Lipids, inflammation and atherosclerosis

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Chapter 7

CHARACTERIZATION OF THE CHOLESTEROL LOWERING EFFECT OF INTERLEUKIN-10 BY MICROARRAY GENE EXPRESSION ANALYSIS OF LIVER PARENCHYMAL CELLS

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ABSTRACT

IL-10 is a pleiotropic cytokine primarily produced by macrophages and T cells. The main function of IL-10 appears to be anti-inflammatory which is underlined by its protective effect on atherosclerosis, a disease characterized by chronic inflammation and affected by many pro- and anti-inflammatory mediators. Although serum IL-10 levels are negatively correlated with cholesterol levels in several studies, no explanation for this effect has been described. In our study, male LDLr deficient mice were put on a Western-type diet for four weeks. After two weeks of diet feeding, the animals were treated with an adenovirus expressing human IL-10 or LacZ as a control. Liver parenchymal cells, which play a dominant role in regulating serum cholesterol homeostasis, were isolated two weeks after the administration of adenovirus. RNA of the parenchymal cells was applied to mouse Genome Survey Microarrays to identify genes affected by IL-10. Serum cholesterol levels increased in the Ad.LacZ treated group during four weeks of Western-type diet feeding. In the Ad.hIL-10 treated group, serum cholesterol, especially LDL, levels were significantly 1.85-fold lower ($p<0.01$). Microarray analysis revealed that in parenchymal cells, Ad.hIL-10 treatment led to a more than 2-fold change in the expression of 913 genes of which 205 were downregulated and 708 were upregulated. In addition to the classical target genes such as TNF-α and IL-6, more genes involved in lipid metabolism were affected by IL-10 than anticipated from statistical expectations. Interestingly, amongst these genes, ABCG5 and ABCG8 were both upregulated upon Ad.hIL-10 treatment. ABCG5 and ABCG8 are involved in the excretion of cholesterol from the body and their upregulation by IL-10 might explain the cholesterol lowering effect observed in LDLr deficient mice on a Western-type diet.

INTRODUCTION

Interleukin (IL)-10, a pleiotropic cytokine, is mainly produced by macrophages, T cells and B cells[1,2]. The main function of IL-10 is anti-inflammatory by inhibiting the macrophage production of pro-inflammatory mediators, inhibiting antigen presentation and inducing a shift in the T-helper (Th)1/Th2 balance toward a Th2-type response. IL-10 affects a broad spectrum of cells and is involved in the pathogenesis of many diseases such as cancer, autoimmune diseases and viral infections[1-4]. In addition, IL-10 plays an important role in the development of atherosclerosis, a disease characterized by chronic inflammation[5,6] and affected by many pro- and anti-inflammatory mediators[7,8]. In previous studies, overexpression of IL-10 resulted in attenuation of atherogenesis, indicating a protective role for endogenous IL-10[9-11]. This effect was underlined by studies in IL-10 deficient mice that showed an enhanced atherosclerotic lesion development[12,13]. The protective role of IL-10 can, partly, be explained by the inhibitory effect of IL-10 on inflammation[10] and on the expression of adhesion molecules[14]. In addition, several animal studies showed that IL-10 influences lipid metabolism resulting in lower serum total cholesterol levels[9,11]. However, the effect of IL-10 on serum cholesterol levels is not yet clarified. Serum cholesterol levels are largely influenced by synthesis and secretion of very low-density lipoprotein (VLDL) and high-density lipoprotein (HDL) and by removal of cholesterol from the body via bile[15,16]. These processes occur in the liver, which therefore is the key organ in serum lipid homeostasis. The liver consists mainly of three cell types: parenchymal cells, endothelial cells and Kupffer cells. Of these cell types, parenchymal cells occupy almost 80% of the total liver volume and perform the majority of the numerous liver functions[17], including cholesterol uptake and metabolism for biliary excretion and VLDL and HDL synthesis and secretion. Therefore, in order to unravel the mechanism behind the cholesterol lowering effect of IL-10, we performed a microarray analysis on liver parenchymal cells of low-density lipoprotein (LDL) receptor (LDLr) deficient mice fed a Western-type diet.

MATERIALS AND METHODS

Recombinant adenoviruses

Replication defective recombinant serotype 5 adenovirus expressing a human IL-10 construct under control of an RSV promoter (Ad.hIL-10)[18] and the corresponding control virus expressing LacZ (Ad.LacZ) were purchased from the University of Iowa Gene Transfer Vector Core.

Animals

Homozygous LDL receptor deficient mice[19,20] were obtained from The Jackson Laboratory as mating pairs and bred at the Gorlaeus Laboratories, Leiden, The Netherlands. All animal work was approved by the regulatory authority of Leiden University and was carried out in compliance with guidelines issued by the Dutch government. Male LDLr deficient mice, 10–12 weeks old, were put on a Western-type diet containing 0.25%

cholesterol and 15% cocoa butter (Special Diet Services, Witham, UK) for four weeks. Diet and water were provided *ad libitum*. After two weeks of diet feeding, the animals were inoculated intravenously with $1*10^9$ pfu of Ad.hIL-10 or Ad.LacZ in 200 µl of phosphate buffered saline. After a total of four weeks of diet feeding, liver parenchymal cells were isolated according to the method of Nagelkerke *et al*[21] as modified for mice by Van Berkel *et al*[22]. The purity and viability of the cells was analysed using trypan blue staining and phase contrast microscopy. The liver parenchymal cell fractions consisted of >99% parenchymal cells with a viability >95%.

