Adoptive transfer of ApoB100 specific T cells in atherosclerosis

Ali Ismail

Degree project in biology, Master of science (2 years), 2013
Examensarbete i biologi 45 hp till masterexamen, 2013
Biology Education Centre, Uppsala University, and Center for Molecular Medicine (CMM), Karolinska Institutet, Stiftelsen CMM, Karolinska Universitetssjukhuset, Solna L8:01 171 76 Stockholm
Supervisors: Daniel Ketelhuth and Anton Gistera
Abstract

Atherosclerosis, the major process leading to cardiovascular diseases, is responsible for 75 to 80% of deaths around the world. Recent research has mapped fundamental stages of atherosclerotic plaque progression. However, prevention and treatment against this life threatening disease remains a challenge for a large part of the population. Atherosclerosis is a chronic inflammatory disease initiated by retention and accumulation of lipoproteins in the artery wall, in special Low-density lipoprotein that contains apolipoprotein B100 (ApoB100), leading to maladaptive immune responses of macrophages and T cells.

The aim of this study was to investigate antigen specific effects of T cells on inflammation and atherosclerosis. Further, using in vitro assays, we attempt to identify potential epitopes recognized by TRVB31 T cells.

Mice that spontaneously develop atherosclerosis were injected with two million ApoB100 reactive T cells from a TRVB31-TCR transgenic mouse (Tg) or two million T cells from wild type mice (WT). A third group of untreated animals was also used as a control (Un). Contrary to initial hypothesis that the adoptive transfer of ApoB100 specific T cells would accelerate disease, the transfer of TRVB31+ Tg T cells induced a reduction in atherosclerosis compared to WT and Un groups. Both WT and Tg mice presented increased macrophage (CD68) and CD4+ T cells infiltration in the plaque. We observed that the correlation between macrophage staining and percentage plaque differ between WT and Tg groups, indicating injection of ApoB100 specific T cells may lead different macrophages responses in the plaque. Results suggests an up regulation of TGF-β in the aorta and spleen for Tg and WT. The absence of differences between the groups on levels of blood cholesterol, triglycerides indicate the effects of the adoptive transfer on atherosclerosis progression may be due to direct effects of T cell derived responses. Indeed, protective mechanisms operating on Tg injected animals may involve Tregs and/or different macrophage repertoires.

We conclude that CD3+ TRVB31 transfer reduces atherosclerosis and that the biological mechanisms may involve the generation of antigen specific Tregs and/or different macrophage subsets. The limited data set from this experiment suggest that further investigation is needed to strengthen this conclusion.

Acknowledgment

I would like to express my heartfelt gratitude and thanks to my supervisors Daniel F.J Ketelhuth and Anton Gisterå, for their never ending support throughout this project. I also would like to thank Professor Göran K Hansson for his inspiration and for his help to initiate this project; Ingrid Törnberg, Anneli Olsson and Linda Haglund for their technical assistance during my experiments; Emilia Johansson for her ability to motivate me and cheer me up. And to all other members in the research group at CMM, I am grateful to have had the opportunity to work with you.
**Abbreviations**

APC – Antigen presenting cell  
Apo B100 – Apolipoprotein B100  
BCR - B cell receptors  
Breg - Regulatory B cells  
ConA- Concavalin A  
CVD – Cardiovascular disease  
DC – Dendritic cell  
EC – Endothelial cell  
ELISA – Enzyme linked immunosorbent assay  
Forkhead box P3 - FoxP3  
HDL – High-density lipoprotein  
hsCRP - High-sensitivity C-reactive Protein  
ICAM-1 - Intercellular Adhesion Molecule 1  
IHC - Immunohistochemistry  
IL- 4 – Interleukin-4  
IL-1 - Interlukin -1  
IL-10 – Interleukin-10  
IL-13 –Interleukin-13  
IL-2 – Interleukin-2  
IL-6 –Interleukin-6  
INF-γ – Interferon –γ  
LDL – Low-density lipoprotein  
Macrophage colony-stimulating factor - M-CSF  
MDA – Malondialdehyde  
MHC Class II - Major histocompatibility complex class II  
MI – Myocardial infarction  
MMP – Matrix metalloproteinase  
OD – Optical density  
oxLDL – Oxidised LDL  
SMC – Smooth muscle cell  
SR-A - Scavenger receptor-A  
TCR - T cell receptors  
Tg – Transgenic  
TGF-β – Transforming growth factor –β  
Th1 - T helper cell 1  
Th2 - T helper cell 2  
TLR – Toll-like receptor  
TNF-α - Tumor necrosis factor-alpha  
Treg - Regulatory T cells  
TRVB31 - T cell receptor variable β chain 31  
Un – Untreated  
VCAM-1 - Vascular cell adhesion molecule 1  
WT – Wild Type
# Table of Contents

Introduction ............................................................................................................................................. 4  
Background to atherosclerosis ............................................................................................................. 4  
Immunity in atherosclerosis ............................................................................................................ 4  
Immunomodulation of atherosclerosis ............................................................................................ 7  
Aims .................................................................................................................................................... 7  
Methodology ........................................................................................................................................... 8  
Animals and experimental design ....................................................................................................... 8  
Lesion and immunohistochemistry analysis ........................................................................................ 9  
RNA extraction and cDNA synthesis .................................................................................................. 9  
Serum cholesterol and triglycerides .................................................................................................. 10  
Real-Time Polymerase Chain Reaction ............................................................................................. 10  
Oxidized low density lipoprotein preparation .................................................................................... 10  
Preparation of soluble ApoB100 ......................................................................................................... 10  
Plasma titers of anti-ApoB, anti-oxLDL and anti-TCR antibodies ................................................... 11  
MHC II epitope mapping .................................................................................................................. 11  
Statistical Analysis ............................................................................................................................ 12  
Results ................................................................................................................................................... 13  
Transfer of ApoB100 reactive T cells reduce the progression of atherosclerotic lesions in hypercholesterolemic mice ................................................................. 13  
Transfer of ApoB100 reactive TRVB31 T cells induce immunity in the vessel wall ....................... 14  
TRVB31+ T cells may reach the vessel wall ...................................................................................... 15  
Plasma lipids and body weight .......................................................................................................... 17  
Antibodies against ApoB100 and oxLDL ......................................................................................... 17  
Transfer of ApoB100 reactive T cells does not induce anti-TCR TRVB31 antibodies ............... 18  
Correlation analysis ........................................................................................................................... 18  
Discussion ............................................................................................................................................. 20  
References ............................................................................................................................................. 22  

