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Adoptive transfer of ApoB100 specific T cells in atherosclerosis

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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

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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].

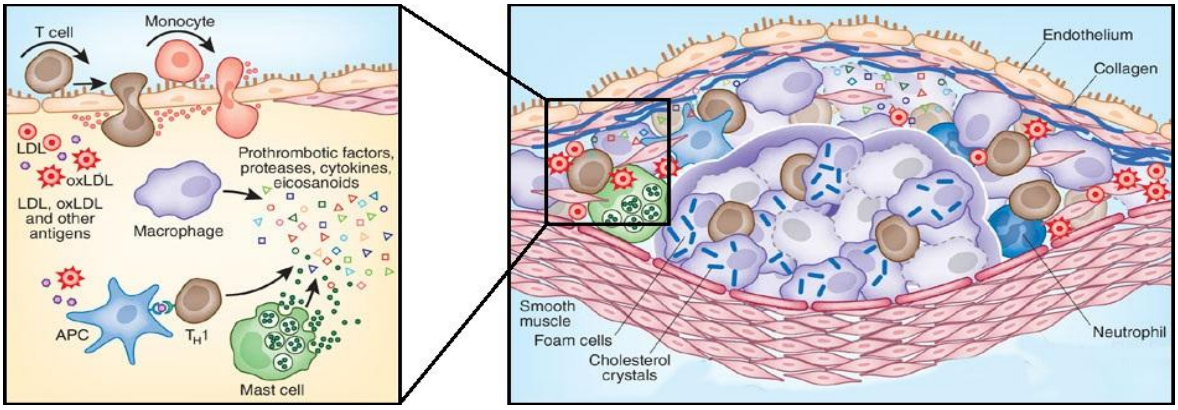


Figure 1: The atherosclerotic process. The zoom in box (to the left) depicts LDL particles that have migrated into the intima, through the mono layer of endothelial cells. Here we see the initial response from oxidized LDL particles that triggers an inflammatory response with expression of adhesion molecules. 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.

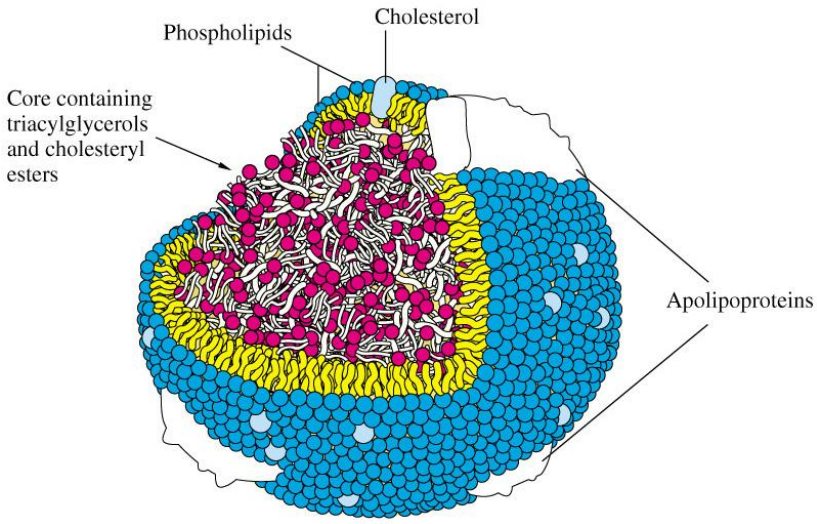


Figure 2: Low-density lipoprotein (LDL). The LDL particle is approximately 22nm in size and has a core of hydrophobic cholesterol esters and triglycerides, which are surrounded by hydrophilic unesterfied cholesterols and phospholipids. The particle has a single copy of ApoB100, a glycoprotein consisting 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 TRVB31, 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-ɣ, 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 CO_2 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^{o}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-ɣ (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) – ΔCT (calibrator = mean CT values of all samples within each group), and ΔCT 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 streptoavidin-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 % H_2SO_4. 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^b 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 reveled 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 10^5 T hybridoma cells in combination with 4 x10^5 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).