Serum analysis

We obtained serum samples after 0, 13, 19 and 27 days of diet feeding. Human IL-10 levels were determined by ELISA (OptEIA kit, PharMingen). Serum concentrations of cholesterol (Roche Diagnostics), phospholipid (Wako) and triglyceride (Roche) were determined using enzymatic colorimetric assays. The cholesterol distribution over the different lipoproteins in serum was analysed by fractionation of 30 µl serum using a Superose 6 column (3.2×30 mm, Smart-system, Pharmacia). Total cholesterol content of the effluent was determined using enzymatic colorimetric assays (Roche Diagnostics).

Microarray analysis

The Mouse Genome Survey Microarrays (Applied Biosystems) used in this study contained 33.012 different probes, which included transcripts from the public domain as well as from the Celera library.

Total RNA from liver parenchymal cells was isolated using TriZol® reagent (Invitrogen Life technologies) according to the manufacturer's instructions. The Applied Biosystems Chemiluminescent RT-IVT Labeling Kit V.2.0 was used to convert 1 µg RNA into 20-100 µg digoxigenin-labeled cRNA. Subsequently, the Applied Biosystems Chemiluminescence Detection Kit was used. Equal amounts of fragmented cRNA (10 µg) were hybridized with microarrays for 16 hours at 55°C. The microarray was incubated with an alkalic phosphatase-linked digoxigenin antibody and the phosphatase activity was initiated to start the chemiluminescent signal. The chemiluminescent (cRNA) and fluorescent (spot background) signals of the cRNA and standard controls spots were scanned for 5 and 25 seconds using an AB1700 Chemiluminescence Analyser (Applied Biosystems). Using the software supplied with the AB1700 apparatus, the spot chemiluminescent signal was normalized over the fluorescent signal of the same spot to obtain the normalized signal value. Spots of poor quality were flagged by the software and removed from the analysis if more than 100 flags were assigned to it. Furthermore, only spots with a signal to noise ratio of at least 2 were considered detectable and used for further analysis. The median value of the normalized signal of two independent arrays was used as an indication for the relative gene expression of parenchymal cells from mice treated with either Ad.LacZ or Ad.hIL-10. Genes were arbitrarily designated as differentially expressed if the gene expression levels of the Ad.LacZ treated and Ad.hIL-10 treated parenchymal cells differed 2-fold or more. Differentially expressed genes were classified into functional

categories based on the Protein ANalysis THrough Evolutionary Relationships (Panther) Classification System (Celera).

Gene expression

Quantitative gene expression analysis on isolated liver parenchymal cells was performed as described before[23]. In short, total RNA was isolated using TriZol® reagent (Invitrogen Life technologies) according to the manufacturer's instructions and reverse transcribed using RevertAid™ reverse transcriptase. Gene expression analysis was performed using real-time SYBR Green technology (Eurogentec) with the primers displayed in **Table 1**. Hypoxanthine-guanine phosphoribosyltransferase (HPRT), β-actin, cyclophilin and acidic ribosomal phosphoprotein P0 (36B4) were used as the standard housekeeping genes. Relative gene expression numbers were calculated by subtracting the threshold cycle number (Ct) of the target gene from the average Ct of housekeeping genes and raising 2 to the power of this difference. The average Ct of four housekeeping genes was used to exclude that changes in the relative expression were caused by variations in the separate housekeeping gene expressions. The average Ct values for housekeeping genes (mean±SEM, n=6) were 19.5±0.2 for Ad.LacZ treated cells and 19.4±0.2 for Ad.hIL-10 treated cells.

Table 1: Primers for quantitative real-time PCR analysis

Gene	GenBank Accession	Forward primer	Reverse Primer
36B4	X15267	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG
B-actin	X03672	AACCGTGAAAAGATGACCCAGAT	CACAGCCTGGATGGCTACGTA
ABCG5	NM031884	TGGCCCTGCTCAGCATCT	ATTTTTAAAGGAATGGGCATCTCTT
ABCG8	NM026180	CCGTCGTCAGATTTCCAATGA	GGCTTCCGACCCATGAATG
Adiponutrin	AY037763	ATTCCCCTCTTCTCTGGCCTA	ATGTCATGCTCACCGTAGAAAGG
C10	NM009139	GGAACAAGGTCATTGCTTGAGAA	TCCTAGGCAGGCGGTCACT
Calmodulin 3	NM007590	TGGGACCTGCCAGCTTTG	TGGGTCTCCTGATTTGCTCTGT
CD36	NM007643	GTTCTTCCAGCCAATGCCTTT	ATGTCTAGCACACCATAAGATGTACAGTT
CrOT	NM023733	TTCATGGACGCCCTGGTT	CTCTGTTCGGCCGTGGTAA
Cyclophilin	AK010338	CCATTTCAAGAAGCAGCGTTT	ATTTTGTCTTAACTGGTGGT
Hba-a1	NM008218	ACCTACTTTCCTCACTTTGATGTAAGC	CGCATCGGCGACCTTCT
HMGCR*	M62766	TCTGGCAGTCAGTGGGAACTATT	CCTCGTCCTTCGATCCAATTT
HO-1	NM010442	CAACAGTGGCAGTGGGAATTTA	CCAGGCAAGATTCTCCCTTACC
HPRT	J00423	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG
IL-6	M20572	GAAGAATTTCTAAAAGTCACTTTGAGATCTAC	CACAGTGAGGAATGTCCACAAAC
LRAT	NM023624	TCACCTCGTACAGAACAGTTGCA	GGACGCAGCTTCCAGCAT
MCP-2	NM021443	GGTGCTGAAAAGCTACGAGAGAAT	TCTGGAAAACCACAGCTTCCA
PLTP	NM011125	ATCAATGCCTCGGCGGGA	CGACCACTGGAATCCTGGG
SR-BI	NM016741	GGCTGCTGTTTGCTGCG	GCTGCTTGATGAGGGAGGG
SREBP-2	AF374267	TGAAGCTGGCCAATCAGAAAA	ACATCACTGTCCACCAGACTGC
TNF-α	X02611	GCC AGC CGA TGG GTT GTA	AGGTTGACTTTCTCCTGGTATGAGA

