Gene therapy strategies to target post-interventional vascular remodeling

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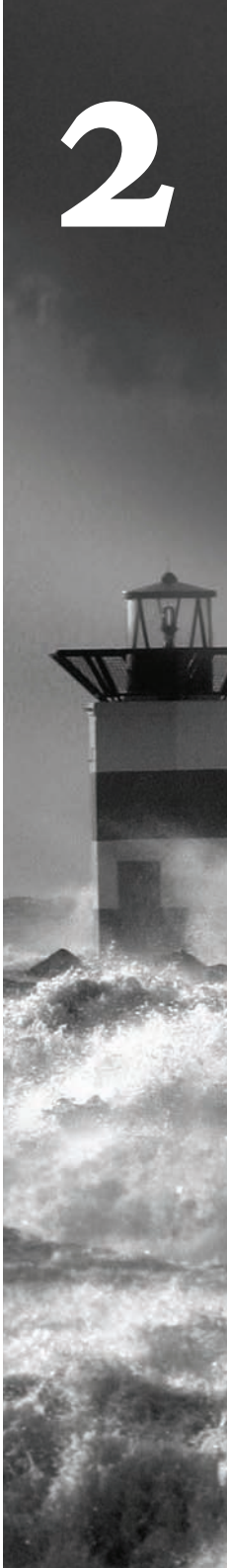

The effect of interleukin-10 knock-out and overexpression on neointima formation in hypercholesterolemic APOE*3Leiden mice

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Abstract

Objective: Inflammatory factors are thought to play a regulatory role in restenosis. Interleukin (IL)-10 is an important anti-inflammatory cytokine with anti-atherogenic potentials. The aim of this study was to assess the effects of IL-10 modulation on cuff-induced neointima formation in hypercholesterolemic APOE*3Leiden mice.

Methods: The involvement of IL-10 in neointima formation was studied in a hypercholesterolemic mouse model of cuff-induced stenosis of the femoral artery by IL-10 knocking-out or overexpression procedures. IL-10$^{+/-}$ mice were crossbred with APOE*3Leiden mice to generate hypercholesterolemic APOE*3LeidenIL-10$^{-/-}$ mice. To achieve IL-10 overexpression in APOE*3Leiden mice, a single intramuscular injection of a murine IL-10 overexpression plasmid was performed followed by electroporation.

Results: Knocking-out IL-10, in hypercholesterolemic APOE*3Leiden mice, resulted in a significant 1.9-fold increase of neointima surface as compared to APOE*3LeidenIL-10$^{+/+}$ littermates (p=0.02). Conversely, a marked 45% inhibition on cuff-induced neointima formation was obtained after IL-10 overexpression (p=0.02). Electrodelivery of IL-10 vector leads to detectable IL-10 serum levels, with a sustained expression over the experimental period of three weeks. IL-10 overexpression reduced plasma cholesterol levels in APOE*3Leiden mice, whereas IL-10 deficiency in these mice did not lead to altered cholesterol levels as compared to the IL-10$^{+/+}$ group. Finally, IL-10 overexpression stimulated endogenous IL-10 mRNA expression in the spleen and reduced the transcriptional responses of several pro-inflammatory cytokines.

Conclusion: Here, we clearly demonstrate the role of IL-10 in the development of neointima formation in hypercholesterolemic mice and the potential therapeutic effect of non-viral electrodelivery of IL-10 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. IL-1β, TNF-α and IFN-γ) 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 (IL)-10 is known as an anti-inflammatory and anti-atherogenous cytokine. In addition, IL-10 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, IL-10 is endogenously produced in the atherosclerotic plaque to modulate the inflammatory process[5-7].

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

In this study, we examined the effect of IL-10 gene knock-out and overexpression on neointima formation in hypercholesterolemic APOE*3Leiden mice. In these APOE*3Leiden mice, a mutated human APOE*3Leiden 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 IL-10 in these hypercholesterolemic mice enables us to study the involvement of IL-10 on neointima formation in comparison to their IL-10$^{+/+}$ littermates.

