CD8+ T-cells in Atherosclerosis: mechanistic studies revealing a protective role in the plaque microenvironment

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CD8$^+$ T-cells contribute to lesion stabilization in advanced atherosclerosis by limiting macrophage content and CD4$^+$ T-cell responses

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ABSTRACT

Aims T lymphocytes play an important role in atherosclerosis development, but the role of the $CD8^+$ T-cells remains debated, especially in the clinically relevant advanced stages of atherosclerosis development. Here, we set out to determine the role of $CD8^+$ T-cells in advanced atherosclerosis.

Methods and Results Human endarterectomy samples analyzed by flow cytometry showed a negative correlation between the percentage of $CD8^+$ T-cells and macrophages, suggesting a possible protective role for these cells in lesion development. To further test this hypothesis, $LDLr^{-/-}$ mice were fed a Western-type diet (WTD) for 10 weeks to induce atherosclerosis, after which they received CD8α-depleting or isotype control antibody for six weeks. Depletion of $CD8^+$ T-cells in advanced atherosclerosis resulted in less stable lesions, with significantly reduced collagen content in the trivalve area, increased macrophage content and increased necrotic core area compared to controls. Mechanistically, we observed that $CD8^+$ depletion specifically increased the fraction of Th1 $CD4^+$ T-cells in the lesions. Treatment of WTD-fed $LDLr^{-/-}$ mice with a FasL-neutralizing antibody resulted in similar changes in macrophages and $CD4^+$ T-cell skewing as $CD8^+$ T-cell depletion.

Conclusion These findings demonstrate for the first time a local, protective role for $CD8^+$ T-cells in advanced atherosclerosis, through limiting accumulation of Th1 cells and macrophages, identifying a novel regulatory mechanism for these cells in atherosclerosis.

1. Introduction

Atherosclerosis is a chronic disorder characterized by inflammation and accumulation of lipids in the vessel wall. Inflammation plays a key role throughout all stages of atherosclerosis development, involving a complex interplay between different inflammatory cell types. Among these are CD8$^+$ T-cells, which could play an important role in all stages of atherogenesis, as they represent 29% of all leukocytes in early human lesions, increasing to 50% in advanced plaques [1]. In accordance, CD8$^+$ T-cell numbers are shown to increase significantly as human lesions become more progressed and vulnerable to rupture but show a decrease in healed plaque ruptures and fibrotic calcified plaques [2]. Also in the circulation, CD8$^+$ T-cells have been linked to atherosclerosis development as the percentage of low-differentiated CD8$^+$CCR7$^+$CD45RA$^+$ T-cells is reduced in patients with advanced coronary atherosclerosis compared to those without significant coronary disease [3] Furthermore, a significant correlation between the total number of CD8$^+$ T-cells in the circulation and the occurrence of myocardial infarction was established [4], suggesting that CD8$^+$ T-cells contribute to lesion growth and instability. Interestingly, the same study demonstrates an inverse correlation between the number of IFN-γ^+CD8$^+$ T-cells and carotid stenosis, suggesting that certain CD8$^+$ T-cell subpopulations slow down lesion progression. The PD-1$^+$TIM-3$^+$CD8$^+$ T-cell subset has been identified in the circulation of atherosclerotic patients, which exhibit an increased production of anti-atherogenic cytokines and decreased pro-atherogenic cytokines, suggesting a regulatory function for these cells in atherogenesis [5].

Several studies in experimental models of atherosclerosis have been conducted to provide insight into the role of CD8$^+$ T-cells in atherogenesis, but the results are thus far conflicting [6–12]. Whereas CD8$^+$ T-cells can induce lesion growth and instability, through lysis of endothelial cells and vascular smooth muscle cells [9], CD8$^+$ T-cell mediated killing of dendritic cells and follicular helper T-cells can reduce plaque formation [11, 12]. Importantly, studies in murine atherosclerotic models focus mainly on initial lesion development, whereas it is clinically more relevant to study advanced and/or unstable lesions, as patients usually experience symptoms related to severe stenosis when lesions are advanced. Therefore, from a drug development perspective, it is most valuable to understand the role of CD8$^+$ T-cells in this stage of disease progression, as this is the stage when pharmacological intervention is possible.

In the present study, we aimed to assess how CD8$^+$ T-cells affect plaque composition and stability of advanced lesions. We show a negative correlation between the percentage of CD8$^+$ T-cells and macrophages in human endarterectomy samples, suggesting a possible protective role for CD8$^+$ T-cells in the more advanced stages of atherogenesis. We next set out to investigate the role of CD8$^+$ T-cells in advanced plaques by CD8$^+$ T-cell depletion in the low-density lipoprotein receptor (LDLr) knockout mouse model. We show here for the first time that CD8$^+$ T-cells contribute to increased plaque stability, as well as to a microenvironment-specific skewing of CD4$^+$ T-cells within the lesions.

2. Materials and methods

2.1. Human studies

7 plaques from the carotid artery and 12 plaques from the femoral artery were obtained during endarterectomy from anonymous individuals, from whom we did not receive any patient details. The patients underwent endarterectomy surgery between July and December 2016 at the Haaglanden Medical Center, Westeinde, The Hague, NL. The handling of all human samples complied with the "Code for Proper Secondary Use of Human Tissue" and conforms with the principles outlined in the Declaration of Helsinki. Single-cell suspensions were obtained from human plaques by cutting the tissue into small pieces, followed by a 2-hour digestion at 37 °C with an enzymatic mix consisting of collagenase IV (Gibco) and DNase (Sigma) as previously described [13]. Cells were stained for flow cytometric analysis as described below.

2.2. Animals

LDLr$^{-/-}$ and apolipoprotein E (apoE)$^{-/-}$ mice were purchased from Jackson Laboratory (Sacramento, CA, USA) and bred in-house. Animals were kept under standard laboratory conditions; food and water were provided *ad libitum*. All animal work was performed in compliance with the Dutch government guidelines and the Directive 2010/63/EU of the European Parliament. Experiments were approved by the Ethics Committee for Animal Experiments of Leiden University.