* HMGCR = HMG-CoA reductase

RESULTS

Serum hIL-10 levels

Male LDLr deficient mice were put on a Western-type diet containing 0.25% cholesterol and 15% cocoa butter for two weeks to induce atherogenic cholesterol levels. Subsequently, mice were injected intravenously with $1*10^9$ pfu of Ad.hIL-10 or Ad.LacZ and Western-type diet was continued for another two weeks. As described earlier by our group[9], intravenous administration of $1*10^9$ pfu of Ad.hIL-10 led to detectable serum levels of

the transgene within 5 days and significant levels of human IL-10 of 1.5±0.4 ng/ml were maintained for the duration of the experiment (**Fig. 1A**). In the serum of mice treated with Ad.LacZ no human IL-10 was detected.

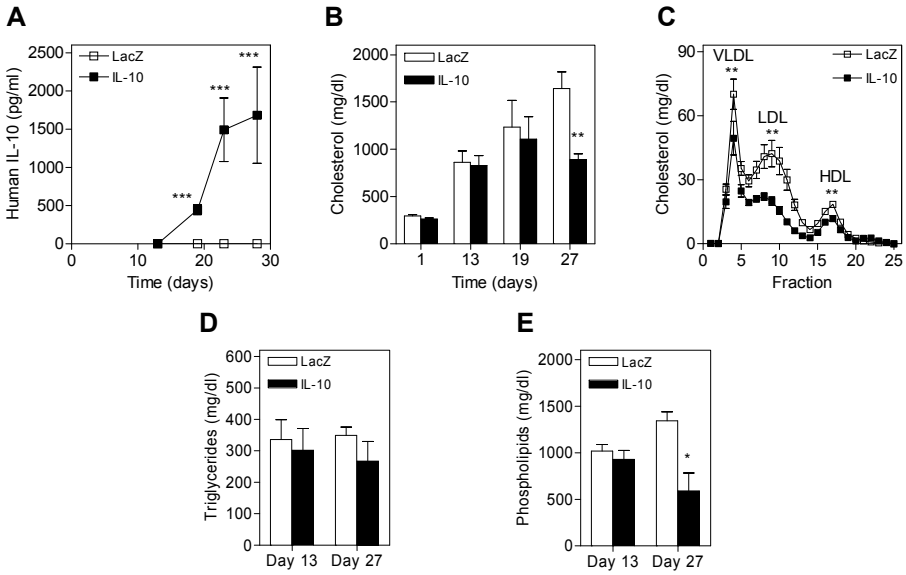

Fig. 1: Serum human IL-10 and lipid levels. LDLr deficient mice were fed a Western-type diet for four weeks and were inoculated with Ad.LacZ or Ad.hIL-10 at day 14. At day 1, 13, 19 and 27 serum samples were collected. All serum samples were analysed for **A** human IL-10, **B** total cholesterol, **D** phospholipid and **E** triglyceride content. **C** Serum samples from day 27 were fractionated using a Superose 6 column. Fractions were analysed for cholesterol content. Fractions 3-7 represent VLDL, fractions 8-15 LDL and fractions 15-19 HDL, respectively. Values are expressed as mean±SEM. Significant differences between Ad.LacZ and Ad.hIL-10 treatment: * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

Serum lipid levels

Two weeks of Western-type diet feeding resulted in a significant increase in serum cholesterol levels of both groups with no significant difference between the groups (**Fig. 1B**). After treatment with Ad.LacZ, total cholesterol levels still increased in the next two weeks of diet feeding. Although serum total cholesterol levels increased during the first days after treatment with the adenovirus encoding IL-10, a clear reduction in total cholesterol levels was observed at 14 days of IL-10 expression. The mice were sacrificed after 4 weeks of Western-type diet, when serum total cholesterol levels of Ad.hIL-10 treated mice had reached 888±65 mg/dl, whereas serum levels of Ad.LacZ treated mice reached 1640±179 mg/dl ($p<0.01$, **Fig. 1B**). After adenoviral treatment and four weeks of diet feeding, significantly lower levels of serum VLDL, HDL (both 1.56-fold) and predominantly LDL (2.5-fold) were observed in the Ad.hIL-10 treated group compared to the Ad.LacZ treated group (all $p<0.01$, **Fig. 1C**). The same pattern as described for total cholesterol was observed for free cholesterol levels in serum (data not shown). Ad.hIL-10 treatment also caused a significant decrease (1.54-fold, $p<0.05$) in serum phospholipid levels (**Fig. 1D**), whereas no differences between the two groups were observed in serum triglyceride levels (**Fig. 1E**).