Opposingly, non-viral intramuscular electroporation mediated gene transfer of interleukin-10 cDNA was used to assess the effect of high circulating

levels of IL-10 protein on neointima formation in APOE*3Leiden 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 transgenes14,15. In this study, we show that intramuscular electroporation mediated delivery of a murine IL-10 expression plasmid results in sufficient IL-10 serum levels and inhibits cuff-induced neointima formation in hypercholesterolemic APOE*3Leiden 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 IL-10 overexpression experiments, Specific Pathogen-Free transgenic APOE*3Leiden mice were crossbred for at least 18 generations with C57BL/6 mice. These mice develop a diet dependent hypercholesterolemia and spontaneous atherosclerosis[11,16]. Male APOE*3Leiden animals, with a mean weight of 28.4±0.6 grams, were allocated randomly to one of the two experimental groups.

For the IL-10 knock-out experiments, experimental mice (APOE*3LeidenIL-$10^{-/-}$ and control littermate APOE*3LeidenIL-$10^{+/+}$ mice) were obtained by crossbreeding IL-$10^{+/-}$ mice (Jackson Laboratory) with heterozygote APOE*3Leiden mice. Mice were characterized by PCR for IL-10 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 IL-10 knock-out study and in the IL-10 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 (IL-10$^{-/-}$ experiment) and 21 days (IL-10 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 IL-10, intramuscular electroporation with a CAGGS plasmid encoding for murine IL-10 (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 vivo20. 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-mIL-10 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 IL-10 concentration was measured using a mouse IL-10 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 previously22. 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 IL-10 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 2.1). TaqMan® Gene Expression Assays were used for IL-6, IL-1β, IFN-γ, IL-4 and IL-10 (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).

For the analysis, the average cycle threshold per time point was subtracted from the average cycle threshold of the housekeeping gene HPRT (ΔCt). ΔΔCt was determined as the difference between ΔCt-values of the IL-10 electroporated mice and the control group (luciferase). Data are presented as mean fold induction compared to the normalized luciferase group, calculated as $2^{-\Delta\Delta Ct}$.

Table 2.1. Taqman Primers and Probes for real-time quantitative RT-PCR

TNF-α	Sense:5' CATCTTCTCAAAATTCGAGTGACAA 3' Antisense: 5' TGGGAGTAGACAAGGTACAACCC 3' Probe: 5' CACGTCGTAGCAAACCACCAAGTGGA 3'
HPRT	Sense: 5'-GGCTATAAGTTCTTTGCTGACCTG-3' Antisense: 5'-AACTTTTATGTCCCCCGTTGA-3' Probe: 5'-CTGTAGATTTTATCAGACTGAAGAGCTACTGTAATGACCA-3'

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 T-Test. A value of $P<0.05$ was considered statistically significant.

Results

Neointima formation in hypercholesterolemic APOE*3LeidenIL-10 knock-out mice

To study the effect of lacking interleukin-10 on neointima formation in mice with a hypercholesterolemic background, IL-10$^{+/-}$ mice were crossbred with APOE*3Leiden mice. Twelve week old APOE*3-LeidenIL-10$^{-/-}$ and control littermate (APOE*3LeidenIL-10$^{+/+}$) 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 IL-10$^{-/-}$ group and 7.2±1.1 mmol/L in the IL-10$^{+/+}$ 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 (IL-10$^{-/-}$ group) versus 7.3±1.9 mmol/L (IL-10$^{+/+}$ 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 IL-10$^{-/-}$ group as compared to the control group (control: 1.1±0.2 mm^2, IL-10$^{-/-}$: 2.1±0.3 mm^2; p=0.02; Figure 2.1, 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 IL-10$^{-/-}$ group (lumen stenosis in control group: 18.7±2.8% and IL-10$^{-/-}$ group: 33.8±4.5%; p=0.01, Figure 2.1, panel B and C).

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

Seven days after intramuscular injection and electroporation with pCAGGS-mIL-10 or pCAGGS-Luciferase, serum levels of mIL-10 were 43.7±4.8 ng/ml and 0.7±0.1 ng/ml, respectively (p<0.001). 21 days after electroporation, mIL-10 concentration in the serum was 2.0±0.3 ng/ml in the IL-10 treated group and 0.3±0.04 ng/ml in the control group (p=0.009, Figure 2.2, panel A). To verify expression of the transfected transgene luciferase, bioluminescence

imaging was performed and luciferase activity was quantified (see Figure 2.2, panel B and C).