2.3. Murine studies

For the pilot study to determine the dosing regimen of the CD8α-depleting antibody, male LDLr$^{-/-}$ mice (n = 3) were injected with 50 µg anti-CD8α antibody (clone 2.43, BioXcell, NH, USA). 100 µL of blood was drawn for a baseline measurement and at 1, 4 and 7 days post injection from the tail vein in EDTA containing tubes (Sarstedt) and subsequently analyzed by flow cytometry. For the CD8 depletion study, male LDLr$^{-/-}$ mice (n = 24) were fed a Western-type diet (WTD) containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, Witham, Essex, UK) for 10 weeks, and subsequently randomized into two groups based on age, weight, and plasma cholesterol levels. WTD feeding was continued for another 6 weeks combined with twice weekly i.p. injections of 50 µg rat IgG2b isotype control (clone LTF-2) or anti-CD8α antibody. During the 6-week injection period, depletion efficiency was monitored by drawing 100 µL of blood from the tail vein in EDTA containing tubes (Sarstedt) from n = 6 mice per group every two weeks the day after injection, alternating each week between the mice. At the end of the experiment, mice were sacrificed via subcutaneous injection with a mix of ketamine (100 mg/ml), sedazine (25 mg/ml) and atropine (0.5 mg/ml) and tissues were harvested after *in situ* perfusion using PBS. Total cholesterol levels were assessed using an enzymatic colorimetric assay (Roche Diagnostics).

To investigate FasL expression on CD8^{+} T-cells of atherosclerotic mice, 3 male apoE$^{-/-}$

mice of 65 weeks old were used, which display advanced atherosclerotic lesions at this age. Mice were sacrificed and spleens and aortas were harvested after *in situ* perfusion with PBS.

For the FasL blocking study, male LDLr$^{-/-}$ mice (n = 20) were fed a WTD for 12 weeks, and subsequently randomized into two groups based on age, weight, and plasma cholesterol levels. WTD feeding was continued for 2 weeks combined with i.p. injections on alternate days of 500 µg FasL-neutralizing antibody (clone MFL4 [14]) or Armenian hamster isotype control (Innovative Research, MI, USA). After a total of 8 injections, mice were sacrificed, and tissues were harvested after *in situ* perfusion with PBS.

2.4. Cell preparation and flow cytometry

Mice were sacrificed and blood, spleens, and aortas were harvested. WBCs were obtained by lysing the blood twice for 2 minutes with lysis buffer (0.15 M NH_4Cl,1 mM $KHCO_3$, 0.1 mM Na_2EDTA; pH 7.3). Single-cell suspensions of spleens were obtained by using a 70 µm cell strainer (Greiner Bio-One). Splenocytes were lysed for 1 minute with lysis buffer to obtain WBCs. Aortas were cleaned of perivascular fat and cut up into small pieces and digested by incubation with digestion mix (collagenase I 450 U mL^{-1}, collagenase XI 250 U ml^{-1}, DNAse 120 U ml^{-1} and hyaluronidase 120 U ml^{-1}; all Sigma-Aldrich) for 30 min at 37 °C while shaking and subsequently strained over a 70 µm strainer. A maximum of 200 000 cells was stained with the appropriate antibodies (Table S1). To stain apoptotic cells, Annexin V Apoptosis Detection Kit (eBioscience) was used according to manufacturer's protocol. For intracellular staining, cells were fixed and permeabilized by using an intracellular staining kit (eBioscience) according to manufacturer's protocol. Flow cytometry analyses were performed on a Beckman Coulter Cytoflex S or BD Biosciences Canto II and FlowJo software (Treestar).

2.5. Histological analysis

All hearts were embedded in O.C.T. compound (Sakura) and sectioned horizontally to the aortic axis and towards the aortic arch. Upon identification of the aortic root, defined by the trivalve leaflets, 10 µm sections were collected. Analysis of lesion size was performed on cryosections of the aortic root lesion stained with Oil-red O and haematoxylin (Sigma-Aldrich). Corresponding sections were stained with Sirius Red (Sigma-Aldrich) to determine collagen content and with Masson's Trichrome staining (Sigma-Aldrich) to determine the necrotic area. Plaque macrophages were stained immunohistochemically by using a monocyte/macrophage (MOMA)-2 antibody (1:1000 rat IgG2b, Serotec Ltd.) as a primary antibody, goat anti-rat IgG alkaline phosphatase conjugate (1:100; Sigma-Aldrich) as a secondary antibody, and nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as enzyme substrates. Furthermore, sections were stained with an antibody against CD4 (1:90 clone RM4-5, BD Biosciences), biotinylated rabbit anti-rat IgG (1:200, Vector) as a secondary antibody, and 3-amino-9-ethyl carbazole (Dako) for visualization. For VCAM-1 staining,

sections were incubated with CD106 antibody (1:100, BD Biosciences), followed by incubation with biotinylated rabbit anti-rat IgG (1:200, Vector) as a secondary antibody, and stained with 3-amino-9-ethyl carbazole (Dako). TUNEL staining was performed using the In Situ Cell Death Detection Kit, POD (Sigma-Aldrich). The average plaque size (in μm^2) was calculated from 5 sequential sections. For all other stainings, three subsequent sections displaying the highest plaque content per mouse were analyzed. All microscopic analyses were performed on a Leica DM-RE microscope using Leica QWin software and were blinded for independent analysis. The percentages of collagen, VCAM-1^+ cells, and macrophages in the atherosclerotic lesions were determined by dividing the area in μm^2 stained positive for collagen, VCAM-1 or MOMA-2 by the total lesion surface area, and calculated as a percentage. The percentage of necrosis was determined by dividing the acellular area by the total lesion surface area and again calculated as a percentage. The total number of $CD4^+$ T-cells or $TUNEL^+$ cells in each stained section were counted, and the average was divided by the total lesion surface area in order to obtain the number of $TUNEL^+$ or $CD4^+$ T-cells per mm^2 of lesion area.

2.6. Cell culture

$CD4^+$ and $CD8^+$ T-cells were isolated from splenocytes of a male $LDLr^{-/-}$ mouse of 18 weeks old using the $CD4^+$ and $CD8^+$ T-cell isolation kits (Miltenyi Biotec). Isolated cells were mixed in a 1:1 ratio and 0.5×106 total cells were plated out in 12-well plates (Greiner Bio-One) and cultured overnight at 37 °C and 5% CO_2 in RPMI 1640 medium containing 25 mM HEPES (Lonza) supplemented with 10% fetal calf serum (FCS), 60 µM β-mercaptoethanol (Sigma), 100 U mL^{-1} mix of penicillin/streptomycin (PAA), 1% non-essential amino acids (NEAA; Gibco), 1% sodium pyruvate (Sigma) and 2% L-glutamine (Lonza). The medium was supplemented with 1 µg mL^{-1} of anti-CD3 and anti-CD28 (ThermoScientific) and 10 µg mL^{-1} of either FasL-neutralizing antibody (clone MFL414) or Armenian hamster isotype control (Innovative Research, MI, USA).