Target genes of IL-10

Parenchymal cells are predominantly responsible for the regulation of serum lipoprotein levels by the liver. It is unknown whether the anti-inflammatory cytokine IL-10 can affect gene expression of liver parenchymal cells under high cholesterol conditions. Therefore, we investigated the effect of Ad.hIL-10 on gene expression of classical target genes tumor necrosis factor (TNF)-α, IL-6 and heme oxygenase (HO)-1. After diet feeding and Ad.hIL-10 treatment, liver parenchymal cells were isolated and RNA from these cells was used for quantitative real-time PCR. In agreement with its anticipated role, Ad.hIL-10 treatment led to a highly significant reduction in the gene expression of both TNF-α (6.67-fold, **Fig. 2A**) and IL-6 (9.09-fold, **Fig. 2B**) in liver parenchymal cells. In addition, a clear trend towards an increased expression of HO-1 was observed (1.8-fold, p=0.11, **Fig. 2C**), which failed to reach significance due to a high induction of HO-1 in mice with high IL-10 levels and a low induction of HO-1 expression in mice with low IL-10 levels. From these data, we conclude that IL-10 affects gene expression in liver parenchymal cells in our experimental set-up. To unravel the mechanism via which IL-10 exerts its cholesterol lowering effects, we performed microarray analysis on RNA samples of these liver parenchymal cells.

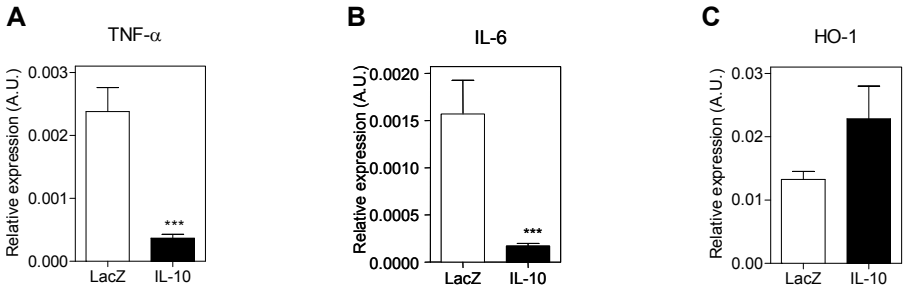

Fig. 2: Effect of IL-10 on TNF-α, IL-6 and HO-1. LDLr deficient mice were fed a Western-type diet for four weeks and were inoculated with Ad.LacZ or Ad.hIL-10 at day 14. At day 28, liver parenchymal cells were isolated and RNA of these cells was isolated. Quantitative real-time PCR analysis was used to determine the mRNA expression of **A** TNF-α, **B** IL-6 and **C** HO-1. Values are expressed as mean±SEM of relative mRNA expression compared to housekeeping gene expression (arbitrary units). Significant differences between Ad.LacZ and Ad.hIL-10 treatment: *** p<0.001.

Microarray analysis

We selected RNA samples of two mice with the highest human IL-10 expression and RNA samples of two arbitrary chosen mice of the Ad.LacZ treated group. RNA was converted into digoxigenin-labeled cRNA, which was hybridized to Mouse Genome Survey Arrays. Only genes with a detectable signal (signal to noise ratio > 2) of good quality (flags < 100) were used for further analysis. The microarray contained a total of 33.012 different probes representing 26.514 genes of which 7896 genes were detected in all microarray samples (30%). Genes were designated as differentially expressed if the gene expression levels of the Ad.LacZ treated and Ad.hIL-10 treated parenchymal cells differed 2-fold or more. Analysis of the microarrays revealed that Ad.hIL-10 treatment resulted in 913

differentially expressed genes of which 208 were downregulated and 705 were upregulated.

Biological processes

The genes detected by the microarray were analysed using the PANTHER classification system, which classifies genes into (sub)families of shared function and subsequently categorizes genes by pathway, biological process and molecular function. The complete set of 7896 genes detected by the microarray was used as a reference for the analysis of the 913 differentially expressed genes. The genes were categorized into 235 biological processes of which 23 processes contained significantly ($p<0.05$) more differentially expressed genes than expected based on the reference (**Table 2**). In addition to several biological processes involved in immunity, other processes like sulfur (redox) metabolism, vitamin/cofactor transport and porphyrin metabolism were highly affected by IL-10. Interestingly, Ad.hIL-10 treatment also led to significantly more differentially expressed genes categorized in lipid and fatty acid binding and in lipid, fatty acid and steroid metabolism than expected.

Table 2: Biological processes significantly affected by IL-10. All detected genes were categorized into biological processes using PANTHER software and used as a reference for the categorization of the differentially expressed genes. Biological processes were considered significantly affected by IL-10, if the amount of observed differentially expressed genes in this category was different than expected based on the reference ($p<0.05$; Binomial test)

Biological Process	Observed	Observed / Expected
Sulfur redox metabolism	6	5.04
Vitamin/cofactor transport	8	3.52
Porphyrin metabolism	5	3.50
Lipid and fatty acid binding	5	2.99
Sulfur metabolism	10	2.33
Macrophage-mediated immunity	14	2.21
B-cell- and antibody-mediated immunity	7	2.17
Complement-mediated immunity	8	2.16
Blood clotting	10	1.99
Chromosome segregation	11	1.96
Ligand-mediated signaling	19	1.92
Coenzyme and prosthetic group metabolism	21	1.83
Cytokine and chemokine mediated signaling pathway	15	1.82
Cell communication	51	1.75
Cell adhesion-mediated signaling	14	1.75
Mitosis	25	1.66
Cation transport	20	1.54
Immunity and defense	88	1.46
Protein biosynthesis	55	1.42
Cell proliferation and differentiation	47	1.34
Cell cycle	53	1.31
Signal transduction	124	1.28
Lipid, fatty acid and steroid metabolism	58	1.25
Biological process unclassified	326	0.86