Appendix 1
Introduction

Cardiovascular disease are responsible for over four million deaths in Europe each year as well as 30% of annual deaths globally. Added together, CVDs cause the death of approximately 17.1 million people every year [1]. Approximately 75-80% of deaths caused by CVDs are due to atherosclerosis. Moreover, CVDs have become the main cause of death among women (52%) in Europe. Financially, CVDs cost the European Union around 196 billion € a year, a figure indicating that CVDs have become one of Europe's most serious health problems [2]. According to the World Health Organization, annual world deaths caused by CVDs are likely to reach 23.6 million by 2030.

Atherosclerotic lesions are asymmetric focal thickenings of the inner layer of the artery wall, the intima. The thickening is caused by an accumulation of lipid-laden cells beneath the endothelium. In the center of an atheroma, foam cells and extracellular lipid droplets form the core region of the atherosclerotic plaque. The core is surrounded by a cap of smooth-muscle cells and a collagen-rich matrix that keeps the plaque from rupturing (Figure 1) [3]. The accumulation of dead cells, foam cells and lipid droplets expand over time and prevents blood flow through the coronary artery. This can lead to either a myocardial infarction or a thrombosis that disrupts the blood supply to the brain.

The major risk factors for atherosclerosis are smoking, high blood pressure, physical inactivity, diabetes, obesity and an unhealthy diet [4]. Direct detection of atherosclerosis is seldom undertaken unless clinical symptoms are apparent. Instead, a cardiovascular risk assessment is done in the clinic [5], and usually involves a blood sample and the detection of plasma markers, i.e. low-density lipoprotein (LDL), high-density lipoprotein (HDL) and high-sensitivity C-reactive protein (hsCRP). Moreover, blood pressure, smoking, gender and age is taken into account in the cardiovascular risk assessment.

Background to atherosclerosis

Immunity in atherosclerosis

In 1904, Felix Marchand created the expression atherosclerosis [6] and in 1913 Nikolai N. Anichkov found the link between cholesterol and swelling in the artery walls [7]. The seminal experiments of Anichkov, involving rabbits fed a non-vegetable diet, introduced a new trend in the studies of CVD. Since these findings, researchers have slowly acknowledged that atherosclerosis is a complex chronic inflammatory disease. Although there is knowledge about the fundamental stages of plaque development, the early stage of plaque formation is still puzzling.
The initial stages of atherosclerosis can be described as a process where LDL migrate from the blood through the monolayer of endothelial cells and in to the intima of the artery (Figure 1), where they can get trapped due to interactions with extracellular matrix protein [8]. Thereafter, the trapped LDL becomes oxidized (oxLDL) through a process of oxygen and enzymatic reactions [9]. The newly modified oxLDL has biological activities that can trigger the expression of leukocyte adhesion molecules, VCAM-1 and ICAM-1 on the endothelial surface [10, 11].

**Figure 1: The atherosclerotic process.** The zoom in box (to the left) depicts LDL particles that have migrated into the intima, through the monolayer of endothelial cells. Here we see the initial response from oxidized LDL particles that triggers an inflammatory response with expression of adhesion molecules. Monocytes transform into macrophages and start to gorge oxLDL particles. T cells, macrophages and mast cells start to produce pro-inflammatory mediators and enzymes. The zoomed out box (to the right) depicts the plaque with its fibrous cap, which consist of a monolayer of endothelium cells, smooth muscle cells (SMC) and fibrous collagen. The accumulated plasma lipoproteins (which build up a core of lipids), cholesterol, and both living and dead cells make up the sub endothelial region in the center of the intima. Picture by Göran K Hansson [12].

Once VCAM-1 has been expressed, monocytes and T cells are recruited through locally produced chemokines [13-16]. The newly recruited monocytes differentiate into macrophages and up regulate their scavenger receptors, i.e. Scavenger receptor-A (SR-A) and CD36. Thereafter, the macrophages start taken up oxLDL particles [17-19] to become macrophages overloaded cholesterol, the latter which are called foam cells. Hence, the foam cells accumulate in the intima and will undergo apoptosis or necrosis [20] causing the initial creation of a lipid-rich necrotic core in the plaque. Further, as a defense mechanism to limit this unwanted process, a fibrous cap, consisting smooth muscle cells (SMC), forms and upholds the integrity of the vessel wall [21], by production of fibrillar collagen and elastin. OxLDL may also interact with Toll Like Receptors (TLRs) expressed on macrophages leading to their activation, and release of pro-inflammatory cytokines like TNF-α, IL-1 and IL-6. The TLRs on macrophages may also stimulate the release of matrix-degrading matrix metalloproteinases (MMPs), which has a role in weakening the fibrous cap.
LDL and its main component, apolipoprotein B100 (ApoB100), (Figure 2) are abundant in macrophages in the plaque and will be presented to CD4+ T-helper cells via the MHC Class II pathway. Once the peptides have been presented to T-helper cells, especially Th1 cells, pro-inflammatory mediators like IFN-γ and TNF-α are released [16, 18, 22, 23]. These cytokines inhibit the production of fibrillar collagen and elastin by SMCs, and increase the production of extracellular matrix-degrading proteins. Such a process accelerates up the destabilization of the fibrous cap and increases the risk for the plaque to rupture, which can lead to a thrombotic events [24], one of the major events leading to CVD clinical symptoms.