2.7. Statistical analysis

Data are presented as individual dot plots with bars denoting the mean, the number of animals in each group is stated in the text. Data were tested for normal distribution and analyzed by using a two-way ANOVA, two-tailed Student's T-test or Mann-Whitney test, as appropriate. Statistical analysis was performed by using Prism (GraphPad). Probability values of $p < 0.05$ were considered significant.

3. Results

3.1. CD8$^+$ T-cell content negatively correlates with macrophage content in human atherosclerosis

With the progression of atherosclerosis, the total number of CD8$^+$ T-cells in the plaque increases [2]. However, other leukocyte populations may also increase in number. We sought to investigate whether there is a correlation between the number of these CD8$^+$ T-cells and other atherogenic cell types in human atherosclerosis. Nineteen endarterectomy samples were obtained from atherosclerosis patients and analyzed by flow cytometry for CD8$^+$ T-cells, CD4$^+$ T-cells and macrophages (Fig. 1A-C, for gating strategy, see Fig. S1). Interestingly, there was a significant inverse correlation between the percentage of CD8$^+$ T-cells and macrophages (p = 0.03, Fig. 1D), which could indicate that CD8$^+$ T-cells limit macrophage content in human atherosclerosis. Importantly, this correlation was not observed between the percentage of CD4$^+$ T-cells and macrophages (Fig. 1E), suggesting that a change in macrophage content does not lead to an increase in every T-cell subset and the correlation between CD8$^+$ T-cells and macrophages may have functional relevance.

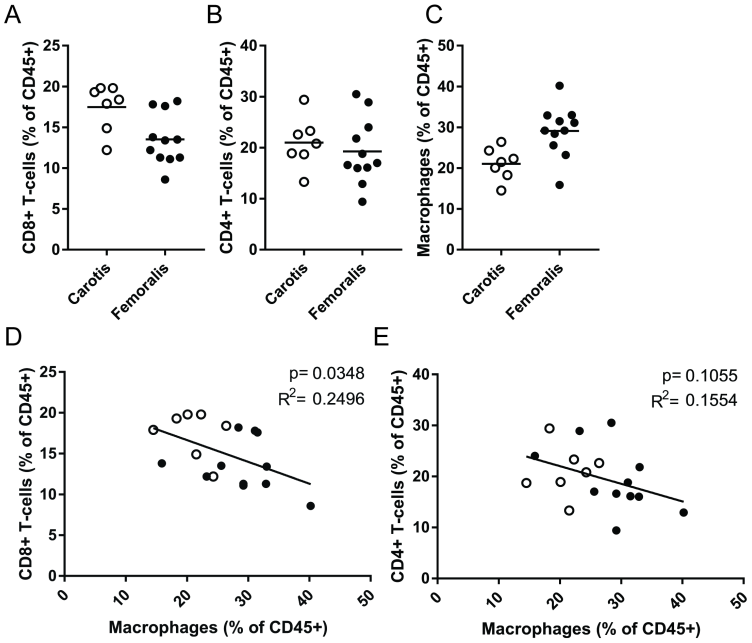

Figure 1: Inverse correlation between the percentages of CD8$^+$ T-cells and macrophages in human atherosclerotic lesions. Flow cytometry analysis of (A) CD8$^+$ T-cells (B) CD4$^+$ T-cells and (C) macrophages in endarterectomy samples from the arteria carotis (n = 7, open circles) or arteria femoralis (n = 12, closed circles) expressed as a percentage of live CD45$^+$ cells. Cells were gated as shown in Fig. S1 (D) Correlation between the percentage of CD8$^+$ T-cells and macrophages in all endarterectomy samples. (E) Correlation between the percentage of CD4$^+$ T-cells and macrophages in all endarterectomy samples. Significance was determined using linear regression analysis.

3.2. Lesion size is not affected by CD8$^+$ T-cell depletion in advanced stages of murine atherosclerosis

To further elucidate the role of CD8$^+$ T-cells in advanced atherosclerosis, we fed LDLr$^{-/-}$ mice a WTD for 10 weeks to establish lesions, followed by another 6 weeks of WTD combined with twice weekly administration of a CD8α-depleting antibody or an isotype control antibody (see Fig. S2A for the experimental setup). The dosing regimen was determined by a pilot experiment, in which 3 LDLr$^{-/-}$ mice were injected with 50 µg of CD8α-depleting antibody. CD8$^+$ T-cells were fully depleted for at least 4 days (Fig. S2B). As we saw a slight increase in the number of CD8$^+$ T-cells 7 days after injection of the anti-CD8 monoclonal antibody, we decided to inject the mice in the atherosclerosis experiment twice weekly. Administration of the CD8 depleting antibody in the atherosclerosis study resulted in successful depletion of CD8$^+$ T-cells in blood throughout the course of the experiment (Fig. S2C). At sacrifice, successful depletion was also observed in the spleen and aorta, whereas the CD8α^+dendritic cell population in the spleen was only slightly affected (Fig. S2D,E). We observed no differences in the percentages of NK cells, neutrophils or monocytes in the blood at sacrifice (Fig. S2F-H). Upon treatment with the CD8-depleting antibody, we did observe a percentual increase in CD4$^+$ T-cells within the T-cell population, as is to be expected, as well as a percentual increase of B-cells in the spleen. However, the absolute numbers of these cells were not different upon treatment (Fig. S2I-L). Depletion of CD8$^+$ T-cells in advanced atherosclerosis did not affect the aortic root lesion size as determined by Oil-red O staining (Fig. 2). We found no difference in body weight at any time during the treatment, nor did we find any differences in serum cholesterol levels (Fig. S2M,N).

