: The involvement of IL10 in neointima formation was studied in a hypercholesterolemic mouse model of cuff-induced stenosis of the femoral artery by IL10 knocking-out or overexpression procedures. $IL10^{+/-}$ mice were crossbred with ApoE3Leiden mice to generate hypercholesterolemic ApoE3Leiden$IL10^{-/-}$ mice. To achieve IL10 overexpression in ApoE3Leiden mice, a single intramuscular injection of a murine IL10 overexpression plasmid was performed followed by electroporation.

Results: Knocking-out IL10, in hypercholesterolemic ApoE3Leiden mice, resulted in a significant 1.9-fold increase of neointima surface as compared to ApoE3Leiden$IL10^{+/+}$ littermates ($p=0.02$). Conversely, a marked 45% inhibition on cuff-induced neointima formation was obtained after IL10 overexpression ($p=0.02$). Electrodelivery of IL10 vector leads to detectable IL10 serum levels, with a sustained expression over the experimental period of three weeks. IL10 overexpression reduced plasma cholesterol levels in ApoE3Leiden mice, whereas IL10 deficiency in these mice did not lead to altered cholesterol levels as compared to the $IL10^{+/+}$ group. Finally, IL10 overexpression stimulated endogenous IL10 mRNA expression in the spleen and reduced the transcriptional responses of several pro-inflammatory cytokines.

Conclusion: Here, we clearly demonstrate the role of IL10 in the development of neointima formation in hypercholesterolemic mice and the potential therapeutic effect of non-viral electrodelivery of IL10 cDNA to inhibit post-angioplasty restenosis.

INTRODUCTION

Inflammation plays an important role in the development of restenosis. Like in atherosclerosis, several pro-inflammatory cytokines (e.g. IL1β, TNFα and IFNγ) and immuno-inflammatory cells (e.g. T-lymphocytes and activated monocytes) are involved in the formation of restenosis in the vessel wall[1-4]. Interfering in this inflammatory process to prevent post-angioplasty restenosis has potential as a therapeutic application.

Interleukin-10 (IL10) is known as an anti-inflammatory and anti-atherogenous cytokine. In addition, IL10 is a potent monocyte deactivator. This cytokine is produced by most of the cellular components of the vessel wall, such as endothelial cells and vascular smooth muscle cells, but also by lymphocytes and macrophages. Moreover, IL10 is endogenously produced in the atherosclerotic plaque to modulate the inflammatory process[5-7].

IL10 protein administration was reported to inhibit post-injury intimal hyperplasia in hypercholesterolemic rabbits and exogenous IL10-protein delivering resulted in a reduced vascular injury response in normocholesterolemic mice and rats[8-10]. However, the effect of IL10 on neointima formation in hypercholesterolemic mice has never been described before.

In this study, we examined the effect of IL10 gene knock-out and overexpression on neointima formation in hypercholesterolemic ApoE3Leiden mice. In these ApoE3Leiden mice, a mutated human ApoE3Leiden gene is cloned and this leads to a defective clearance of ApoE-rich lipoproteins by the liver. Therefore these mice develop a diet dependent hyperlipidemia and diet induced atherosclerosis[11]. After placement of a non-constricting polyethylene cuff around the femoral artery in these transgenic mice, restenosis with signs of accelerated atherosclerosis develops within two to three weeks[12]. This model for cuff-induced neointima formation is very suitable to investigate the role of inflammatory factors in stenosis[4;13]. The lesions formed after cuff placement in the hypercholesterolemic mice contain both smooth cells and macrophages that might become foam cells and therefore this model mimics both restenosis as it occurs in hypercholesterolemic patients as well as the very early steps of atherosclerotic plaque formation.

Knocking-out IL10 in these hypercholesterolemic mice enables us to study the involvement of IL10 on neointima formation in comparison to their $IL10^{+/+}$ littermates.

Opposingly, non-viral intramuscular electroporation mediated gene transfer of interleukin-10 cDNA was used to assess the effect of high circulating levels of IL10 protein on neointima formation in ApoE3Leiden mice. With this method, disadvantages of viral gene delivery (like inflammatory and immunological responses) are circumvented. Moreover, in stead of daily protein administration, a single intervention will give long-term expression of transgenes[14;15]. In this study, we show

that intramuscular electroporation mediated delivery of a murine IL10 expression plasmid results in sufficient IL10 serum levels and inhibits cuff-induced neointima formation in hypercholesterolemic ApoE3Leiden mice.