**Figure 2: Low-density lipoprotein (LDL).** The LDL particle is approximately 22nm in size and has a core of hydrophobic cholesterol esters and triglycerides, which are surrounded by hydrophilic unesterified cholesterol and phospholipids. The particle has a single copy of ApoB100, a glycoprotein consisting of 4536 amino acid residues and with a total weight of 550 kDa [25]. ApoB100 girds around the whole particle and acts as homing molecule that facilitates harvesting of lipoproteins by receptor mediated endocytosis [26]. Picture by Mark Sisson [27].

As mentioned, the adaptive immunity is activated when precise peptides derived from LDL or other antigens are identified by receptors on the surface of lymphocytes, such as T cell receptors (TCR) and B cell receptors (BCR). In the adaptive immunity B cells have an important role in regulating the adaptive immunity. They produce antibodies and can modulate the immune system through cytokines and antigen presentation [16]. Studies suggest the existence of B regulatory cells (Breg) that produce interleukin-10 (IL-10), an anti-inflammatory cytokine that can support regulatory T cell (Treg) differentiation, the latter playing a major role in immune-regulation and anti-atherosclerotic responses [28]. Other studies point out that plaque development is partly impaired through the secretion of IL-4, IL-5, and IL-10 from Th2 cells [29, 30]. However, whether Th2 cells play a major role in atherosclerotic plaques has been debated. It is known that cytokines like IL-4 promote Th2 cell
differentiation through the induction of Gata-3 transcription factor, which in turn leads to an up regulation of IL-4 and IL-5. The latter two will down regulate the pro-inflammatory cytokine INF-γ from Th1 cells [31]. To what extent this mechanism operates in the plaque is unclear.

**Immunomodulation of atherosclerosis**

Atherosclerosis is a chronic inflammatory disease regulated through the innate and the adaptive immune systems [32]. Immune-modulation is a promising area of research when developing methods to counteract the development of the disease. Experimental studies show that modulation of the inflammatory response can decrease lesion size in hypercholesterolemic animals. Current research has shown that, plaque sizes in hypercholesterolemic mice have been reduced by injections of ApoB100-loaded dendritic cells (DC) made tolerogenic with IL-10 stimulation. The effect of the pulsed DCs led to an increase in FoxP3+ Tregs, which counteracted the response of the pro-inflammatory T cells [33]. An inhibition of T cell response to LDL has also been proven effective through immunization with TRVB31 (T cell receptor variable β chain 31) peptides in hypercholesterolemic mice [34]. Other noteworthy studies using hypercholesterolemic animals have confirmed a reduction in plaque development through immunomodulation with β2-glycoprotein-1b (Apo-H) and heat-shock protein 60/65 [35, 36]. Interestingly, another study, conducted in 2006, showed that patients with rheumatoid arthritis who were treated with anti-TNF-α for six months had a lower probability of myocardial infarction than the control group that received no anti-TNF-α [37]. Hence, modulation of immune response is an attractive approach to suppress the effect of the chronic inflammatory response of atherosclerotic development.

**Aims**

The aim of this project was to investigate T cell dependent effects on inflammation and atherosclerosis. Based on previous findings that mice treated with CD4+ T cells have accelerated atherosclerosis [38], we hypothesize that hypercholesterolemic mice transferred with CD3+ transgenic T cells recognizing ApoB100, T cells carrying TRVB31-TCR, will show effects in inflammation and plaque development. Further, we aim in this project to investigate potential epitopes activating TRVB31+ T cells.
Methodology

Animals and experimental design

All animal experiments were approved by the local ethical committee, Stockholms Norra djurförsöksetiska nämnd. Eighteen week old male mice, expressing human ApoB100 as a transgene and lacking the gene for low density lipoprotein (LDL) receptor, (huApoB100tg x Ldlr-/-) were used in this experiment. All animals used are on C57Bl/J background, backcrossed for ten generations or derived in a fashion with such a background. The mice were divided in to three groups. The first group (Tg) was injected with two million CD3+ T cells originated from a TCR transgenic mouse (a CD2-Promoter driving TCR48:33 expressions), they were five in total. The TCR transgenic mice carry a transgene with a specific variant of the T cell receptor TRV4B31, and around 80% of the CD4+ T cells (see appendix 1) express this specific receptor. The receptor is auto-reactive to human ApoB100, the protein part of LDL. The second WT group that were six in total were injected with two million CD3+ T cells originated from wild type mice (regular C57-B6/J). The wild type mice have a normal repertoire of T cells. The goal of this project is to find out whether this ApoB100 reactive T cell receptor impacts atherosclerosis development and inflammation. Atherosclerosis development was investigated eight weeks after the treatment through microscopic evaluation of plaque size. Inflammation was assessed at the same time-point through immunohistological chemistry stainings in plaques and gene expression. The latter was covered with cytokines such as, INF-γ, IL-10, and TGF-β, and transcription factors such asTbet (Th1), GATA3 (Th2), FoxP3 (Treg), and RORγt (Th17). The last six mice were the Un group they were left as an untreated control group. In total seventeen huApoB100tg x Ldlr-/- were used in this experiment.