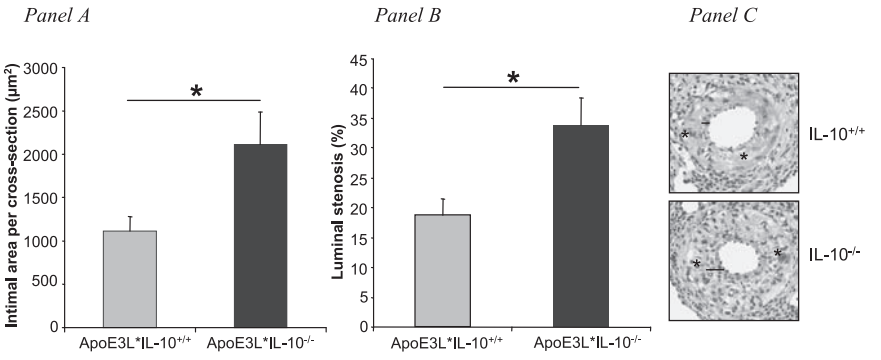

Figure 2.1. Effect of IL-10 knock-out on neointimal formation in hypercholesterolemic mice.

Total intimal thickening (Panel A) and percentage of lumenstenosis (B) of cuffed femoral arteries in APOE*3LeidenIL-10-/- and their IL-10+/+ 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 IL-10 knock-out group. Asterisks (*) indicate macrophage-derived foam cells (magnification 250x).

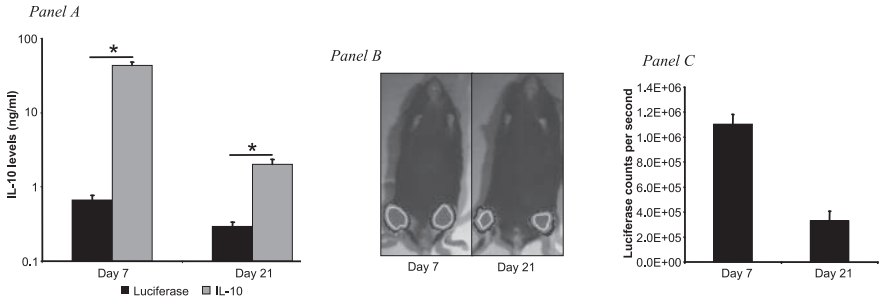

Figure 2.2. Expression of IL-10 and Luciferase after intramuscular, non-viral gene therapy.

Panel A: Murine IL 10 serum levels in ng/ml, one and three weeks after intramuscular electroporation of mIL-10 cDNA or Luciferase as a control (n=5 per group, *p<0.01). IL-10 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).

Effect of interleukin-10 overexpression on neointima formation in APOE*3Leiden mice

To study the effect of IL-10 on neointima formation in hypercholesterolemic mice, femoral arterial non-constricting cuffs were placed in male APOE*3Leiden 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 IL-10 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 IL-10 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 IL-10 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).

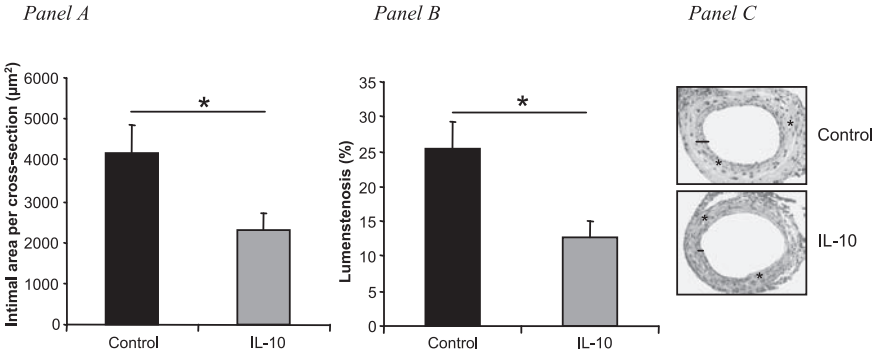

Figure 2.3. Effect of IL-10 overexpression on neointima formation in hypercholesterolemic APOE*3Leiden mice.