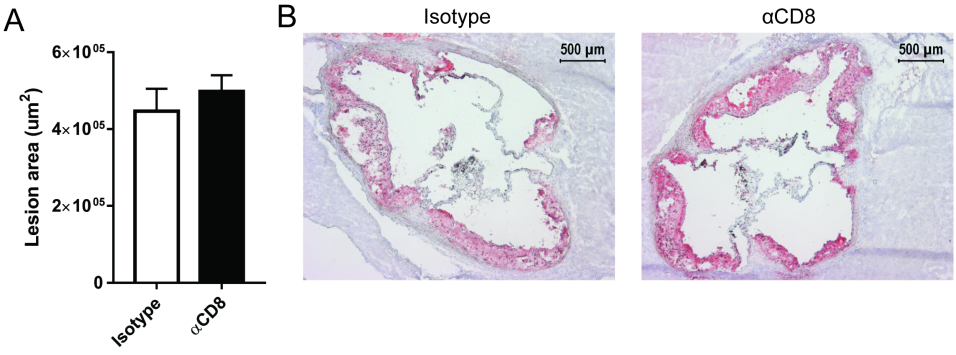

Figure 2: CD8$^+$ T-cell depletion does not affect lesion size in advanced atherosclerosis. (A) Quantification of lesion size in the aortic roots of LDLr$^{-/-}$ mice treated with CD8-depleting or isotype antibody by Oil-red O staining, n = 12 mice per group. Significance was determined using an unpaired T-test. (B) Representative images of ORO staining.

3.3. Advanced atherosclerotic lesions show decreased plaque stability upon CD8$^+$ T-cell depletion and increased inflammatory CD4$^+$ T-cells responses

Although lesion size was not significantly affected by CD8$^+$ T-cell depletion in advanced atherosclerosis, we investigated whether plaque stability and composition were altered in the aortic root lesions of these mice. The collagen content, assessed by Sirius Red staining, showed a significant decrease of 18% upon depletion of CD8$^+$ T-cells ($p = 0.02$, Fig. 3A,B). In addition, we found a 42% increase in necrotic core formation in the CD8$^+$ T-cell depleted group ($p = 0.04$, Fig. 3C,D). The content of total apoptotic cells in the lesions was not significantly different between both groups, as measured by TUNEL staining ($p = 0.48$, Fig. S3A). Regarding the monocyte/macrophage positive area, we observed a 27% increase upon CD8$^+$ T-cell depletion in the total area stained positive for MOMA-2 ($p = 0.045$, Fig. 3E,F). The most likely reasons for the increasing number of macrophages and decreased stability of the lesions after CD8$^+$ T-cell depletion would be an enhanced influx of monocytes or reduced cell death of macrophages in the lesion. Regarding monocyte influx, VCAM-1 is known to play an important role in monocyte adhesion upon the endothelium of the atherosclerotic lesion [15]. However, the expression of VCAM-1 in the aortic root endothelium was not found to be significantly different between both groups ($p = 0.14$, Fig. S3B). Furthermore, the percentage of monocytes in the blood did not show any differences between the two groups ($p = 0.86$, Fig. S3C). Based on these results, we cannot exclude effects of CD8$^+$ T-cell depletion on macrophage recruitment or adhesion. However, it is likely that other, lesion localized, mechanisms may be involved in the observed increase in lesional macrophage content.

Besides macrophages and CD8$^+$ T-cells, CD4$^+$ T-cells represent a third major leukocyte population in atherosclerotic lesions. Especially IFN-γ-producing Th1 cells have been associated with macrophage activation and plaque instability [16]. To determine whether CD4$^+$ T-cells play a role in destabilizing the plaques upon depletion of CD8$^+$ T-cells, we analyzed the presence and phenotype of CD4$^+$ T-cells in the lesions. We observed no difference in the number of CD4$^+$ T-cells in the aortic root lesions of these mice by immunohistochemistry ($p = 0.44$, Fig. 4A). However, we observed a skewing towards a more inflammatory Th1 phenotype in the aortic plaques of the CD8-depleted mice compared to the controls. The T-bet expression in CD4$^+$ T-cells in this group was significantly increased by 25% resulting in an over two-fold increase in the T-bet/GATA3 ratio ($p = 0.01$, Fig. 4B, Fig. S3D), indicating a shift from the Th2 towards the Th1 phenotype. Interestingly, this skewing of CD4$^+$ T-cell responses was not observed in the circulation (data not shown), whereas opposite trends were observed for T-bet expression ($p = 0.02$) and the T-bet/GATA3 ratio in the splenic compartment ($p = 0.09$, Fig. 4C, Fig. S3D). Total CD4$^+$ T-cell numbers in the spleen were not significantly different between both treatment groups ($p = 0.15$, Fig. 4D). Taken together, these results suggest a local, anti-inflammatory and lesion-stabilizing role of CD8$^+$ T-cells in advanced atherosclerosis.