The huApoB100tg x Ldlr-/- mice only expresses human ApoB100 due to a mutation from CAA to CTA in codon 2153 that effectively obstructs the formation of Apo48 [39]. Therefore, at birth the huApoB100tg x Ldlr-/- mice have significantly higher levels of LDL cholesterol and human ApoB100 than normal mice [40]. For that reason huApoB100tg x Ldlr-/- mice tend to develop atherosclerotic plaques more easily than regular Ldlr-/- mice [41, 42].

The mice were fed with a standard chow diet. Eight weeks after the injection of CD3+ T cells, the mice were euthanized under CO2 anesthesia. Blood from each mouse was collected through cardiac puncture in heparinized tubes and then plasma was separated by centrifugation (1500g, 15 minutes) and stored at -80°C. Mice were then perfused with sterile PBS before organ harvesting. The abdominal aorta, the spleen and the lymph nodes were harvested and snap frozen for RNA analysis. The hearts
and the aortic root were collected and preserved for cryo-sectioning, and later lesion size and immunohistochemistry analysis.

**Lesion and immunohistochemistry analysis**

The aorta was treated with 4% formaldehyde for preservation and then dissected and pinned onto black wax plates. Directly afterwards the pinned samples were stained with Sudan IV (Merck AG, Darmstadt) for an en face staining of the lipids, triglycerides and the lipoproteins in the tissue, all according to previous studies [43]. Pictures were then taken with DC480 camera with a MZ6 stereo microscope (Leica) and then analyzed with the software ImageJ (NIH). The total area of all plaques in the aortic arch (branching vessels were excluded) were calculated as a percentage of the total surface area in the arch.

The hearts with the aortic roots facing upward were embedded in OCT for cryosectioning. Cryosections of 10 μm thickness was collected for the beginning 1 mm of the aortic root and then placed onto microscope slides. Slides for IHC were acetone fixed, while the slides for Oil Red O (ORO) were fixated in 10 % formalin.

The primary monoclonal antibodies used for IHC were all obtained from BD-PharMingen: CD68 (macrophage marker), CD4 (Th cells) and I-A^b_ (MHC-II). For detection an ABC peroxidase kit (Vector Laboratories) was used. Avidin/Biotin Blocking Kit (SP-2001, Vector Laboratories) were used to block endogenous biotin and reduce background. The IHC slides were analyzed with a DM-LB2 microscope (Leica) equipped with a 20×/0.9 objective and a DC300 camera (Leica). The formalin slides were treated with ORO and hematoxylin, according to prior studies [44]. The slides were then mounted with glass cover sheets and pertex. Pictures of the slides were then taken with the DM-LB2 for analyzing, where the surface areas of the lesion and of the entire vessel were measured with the software Qwin (Leica).

**RNA extraction and cDNA synthesis**

The RNA was extracted from the aorta and lymph nodes using AllPrep DNA/RNA/protein Kit (Qiagen). The protein and the DNA from the aorta and the spleen were saved but not used in this study. The RNA from the spleen was extracted with RNeasy columns (Qiagen) and the use of a QiaCube (Qiagen) (QIAGEN RNeasy handbook). All RNA samples were tested on the NanoDrop 1000 for quantification before complementary DNA synthesis.
The cDNA was synthesized using the SuperScript® II Reverse Transcriptase (Invitrogen) from 1 µg RNA. All procedures were carried out in accordance with the handbooks complementing each kit.

**Serum cholesterol and triglycerides**

Total plasma cholesterol and plasma triglycerides were quantified with enzymatic-colorimetric assays, Randox Cholesterol and Triglycerides kits (Randox).

**Real-Time Polymerase Chain Reaction**

The polymerase chain reaction was performed on an Applied Biosystems 7900HT Fast Real-Time PCR System using TaqMan® Fast Universal PCR Master Mix (Invitrogen). All the following assay on demands were bought from Applied Biosystems: TGF-β (mm00441726_m1), INF-γ (mm0116813_m1), GATA3 (mm00484683_m1), RORγT (mm0041144_g1), Tbx21/Tbet (mm00450960_m1), FoxP3 (mm00475156_m1), IL-10 (mm01288386_m1). HPRT (mm00446968) was used to normalize the sample amplifications.

The collected data from was analyzed with the softwares SDS 2.3 and Microsoft Excel. The $2^{-\Delta \Delta CT}$ formula was implemented for messenger RNA level comparisons. $\Delta \Delta CT = \Delta CT$ (sample) – $\Delta CT$ (calibrator = mean CT values of all samples within each group), and $\Delta CT$ is the CT of the housekeeping gene (HPRT) that is subtracted from the CT of the target gene.

**Oxidized low density lipoprotein preparation**

The LDL was obtained by means of ultracentrifugation from pooled plasma of healthy donors. The method was follow as previously described by Richard J. Havel [45]. The concentration of obtained LDL was measured with a Bradford assay (Bio-Rad Laboratories) and the use of a microplate reader (VersaMax; MDS Analytical Devices) at 592 nm. The modification of LDL was carried out by incubating 1 ml of LDL with 20 µM CuSO4 for 18 hour at 37°C.

**Preparation of soluble ApoB100**

ApoB100 was isolated by using a modification of previous methods [46, 47]. With 0.1 ml of LDL (1 mg/ml) 0.4 ml methanol, 0.1 ml chloroform, and 0.3 ml water were added. The whole solution was then vortexed and put at centrifugation for 1 min at 9,000 g. This left the solution in three phases,
whereas the upper phase was removed and replaced with 0.3 ml of methanol. The whole solution was then again centrifuged at 9,000 g for 2 min to produce a pellet. The remaining liquids were tipped off and the pellet was resuspended in a minimum volume of 10% SDS (Bio-Rad Laboratories). To remove excess SDS the soluble proteins were filtered through a PD-10 column (GE Healthcare). The solution was then further purified through a Superdex-200 size-exclusion column (0.5 ml/min, in Tris-HCl, pH 7.4). The concentration of the final isolate of ApoB100 protein was measured with a Bradford assay (Bio-Rad Laboratories) on a microplate reader (VersaMax; MDS Analytical Devices) at 592 nm.