Total intimal area (Panel A) and percentage of lumenstenosis (B) of cuffed femoral arteries in hypercholesterolemic APOE*3Leiden mice, three weeks after electroporation of pCAGGS-mIL-10 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. IL-10 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).

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 IL-10 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 IL-10 treated group as compared to the control group (control: 4.2±0.7 mm^2 and IL-10: 2.3±0.4 mm^2; p=0.02; Figure 2.3, panel A and C). The medial area was not changed significantly in both groups (control: 11.0±1.0 mm^2 and IL-10: 9.5±0.7 mm^2). The luminal stenosis was inhibited with 50.5% in the IL-10 treated group (control: 25.5±3.8 % and IL-10: 12.6±2.5 %, p=0.006, Figure 2.3, panel B and C).

To study the effect of IL-10 modulation on the cellular composition of the lesion in the cuffed arterial wall, immunohistochemical analysis for the presence of smooth muscle cells and macrophage-derived foam-cells of both, IL-10 knock-out and IL-10 overexpression experiments was performed (Figure 2.4). 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 IL-10 knock-out experiment were: IL-$10^{+/+}$: 40±5% and IL-$10^{-/-}$: 40±6%, p=0.49 and in the IL-10 overexpression experiment the relative SM α-actin positive areas were: IL-10 overexpression: 23±5% and control: 25±4%, p=0.36. Also the relative macrophage positive areas did not change significantly after knocking-out IL-10 as compared to IL-$10^{+/+}$ littermates (IL-$10^{+/+}$: 2±1% and IL-$10^{-/-}$: 3±1%, p=0.25). In addition, IL-10 overexpression did not affect the relative macrophage positive areas either (IL-10 overexpression: 26±5% and control: 27±5%, p=0.46).

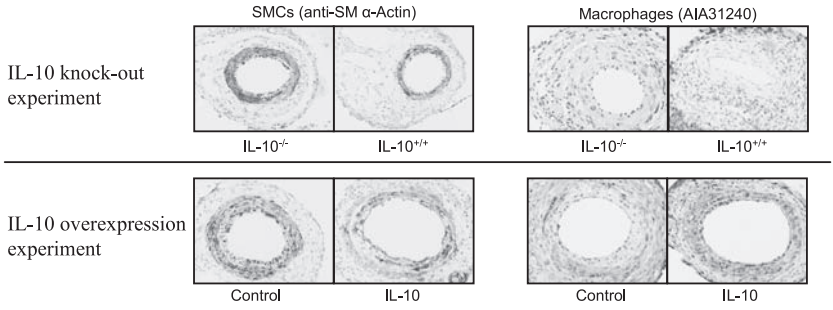

Figure 2.4. Effect of IL-10 on relative smooth muscle cell and macrophage content of medial and intimal areas.

Representative cross-sections of cuffed femoral arteries of both, IL-10 knock-out and IL-10 overexpression experiments, immunohistochemically stained for smooth muscle cells (SMCs; anti-SM α-Actin) and macrophages (AIA31240). Magnification 200x.

In spite of an increase of neointima formation in the IL-10$^{-/-}$ mice and a reduction of neointima formation after IL-10 overexpression, differences in the relative SM α-actin positive area in the neointima were neither significant in the IL-10$^{-/-}$ mice as compared to their control littermates (IL-10$^{+/+}$: 18±4% and IL-10$^{-/-}$: 18±1%, p=0.50) nor in the IL-10 overexpression group as compared to their controls (IL-10 overexpression: 19±3% and control: 24±3%, p=0.17). The relative macrophage positive areas were also similar in the IL-10 knock-out experiment (IL-10$^{+/+}$: 9±4% and IL-10$^{-/-}$: 7±2%, p=0.40) and after IL-10 overexpression (IL-10 overexpression: 20±2% and control: 19±3%, p=0.41).