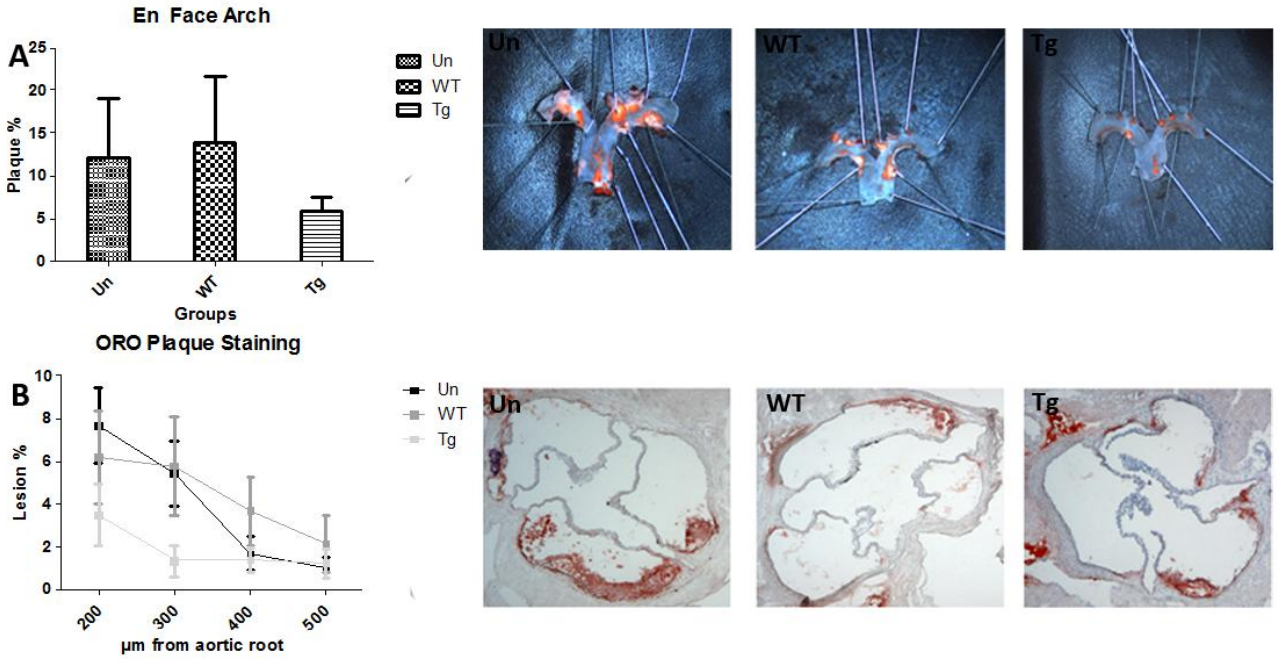


Figure 3: (A) Percentage of plaque in Un, WT and Tg mice and (B) percentage of lesion in the aortic root. Picture (A) depicts the En Face results, on the X-axis the results for the three different groups of mice are presented, and each bar represents the plaque size corresponding to the percentage which 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.