Differentially expressed genes

The gene expression determined by the microarray was verified by quantitative real-time PCR. All six liver parenchymal cell RNA samples of both groups were used for this analysis. To find the mechanism via which IL-10 affects serum cholesterol levels, we investigated the gene expression of known mediators of cholesterol homeostasis. Scavenger receptor class B, type I (SR-BI) and CD36, both involved in lipoprotein uptake, were significantly lower expressed in parenchymal cells of Ad.hIL-10 treated mice than of Ad.LacZ treated cells (1.51-fold and 1.82-fold respectively, **Fig. 3A/B**). In contrast, the gene expression of LDLr related protein (LRP)-1, also involved in lipoprotein uptake, was not affected by IL-10 (data not shown). Several ATP-binding cassette (ABC) transporters have been known to be important mediators of cholesterol efflux to serum and bile. In our study, ABCA1 and ABCG1 were not changed (data not shown), whereas ABCG5 and ABCG8 were 1.63-fold and 1.97-fold upregulated upon Ad.hIL-10 treatment respectively (**Fig. 3C/D**). The transcription of a variety of genes involved in lipid metabolism such as the genes discussed above, is regulated by peroxisome proliferator-activated receptors (PPAR), liver X receptors (LXR) and sterol regulatory element binding proteins (SREBP). However, only SREBP-2 gene expression was 1.39-fold downregulated by Ad.hIL-10 treatment (**Fig. 3E**), while PPARα, PPARγ, PPARδ, LXRα, LXRβ and SREBP-1 gene expression levels were not affected (data not shown).

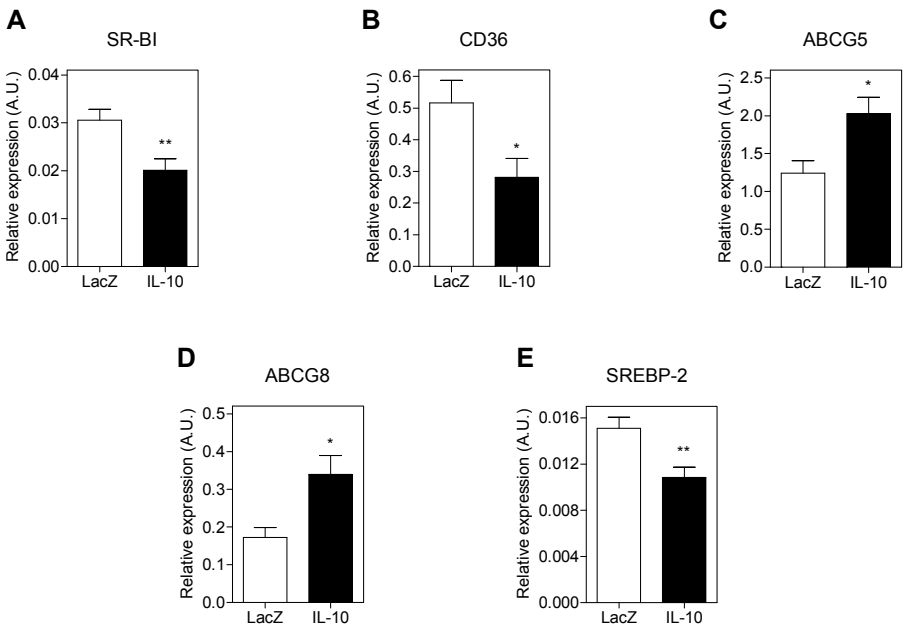

Fig. 3: Effect of IL-10 on SR-BI, CD36, ABCG5, ABCG8 and SREBP-2. LDLr deficient mice were fed a Western-type diet for four weeks and were inoculated with Ad.LacZ or Ad.hIL-10 at day 14. At day 28, liver parenchymal cells were isolated and RNA of these cells was isolated. Quantitative real-time PCR analysis was used to determine the mRNA expression of **A** SR-BI, **B** CD36, **C** ABCG5, **D** ABCG8 and **E** SREBP-2. Values are expressed as mean±SEM of relative mRNA expression compared to housekeeping gene expression (arbitrary units). Significant differences between Ad.LacZ and Ad.hIL-10 treatment: * $p<0.05$ and ** $p<0.01$.

In addition to transcription regulators, numerous enzymes play an important role in maintaining cholesterol homeostasis. For instance, intracellular fatty acids, cholesterol and sterols are metabolized by cytochrome P-450 (CYP) family members CYP4A14, CYP7A1 and CYP27A1 respectively. Although the activity of several CYPs is regulated by cytokines such as IL-1β and TNF-α[24-26], none of the CYPs mentioned above had a different gene expression in the IL-10 treated group (data not shown). IL-10 also did not affect the gene expression of acyl-CoA:cholesterol acyltransferase (ACAT) 1 and 2, which esterify excess intracellular cholesterol (data not shown). In contrast, the gene expression of 3-hydroxy-3-methyglutaryl (HMG)-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis, was 2.2-fold higher in the Ad.hIL-10 treated group (**Fig. 4A**). Furthermore, the gene expression of phospholipid transfer protein (PLTP), an enzyme involved in remodeling of lipoproteins, was 3.10-fold increased (**Fig. 4B**) and the gene expression of carnitine octanoyltransferase (CrOT), which is involved in peroxisomal fatty acid oxidation, was 2.86-fold decreased (**Fig. 4C**). Adiponutrin is a recently identified enzyme that may play a role in both lipogenesis and lipolysis. Adiponutrin gene expression is positively correlated with feeding status in several studies and is associated with obesity. Although Ad.hIL-10 treatment resulted in lower serum cholesterol levels, adiponutrin gene expression in liver parenchymal cells was 6.32-fold higher (**Fig. 4D**).