Effect of interleukin-10 overexpression on inflammatory-related cytokines

IL-10 is known as an anti-inflammatory and atheroprotective cytokine[5]. Because inflammation is thought to play an important role in the restenotic process[2,23], we investigated the systemic effect of IL-10 on inflammatory-related cytokines after electroporation mediated IL-10 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 IL-10 overexpression. IL-1β, IL-6, TNF-α and IFN-γ were chosen as prototypes for pro-atherogenic cytokines. As anti-inflammatory genes, IL-4 and IL-10 were selected. Both in the IL-10 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.

Figure 2.5 shows the fold inductions of mRNA levels of inflammatory related genes in the IL-10 electroporated group as compared to the normalized

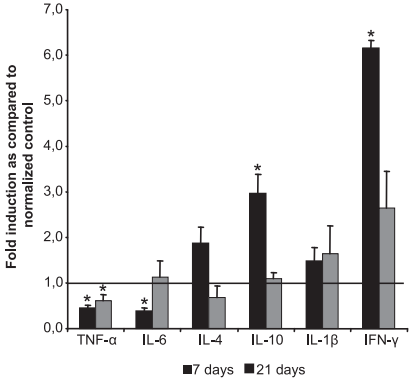

Figure 2.5. Relative mRNA expression of inflammatory-related cytokines after IL-10 overexpression.

control group (fold induction in control group set as 1). Seven days after IL-10 overexpression, mRNA expression in the spleen of two important pro-atherogenic cytokines, TNF-α and IL-6, was strongly reduced (55±7% ($p=0.006$) and 62±7% ($p=0.02$), respectively) in the IL-10 treated group. Expression of TNF-α mRNA remained low after 21 days (40±13% as compared to the control group, $p=0.02$) and mRNA levels of IL-6 normalized to control group level.

Both 7 as well as 21 days after electroporation, mRNA expression of IL-1β was not significantly altered. However, mRNA levels of IFN-γ showed a 6.1±0.2 fold increase 7 days after IL-10 treatment ($p<0.001$). After three weeks, no significant difference was seen anymore in the expression of IFN-γ mRNA as compared to observed levels in the control group. The mRNA expression of the anti-inflammatory gene IL-10 was 3.0±0.4 fold increased in the IL-10 treated group as compared to the normalized control, 7 days after electroporation and surgery ($p=0.01$). Remarkable is that endogenous IL-10 production in the spleen can be enhanced after exogenous administration of IL-10 gene. IL-10 mRNA expression normalized within 3 weeks. The levels of IL-4 mRNA did not alter significantly after IL-10 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[24]. Since inflammation is thought to play an important role in the development of restenosis, anti-inflammatory approaches are promising alternative strategies. IL-10 is a well-known anti-inflammatory and anti-atherogenic cytokine, therefore this cytokine is attractive to use in the prevention of post-angioplasty restenosis.

In this study, we demonstrate that interleukin-10 has a beneficial effect on restenosis in hypercholesterolemic mice. Knocking-out IL-10 in APOE*3Leiden mice results in a 1.9-fold increase of neointima formation in the femoral artery after cuff placement. On the other hand, IL-10 overexpression by non-viral gene transfer leads to a 45% reduction of neointima formation after cuff placement. Furthermore, this study shows that systemic IL-10 overexpression inhibits the expression of several important pro-atherogenic cytokines and enhances the expression of endogenous IL-10 mRNA in the spleen.

It has previously been reported that IL-10 deficiency resulted in augmented intimal hyperplasia after carotid artery wire injury in normocholesterolemic

mice[9]. In our study, we crossbred APOE*3Leiden mice with IL-10$^{+/-}$ mice to generate diet-dependent hypercholesterolaemic IL-10 knock-out mice and demonstrate that also the injury induced accelerated atherosclerosis is strongly reduced in the restenotic lesions. Caligiuri and colleagues described that IL-10 deficiency in ApoE$^{-/-}$ mice did increase spontaneous atherosclerosis and low-density lipoproteins, but not cholesterol levels and triglycerides[25]. Also in APOE*3Leiden mice, after feeding a high-fat cholesterol rich diet up to six weeks, IL-10 deficiency did not lead to significant higher cholesterol levels as compared to the levels in the IL-10$^{+/+}$ group. Therefore, the aggravation of neointima formation in the IL-10 deficient mice is thought to be largely attributed to the lack of interleukin-10, rather than via indirect effect of IL-10 such as modulation of plasma cholesterol levels.