MATERIALS AND METHODS

Mice.
All animal experimental protocols were reviewed and approved by the animal welfare committee of the Netherlands Organization for Applied Scientific Research (TNO, Leiden, The Netherlands). The investigation conforms with the *Guide for Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). For the IL10 overexpression experiments, Specific Pathogen-Free transgenic ApoE3Leiden mice were crossbred for at least18 generations with C57BL/6 mice. These mice develop a diet dependent hypercholesterolemia and spontaneous atherosclerosis[11;16]. Male ApoE3Leiden animals, with a mean weight of 28.4±0.6 grams, were allocated randomly to one of the two experimental groups.

For the IL10 knock-out experiments, experimental mice (ApoE3LeidenIL10$^{-/-}$ and control littermate ApoE3Leiden IL10$^{+/+}$ mice) were obtained by crossbreeding IL10$^{+/-}$ mice (Jackson Laboratory) with heterozygote ApoE3Leiden mice. Mice were characterized by PCR for IL10 from tail biopsies (Primers; forward: 5'- GCC TTC AGT ATA AAA GGG GGA CC-3', NEO primer: 5'-CCT GCG TGC AAT CCA TCT TG-3'and reverse: 5'-GTG GGT GCA GTT ATT GTC TTC CCG-3')[17]. Mean weight of all experimental male mice was 28.0±0.5 grams. No differences in weights were found between both groups.

Both in the IL10 knock-out study and in the IL10 overexpression study, animals were fed with a cholesterol-enriched high-fat diet (diet W, containing 1% cholesterol and 0.01% cholate, Arie Blok B.V.[18]), starting 4 weeks prior to surgery and continued during the whole experiment. All mice received water and food ad libitum.

Cholesterol levels in serum were determined one week before surgery and at sacrifice. Before electroporation and cuff placement, mice were anaesthetized by a combination of Midazolam (5 mg/kg; Roche), Medetomide (0.5 mg/kg; Orion) and Fentanyl (0.05 mg/kg; Janssen).

Cuff-induced neointima formation.
Neointima formation and accelerated atherosclerosis was induced by means of vascular injury through cuff placement around the femoral artery of mice on a hypercholesterolemic diet as described previously[12;19]. Briefly, femoral arteries were isolated from surrounding tissues and a non-constricting 3 mm polyethylene cuff (internal diameter 0.4 mm) was placed around the arteries. With this intervention, a profound neointima formation with signs of accelerated atherosclerosis develops within two to three weeks. Animals were sacrificed 14 days (IL10$^{-/-}$ experiment) and

21 days (IL10 overexpression experiment) after cuff placement. Tissue segments were harvested after perfusion fixation and paraffin-embedded.

Quantification of neointima formation and immunohistochemistry.
To quantify the intimal thickening, elastic laminae were visualized with Weigert's elastin staining. Six sequential representative sections per vessel segment were used to quantify neointima formation, using image analysis software (Qwin, Leica).

The composition of neointima formation was visualized by haematoxylin-phloxine-saffron (HPS) staining and immunohistochemistry. Smooth muscle cells were visualized with α-smooth muscle cell actin staining (anti-SM α-actin, dilution 1:1600, Roche). AIA31240 (dilution 1:3000, Accurate Chemical) staining was used to detect monocytes/macrophages. The smooth muscle cells positive area and macrophages positive area in the neointima and media of the cuffed arteries were calculated as a percentage of the total intimal and medial area by means of image analysis software (Qwin, Leica).

Interleukin-10 overexpression.
For overexpression of IL10, intramuscular electroporation with a CAGGS plasmid encoding for murine IL10 (kindly provided by Dr. Miyazaki, Division of Stem Cell Regulation Research, Osaka University Medical School, Osaka, Japan) was applied one day before cuff placement. As a control, *pCAGGS-Luciferase* was used. Both CAGGS plasmids contain a cytomegalovirus immediate-early enhancer-chicken β-actin hybrid promoter, to obtain a long-lasting expression *in vivo*[20]. All plasmid DNA was prepared using DH5α E.coli (Invitrogen) and QIAfilter Plasmid Giga Kits (Qiagen). Plasmid DNA was dissolved in Endofree Tris-EDTA buffer (Qiagen) at a final concentration of 3.5 mg/ml.