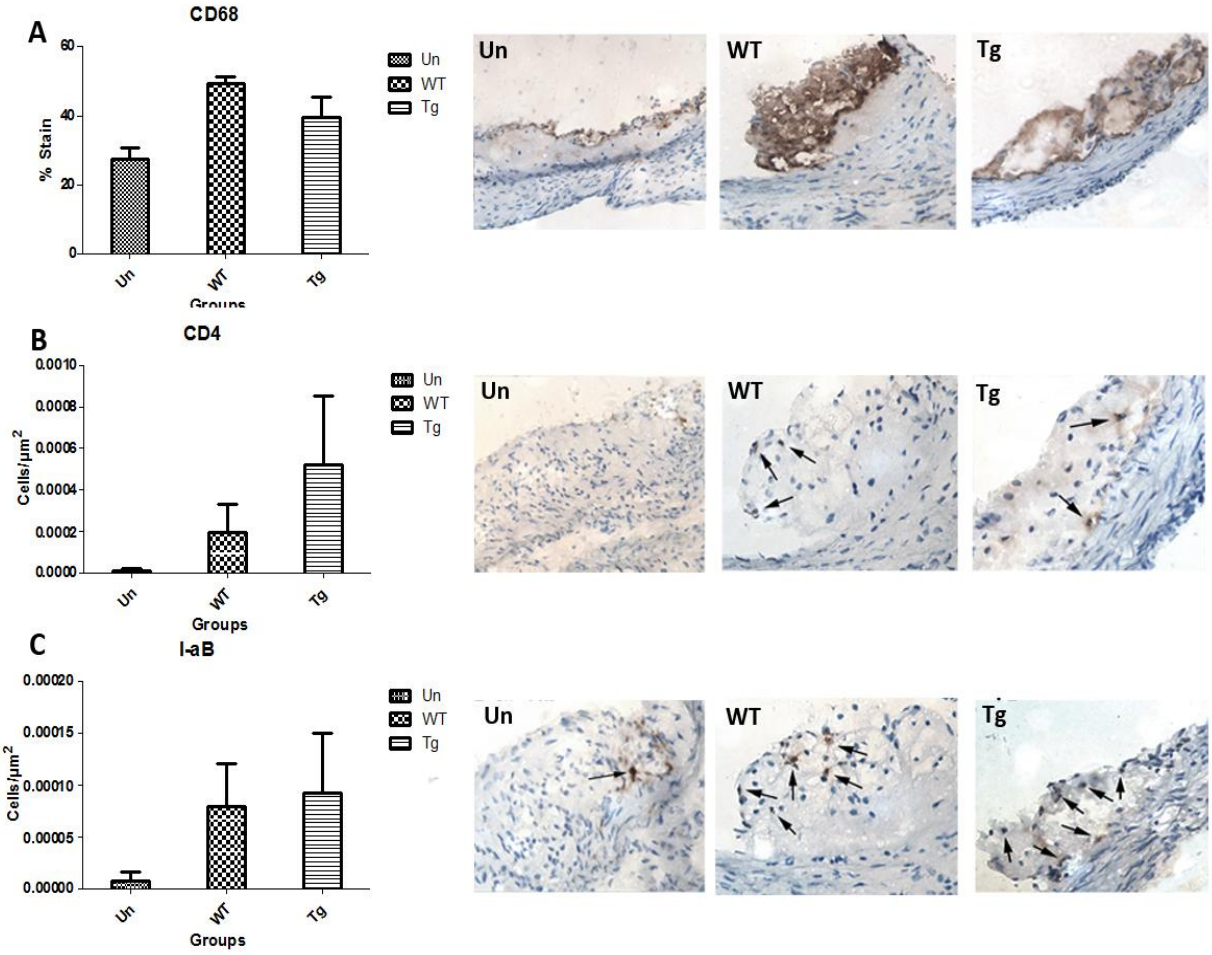


Figure 4: (A) IHC Staining with macrophage marker CD68 for Un, WT and Tg, (B) Amount of CD4+ per µm^2 for Un, WT and Tg and (C) Amount of MHC II per µm^2 for Un, WT and Tg Picture figure 4A depicts the IHC staining with the macrophage marker CD68. Each bar represents the total area, in percentage of 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).

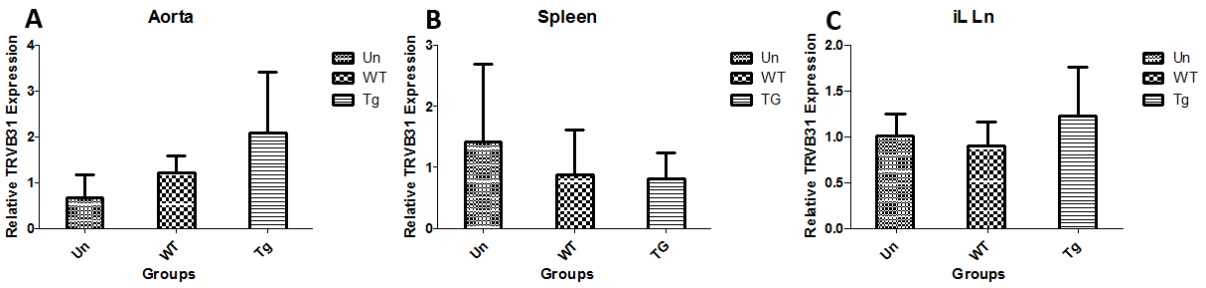


Figure 5: Relative TRVB31 for Un WT and Tg in (A) the aorta, (B) the spleen and (C) the Il Ln Each bar in the graph bars represents the relative TRVB31 expression in the aorta (A), the spleen (B) and the Il Ln (C) throughout the groups. The values from the PCR were calculated with the ΔΔCT-formula. All PCR reactions were normalized with the HPRT house keeping gene.

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

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

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 IFNɣ 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

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

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

iLn	Un	WT	Tg	P value Tg vs. Un	P value Tg vs. WT
Tbx21	1,1 ± 0,20	1,1 ± 0,14	1,4 ± 0,13	0,14	0,23
GATA3	1,1 ± 0,24	1,1 ± 0,22	1,4 ± 0,29	0,62	0,52
FoxP3	1,0 ± 0,11	1,1 ± 0,11	1,1 ± 0,12	0,54	0,64
RORγt	1,1 ± 0,20	1,3 ± 0,07	1,7 ± 0,22	0,07	0,14
INF-ɣ	1,1 ± 0,30	1,7 ± 0,82	1,0 ± 0,03	0,53	0,39
IL-10	1,1 ± 0,23	0,72 ± 0,23	0,72 ± 0,11	0,10	0,99
TGF-β	1,0 ± 0,10	1,1 ± 0,07	1,1 ± 0,10	0,46	0,55

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.