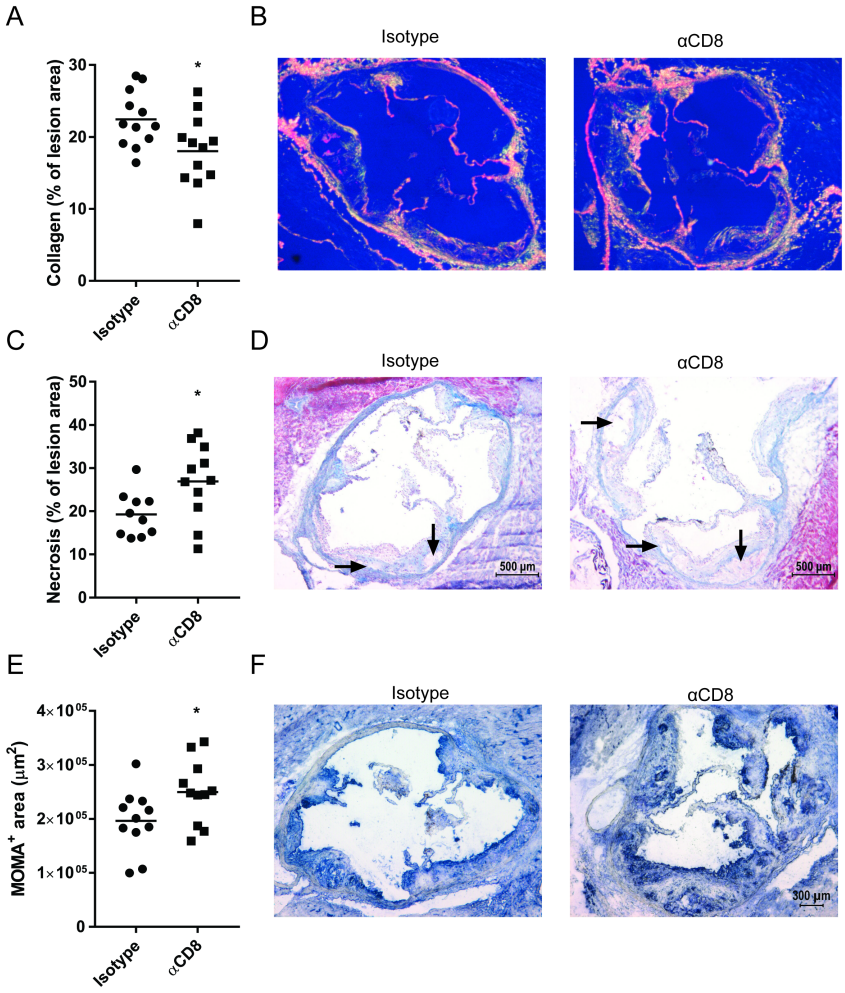

Figure 3: CD8$^+$ T-cell depletion in advanced lesions reduces plaque stability and increases necrosis and macrophage content. (A) Quantification of collagen content by Sirius Red staining in the aortic roots of LDLr$^{-/-}$ mice treated with CD8-depleting or isotype antibody. Significance was determined using a Mann-Whitney test. (B) Representative images of Sirius Red staining. (C) Quantification of necrosis in the aortic roots of the LDLr$^{-/-}$ mice. Significance was determined using an unpaired T-test (D) Representative images of the Masson's Trichrome staining, necrotic areas are indicated by arrows. (E) Macrophage quantification in the aortic roots of the LDLr$^{-/-}$ mice. (F) Representative images of MOMA-2 staining. n = 12 mice per group. Significance was determined using an unpaired T-test. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

3.4. FasL blockade increases inflammation in advanced atherosclerotic lesions

Although CD8$^+$ T-cells are foremost known as a pro-inflammatory cell type, various reports also support an immune regulatory role for these cells [11, 12, 17]. For instance, CD8$^+$ T-cells can kill dendritic cells in an antigen-specific, perforin-dependent man-

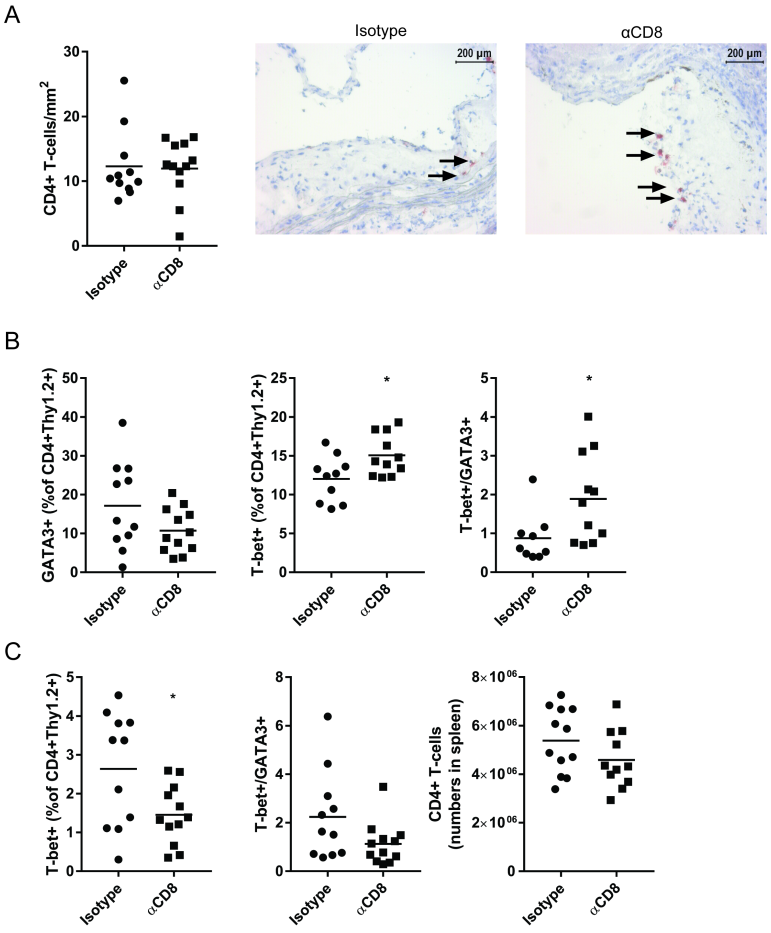

Figure 4: CD8$^+$ T-cell depletion in advanced lesions skews CD4$^+$ T-cell responses towards a more inflammatory phenotype specifically in the aortic microenvironment. (A) Quantification of CD4$^+$ T-cell staining per mm^2 of lesion in the aortic roots of LDLr$^{-/-}$ mice treated with CD8-depleting or isotype antibody, and representative images of the CD4 staining, arrows indicate CD4$^+$ cells. (B) Flow cytometry analysis of percentages of aortic CD4$^+$ T-cells expressing GATA3 and T-bet, as well as the T-bet$^+$/GATA3$^+$ ratio. (C) Flow cytometry analysis of the percentages of splenic CD4$^+$ T-cells expressing T-bet, as well as the T-bet$^+$/GATA3$^+$ ratio. (D) The total amount of CD4$^+$ T-cells in the spleens at the time of sacrifice. Cells were pregated on Live Thy1.2$^+$CD4$^+$ cells. n = 12 mice per group. All significance was determined using unpaired T-tests. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

ner [18] and can regulate T-cell homeostasis by killing activated T-cells via a Fas-FasL-mediated mechanism [19]. Interestingly, a deficiency in Fas on hematopoietic cells in LDLr$^{-/-}$ mice results in an enhanced inflammatory state [20] and a decrease in lesion stability [21], suggesting Fas-FasL interaction is an important immune regulatory pathway in the context of hypercholesteremia. As Th1 cells are more susceptible to FasL-induced apoptosis than Th2 cells [22], we hypothesized that the Th1 skewing effect we observed specifically in the lesions of CD8$^+$ T-cell depleted mice, was in part mediated