For optimal transfection efficiency both calf muscles were injected with 30µl of hyaluronidase (13.5U, Sigma), one hour before electroporation, as described previously by McMahon et al.[21]. Subsequently, 50 µg of either pCAGGS-mIL10 or pCAGGS-Luciferase as a control, dissolved in 30 µl TE buffer, was injected in both calf muscles followed by eight electrical pulses of 10 milliseconds of 200V/cm with an interval of one second. The pulses were generated with a Square Wave Electroporator ECM 830 (BTX, Harvard Apparatus) using Caliper Electrodes.

At 7 and 21 days after electroporation, serum samples were collected and IL10 concentration was measured using a mouse IL10 ELISA kit (Endogen).

Furthermore, transfection efficiency was analyzed by luciferase activity quantification in the control mice, 7 and 21 days after electroporation with pLuciferase. For this purpose, mice were anesthetized and injected i.p. with luciferin (90 mg/kg body weight, Synchem OHG). Five minutes after injection, luciferase activity was measured with a cooled charged-coupled device (CCCD) bioluminescence camera (The Night-OWL LB 981 UltraSens Frontlit, Berthold Technologies) as described previously[22].

Briefly, a gray-scale image of mice was recorded after placing the mice in the dark chamber. Hereafter, photon emission was integrated over a period of 30 seconds and recorded as pseudo-color images (pixel binning 7 x 7). For co-localization of the bioluminescent photon emission on the calf muscles, gray-scale and pseudo-color images were combined by using WinLight software (Berthold Technologies). Localization and measurement of luminescence emitted from the muscles was performed by using the overlay of the real image and the luminescence scan. Data were expressed as photon flux (counts/s).

RNA isolation and Real-Time RT-PCR procedure.
To assess the systemic regulation of inflammation-related genes after IL10 overexpression, mRNA from spleens was isolated after three weeks (n=5). For the 7 days time point, five extra mice were electroporated and sacrificed one week later. Directly after harvesting, spleens were snap-frozen in liquid nitrogen. Next, spleens were ground with pestle and mortar. RNA isolation was performed using Trizol (Invitrogen) according to manufacturer's protocol. Synthesis of cDNA was performed by means of Ready-To-Go Beads (Amersham Biosciences).

For TNF-α and HPRT, intron-spanning primer-probe sets were designed using Primer Express™ 1.5 software (Applied Biosystems, Table 3.1). TaqMan® Gene Expression Assays were used for IL6, IL1β, IFNγ, IL4 and IL10 (Applied Biosystems). HPRT was used as a housekeeping gene. The PCR reaction was performed using qPCR Mastermix (Eurogentec). Analysis of mRNA expression by real time-PCR was performed on an ABI Prism™ 7700 sequence detection system (Perkin Elmer Biosystems).

Table 3.1

Taqman Primers and Probes for real-time quantitative RT-PCR	
TNFα	Sense:5' CATCTTCTCAAAATTCGAGTGACAA 3' Antisense: 5' TGGGAGTAGACAAGGTACAACCC 3' Probe: 5' CACGTCGTAGCAAACCACCAAGTGGA 3'
HPRT	Sense: 5'-GGCTATAAGTTCTTTGCTGACCTG-3' Antisense: 5'-AACTTTTATGTCCCCCGTTGA-3' Probe: 5'-CTGTAGATTTTATCAGACTGAAGAGCTACTGTAATG ACCA-3'

For the analysis, the average cycle threshold per time point was subtracted from the average cycle threshold of the housekeeping gene HPRT (dCt). ddCt was determined as the difference between dCt-values of the IL10 electroporated mice and the control group (luciferase). Data are presented as mean fold induction compared to the normalized luciferase group, calculated as 2^{-ddCt}.

Statistical analysis.
Results are expressed as mean±SEM. Statistical significance was calculated in SPSS 11.5 for Windows. Groups were compared to their controls and significant differences were determined using the Student's Tests. A value of $P<0.05$ was considered statistically significant.