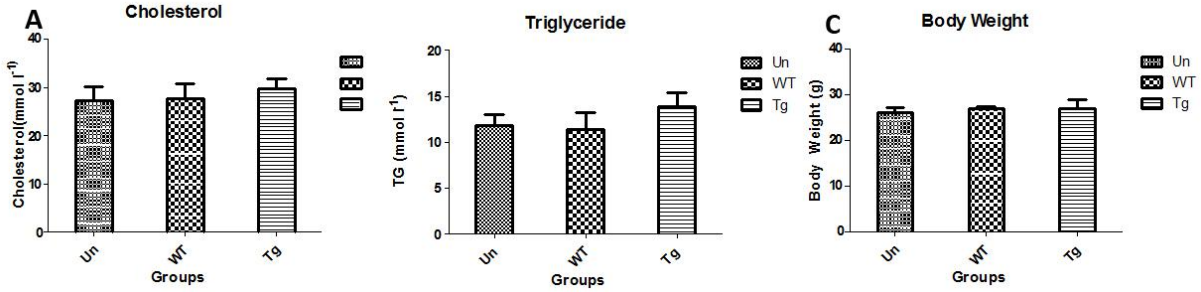


Figure 6: Plasma lipids and body weight for Un, WT and Tg mice; (A) Cholesterol, (B) Triglyceride and (C) Body Weight. The bars in the graph bars represent their corresponding groups. The plasma cholesterol (A), and triglyceride (B) are measured in mmol l^{-1}. The body weight (C) for each mouse is measured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

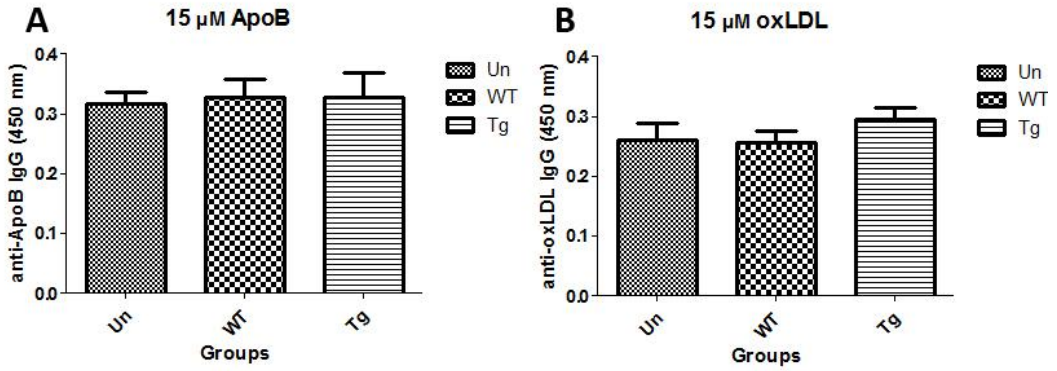


Figure 7: IgG in mmol l-1 against (A) ApoB100 and (B) OxLDL for Un, WT and Tg. Picture (A) represents the three different groups, where each bar shows the levels of IgG measured at 450 nm OD against ApoB100 in serum (diluted 1:15). Picture (B) represents the three different groups, where each bar shows 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.

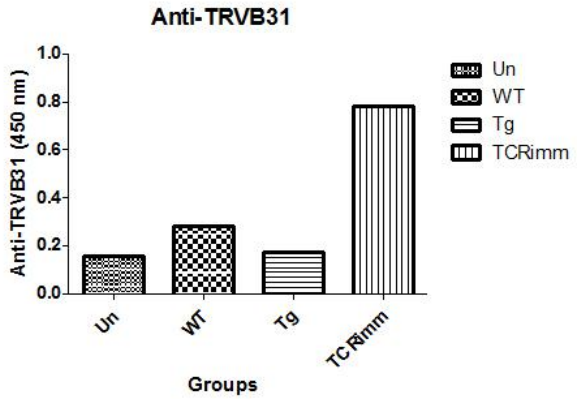


Figure 8: IgG in µg ml-1 against anti-TRVB31 in serum for Un, WT, Tg and the ImM control. The X axis, presented in the bar graph, represents the three different groups and the TCRimm control. Each bar shows the levels of IgG measured at 450 nm OD against anti-TRVB31 in serum (diluted 1:15).

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).