through the lack of FasL-induced apoptosis. Therefore, we first set out to determine whether $CD8^+$ T-cells in the lesions of advanced atherosclerotic mice express increased levels of FasL compared to their counterparts in the spleen. To this end, we isolated the spleens and aortas of mice with advanced atherosclerosis. Flow cytometry analysis confirmed that the mean fluorescence intensity (MFI) for FasL was 1.7-fold higher in the aortas compared to the spleens ($p = 0.008$, Fig. 5A, Fig. S4A). To assess whether FasL-mediated killing by $CD8^+$ T-cells specifically affects the Th1 $CD4^+$ T-cells in our model, $CD8^+$ and $CD4^+$ T-cells from $LDLr^{-/-}$ mice were isolated and stimulated *in vitro* in the presence of a blocking anti-FasL antibody or isotype control antibody and stained for apoptosis using Annexin V. Whereas apoptosis decreased only slightly and not significantly in Th2 cells upon anti-FasL treatment (1.5 fold, $p = 0.07$, Fig. 5B), there is a striking 4.1 fold decrease in apoptotic Th1 cells ($p = 0.0001$, Fig. 5C, Fig. S4B). This confirms previous work [22] and shows that FasL expressed on $CD8^+$ T-cells preferentially targets Th1 cells for apoptosis. Finally, we proceeded to block FasL activation in $LDLr^{-/-}$ mice in an advanced stage of lesion formation in order to assess whether this affected the $CD4^+$ T-cell responses and lesion development in a similar fashion as $CD8^+$ T-cell depletion. In agreement with the $CD8^+$ T-cell depletion study, treatment with anti-FasL antibody resulted in a significant increase in T-bet-expressing $CD4^+$ T-cells in the aortic lesion ($p = 0.04$, Fig. 5D) and not in the spleen (Fig. S4C), although GATA3 expressing $CD4^+$ T-cells were not changed ($p = 0.98$, Fig. 5E, Fig. S4D). We did observe a small, non-significant 1.3 fold increase in the T-bet/GATA3 ratio in the aortas of the treated mice ($p = 0.29$, Fig. 5F). Moreover, in agreement with the $CD8^+$ T-cell depletion study, we observed no changes in lesion size ($p = 0.09$, Fig. 5G), but immunohistochemical analysis of the aortic root lesions revealed a 69% increase in the monocyte/macrophage positive area upon treatment with the anti-FasL antibody ($p = 0.04$, Fig. 5H). Together, these data suggest that FasL-mediated interactions play an important immune regulatory role in atherosclerosis by decreasing Th1 $CD4^+$ T-cells and macrophages within the lesion.

4. Discussion

In this study, we show that $CD8^+$ T-cells may be protective in advanced stages of atherosclerotic lesion development. The negative correlation we observed between the percentages of $CD8^+$ T-cells and macrophages in human atherosclerosis indicates that $CD8^+$ T-cells may play a protective role by reducing plaque macrophage content. Additionally, in a murine model, we show that $CD8^+$ T-cells contribute to increased plaque stability in advanced atherosclerotic lesions, by restricting the accumulation of macrophages and pro-inflammatory Th1 cells. Importantly, we show that the effect of $CD8^+$ T-cells on Th1 cells is specific to the microenvironment of the lesion, as such effects are not observed in splenic tissue. Our results are in agreement with previously published studies, demonstrating that antigen-specific $CD8^+$ cells are protective against atherosclerosis by mounting a cytolytic response against antigen-presenting dendritic cells [11, 23]. Previous studies have shown that $apoE^{-/-}CD8^{-/-}$ mice show no difference in early or late atherosclerotic lesion development compared to $apoE^{-/-}$ mice [6]. Additionally, full body knockout of the antigen peptide transporter TAP1

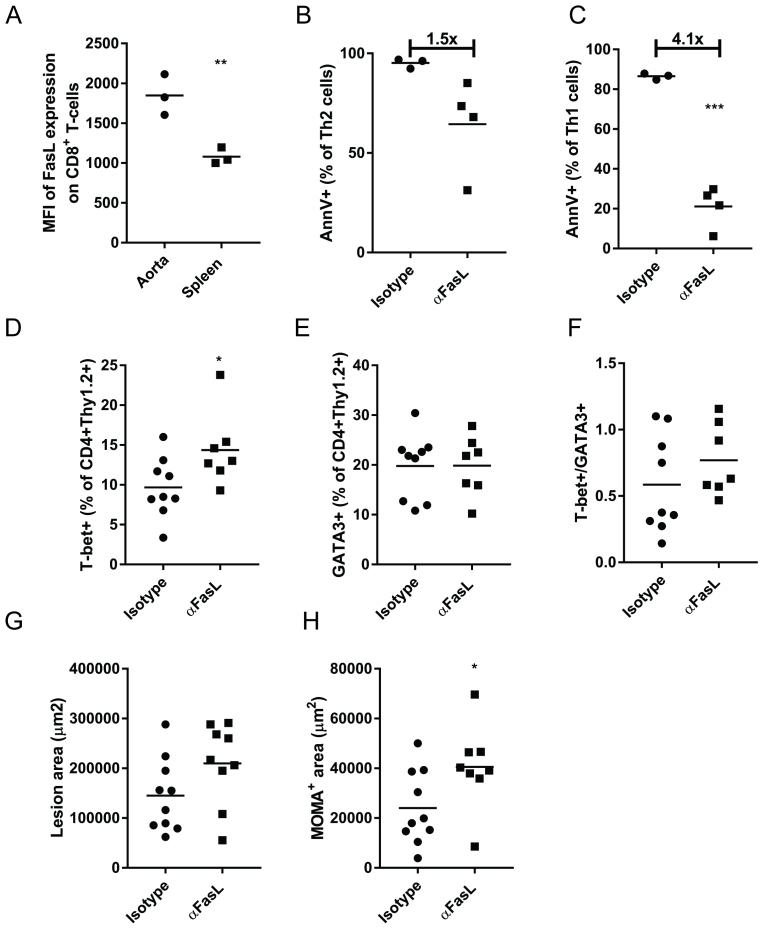

Figure 5: FasL blockade preferentially inhibits apoptosis of Th1 compared to Th2 CD4$^+$ T-cells and increases inflammatory CD4$^+$ T-cell responses in advanced atherosclerosis. (A) Flow cytometry analysis of FasL MFI on CD8$^+$ T-cells in single-cell suspensions obtained from the indicated organs of 3 male apoE$^{-/-}$ mice of 65 weeks old. (B-C) LDLr$^{-/-}$ derived CD4$^+$ and CD8$^+$ T-cells were cultured in a 1:1 ratio for 24h and stimulated with anti-CD3 and anti-CD28 antibodies in the presence of either anti-FasL or isotype antibody. Flow cytometry analysis of Annexin V staining on Thy1.2$^+$CD4$^+$GATA3$^+$ (B) and Thy1.2$^+$CD4$^+$T-bet$^+$ (C) cells, n = 4. (D-F) Flow cytometry analysis of aortic cells derived from LDLr$^{-/-}$ mice treated with anti-FasL or isotype antibody. Percentages of aortic CD4$^+$ T-cells expressing T-bet (D) and GATA3 (E), as well as the T-bet$^+$/GATA3$^+$ (F) ratio. Cells were pregated on Live Thy1.2$^+$CD4$^+$ cells. (G) Quantification of lesion size in the aortic roots of the LDLr$^{-/-}$ mice treated with anti-FasL or isotype antibody by Oil-red O staining. (H) Macrophage quantification in the aortic roots of the LDLr$^{-/-}$ mice. n = 9 in αFASL group, n = 8 in isotype group. All significance was determined using unpaired T-tests $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