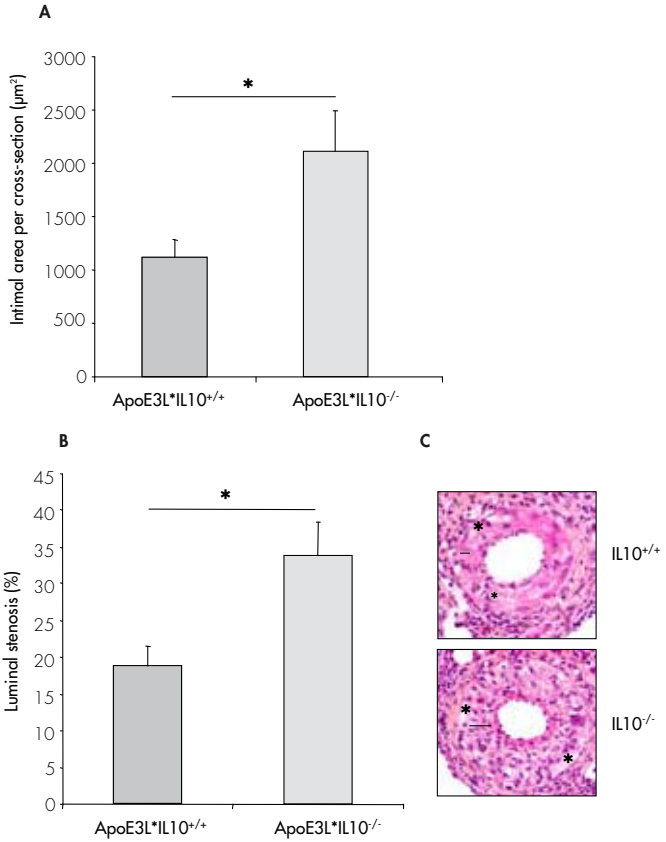
RESULTS

Neointima formation in hypercholesterolemic ApoE3LeidenIL10 knock-out mice.
To study the effect of lacking interleukin-10 on neointima formation in mice with a hypercholesterolemic background, $IL10^{+/-}$ mice were crossbred with ApoE3Leiden mice. Twelve week old ApoE3Leiden$IL10^{-/-}$ and control littermate (ApoE3Leiden$IL10^{+/+}$) mice were fed with a mild-type western-type diet for 4 weeks. One week prior to surgery, serum cholesterol levels were 8.4±0.6 mmol/L in the $IL10^{-/-}$ group and 7.2±1.1 mmol/L in the $IL10^{+/+}$ mice (difference not significant). At sacrifice, serum cholesterol levels were not changed significantly between both groups as well as compared to pre-surgery: 8.1±0.3 mmol/L ($IL10^{-/-}$ group) versus 7.3±1.9 mmol/L ($IL10^{+/+}$ group).

All mice underwent femoral artery cuff placement (n=8) to induce neointimal hyperplasia and subsequent accelerated foam cell accumulation. Lesions were composed of smooth muscle cells in combination with macrophages/foam cells. Mean bodyweights in both groups were comparable and no significant changes were registered in any of the animals during the whole experiment (data not shown).

Two weeks after surgery, femoral arteries were harvested and histomorphometric analysis showed a 1.9 fold increase of neointima formation in the $IL10^{-/-}$ group as compared to the control group (control: 1.1±0.2 mm^2, $IL10^{-/-}$: 2.1±0.3 mm^2; p=0.02; Figure 3.1, panel A and C). Medial area was similar in both groups (both 5.8±0.6 mm^2, data not shown). Finally, the lumens of the vessels were reduced by a factor 1.81 in the $IL10^{-/-}$ group (lumen stenosis in control group: 18.7±2.8% and $IL10^{-/-}$ group: 33.8±4.5%; p=0.01, Figure 3.1, panel B and C).

Figure 3.1: Effect of IL10 knock-out on neointimal formation in hypercholesterolemic mice. *Total intimal thickening (Panel A) and percentage of lumenstenosis (B) of cuffed femoral arteries in ApoE3LeidenIL10$^{-/-}$ and their IL10$^{+/+}$ control littermates, 14 days after cuff placement (n=8 per group, *p<0.02). Panel C represents haematoxylin-phloxine-saffron (HPS) staining of cuffed femoral arteries of both groups. Neointimal surface (indicated by black line) is clearly increased in the IL10 knock-out group. Asterisks (*) indicate macrophage-derived foam cells (magnification 250x).*



Expression of interleukin-10 and luciferase after electroporation mediated gene transfer.

Seven days after intramuscular injection and electroporation with pCAGGS-mIL10 or pCAGGS-Luciferase, serum levels of mIL10 were 43.7±4.8 ng/ml and 0.7±0.1 ng/ml, respectively (p<0.001). 21 days after electroporation, mIL10 concentration in the serum was 2.0±0.3 ng/ml in the IL10 treated group and 0.3±0.04 ng/ml in the control group (p=0.009, Figure 3.2, panel A). To verify expression of the transfected

transgene luciferase, bioluminescence imaging was performed and luciferase activity was quantified (see Figure 3.2, panel B and C).