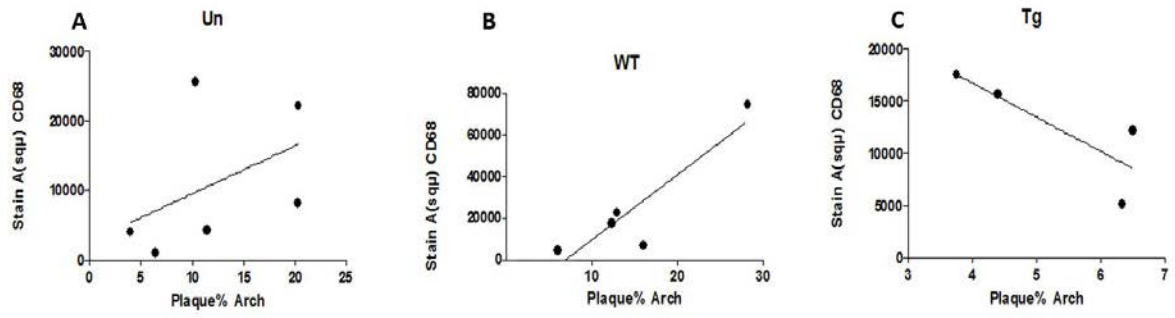


Figure 9: Correlation between IHC CD68 staining and percentage plaque for (A) Un, (B) WT, and (C) Tg. Picture (A) shows the plaque percentage against CD68 in the Un group, $R^2 = 0{,}2071$. Picture (B) shows the plaque percentage against CD68 in the WT group, $R^2 = 0{,}8142$. Picture (C) shows the plaque percentage against CD68 in the WT group, $R^2 = 0{,}6711$.

Epitope mapping does not reveal the epitope for TRVB31+ cells

Using hybridoma cells expressing the TRVB31, the cell from which TCRs were cloned and applyed 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 *in vitro* evaluation of the ninthen peptides that had a top scored peptide binding propability towards IA-b, dident induce the release of IL-2 by hybridomal T cells (Fig 10). On the other hand, soluble ApoB100 as well as the positive control ConA induce a robust response of these cells.

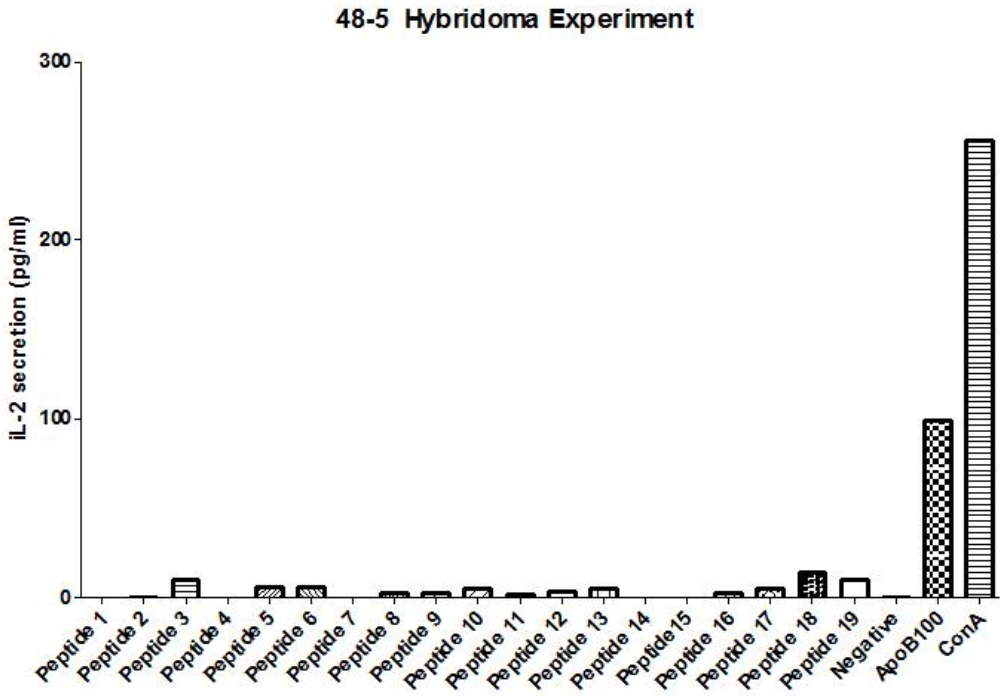


Figure 10: ApoB100 eppitope mapping. The 48-5 hybidoma cells were cultured in vitro with irradiated APCs in the presence of nineteen different peptides predicted to be IA-b high affinity binders. ApoB100 and ConA were used as positive controls. Bars show the average IL-2 levels for each sample.

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 in to 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-A^b 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.