in apoE$^{-/-}$ mice, resulting in deficient MHC-I antigen presentation, did not affect lesion development at either early or late stages of atherosclerosis [7]. However, these mutations are able to affect other cell types besides CD8$^+$ T-cells, and therefore do not provide conclusive evidence about CD8$^+$ T-cell function in atherogenesis. Other work has shown pro-atherogenic roles for CD8$^+$ T-cells in atherosclerosis, based on reduced

monopoiesis in the absence of $CD8^+$ T-cells [8]. In contrast to the data presented here, however, the aforementioned study focused on early stages of atherosclerotic lesion development, which suggests the role of $CD8^+$ T-cells may depend on the stage of atherogenesis. Indeed, another study investigating CD8α- and CD8β-depletion on initial lesion development in $apoE^{-/-}$ mice also showed reductions in lesion area, macrophage accumulation and necrotic core formation [9]. Together, this indicates a pro-atherogenic role for $CD8^+$ T-cells in initial atherosclerosis, whereas our data suggest a protective role for these cells in advanced atherosclerosis. In agreement with our findings, C57BL/6J mice that are deficient for MHC class I demonstrated a threefold increase in atherosclerotic lesion area compared to WT mice after 15 weeks on an atherogenic diet [10], suggesting a protective role for $CD8^+$ T-cells. Several regulatory $CD8^+$ T-cell subsets have been described that could exert protective effects on lesion development [12, 17], but as we used a CD8α-depleting antibody, we depleted all $CD8^+$ T-cell subsets and were therefore unable to identify which $CD8^+$ T-cell subset is responsible for the protective effects observed here.

The lesion size in advanced stages of atherosclerosis was not affected by $CD8^+$ T-cell depletion in our study. As we only started depleting the $CD8^+$ T-cells after 10 weeks of WTD feeding, upon which lesions are already established, we expected the treatment to mainly affect lesion composition. Indeed, we did observe a decreased plaque stability upon depletion in this stage. Specifically, $CD8^+$ T-cell depletion decreased collagen content, whereas it increased necrotic core formation and plaque macrophages. We observed a microenvironment-specific skewing of $CD4^+$ T-cells towards the Th1 phenotype, which may explain the observed effects on plaque stability. Th1 cells are known to produce high levels of IFN-γ, which has been shown to inhibit collagen synthesis by vascular smooth muscle cells [24] and may explain the reduced collagen content observed here. Indeed, vaccination against IL-12, a cytokine known to favor the development of Th1 cells, was previously shown to inhibit atherosclerosis development and promote lesion stability via a Th1/Th2 switch and the associated reduction in IFN-γ levels [25]. A Th1 shift and an increase IFN-γ may explain why we observed an increase in plaque resident macrophages upon CD8 depletion. Monocyte transmigration into atherosclerotic lesions is mediated by upregulation of IFN-γ inducible VCAM-1 and ICAM-1 [26]. Even though we found no increases in VCAM-1 expression at the moment of sacrifice, we cannot exclude regulation of these adhesion molecules at earlier time points during the experiment. Furthermore, we have not measured ICAM-1 expression, which may have contributed to the increased MOMA-2 staining observed in this study. Alternatively, the increased macrophage accumulation may be caused by decreased apoptosis of macrophages, however, we did not observe any changes in the number apoptotic ($TUNEL^+$) cells in lesions of $CD8^+$ T-cell depleted mice. Importantly, we provide data suggesting a direct role of $CD8^+$ T-cells in the regulation of Th1 $CD4^+$ T-cells and macrophages through Fas-FasL-mediated apoptosis. Fas-FasL interaction is a major contributor to apoptosis of activated $CD4^+$ T-cells, a process referred to as activation-induced cell death [27]. We propose that $FasL^+CD8^+$ T-cells may be able to regulate $CD4^+$ T-cell responses via Fas-FasL-induced apoptosis of Th1 cells. Fas expression on bone marrow-derived cells has been shown to play a protective role in atherosclerosis development as bone marrow transplantation of cells derived

from Fas-mutant lpr mice into LDLr$^{-/-}$ mice resulted in less fibrous lesions compared to transplantation with WT bone marrow, suggesting Fas expression contributes to a more stable lesion phenotype [21]. Here, we show that CD8$^+$ T-cells within atherosclerotic lesions express higher levels of FasL than their counterparts in the spleen, suggesting that the atherosclerotic microenvironment may affect CD8$^+$ T-cell phenotype and function. Th1 cells have an increased susceptibility to FasL-induced cell death, as they express lower levels of Fas-associated phosphatase 1, which plays an important role in inhibiting FasL-induced cell death by attenuating Fas export to the cell surface [22, 28]. Indeed, we observed that upon *in vitro* treatment of activated LDLr$^{-/-}$ derived CD4$^+$ and CD8$^+$ T-cells with a FasL-neutralizing antibody, the decrease in apoptotic cells was much larger in the Th1 subset compared to the Th2 subset. Upon *in vivo* treatment of atherosclerotic mice with a neutralizing FasL antibody, we observed a similar skewing of CD4$^+$ T-cell subsets in favor of Th1 CD4$^+$ T-cells as we did in CD8-depleted mice. Although this *in vivo* experiment cannot rule out contributions of other FasL$^+$ cell types; in light of the upregulation of FasL on CD8$^+$ T-cells and the increased susceptibility of Th1 cells to FasL-induced cell death these data suggest that FasL is an important effector molecule for CD8$^+$ T-cells to limit Th1 accumulation in the plaque.