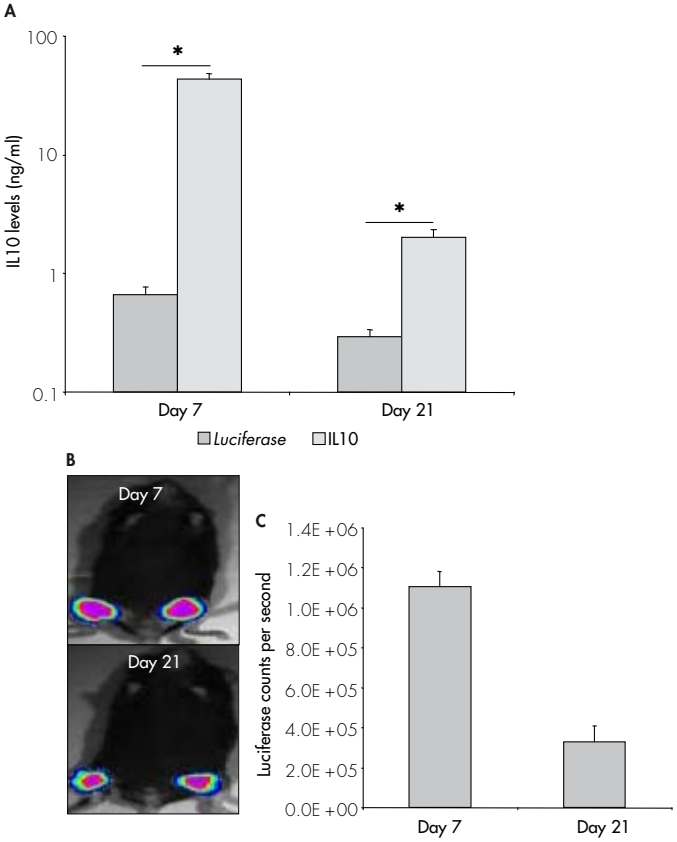
Effect of interleukin-10 overexpression on neointima formation in ApoE3Leiden mice.

To study the effect of IL10 on neointima formation in hypercholesterolemic mice, femoral arterial non-constricting cuffs were placed in male ApoE3Leiden mice (n=8 per group). Also these mice were fed with a mild-type western-type diet for 4 weeks before surgery and electroporation. Serum cholesterol levels were 14.8±1.4 mmol/L in the IL10 treated group and 14.0±0.4 mmol/L in the control mice (difference not significant). One day prior to surgery, the hindlimbs of the mice were electroporated with either an IL10 expression vector or a luciferase vector as a control. Three weeks after electroporation and surgery, mean cholesterol levels were reduced with 41.4% (8.7±0.5 mmol/L, $p<0.001$) in the IL10 treated group. The cholesterol levels in the control group were not changed significantly (14.7±1.2 mmol/L). No effects on body weights of all mice were observed (data not shown).

Histomorphometric analysis was performed on femoral arteries harvested 21 days after electroporation and surgery. It was anticipated to observe less neointima than in the experiment with the IL10 knock-out mice, therefore we decided to harvest the femoral arteries after three weeks instead of two weeks. A 45.4% reduction of neointima was found in the IL10 treated group as compared to the control group (control: 4.2±0.7 mm^2 and IL10: 2.3±0.4 mm^2; p=0.02; Figure 3.3, panel A and C). The medial area was not changed significantly in both groups (control: 11.0±1.0 mm^2 and IL10: 9.5±0.7 mm^2). The luminal stenosis was inhibited with 50.5% in the IL10 treated group (control: 25.5±3.8 % and IL10: 12.6±2.5 %, p=0.006, Figure 3.3, panel B and C).

To study the effect of IL10 modulation on the cellular composition of the lesion in the cuffed arterial wall, immunohistochemical analysis for the presence of SMCs and macrophage-derived foam-cells of both, IL10 knock-out and IL10 overexpression experiments was performed (Figure 3.4). Equally to the medial surface, the cellular composition of the media was not affected in both experiments. Relative SM α-actin positive areas in the IL10 knock-out experiment were: $IL10^{+/+}$: 40±5% and $IL10^{-/-}$: 40±6%, p=0.49 and in the IL10 overexpression experiment the relative SM α-actin positive areas were: IL10 overexpression: 23±5% and control: 25±4%, p=0.36. Also the relative macrophage positive areas did not change significantly after knocking-out IL10 as compared to $IL10^{+/+}$ littermates ($IL10^{+/+}$: 2±1% and $IL10^{-/-}$: 3±1%, p=0.25). In addition, IL10 overexpression did not affect the relative macrophage positive areas either (IL10 overexpression: 26±5% and control: 27±5%, p=0.46).