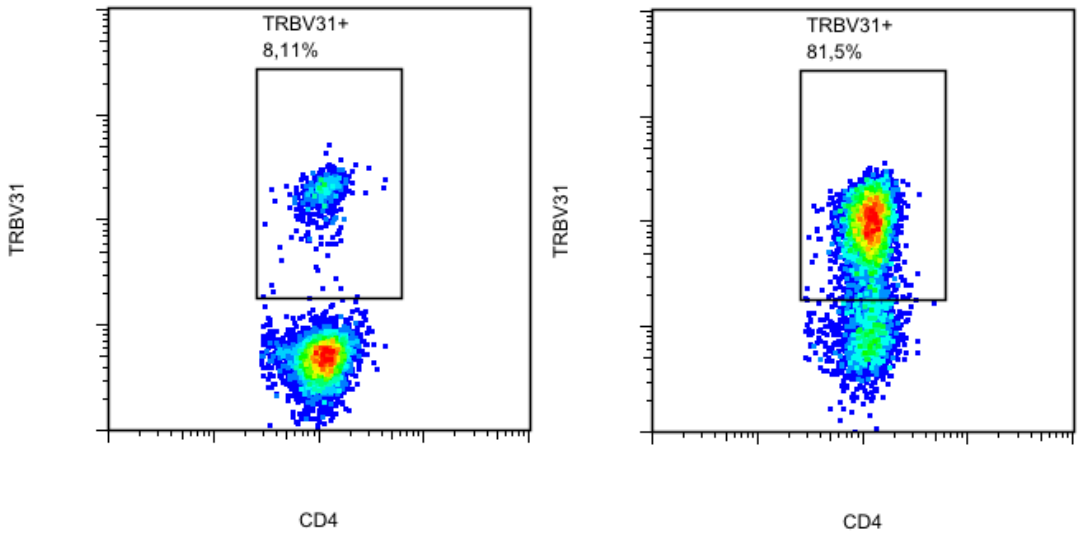
References

1. World Health Organization., *Global health risks : mortality and burden of disease attributable to selected major risks*2009, Geneva, Switzerland: World Health Organization. vi, 62 p.
2. Nichols M, T.N., Scarborough P, Luengo-Fernandez R, Leal J, Gray A, Rayner M, *European Cardiovascular Disease Statistics 2012.* European Heart Network, Brussels, European Society of Cardiology, Sophia Antipolis, 2012.
3. Hansson, G.K., *Inflammation, atherosclerosis, and coronary artery disease.* N Engl J Med, 2005. **352**(16): p. 1685-95.
4. Gaziano, T.A., *Cardiovascular disease in the developing world and its cost-effective management.* Circulation, 2005. **112**(23): p. 3547-53.
5. Conroy, R.M., et al., *Estimation of ten-year risk of fatal cardiovascular disease in Europe: the SCORE project.* European Heart Journal, 2003. **24**(11): p. 987-1003.
6. Fishbein, G.A. and M.C. Fishbein, *Arteriosclerosis: rethinking the current classification.* Arch Pathol Lab Med, 2009. **133**(8): p. 1309-16.
7. *Classics in arteriosclerosis research: On experimental cholesterin steatosis and its significance in the origin of some pathological processes by N. Anitschkow and S. Chalatow, translated by Mary Z. Pelias, 1913.* Arteriosclerosis, 1983. **3**(2): p. 178-82.
8. Tabas, I., K.J. Williams, and J. Boren, *Subendothelial lipoprotein retention as the initiating process in atherosclerosis: update and therapeutic implications.* Circulation, 2007. **116**(16): p. 1832-44.
9. Glass, C.K. and J.L. Witztum, *Atherosclerosis. the road ahead.* Cell, 2001. **104**(4): p. 503-16.
10. Cybulsky, M.I. and M.A. Gimbrone, Jr., *Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis.* Science, 1991. **251**(4995): p. 788-91.
11. Nakashima, Y., et al., *Upregulation of VCAM-1 and ICAM-1 at atherosclerosis-prone sites on the endothelium in the ApoE-deficient mouse.* Arterioscler Thromb Vasc Biol, 1998. **18**(5): p. 842-51.
12. Hansson, G.K. and A. Hermansson, *The immune system in atherosclerosis.* Nat Immunol, 2011. **12**(3): p. 204-12.
13. Huang, D.R., et al., *Absence of monocyte chemoattractant protein 1 in mice leads to decreased local macrophage recruitment and antigen-specific T helper cell type 1 immune response in experimental autoimmune encephalomyelitis.* J Exp Med, 2001. **193**(6): p. 713-26.
14. Gu, L., et al., *Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice.* Mol Cell, 1998. **2**(2): p. 275-81.
15. Heller, E.A., et al., *Chemokine CXCL10 promotes atherogenesis by modulating the local balance of effector and regulatory T cells.* Circulation, 2006. **113**(19): p. 2301-12.
16. Andersson, J., P. Libby, and G.K. Hansson, *Adaptive immunity and atherosclerosis.* Clin Immunol, 2010. **134**(1): p. 33-46.
17. Edfeldt, K., et al., *Expression of toll-like receptors in human atherosclerotic lesions: a possible pathway for plaque activation.* Circulation, 2002. **105**(10): p. 1158-61.
18. Libby, P., P.M. Ridker, and G.K. Hansson, *Inflammation in atherosclerosis: from pathophysiology to practice.* J Am Coll Cardiol, 2009. **54**(23): p. 2129-38.
19. Seneviratne, A.N., B. Sivagurunathan, and C. Monaco, *Toll-like receptors and macrophage activation in atherosclerosis.* Clin Chim Acta, 2012. **413**(1-2): p. 3-14.
20. Schrijvers, D.M., et al., *Phagocytosis of apoptotic cells by macrophages is impaired in atherosclerosis.* Arterioscler Thromb Vasc Biol, 2005. **25**(6): p. 1256-61.