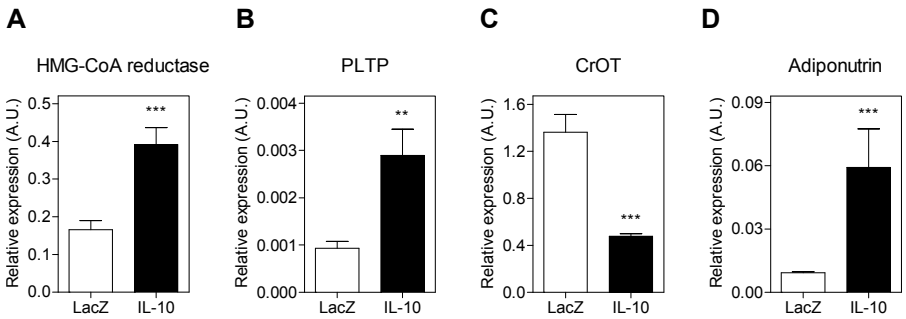

Fig. 4: Effect of IL-10 on HMG-CoA reductase, PLTP, CrOT and adiponutrin. LDLr deficient mice were fed a Western-type diet for four weeks and were inoculated with Ad.LacZ or Ad.hIL-10 at day 14. At day 28, liver parenchymal cells were isolated and RNA of these cells was isolated. Quantitative real-time PCR analysis was used to determine the mRNA expression of **A** HMG-CoA reductase, **B** PLTP, **C** CrOT and **D** adiponutrin. Values are expressed as mean ± SEM of relative mRNA expression compared to housekeeping gene expression (arbitrary units). Significant differences between Ad.LacZ and Ad.hIL-10 treatment: ** $p < 0.01$ and *** $p < 0.001$.

IL-10 also affected the expression of genes not directly related to lipid homeostasis. Lecithin:retinol acyltransferase (LRAT), which esterifies retinol for storage, was 1.67-fold lower expressed (**Fig. 5A**), while Ad.hIL-10 treatment resulted in a 2.28-fold higher gene expression of calmodulin 3, a highly versatile and ubiquitously expressed Ca2+ sensor that regulates the function of many enzymes and ion channels (**Fig. 5B**). In addition, gene expression of C10 and monocyte chemoattractant protein (MCP)-2, two CC chemokines involved in leukocyte recruitment, was higher after Ad.hIL-10 treatment (2.17-fold and 5.05-fold respectively, **Fig. 5C/D**). Interestingly,

Ad.hIL-10 treatment resulted in a 7.36-fold increase in the gene expression of hemoglobin alpha, adult chain 1 (Hba-a1) which is involved in erythrocyte oxygen transport (**Fig. 5E**).

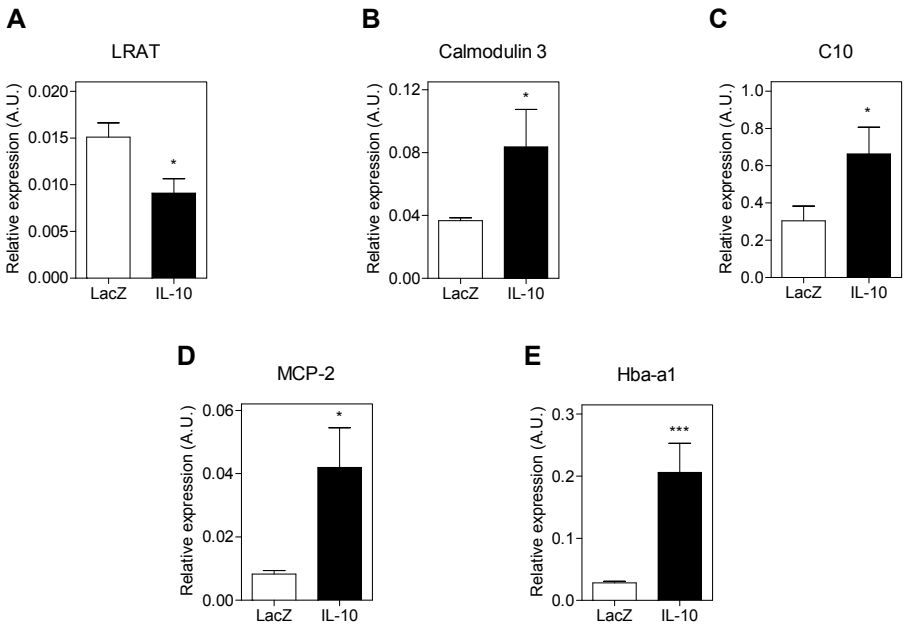

Fig. 5: Effect of IL-10 on LRAT, calmodulin 3, C10, MCP-2 and Hba-a1. LDLr deficient mice were fed a Western-type diet for four weeks and were inoculated with Ad.LacZ or Ad.hIL-10 at day 14. At day 28, liver parenchymal cells were isolated and RNA of these cells was isolated. Quantitative real-time PCR analysis was used to determine the mRNA expression of **A** LRAT, **B** Calmodulin 3, **C** C10, **D** MCP-2 and **E** Hba-a1. Values are expressed as mean±SEM of relative mRNA expression compared to housekeeping gene expression (arbitrary units). Significant differences between Ad.LacZ and Ad.hIL-10 treatment: * $p<0.05$ and *** $p<0.001$.