Figure 3.2: Expression of IL10 and Luciferase after intramuscular, non-viral gene therapy. *Panel A: Murine IL 10 serum levels in ng/ml, one and three weeks after intramuscular electroporation of mIL10 cDNA or Luciferase as a control (n=5 per group, *p<0.01). IL10 protein levels are significantly increased as compared to the control group after electroporation at both time points. Panel B: Representative bioluminescence images of intramuscular luciferase expression at t=7 and 21 days after electrodelivery of Luciferase. Panel C: Quantitative reproduction of luciferase expression as measured with bioluminescence imaging (n=3).*



In spite of an increase of neointima formation in the IL10$^{-/-}$ mice and a reduction of neointima formation after IL10 overexpression, differences in the relative SM α-actin positive area in the neointima were neither significant in the IL10$^{-/-}$ mice as compared to their control littermates (IL10$^{+/+}$: 18±4% and IL10$^{-/-}$: 18±1%, p=0.50) nor in the IL10 overexpression group as compared to their controls (IL10 overexpression: 19±3%

and control: 24±3%, p=0.17). The relative macrophage positive areas were also similar in the IL10 knock-out experiment ($IL10^{+/+}$: 9±4% and $IL10^{-/-}$: 7±2%, p=0.40) and after IL10 overexpression (IL10 overexpression: 20±2% and control: 19±3%, p=0.41).

Effect of interleukin-10 overexpression on inflammatory-related cytokines.
IL10 is known as an anti-inflammatory and atheroprotective cytokine[5]. Because inflammation is thought to play an important role in the restenotic process[2;3], we investigated the systemic effect of IL10 on inflammatory-related cytokines after electroporation mediated IL10 overexpression. Important inflammatory factors and mediators, like T-cells and several cytokines are matured and/or produced in the spleen. Therefore, mRNA of the spleen was isolated to assess effects of systemic IL10 overexpression. IL1β, IL6, TNFα and IFNγ were chosen as prototypes for pro-atherogenic cytokines. As anti-inflammatory genes, IL4 and IL10 were selected. Both in the IL10 treated and in the control mice, transcriptional responses of these inflammatory related genes were determined with real-time RT-PCR, 7 and 21 days after electroporation.

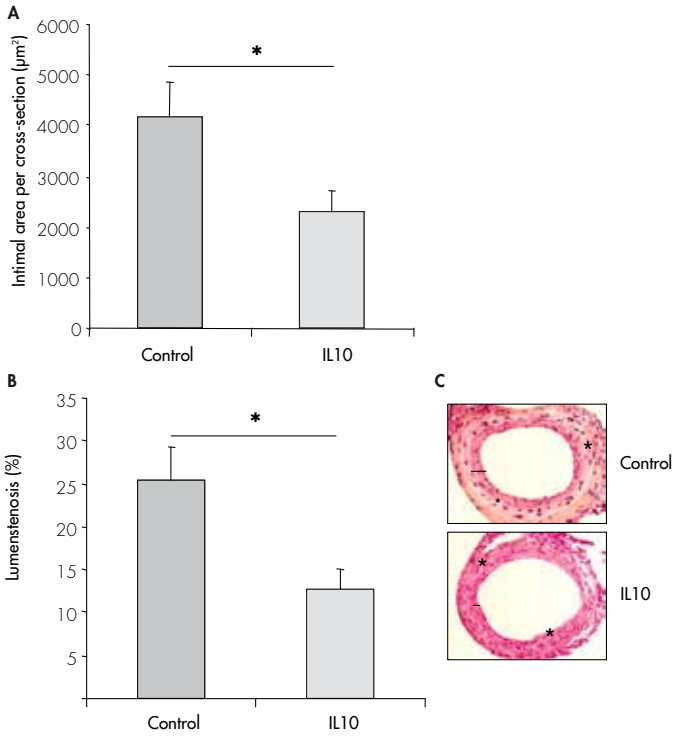
Seven days after IL10 overexpression, mRNA expression in the spleen of two important pro-atherogenic cytokines, TNFα and IL6, was strongly reduced (55±7% (p=0.006) and 62±7% (p=0.02), respectively) in the IL10 treated group. Expression of TNFα mRNA remained low after 21 days (40±13% as compared to the control group, p=0.02) and mRNA levels of IL6 normalized to control group level.