21. Libby, P., *Role of inflammation in atherosclerosis associated with rheumatoid arthritis.* Am J Med, 2008. **121**(10 Suppl 1): p. S21-31.
22. Libby, P., *Inflammation in atherosclerosis.* Arterioscler Thromb Vasc Biol, 2012. **32**(9): p. 2045-51.
23. Zhou, X., et al., *Transfer of CD4(+) T cells aggravates atherosclerosis in immunodeficient apolipoprotein E knockout mice.* Circulation, 2000. **102**(24): p. 2919-22.
24. Libby, P. and M. Aikawa, *New insights into plaque stabilisation by lipid lowering.* Drugs, 1998. **56 Suppl 1**: p. 9-13; discussion 33.
25. Segrest, J.P., et al., *Structure of apolipoprotein B-100 in low density lipoproteins.* J Lipid Res, 2001. **42**(9): p. 1346-67.
26. Goldstein, J.L., et al., *Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition.* Proc Natl Acad Sci U S A, 1979. **76**(1): p. 333-7.
27. Sisson, M.; Available from: http://www.marksdailyapple.com.
28. Mauri, C. and A. Bosma, *Immune regulatory function of B cells.* Annu Rev Immunol, 2012. **30**: p. 221-41.
29. Schulte, S., G.K. Sukhova, and P. Libby, *Genetically programmed biases in Th1 and Th2 immune responses modulate atherogenesis.* Am J Pathol, 2008. **172**(6): p. 1500-8.
30. Binder, C.J., et al., *IL-5 links adaptive and natural immunity specific for epitopes of oxidized LDL and protects from atherosclerosis.* J Clin Invest, 2004. **114**(3): p. 427-37.
31. Ferber, I.A., et al., *GATA-3 significantly downregulates IFN-gamma production from developing Th1 cells in addition to inducing IL-4 and IL-5 levels.* Clin Immunol, 1999. **91**(2): p. 134-44.
32. Hansson, G.K., et al., *Innate and adaptive immunity in the pathogenesis of atherosclerosis.* Circ Res, 2002. **91**(4): p. 281-91.
33. Klingenberg, R., et al., *Intranasal immunization with an apolipoprotein B-100 fusion protein induces antigen-specific regulatory T cells and reduces atherosclerosis.* Arterioscler Thromb Vasc Biol, 2010. **30**(5): p. 946-52.
34. Hermansson, A., et al., *Inhibition of T cell response to native low-density lipoprotein reduces atherosclerosis.* J Exp Med, 2010. **207**(5): p. 1081-93.
35. George, J., et al., *Suppression of early atherosclerosis in LDL-receptor deficient mice by oral tolerance with beta 2-glycoprotein I.* Cardiovasc Res, 2004. **62**(3): p. 603-9.
36. Maron, R., et al., *Mucosal administration of heat shock protein-65 decreases atherosclerosis and inflammation in aortic arch of low-density lipoprotein receptor-deficient mice.* Circulation, 2002. **106**(13): p. 1708-15.
37. Dixon, W.G., et al., *Reduction in the incidence of myocardial infarction in patients with rheumatoid arthritis who respond to anti-tumor necrosis factor alpha therapy: results from the British Society for Rheumatology Biologics Register.* Arthritis Rheum, 2007. **56**(9): p. 2905-12.
38. Zhou, X., et al., *Adoptive transfer of CD4+ T cells reactive to modified low-density lipoprotein aggravates atherosclerosis.* Arterioscler Thromb Vasc Biol, 2006. **26**(4): p. 864-70.
39. Boren, J., et al., *Identification of the low density lipoprotein receptor-binding site in apolipoprotein B100 and the modulation of its binding activity by the carboxyl terminus in familial defective apo-B100.* J Clin Invest, 1998. **101**(5): p. 1084-93.
40. Plump, A.S., et al., *Increased atherosclerosis in ApoE and LDL receptor gene knock-out mice as a result of human cholesteryl ester transfer protein transgene expression.* Arterioscler Thromb Vasc Biol, 1999. **19**(4): p. 1105-10.
41. Powell-Braxton, L., et al., *A mouse model of human familial hypercholesterolemia: markedly elevated low density lipoprotein cholesterol levels and severe atherosclerosis on a low-fat chow diet.* Nat Med, 1998. **4**(8): p. 934-8.