DISCUSSION

The role of inflammation in atherosclerosis is well established. The cytokine IL-10 protects against the development of atherosclerosis in several mouse models[9-13] and polymorphisms in the IL-10 gene were associated with a different prevalence of atherosclerosis in hemodialysis patients[27]. In addition to the inhibitory effect of IL-10 on adhesion molecules and inflammation[10,14], several animal studies showed lower serum cholesterol levels in IL-10 treated groups[9-11]. However, the mechanism of this effect of IL-10 on serum cholesterol levels has not been elucidated. In our study, we confirm that mice treated with Ad.hIL-10 had significantly lower serum cholesterol levels than the mice treated with Ad.LacZ. VLDL, HDL and especially LDL serum levels were reduced by Ad.hIL-10 treatment. To unravel the mechanism of the effect of enhanced IL-10 serum levels on cholesterol levels we performed microarray analysis on liver parenchymal cells. Liver parenchymal cells are the predominant cells in the liver and they are responsible for the metabolic function of the liver, because they are able

to synthesize and secrete VLDL and HDL and clear excess cholesterol from the blood by secreting it into the bile[15-17].

Known target genes of IL-10, such as TNF-α, IL-6 and HO-1[28-30], were clearly affected in liver parenchymal cells by Ad.hIL-10 treatment, indicating that in addition to many other cell types, parenchymal cells are target cells for IL-10. This is in agreement with a study of Santiago-Lomeli *et al* in which IL-10 affects TNF-α and cycloheximide induced apoptosis in human HepG2 hepatoblastoma cells and glutathion depletion and ethanol induced apoptosis in rat hepatocytes[31]. The mRNA expression of CC chemokine C10 (mouse homologue of CCL6) is found to be regulated by IL-10 in bone marrow derived macrophages[32]. Although C10 expression in parenchymal cells was never reported before, we did observe low expression levels in the control group and the expression was increased in the Ad.hIL-10 treated group (2.17-fold, $p<0.05$). In addition, gene expression of another CC chemokine, MCP-2 (CCL8), was also increased by Ad.hIL-10 treatment. It is known that MCP-2 is involved in the recruitment of leukocytes[33], but its function in the liver is not yet understood. Further research is needed to investigate whether upregulation of these chemokines in liver parenchymal cells leads to an influx of leukocytes in the liver.

Analysis of the differentially expressed genes using PANTHER revealed that biological processes involved in immunity, sulfur (redox) metabolism, vitamin/cofactor transport and porphyrin metabolism were highly affected by Ad.hIL-10 treatment. Interestingly, Ad.hIL-10 treatment also affected more genes categorized in lipid and fatty acid binding and in lipid, fatty acid and steroid metabolism than statistically expected. The differentially expressed genes were validated by quantitative real-time PCR.

Yoshioka *et al* suggest that IL-10 may have direct effects on cholesterol metabolism through the HMG-CoA reductase pathway[11]. They observed that IL-10 treatment of human HepG2 hepatoblastoma cells resulted in a decreased expression of HMG-CoA reductase. In contrast, Ad.hIL-10 treatment led to a significant 2.4-fold increase in HMG-CoA reductase gene expression in our study. Moreover, in earlier studies, we did not observe any effect of IL-10 on the *in vivo* production of VLDL[9]. Therefore, we suggest that the effect of IL-10 on serum lipoprotein levels is not due to an inhibition of cholesterol biosynthesis. The discrepancy between the results from our study and the study from Toshioka *et al* can be explained by the different cell types used: *in vitro* human hepatocyte cell line in the absence of lipoproteins vs. *in vivo* mouse liver parenchymal cells with enhanced serum cholesterol levels.

In addition, it is unlikely that IL-10 exerts its serum cholesterol lowering effect by inducing lipoprotein uptake by parenchymal cells, because the genes that are primarily involved in lipoprotein uptake in the liver were not upregulated upon Ad.hIL-10 treatment. In fact, LRP-1 gene expression was not affected by IL-10 and the gene expression of both SR-BI (1.51-fold) and CD36 (1.89-fold) was even significantly decreased.

Cholesterol efflux pumps ABCA1 and ABCG1 were not significantly affected by IL-10, but IL-10 did increase the gene expression of ABCG5 and ABCG8. These members of the ABC transporter family promote biliary cholesterol excretion[34], suggesting a role for IL-10 in the regulation of the elimination of cholesterol from the body via bile. Cholesterol secretion leads

to lower intracellular cholesterol concentrations to which the cell responds by enhancing cholesterol synthesis via, for instance, upregulation of HMG-CoA reductase. Upon Ad.hIL-10 treatment, we observed a higher expression of HMG-CoA reductase in liver parenchymal cells, suggesting that these cells have lower intracellular cholesterol levels possibly due to a higher cholesterol secretion. Transgenic LDLr deficient mice expressing human ABCG5 and ABCG8 on a Western-type diet had lower serum cholesterol levels (VLDL and LDL) and the atherosclerotic lesion area in these mice were significantly smaller[35] which is in agreement with the effect of Ad.hIL-10 in our studies. Interestingly, Khovindhunkit *et al* showed that intraperitoneal injection of lipopolysaccharide (LPS) downregulates ABCG5 and ABCG8 in mouse liver[36]. LPS activates monocytes and macrophages to produce cytokines such as TNF-α and IL-6, which, in turn, serve as endogenous mediators of inflammation through interactions with various target cells[37]. We showed that in liver parenchymal cells TNF-α and IL-6 are downregulated, which might explain the coinciding upregulation of ABCG5 and ABCG8 upon Ad.hIL-10 treatment.

The transcription of a variety of genes involved in lipid metabolism such as the genes mentioned before, is regulated by PPARs, LXR and SREBPs[38,39]. However, PPARα, PPARγ, PPARδ, LXRα, LXRβ and SREBP-1 gene expression levels were not affected by IL-10. Apparently, the moderate 1.39-fold decrease of SREBP-2 expression observed upon Ad.hIL-10 treatment was not sufficient to result in a reduction of HMG-CoA reductase, a target gene of SREBP-2[40], as the expression of HMG-CoA reductase was increased by IL-10.