Both 7 as well as 21 days after electroporation, mRNA expression of IL1β was not significantly altered. However, mRNA levels of IFNγ showed a 6.1±0.2 fold increase 7 days after IL10 treatment (p<0.001). After three weeks, no significant difference was seen anymore in the expression of IFNγ mRNA as compared to observed levels in the control group. The mRNA expression of the anti-inflammatory gene IL10 was 3.0±0.4 fold increased in the IL10 treated group as compared to the normalized control, 7 days after electroporation and surgery (p=0.01). Remarkable is that endogenous IL10 production in the spleen can be enhanced after exogenous administration of IL10 gene. IL10 mRNA expression normalized within 3 weeks. The levels of IL4 mRNA did not alter significantly after IL10 overexpression.

DISCUSSION

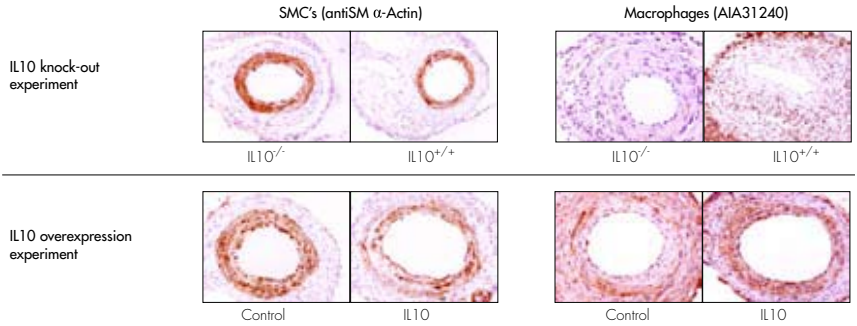
In spite of new therapies, like anti-proliferative drug eluting stents, restenosis still remains a clinical problem. For example, drug eluting stents are not that effective when applied in the femoropopliteal segment[23]. Since inflammation is thought to play an important role in the development of restenosis, anti-inflammatory approaches are promising alternative strategies. IL10 is a well-known anti-inflammatory and anti-atherogenic cytokine, therefore this cytokine is attractive to use in the prevention of post-angioplasty restenosis.

Figure 3.3: Effect of IL10 overexpression on neointima formation in hypercholesterolemic ApoE3Leiden mice. *Total intimal area (Panel A) and percentage of lumenstenosis (B) of cuffed femoral arteries in hypercholesterolemic ApoE3Leiden mice, three weeks after electroporation of pCAGGS-mIL10 and pCAGGS-Luciferase as a control (n=8 per group, *p<0.02). Panel C represents haematoxylin-phloxine-saffron (HPS) staining of cuffed femoral arteries after electroporation. IL10 overexpression results in a marked reduction of neointima formation (intimal area is indicated by black line and asterisks (*) indicate macrophage-derived foam cells, magnification 200x).*



In this study, we demonstrate that interleukin-10 has a beneficial effect on restenosis in hypercholesterolemic mice. Knocking-out IL10 in ApoE3Leiden mice results in a 1.9-fold increase of neointima formation in the femoral artery after cuff placement. On the other hand, IL10 overexpression by non-viral gene transfer leads to a 45% reduction of neointima formation after cuff placement. Furthermore, this study shows that systemic IL10 overexpression inhibits the expression of several important pro-atherogenic cytokines and enhances the expression of endogenous IL10 mRNA in the spleen.

Figure 3.4: ***Effect of IL10 on relative SMC and macrophage content of medial and intimal areas.*** *Representative cross-sections of cuffed femoral arteries of both, IL10 knock-out and IL10 overexpression experiments, immunohistochemically stained for smooth muscle cells (antiSM α-Actin) and macrophages (AIA31240). Magnification 200x.*



It has previously been reported that IL10 deficiency resulted in augmented intimal hyperplasia after carotid artery wire injury in normocholesterolemic mice[9]. In our study, we crossbred ApoE3Leiden mice with IL10$^{+/-}$ mice to generate diet-dependent hypercholesterolaemic IL10 knock-out mice and demonstrate that also the injury induced accelerated atherosclerosis is strongly reduced in the restenotic lesions. Caligiuri and colleagues described that IL10 deficiency in ApoE$^{-/-}$ mice did increase spontaneous atherosclerosis and low-density lipoproteins, but not cholesterol levels and triglycerides[24]. Also in ApoE3Leiden mice, after feeding a high-fat cholesterol rich diet up to six weeks, IL10 deficiency did not lead to significant higher cholesterol levels as compared to the levels in the IL10$^{+/+}$ group. Therefore, the aggravation of neointima formation in the IL10 deficient mice is thought to be largely attributed to the lack of interleukin-10, rather than via indirect effect of IL10 such as modulation of plasma cholesterol levels.