To investigate the therapeutic potential of IL-10 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 IL-10 protein administration on in-stent restenosis and vessel injury[8-10]. Also the effect of viral gene delivery of IL-10 on atherosclerosis has been reported[26,27]. 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 IL-10 on restenosis in hypercholesterolemic mice. To address this issue, we used in our study single, non-viral administration of IL-10 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 IL-10 expression could be detected for at least 21 days after electroporation. A clear reduction in neointima formation and the accelerated atherosclerosis was observed (Figure 2.3).

In contrast to the IL-10 knock-out experiments, the plasma cholesterol levels did alter after electroporation with the IL-10 plasmid. This is in line with the findings of Von der Thüsen *et al.* and Yoshioka *et al.*[27,28]. The latter showed that IL-10 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 IL-10 gene transfer[26], probably due to differences in circulating IL-10 levels after gene transfer.

Although IL-10 overexpression affects plasma cholesterol levels in our study, this reduction of cholesterol levels certainly does not explain the entirely attenuation of neointima formation after IL-10 overexpression. In the IL-10$^{-/-}$

mice, neointima formation increased without a significant rise of cholesterol levels. Most likely, as described in literature, the anti-inflammatory effect of IL-10 contributes to the inhibitory effect of IL-10 on neointima formation[8,10]. To support this hypothesis, we assessed the effect of IL-10 overexpression on several inflammatory-related cytokines. The mRNA expression of pro-inflammatory cytokines, like TNF-α and IL-6, was diminished after IL-10 overexpression, whereas the expression of IL-1β was hardly affected. The expression of anti-inflammatory cytokine IL-10 was augmented. Taken together, these data suggest that IL-10 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[25,29]. Nevertheless, the IFN-y response is not completely understood.

In contrast to the alterations of neointima formation due to the IL-10 modulation in the hypercholesterolemic APOE*3Leiden mice, the cellular composition of the neointimal area did not change as compared to their controls, i.e. the relative contribution of smooth muscle cells 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 IL-10. A similar inhibitory effect of IL-10 on smooth muscle cell activation was previously described and might be a result of NF-kappaB inactivation via IL-10[10].

Also in patients, the relationship between IL-10 and restenosis becomes more and more clear. When examining the correlation of four known polymorphisms of the IL-10 gene on the development of restenosis in the previously published population of the GENetic DEterminants of Restenosis (GENDER) project[4,30] we were able to demonstrate that three out of four polymorphisms of the IL-10 gene correlated with an increased risk of developing restenosis[31]. Koss *et al.* demonstrated that one these polymorphisms was associated with decreased IL-10 production[32]. These data corroborate the hypothesis that lower levels of IL-10 may increase the risk of developing restenosis.

In conclusion, in the present study we demonstrate both by knocking-out and by overexpressing the IL-10 gene, that IL-10 is significantly involved in the regulation of neointima formation and accelerated atherosclerosis in hypercholesterolemic APOE*3Leiden mice. The therapeutic potential of IL 10 overexpression was demonstrated by the inhibitory effect of non-viral, electroporation mediated intramuscular delivery of IL-10 cDNA on neointima formation and accelerated atherosclerosis in APOE*3Leiden mice. Because

of the high and prolonged gene expression after a single intramuscular electroporation, non-viral IL-10 gene delivery is a potential therapeutic approach to prevent post-angioplasty restenosis.