**Plasma titers of anti-ApoB, anti-oxLDL and anti-TCR antibodies**

The concentration of antibodies against ApoB100, oxLDL, and TCR-derived peptides were evaluated by enzyme-linked immunosorbent assay (ELISA). ApoB100 and oxLDL was prepared as described above. A coaster 96 well plate (Corning, USA) was coated with 30μl ApoB100 or oxLDL at 4°C overnight. The plates were then washed two times on a Wellwash4 (Denley) with PBS and blocked with 1 % gelatin in PBS for one hour at room temperature. Plates were washed three times with PBS-Tween and incubated with standards and with different dilutions of mouse plasma diluted in TBS-Gelatin 0,1 % (1:15) for two hours at room temperature. After incubation the plates were washed three times with PBS-Tween. After the wash the plates were incubated for 1 hour with biotinylated antibody horse anti-mouse IgG (BD). The plate was then again subjected to a three times of wash with PBS-Tween. The samples were then treated with streptavidin-HRP for 30 minutes, washed again three times and then developed with TMB (BD). After the samples had reached a satisfying color shift of the standards the TMB reactions were stopped with 10 % H2SO4. The absorbance was measured at 450 nm using a micro plate reader (VersaMax; MDS Analytical Devices).

**MHC II epitope mapping**

An epitope mapping of the ApoB100 derived epitope was carried out in parallel to the in vivo project. Specifically, we aimed to identify the exact peptide sequence that antigen presenting cells process and present through the MHC-II to T cells. To assist us in this project we used a T cell hybridoma cell, named 48-5, which expresses the same TCR as the one found on TCR transgenic mice (TRVB31), that has specificity towards human ApoB100 [48]. The T cell hybridomas 48-5 cells were created through fusion of lymphocytes and thymoma cells according to Kapplers protocol [49]. The cells were then maintained in culture with Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 5% fetal calf serum (FCS).
Four prediction methods were employed for the prediction of I-A<sup>b</sup> binding peptides in human ApoB100 precursor, RANKPEP [1], IEDB SMM [2] and ANN methods [3], and a SVM (Support Vector Machine) model. Predicted as binding peptides by RANKPEP and at least two other methods, 18 sequences were synthesized and used experimentally. Because of the patentable potential these amino acid sequences of predicted peptides are not revealed in this report. However, they are elaborated on in these studies [50-52].

The hybridoma reactivity to the selected peptides was evaluated through detection of IL-2 secretion in supernatants by ELISA, according to manufacturer’s instruction (Mabtech). Briefly, 1 x 10<sup>5</sup> T hybridoma cells in combination with 4 x10<sup>5</sup> irradiated (1.6 Gy) murine splenocytes used as antigen presenting cells (APC), were challenged in vitro with single peptides, ApoB100 or Concavalin A (ConA) that was used as a positive control. The experimental procedure was carried out according to previous study by A. Hermansson and D. Ketelhuth [34]. IL-2 was used as a readout of T cell activation.

**Statistical Analysis**

Quantitative data are expressed as mean ± standard error of the mean (SEM). The nonparametric Mann-Whitney U test was used for 2-group comparisons, Kruskal-Wallis test for multigroup comparisons. Spearman’s rank correlation test was performed to assess correlations. Differences between groups were considered significant at P values < 0.05.
Results

Transfer of ApoB100 reactive T cells reduce the progression of atherosclerotic lesions in hypercholesterolemic mice

We hypothesized the transfer of ApoB100 auto reactive T cells would enhance inflammation and accelerate atherosclerosis. Surprisingly, mice that received a single intraperitoneal injection of two million CD3+ cells from TRVB31-TCR transgenic mice (Tg), showed a smaller plaques compared to both mice receiving the wild-type T cells (WT) or untreated (Un) control group (Figures 1A and 1B). Although not significant, Tg injected group presented 42% smaller average plaque size compared to the WT injected group. This trend was prevalent in both the aortic arch (Figure 1A) and in the aortic sinus (Figure 1B).

Figure 3: (A) Percentage of plaque in Un, WT and Tg mice and (B) percentage of lesion in the aortic root. Picture (A) depicts the En Face results, on the X-axis the results for the three different groups of mice are presented, and each bar represents the plaque size corresponding to the percentage which is shown of the Y axis. To the right of the graph the aortic arches from each group of mice are shown. The aortic arches were stained with Sudan IV, which stains the lipids, triglycerides and lipoproteins. The P-Value for Tg vs. WT is 0.0532, Un n= 6, WT n = 6 and Tg n = 5. Picture (B) shows atherosclerotic lesion size in the proximal aorta represented as serial sections from 200 μm to 500 μm from the aortic root. The figure illustrates cross-sectional lesion size in relationship to the total vessel area, presented as lesion percentage. The line shows means of the five cross-sectional lesion sizes for each group. To the right of the graph, micrographs of aortic roots from each group are presented. Each section has been stained with ORO for cholesterol esters and neutral triglycerides, and counter-stained with hematoxylin for visualization of the cell nuclei.
Transfer of ApoB100 reactive TRVB31 T cells induce immunity in the vessel wall

Immunohistochemical analysis shows that the transfer of CD3+ T cells increases the infiltration of CD68+ macrophages to the plaques irrespective of its source, either Tg or WT donor mice (Figure 4). Similarly, in figure 4B and 4C, we show that the mice receiving Tg and WT cells have an increase number of CD4+ cells and MHC-II expression compared to untreated mice, while we also find a trend towards increased CD4 staining in Tg injected animals compared to WT injected.