To investigate the therapeutic potential of IL10 to prevent restenosis and post-interventional accelerated atherosclerosis in more detail, this cytokine was overexpressed by electroporation mediated non-viral gene therapy. Previous studies described the beneficial effect of IL10 protein administration on in-stent restenosis and vessel injury[8-10]. Also the effect of viral gene delivery of IL10 on atherosclerosis has been reported[25;26]. These studies used either frequent protein injection or they used viral gene therapy, with all its drawbacks including the induction of an inflammatory response. None of these studies examined the effect of IL10 on restenosis in hypercholesterolemic mice. To address this issue, we used in our study single, non-viral administration of IL10 cDNA to asses the effect on cuff-induced neointima formation in hypercholesterolemic mice. With electroporation mediated non-viral gene transfer, drawbacks of daily protein administration and viral gene delivery can be prevented, while long term transgene expression can

be achieved. Luciferase and IL10 expression could be detected for at least 21 days after electroporation. A clear reduction in neointima formation and the accelerated atherosclerosis was observed (Figure 3.3).

In contrast to the IL10 knock-out experiments, the plasma cholesterol levels did alter after electroporation with the IL10 plasmid. This is in line with the findings of Von der Thüsen et. al and Yoshioka et al.[26;27]. The latter showed that IL10 overexpression has a direct inhibitory effect on HMG-CoA reductase expression. Conversely, Namiki and colleagues did not find any differences in plasma cholesterol levels after IL10 gene transfer[25], probably due to differences in circulating IL10 levels after gene transfer.

Although IL10 overexpression affects plasma cholesterol levels in our study, this reduction of cholesterol levels certainly does not explain the entirely attenuation of neointima formation after IL10 overexpression. In the $IL10^{-/-}$ mice, neointima formation increased without a significant rise of cholesterol levels. Most likely, as described in literature, the anti-inflammatory effect of IL10 contributes to the inhibitory effect of IL10 on neointima formation[8;10]. To support this hypothesis, we assessed the effect of IL10 overexpression on several inflammatory-related cytokines. The mRNA expression of pro-inflammatory cytokines, like TNFα and IL6, was diminished after IL10 overexpression, whereas the expression of IL1β was hardly affected. The expression of anti-inflammatory cytokine IL10 was augmented. Taken together, these data suggest that IL10 overexpression leads to a specific shift towards a Th2 phenotype. This Th2 phenotype has been reported to be more protective for inflammation related vascular remodeling[24;28]. Nevertheless, the IFNy response is not completely understood. In contrast to the alterations of neointima formation due to the IL10 modulation in the hypercholesterolemic ApoE3Leiden mice, the cellular composition of the neointimal area did not change as compared to their controls, i.e. the relative contribution of SMCs and macrophages was similar. Also the composition and size of the media was not affected in all groups. This suggests, next to a reducing effect on macrophages and monocytes influx, a direct inhibitory effect on smooth muscle cell activation of IL10. A similar inhibitory effect of IL10 on SMC activation was previously described and might be a result of NF-kappaB inactivation via IL10[10].

Also in patients, the relationship between IL10 and restenosis becomes more and more clear. When examining the correlation of four known polymorphisms of the IL10 gene on the development of restenosis in the previously published population of the GENetic DEterminants of Restenosis (GENDER) project[4;29] we were able to demonstrate that three out of four polymorphisms of the IL10 gene correlated with an increased risk of developing restenosis (unpublished data). Koss et al. demonstrated that one these polymorphisms was associated with decreased IL10 production[30]. These data corroborate the hypothesis that lower levels of IL10 may increase the risk of developing restenosis.

In conclusion, in the present study we demonstrate both by knocking-out and by overexpressing the IL10 gene, that IL10 is significantly involved in the regulation

of neointima formation and accelerated atherosclerosis in hypercholesterolemic ApoE3Leiden mice. The therapeutic potential of IL 10 overexpression was demonstrated by the inhibitory effect of non-viral, electroporation mediated intramuscular delivery of IL10 cDNA on neointima formation and accelerated atherosclerosis in ApoE3Leiden mice. Because of the high and prolonged gene expression after a single intramuscular electroporation, non-viral IL10 gene delivery is a potential therapeutic approach to prevent post-angioplasty restenosis.

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