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References

1. Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med 1999;340:115-126.
2. Serrano CV, Jr., Ramires JA, Venturinelli M, Arie S, D'Amico E, Zweier JL, Pileggi F, da Luz PL. Coronary angioplasty results in leukocyte and platelet activation with adhesion molecule expression. Evidence of inflammatory responses in coronary angioplasty. *J Am Coll Cardiol* 1997;29:1276-1283.
3. Welt FG, Rogers C. Inflammation and restenosis in the stent era. *Arterioscler Thromb Vasc Biol* 2002;22:1769-1776.
4. Monraats PS, Pires NM, Schepers A, Agema WR, Boesten LS, de Vries MR, Zwinderman AH, de Maat MP, Doevendans PA, de Winter RJ, Tio RA, Waltenberger J, 't Hart LM, Frants RR, Quax PH, van Vlijmen BJ, Havekes LM, van der LA, van der Wall EE, Jukema JW. Tumor necrosis factor-alpha plays an important role in restenosis development. *FASEB J* 2005;19:1998-2004.
5. de Waal MR, Yssel H, Roncarolo MG, Spits H, de Vries JE. Interleukin-10. *Curr Opin Immunol* 1992;4:314-320.
6. Mallat Z, Besnard S, Duriez M, Deleuze V, Emmanuel F, Bureau MF, Soubrier F, Esposito B, Duez H, Fievet C, Staels B, Duverger N, Scherman D, Tedgui A. Protective role of interleukin-10 in atherosclerosis. *Circ Res* 1999;85:e17-e24.
7. Mallat Z, Heymes C, Ohan J, Faggin E, Leseche G, Tedgui A. Expression of interleukin-10 in advanced human atherosclerotic plaques: relation to inducible nitric oxide synthase expression and cell death. *Arterioscler Thromb Vasc Biol* 1999;19:611-616.
8. Feldman LJ, Aguirre L, Ziol M, Bridou JP, Nevo N, Michel JB, Steg PG. Interleukin-10 inhibits intimal hyperplasia after angioplasty or stent implantation in hypercholesterolemic rabbits. *Circulation* 2000;101:908-916.
9. Zimmerman MA, Reznikov LL, Raeburn CD, Selzman CH. Interleukin-10 attenuates the response to vascular injury. *J Surg Res* 2004;121:206-213.
10. Mazighi M, Pelle A, Gonzalez W, Mtairag eM, Philippe M, Henin D, Michel JB, Feldman LJ. IL-10 inhibits vascular smooth muscle cell activation in vitro and in vivo. *Am J Physiol Heart Circ Physiol* 2004;287:H866-H871.
11. van den Maagdenberg AM, Hofker MH, Krimpenfort PJ, de B, I, van Vlijmen B, van der BH, Havekes LM, Frants RR. Transgenic mice carrying the apolipoprotein E3-Leiden gene exhibit hyperlipoproteinemia. *J Biol Chem* 1993;268:10540-10545.
12. Lardenoye JH, Delsing DJ, de Vries MR, Deckers MM, Princen HM, Havekes LM, van Hinsbergh VW, van Bockel JH, Quax PH. Accelerated atherosclerosis by placement of a perivascular cuff and a cholesterol-rich diet in ApoE*3Leiden transgenic mice. *Circ Res* 2000;87:248-253.
13. Pires NM, Schepers A, van der Hoeven BL, de Vries MR, Boesten LS, Jukema JW, Quax PH. Histopathologic alterations following local delivery of dexamethasone to inhibit restenosis in murine arteries. *Cardiovasc Res* 2005;68:415-424.
14. McMahon JM, Wells DJ. Electroporation for gene transfer to skeletal muscles: current status. *BioDrugs* 2004;18:155-165.
15. Andre F, Mir LM. DNA electrotransfer: its principles and an updated review of its therapeutic applications. *Gene Ther* 2004;11 Suppl 1:S33-S42.
16. van Vlijmen BJ, van den Maagdenberg AM, Gijbels MJ, van der BH, HogenEsch H, Frants RR, Hofker MH, Havekes LM. Diet-induced hyperlipoproteinemia and atherosclerosis in apolipoprotein E3-Leiden transgenic mice. *J Clin Invest* 1994;93:1403-1410.

17. Kuhn R, Lohler J, Rennick D, Rajewsky K, Muller W. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 1993;75:263-274.

18. Lardenoye JH, de Vries MR, Lowik CW, Xu Q, Dhore CR, Cleutjens JP, van Hinsbergh VW, van Bockel JH, Quax PH. Accelerated atherosclerosis and calcification in vein grafts: a study in APOE*3 Leiden transgenic mice. *Circ Res* 2002;91:577-584.