Figure 4: (A) IHC Staining with macrophage marker CD68 for Un, WT and Tg, (B) Amount of CD4+ per µm² for Un, WT and Tg and (C) Amount of MHC II per µm² for Un, WT and Tg Picture figure 4A depicts the IHC staining with the macrophage marker CD68. Each bar represents the total area, in percentage of the CD68 stained area in the plaque. The P-Value for these results was calculated to 0,0141 with a Kruskal-Wallis test. On the right to the bar graph representative photomicrographs for each group are presented. The pictures were taken at 20X magnification with the DM-LB2 microscope. Figure 4B represents the amount of CD4+ expressing cells, for each group, per µm² in the plaque. To the right of the bar graph, pictures representing a 40X magnification for each group are presented. The arrows mark the positive staining in the groups. Figure 4C shows the amount of cells that expresses MHC II per µm² in the plaque. To the right of the graph the corresponding pictures for each group, taken at 40X magnification, are shown. All IHC pictures are taken at the same distance from the aortic root.
TRVB31+ T cells may reach the vessel wall

Figure 5A presents the elevated mRNA levels on TRVB31 in the aorta for the Tg injected group compared to the WT injected and control groups. In the spleen, (figure 5B) bigger variations on expression and no apparent trends were observed. Similarly, within the inguinal lymph nodes, the TRVB31 expression did not differ between groups (figure 5C).

As increased number of CD4+ T cells and TRVB31 mRNA were found in Tg injected mice we evaluated genes corresponding to different T cell subtypes in the harvested tissue. Table 1 show that the Tg group has trend towards increased mRNA levels of the regulatory T cell marker FoxP3, and the anti-inflammatory cytokine IL-10 in the aorta in comparison to Un. Interestingly, IL-10 was also seen increased on WT injected animals.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Aorta</th>
<th>Un</th>
<th>WT</th>
<th>Tg</th>
<th>P value Tg vs. Un</th>
<th>P value Tg vs. WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tbx21</td>
<td></td>
<td>0,68 ± 0,19</td>
<td>1,5 ± 0,20</td>
<td>1,2 ± 0,24</td>
<td>0,14</td>
<td>0,34</td>
</tr>
<tr>
<td>GATA3</td>
<td></td>
<td>0,86 ± 0,32</td>
<td>0,94 ± 0,09</td>
<td>0,90 ± 0,14</td>
<td>0,94</td>
<td>0,83</td>
</tr>
<tr>
<td>FoxP3</td>
<td></td>
<td>0,56 ± 0,18</td>
<td>1,7 ± 0,49</td>
<td>1,9± 0,59</td>
<td>0,08</td>
<td>0,86</td>
</tr>
<tr>
<td>RORγt</td>
<td></td>
<td>0,81 ± 0,24</td>
<td>1,9 ± 0,30</td>
<td>1,4 ± 0,25</td>
<td>0,11</td>
<td>0,28</td>
</tr>
<tr>
<td>INF-γ</td>
<td></td>
<td>0,72 ± 0,30</td>
<td>1,5 ± 0,31</td>
<td>1,3 ± 0,33</td>
<td>0,22</td>
<td>0,63</td>
</tr>
<tr>
<td>IL-10</td>
<td></td>
<td>0,63 ± 0,18</td>
<td>1,2 ± 0,12</td>
<td>1,2 ± 0,20</td>
<td>0,05</td>
<td>0,84</td>
</tr>
<tr>
<td>TGF-β</td>
<td></td>
<td>0,70 ± 0,21</td>
<td>0,98 ± 0,07</td>
<td>1,0 ± 0,12</td>
<td>0,29</td>
<td>1,00</td>
</tr>
</tbody>
</table>

The PCR values from the aorta are analysed with the ΔΔCT-formula, and are presented as mean value ± standard error of mean (SEM). The p-value was calculated with a Mann-Whitney U test and concerns the relation between Tg vs. Un and Tg vs. WT.
Surprisingly, analysis of spleen (Table 2) shows significantly higher mRNA levels of the pro-atherogenic cytokine IFNγ on Tg group compared to Un. No differences on anti-inflammatory genes were observed.

Table 1: PCR results from the spleen for Un, WT and TG

<table>
<thead>
<tr>
<th>Spleen</th>
<th>Un</th>
<th>WT</th>
<th>Tg</th>
<th>P value Tg vs. Un</th>
<th>P value Tg vs. WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tbx21</td>
<td>3,5 ± 2,9</td>
<td>0,42 ± 0,09</td>
<td>3,63 ± 1,33</td>
<td>0,81</td>
<td>0,32</td>
</tr>
<tr>
<td>GATA3</td>
<td>1,2 ± 1,7</td>
<td>2,4 ± 0,71</td>
<td>3,4 ± 0,97</td>
<td>0,15</td>
<td>0,88</td>
</tr>
<tr>
<td>FoxP3</td>
<td>1,4 ± 0,5</td>
<td>0,92 ± 0,33</td>
<td>1,19 ± 0,28</td>
<td>0,71</td>
<td>0,55</td>
</tr>
<tr>
<td>RORγT</td>
<td>1,2 ± 0,37</td>
<td>0,64 ± 0,10</td>
<td>0,80 ± 0,26</td>
<td>0,32</td>
<td>0,60</td>
</tr>
<tr>
<td>INF-γ</td>
<td>1,0 ± 0,2</td>
<td>1,2 ± 0,17</td>
<td>1,86 ± 0,24</td>
<td>0,04</td>
<td>0,08</td>
</tr>
<tr>
<td>IL-10</td>
<td>Na ± Na</td>
<td>Na ± Na</td>
<td>Na ± Na</td>
<td>Na</td>
<td>Na</td>
</tr>
<tr>
<td>TGF-β</td>
<td>2,3 ± 0,58</td>
<td>3,1 ± 0,38</td>
<td>3,62 ± 1,33</td>
<td>0,41</td>
<td>0,74</td>
</tr>
</tbody>
</table>

The PCR values from the spleen are analysed with the ΔΔCT-formula, and are presented as mean value ± standard error of mean (SEM). The p-value was calculated with a Mann-Whitney U test and concerns the relation between Tg vs. Un and Tg vs. WT.