Members of the CYP family such as CYP4A14, CYP7A1 and CYP27A1 metabolize intracellular fatty acids, cholesterol and sterols, respectively. Although the activity of several CYPs is known to be regulated by cytokines such as TNF-α and IL-1β[24-26], none of the CYPs mentioned were affected by Ad.hIL-10 treatment. Moreover, IL-10 also did not affect the gene expression of ACAT1 and ACAT2, which esterify excess intracellular cholesterol for storage, suggesting that intracellular cholesterol storage and metabolism do not contribute to the effect of IL-10 on serum cholesterol levels. On the other hand, gene expression of CrOT, which is involved in peroxisomal fatty acid oxidation[41], was downregulated by IL-10. Alterations in hepatic fatty acid oxidation modify the availability of fatty acids for VLDL synthesis, but in earlier studies IL-10 did not affect the synthesis of VLDL[9], indicating that the effect on CrOT gene expression alone is not sufficient to induce differences in VLDL secretion.

PLTP promotes the transfer of phospholipids between lipoproteins and modulates HDL size and composition, thereby having a crucial role in HDL metabolism. PLTP overexpression is shown to increase susceptibility to atherosclerosis by lowering HDL levels[42,43]. Consistently, Ad.hIL-10 treatment resulted in a higher gene expression of PLTP in liver parenchymal cells and a decrease in serum HDL levels. In contrast to PLTP overexpression, high levels of IL-10 also reduced serum levels of VLDL and especially LDL, which may explain why IL-10 treatment reduces and overexpression of PLTP induces atherosclerosis development. Furthermore, elevation of PLTP activity is known to result in rapid disposal of cholesterol from the body via increased conversion into bile acids and

subsequent excretion[44], which is promoted by ABCG5 and ABCG8. Further research is necessary to investigate whether IL-10 influences removal of cholesterol from the body in a PLTP and/or an ABCG5 and ABCG8 mediated manner.

Interestingly, expression of the recently discovered gene adiponutrin was highly upregulated by Ad.hIL-10 treatment. The function of adiponutrin in the liver is not fully understood yet. However, two polymorphisms in the adiponutrin gene are associated with altered regulation of adiponutrin mRNA expression and with obesity in humans[45]. Under normal conditions this gene is hardly detectable in liver, but it is upregulated upon high calorie intake[46]. Although the mice treated with IL-10 had lower serum cholesterol levels, we did observe an even higher gene expression of adiponutrin upon Ad.hIL-10 treatment. Apart from nutritional status, little is known about the regulation of adiponutrin gene expression. The results from this study suggest that IL-10 might play a regulatory role.

Many genes involved in the homeostasis of serum lipoprotein levels were affected by Ad.hIL-10 treatment in our study. No conclusive pattern could be observed that completely explains the serum cholesterol lowering effect of IL-10. However, the observed combined increase in ABCG5 and ABCG8, potentially leading to increased cholesterol secretion into the bile, forms the most likely connection, as cholesterol secretion is closely linked to serum cholesterol levels.

The downregulation of LRAT in liver parenchymal cells by Ad.hIL-10 points in the direction of a lower absorption of cholesterol in the intestine. Liver LRAT is expressed dose-dependently across a wide range of dietary retinol in long-term feeding studies[47]. Dietary retinol, like cholesterol, is absorbed in the intestines via chylomicrons. The downregulation of liver LRAT expression indicates an impaired retinol uptake via chylomicrons, which, in turn, may suggest a lower cholesterol uptake via chylomicrons in the intestines.

Lately, the possible role of IL-10 in insulin resistance became of interest. Variants of the IL-10 promoter gene have been associated with obesity and insulin resistance[48]. Furthermore, a positive correlation between IL-10 levels and whole-body insulin action was observed in a population study with healthy subjects[49]. However, Ad.hIL-10 treatment did not affect fasted insulin nor glucose levels in our earlier experiment[9]. In agreement, a recent study showed that during high fat feeding, IL-10 deficiency altered hepatic lipid metabolism, but did not improve insulin sensitivity[50].

We observed an increase in Hba-a1 gene expression in liver parenchymal cells upon Ad.hIL-10 treatment, especially in the mice with the highest IL-10 levels. A study in patients suffering from Crohn's disease showed that IL-10 treatment causes a dose-dependent, asymptomatic and reversible anemia with corresponding lower hemoglobin levels[51]. Ad.hIL-10 treatment in our study may also have led to lower hemoglobin levels to which the liver parenchymal cells responded by synthesizing more Hba-a1. In addition, HO-1, a well-known target gene of IL-10, is involved in the degradation of heme. As reviewed by Atac *et al*, several studies show hypocholesterolemia in patients suffering from anemia[52], indicating that IL-10 might influence serum cholesterol indirectly via inducing anemia. Also other processes like

calcium signaling might be involved, because Ad.hIL-10 treatment induced the expression of calmodulin 3.
In conclusion, Ad.hIL-10 caused lower cholesterol, in particular LDL, levels in serum of LDLr deficient mice on a Western-type diet. Microarray analysis of liver parenchymal cells revealed several new IL-10 target genes, which may influence lipid metabolism directly or indirectly. Interestingly, Ad.hIL-10 affected more genes involved in lipid metabolism than anticipated from statistical expectations. The observed combined increase in ABCG5 and ABCG8, potentially coupled to increased cholesterol secretion to the bile, might explain the cholesterol lowering effect of Ad.hIL-10 in LDLr deficient mice on a Western-type diet.

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