19. Moroi M, Zhang L, Yasuda T, Virmani R, Gold HK, Fishman MC, Huang PL. Interaction of genetic deficiency of endothelial nitric oxide, gender, and pregnancy in vascular response to injury in mice. *J Clin Invest* 1998;101:1225-1232.

20. Nitta Y, Tashiro F, Tokui M, Shimada A, Takei I, Tabayashi K, Miyazaki J. Systemic delivery of interleukin 10 by intramuscular injection of expression plasmid DNA prevents autoimmune diabetes in nonobese diabetic mice. *Hum Gene Ther* 1998;9:1701-1707.

21. McMahon JM, Signori E, Wells KE, Fazio VM, Wells DJ. Optimisation of electrotransfer of plasmid into skeletal muscle by pretreatment with hyaluronidase -- increased expression with reduced muscle damage. *Gene Ther* 2001;8:1264-1270.

22. Pillon A, Servant N, Vignon F, Balaguer P, Nicolas JC. In vivo bioluminescence imaging to evaluate estrogenic activities of endocrine disrupters. *Anal Biochem* 2005;340:295-302.

23. Welt FG, Rogers C. Inflammation and restenosis in the stent era. *Arterioscler Thromb Vasc Biol* 2002;22:1769-1776.

24. Palmaz JC. Intravascular stents in the last and the next 10 years. *J Endovasc Ther* 2004;11 Suppl 2:II200-II206.

25. Caligiuri G, Rudling M, Ollivier V, Jacob MP, Michel JB, Hansson GK, Nicoletti A. Interleukin-10 deficiency increases atherosclerosis, thrombosis, and low-density lipoproteins in apolipoprotein E knockout mice. *Mol Med* 2003;9:10-17.

26. Namiki M, Kawashima S, Yamashita T, Ozaki M, Sakoda T, Inoue N, Hirata K, Morishita R, Kaneda Y, Yokoyama M. Intramuscular gene transfer of interleukin-10 cDNA reduces atherosclerosis in apolipoprotein E-knockout mice. *Atherosclerosis* 2004;172:21-29.

27. Yoshioka T, Okada T, Maeda Y, Ikeda U, Shimpo M, Nomoto T, Takeuchi K, Nonaka-Sarukawa M, Ito T, Takahashi M, Matsushita T, Mizukami H, Hanazono Y, Kume A, Ookawara S, Kawano M, Ishibashi S, Shimada K, Ozawa K. Adeno-associated virus vector-mediated interleukin-10 gene transfer inhibits atherosclerosis in apolipoprotein E-deficient mice. *Gene Ther* 2004;11:1772-1779.

28. der Thusen JH, Kuiper J, Fekkes ML, De Vos P, Van Berkel TJ, Biessen EA. Attenuation of atherogenesis by systemic and local adenovirus-mediated gene transfer of interleukin-10 in LDLr-/- mice. *FASEB J* 2001;15:2730-2732.

29. Mallat Z, Tedgui A. Immunomodulation to combat atherosclerosis: the potential role of immune regulatory cells. *Expert Opin Biol Ther* 2004;4:1387-1393.

30. Monraats PS, Pires NM, Agema WR, Zwinderman AH, Schepers A, de Maat MP, Doevendans PA, de Winter RJ, Tio RA, Waltenberger J, Frants RR, Quax PH, van Vlijmen BJ, Atsma DE, van der LA, van der Wall EE, Jukema JW. Genetic inflammatory factors predict restenosis after percutaneous coronary interventions. *Circulation* 2005;112:2417-2425.

31. Monraats PS, Kurreeman FA, Pons D, Sewgobind VD, de Vries FR, Zwinderman AH, de Maat MP, Doevendans PA, de Winter RJ, Tio RA, Waltenberger J, Huizinga TW, Eefting D, Quax PH, Frants RR, van der LA, van der Wall EE, Jukema JW. Interleukin 10: a new risk marker for the development of restenosis after percutaneous coronary intervention. *Genes Immun* 2007;8:44-50.

32. Koss K, Satsangi J, Fanning GC, Welsh KI, Jewell DP. Cytokine (TNF alpha, LT alpha and IL-10) polymorphisms in inflammatory bowel diseases and normal controls: differential effects on production and allele frequencies. *Genes Immun* 2000;1:185-190