Also puzzling, the expression of RORγT mRNA on iLn was found higher in the Tg group compared to Un controls (table 3).

Table 3: PCR results from iLn for Un, WT and Tg

<table>
<thead>
<tr>
<th>iLn</th>
<th>Un</th>
<th>WT</th>
<th>Tg</th>
<th>P value Tg vs. Un</th>
<th>P value Tg vs. WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tbx21</td>
<td>1,1 ± 0,20</td>
<td>1,1 ± 0,14</td>
<td>1,4 ± 0,13</td>
<td>0,14</td>
<td>0,23</td>
</tr>
<tr>
<td>GATA3</td>
<td>1,1 ± 0,24</td>
<td>1,1 ± 0,22</td>
<td>1,4 ± 0,29</td>
<td>0,62</td>
<td>0,52</td>
</tr>
<tr>
<td>FoxP3</td>
<td>1,0 ± 0,11</td>
<td>1,1 ± 0,11</td>
<td>1,1 ± 0,12</td>
<td>0,54</td>
<td>0,64</td>
</tr>
<tr>
<td>RORγT</td>
<td>1,1 ± 0,20</td>
<td>1,3 ± 0,07</td>
<td>1,7 ± 0,22</td>
<td>0,07</td>
<td>0,14</td>
</tr>
<tr>
<td>INF-γ</td>
<td>1,1 ± 0,30</td>
<td>1,7 ± 0,82</td>
<td>1,0 ± 0,03</td>
<td>0,53</td>
<td>0,39</td>
</tr>
<tr>
<td>IL-10</td>
<td>1,1 ± 0,23</td>
<td>0,72 ± 0,23</td>
<td>0,72 ± 0,11</td>
<td>0,10</td>
<td>0,99</td>
</tr>
<tr>
<td>TGF-β</td>
<td>1,0 ± 0,10</td>
<td>1,1 ± 0,07</td>
<td>1,1 ± 0,10</td>
<td>0,46</td>
<td>0,55</td>
</tr>
</tbody>
</table>

The PCR values from the iLn are analysed with the ΔΔCT-formula, and are presented as mean value ± standard error of mean (SEM). The p-value was calculated with a Mann-Whitney U test and concerns the relation between Tg vs. Un and Tg vs. WT.
Plasma lipids and body weight

Also relevant to atherosclerosis, plasma lipids and body weight were evaluated on the studied groups. The results shown in figure 6 revealed no change in plasma cholesterol, triglycerides or body weight between groups.

Figure 6: Plasma lipids and body weight for Un, WT and Tg mice; (A) Cholesterol, (B) Triglyceride and (C) Body Weight. The bars in the graph bars represent their corresponding groups. The plasma cholesterol (A), and triglyceride (B) are measured in mmol l\(^{-1}\). The body weight (C) for each mouse is measured in gram. All mice were fed with a standard chow diet.

Antibodies against ApoB100 and oxLDL

T cells can help B cells to class switch and secretion of antibodies. We evaluated whether Tg or WT cells affected the response to ApoB100 and oxLDL, both relevant antigens in atherosclerosis. The levels of IgG anti-ApoB100 and anti-oxLDL, measured by ELISA, in plasma did not differ between groups (figure 7A and 7B).

Figure 7: IgG in mmol l\(^{-1}\) against (A) ApoB100 and (B) OxLDL for Un, WT and Tg. Picture (A) represents the three different groups, where each bar shows the levels of IgG measured at 450 nm OD against ApoB100 in serum (diluted 1:15). Picture (B) represents the three different groups, where each bar shows the levels of IgG measured at 450 nm OD against oxLDL in serum (diluted 1:15).
Transfer of ApoB100 reactive T cells does not induce anti-TCR TRVB31 antibodies

Subcutaneous immunization with TRVB31 induces antibodies that inhibit TRVB31+ T cells and protect mice against atherosclerosis[34]. Here, we investigated whether Tg injection could have induced similar protective antibodies and explain the surprising effects on lesion size. No differences between anti-TRVB31 IgG expressions between groups were observed. Further, antibody levels on serum from studied mice were 4 times lower than those observed on TRVB31-immunized mice (TCRimm). The concentration was measured from pooled serum for each group.

Figure 8: IgG in µg ml⁻¹ against anti-TRVB31 in serum for Un, WT, Tg and the ImM control. The X axis, presented in the bar graph, represents the three different groups and the TCRimm control. Each bar shows the levels of IgG measured at 450 nm OD against anti-TRVB31 in serum (diluted 1:15).

Correlation analysis

In order to explore connections between analyzed parameters and identify possible pathways explaining our results, a series of correlations were performed. The results in figure 9A and 9B show a hint of a significant positive correlation between percentage of plaque and macrophage staining (CD68). Interestingly, analogous correlations on Tg injected mice showed a negative correlation between the same parameters (figure 9C).
Epitope mapping does not reveal the epitope for TRVB31+ cells

Using hybridoma cells expressing the TRVB31, the cell from which TCRs were cloned and applied to generate Tg mice. The Tg mice were utilized to screen mathematically predicted peptides that could associate to MHC-II on mice of C57BL6/J. The \textit{in vitro} evaluation of the nineteen peptides that had a top scored peptide binding probability towards IA-b, did not induce the release of IL-2 by hybridoma T cells (Fig 10). On the other hand, soluble ApoB100 as well as the positive control ConA induce a robust response of these cells.