42. Sanan, D.A., et al., *Low density lipoprotein receptor-negative mice expressing human apolipoprotein B-100 develop complex atherosclerotic lesions on a chow diet: no accentuation by apolipoprotein(a).* Proc Natl Acad Sci U S A, 1998. **95**(8): p. 4544-9.
43. Palinski, W., et al., *ApoE-deficient mice are a model of lipoprotein oxidation in atherogenesis. Demonstration of oxidation-specific epitopes in lesions and high titers of autoantibodies to malondialdehyde-lysine in serum.* Arterioscler Thromb, 1994. **14**(4): p. 605-16.
44. Nicoletti, A., et al., *Immunoglobulin treatment reduces atherosclerosis in apo E knockout mice.* J Clin Invest, 1998. **102**(5): p. 910-8.
45. Havel, R.J., H.A. Eder, and J.H. Bragdon, *The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum.* J Clin Invest, 1955. **34**(9): p. 1345-53.
46. Steele, J.C., Jr. and J.A. Reynolds, *Characterization of the apolipoprotein B polypeptide of human plasma low density lipoprotein in detergent and denaturation solutions.* J Biol Chem, 1979. **254**(5): p. 1633-8.
47. Wessel, D. and U.I. Flugge, *A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids.* Anal Biochem, 1984. **138**(1): p. 141-3.
48. Paulsson, G., et al., *Oligoclonal T cell expansions in atherosclerotic lesions of apolipoprotein E-deficient mice.* Arterioscler Thromb Vasc Biol, 2000. **20**(1): p. 10-7.
49. Kappler, J.W., et al., *Antigen-inducible, H-2-restricted, interleukin-2-producing T cell hybridomas. Lack of independent antigen and H-2 recognition.* J Exp Med, 1981. **153**(5): p. 1198-214.
50. Reche, P.A., et al., *Enhancement to the RANKPEP resource for the prediction of peptide binding to MHC molecules using profiles.* Immunogenetics, 2004. **56**(6): p. 405-19.
51. Peters, B. and A. Sette, *Generating quantitative models describing the sequence specificity of biological processes with the stabilized matrix method.* BMC Bioinformatics, 2005. **6**: p. 132.
52. Lundegaard, C., et al., *NetMHC-3.0: accurate web accessible predictions of human, mouse and monkey MHC class I affinities for peptides of length 8-11.* Nucleic Acids Res, 2008. **36**(Web Server issue): p. W509-12.
53. Nicholls, S.J., et al., *Lowering the triglyceride/high-density lipoprotein cholesterol ratio is associated with the beneficial impact of pioglitazone on progression of coronary atherosclerosis in diabetic patients: insights from the PERISCOPE (Pioglitazone Effect on Regression of Intravascular Sonographic Coronary Obstruction Prospective Evaluation) study.* J Am Coll Cardiol, 2011. **57**(2): p. 153-9.
54. Sasaki, N., et al., *Oral anti-CD3 antibody treatment induces regulatory T cells and inhibits the development of atherosclerosis in mice.* Circulation, 2009. **120**(20): p. 1996-2005.
55. van Puijvelde, G.H., et al., *Induction of oral tolerance to oxidized low-density lipoprotein ameliorates atherosclerosis.* Circulation, 2006. **114**(18): p. 1968-76.
56. Hermansson, A., et al., *Immunotherapy with tolerogenic apolipoprotein B-100-loaded dendritic cells attenuates atherosclerosis in hypercholesterolemic mice.* Circulation, 2011. **123**(10): p. 1083-91.
57. Robertson, A.K., et al., *Disruption of TGF-beta signaling in T cells accelerates atherosclerosis.* J Clin Invest, 2003. **112**(9): p. 1342-50.
58. Grainger, D.J., *Transforming growth factor beta and atherosclerosis: so far, so good for the protective cytokine hypothesis.* Arterioscler Thromb Vasc Biol, 2004. **24**(3): p. 399-404.

Appendix.1



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.