![Figure 10: ApoB100 epitope mapping. The 48-5 hybridoma cells were cultured in vitro with irradiated APCs in the presence of nineteen different peptides predicted to be IA-b high affinity binders. ApoB100 and ConA were used as positive controls. Bars show the average IL-2 levels for each sample.](image)
Discussion

Understanding the underlying mechanisms and immunological responses in atherosclerosis can lead to the development of future drugs and vaccines to treat and prevent CVDs. Therefore, immunomodulation has become a popular approach when trying to fight this chronic inflammatory disease.

The main hypothesis of this study was that plaque development could be accelerated in huApoB100~Tg~ x Ldlr~−/−~ mice upon the adoptive transfer of ApoB100 specific T cells. Unexpectedly, our primary results showed that transfer of TRVB31+ Tg T cells resulted in decreased disease compared to WT T cells injected and untreated mice.

Both animal and clinical studies have shown that elevated levels of circulating cholesterol can promote atherosclerosis [12, 53]. Evaluation of blood cholesterol and triglyceride levels showed no differences between groups. Similarly, no differences on antibody levels to ApoB100 and oxLDL could be detected. These results suggest that atherosclerosis development would rely on the direct effects of T cell derived responses rather than changes in plasma lipids or affecting B cell responses and the release of antibodies. Indeed, only background levels of anti-TRVB31 IgG could be detected in serum of Tg group.

The immunohistochemical analysis of acetone-fixed cryosections of the aortic root showed clear tendencies towards increased macrophage (CD68) infiltration in the plaque of WT and Tg groups compared to control untreated mice. Macrophages have are major components of the innate immunity where they play a central role in inflammation and healing. Once monocytes enter the plaque they differentiate into macrophages and through different polarization factors can become either M1 or M2 macrophages. The M1 polarization is usually dependent of pro-inflammatory cytokines INF-γ, TNF-α, IL-12 and GM-CSF (granulocyte-macrophage colony-stimulating factor) [1, 2]. In fact, this polarization could be followed after the triggering of macrophages via TLRs by LDL and oxLDL [3]. M1 are considered to be the pro-inflammatory macrophages. Contrasting, M2 is seen as the anti-inflammatory macrophage that stimulates tissue healing and recovery [4]. Different subtypes of M2 exist (M2a, M2b and M2) and they can be induced through factors released by Th2 cells, IL-4, IL-10, IL-13 and TGF-β, and M-CSF (Macrophage colony-stimulating factor) [2-4]. Indeed, we observed an inverse correlation between macrophage staining and percentage plaque on Tg group while a positive correlation between the same parameters is seen for the other groups. Altogether the data suggest differences on the injection of antigen specific T cells may lead to different macrophages responses in
the plaque, speculatively the anti-inflammatory M2. However, further investigation will be needed to confirm this hypothesis.

Parallel to the increased infiltration of macrophages, Tg and WT CD3+ injected mice presented increased numbers of CD4+ cells compared to the Un group. This result could well be explained by the increased pool of T cells achieved upon adoptive transfer. However, the trends towards slightly higher numbers of CD4+ T cells on Tg compared to WT, the latter event that is mirrored by increased mRNA levels of TRVB31, FoxP3 and IL-10 in aortic samples, suggest specificity on T cells was needed to induce protection. Studies have linked IL-10 and Tregs as protective mechanisms against atherosclerosis in mice [54, 55]. In fact, Hermansson et al., 2011 showed that only specific Tregs induced by the transfer of DCs pulsed with ApoB100 and IL-10, but not DCs pulsed with IL-10 alone, can affect disease progression [56]. Further, both the WT and Tg groups indicated a small up regulation of TGF-β in the aorta, as well as in the spleen, when compared to the Un group. TGF-β has been suggested as an important mediator by how Tregs regulate atherosclerotic lesion progression [57, 58].

Last in this study, we aimed to explore epitopes recognized by TRVB31+ T cells. It has been shown that clones expressing TRVB31 can recognize ApoB100 of LDL [34]. In the same study, it was shown that a significant reduction in atherosclerotic lesions could be achieved by inducing anti-TRVB31 immunity, the latter which was mirrored by reduction in macrophage infiltration and MHC II expression in the atherosclerotic plaques [34]. Therefore, finding the epitope activating TRVB31 T cells may expand our basis for the development of new therapies against CVD. Peptides of ApoB100 predicted to bind I-Ab were synthesized and used in vitro to challenge TRVB31+ T cell clones. Unfortunately, none of the peptides induced IL-2 secretion, while the same robustly responded to ApoB100 and ConA. Different explanations for these lack of activation can be speculated, for example the simple fact that the peptides may have not be taken up by the APCs and present the peptides to TRVB31+ cells. A possible solution to such problem could be potentially solved by the generation of peptides fused to liposomes, which will be explored in the future.

A clear limitation of our study was the number of animals composing each of the groups. Therefore, complementing studies will be needed in order to detect stronger statistical differences and to draw final mechanistic conclusions. Therefore, we can only conclude that current studies suggest that CD3+ TRVB31 transfer reduces atherosclerosis, and that the biological mechanisms may rely on the generation of antigen specific Tregs and/or different macrophage subsets. Ongoing and future research will be necessary to conclude these investigations.
References

Appendix 1 FACS analysis of spleen cells stained with anti-CD4-APCH7 and anti-TRVB31-FITC. CD4+ T cells from a wild-type mouse to the left, and from a TRVB31-trangenic mouse on the right.