Alternatively to direct CD4$^+$ T-cell inhibition, indirect effects on CD4$^+$ T-cell skewing can be exerted via cytolytic killing of macrophages by CD8$^+$ T-cells. Inflammatory macrophages are known to secrete cytokines that can recruit CD4$^+$ T-cells toward the lesion site and skew them towards the Th1 phenotype [29, 30]. CD8$^+$ T-cells have long been established to have the capacity to kill virus- or bacteria-infected APCs [31]. Inflammatory stimuli such as IFN-γ are able to increase Fas expression on cultured macrophages, which increases their susceptibility to Fas-mediated apoptosis [32]. Additionally, free cholesterol loading in WT macrophages was previously shown to result in caspase-induced apoptosis, which is much less pronounced in either gld or lpr macrophages [33]. This suggests that macrophage foam cells could be killed by CD8$^+$ T-cells in a Fas-FasL mediated fashion. As CD8$^+$ T-cell depletion in our study resulted in an increased lesional macrophage content, CD8$^+$ T-cells could potentially regulate lipid-loaded macrophage numbers in advanced atherosclerosis directly. Interestingly, upon treatment with an anti-FasL antibody, we observed an increase in lesion macrophage content as well, suggesting that FasL-induced apoptosis of macrophages by CD8$^+$ T-cells may contribute to their protective effect against atherosclerosis. Notably, we observed a link between CD8$^+$ T-cell percentages and the percentage of macrophages in human lesions, suggesting that the regulatory role of CD8$^+$ T-cells we describe here for a murine model of atherosclerosis, may hold true in humans as well.

Finally, our results indicate a microenvironment-specific role for CD8$^+$ T-cells in controlling Th1 responses in atherosclerotic lesions, as we observed this effect only in the aorta and not in the blood or spleen. This finding illustrates the importance of investigating local immune responses, in addition to systemic immune responses. We hypothesize that the atherosclerotic microenvironment contains many lipid-derived and inflammatory stimuli that alter the CD8$^+$ T-cell phenotype specifically at this site. A recent clinical trial with IL-1β-neutralizing antibodies (canakinumab) showed that systemic anti-inflammatory responses significantly reduce cardiovascular events [34], al-

though administration of this drug could also cause neutropenia and was associated with fatal infection. As the anti-inflammatory effect of $CD8^+$ T-cells appears to act locally, expanding these T-cells may provide an interesting strategy to lower inflammation associated with atherosclerosis without unwanted systemic immune suppression.

5. Conclusion

In conclusion, these *in vivo* experiments demonstrate a protective effect of $CD8^+$ T-cells in advanced atherosclerotic lesions via a reduction of macrophages and Th1 cells and show an immune modulatory role for FasL. The protective effect of $CD8^+$ T-cells may be exploited by stimulating $CD8^+$ T-cell responses in advanced stages of atherogenesis, which could translate into the suppression of atherosclerosis in humans.

Funding

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Supplementary information

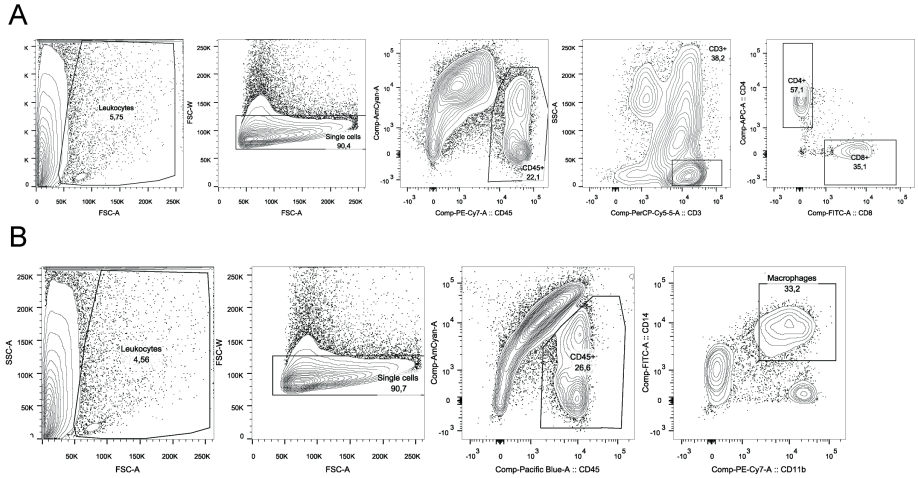

Figure S1: Flow cytometry gating strategy for human atherosclerotic lesions. (A) Flow cytometry gating strategy for determining the percentage of $CD4^+$ and $CD8^+$ T-cells in endarterectomy samples, cells were gated on leukocytes, $CD45^+$, $CD3^+$ and on $CD4^+$ or $CD8^+$. (B) Flow cytometry gating strategy for determining the percentage of macrophages cells in endarterectomy samples, cells were gated on leukocytes, $CD45^+$, and $CD14^+CD11b^+$. $CD45^+$ cells were gated against an empty channel in order to exclude autofluorescent cells.

Table S1: Antibodies used for flow cytometric analysis.

Antibody	Fluorochrome	Clone	Supplier
Anti-Mouse CD3	PerCP	145-2C11	BD Biosciences
Anti-Mouse CD4	Efluor450	GK1.5	eBioscience
Anti-Mouse CD4	PEcy5	GK1.5	eBioscience
Anti-Mouse CD45.2	APC	104	eBioscience
Anti-Mouse CD8	FITC	53-6.7	eBioscience
Anti-Mouse CD8	PerCP	53-6.7	BD Biosciences
Anti-Mouse CD8	PE-TR	5H10	ThermoScientific
Anti-Mouse CD11b	Efluor450	M1/70	eBioscience
Anti-Mouse FasL	Fitc	MFL3	eBioscience
Anti-Mouse GATA3	PE	TWAJ	eBioscience
Anti-Mouse Ly-6C	PerCP/cy5.5	HK1.4	eBioscience
Anti-Mouse Ly-6G	PE	1A8	BD Biosciences
Anti-Mouse NK1.1	FITC	PK136	BD Biosciences
Anti-Mouse T-bet	PEcy7	4B10	eBioscience
Anti-Mouse T-bet	Efluor660	4B10	eBioscience
Anti-Mouse Thy1.2	PerCP/cy5.5	53-2.1	Biolegend
Anti-Mouse Thy1.2	Pecy7	53-2.1	eBioscience
Anti-Human CD3	PerCP/cy5.5	OKT3	eBioscience
Anti-Human CD45	PEcy7	2D1	eBioscience
Anti-Human CD45	Efluor450	2D1	eBioscience
Anti-Human CD8	FITC	SK1	eBioscience
Anti-Human CD4	APC	RPA-T4	Biolegend
Anti-Human CD14	FITC	61D3	eBioscience
Anti-Human CD11b	PEcy7	ICRF44	eBioscience
Fixable Viability Dye	Amcyan	-	eBioscience
Fixable Viability	Efluor780	-	eBioscience

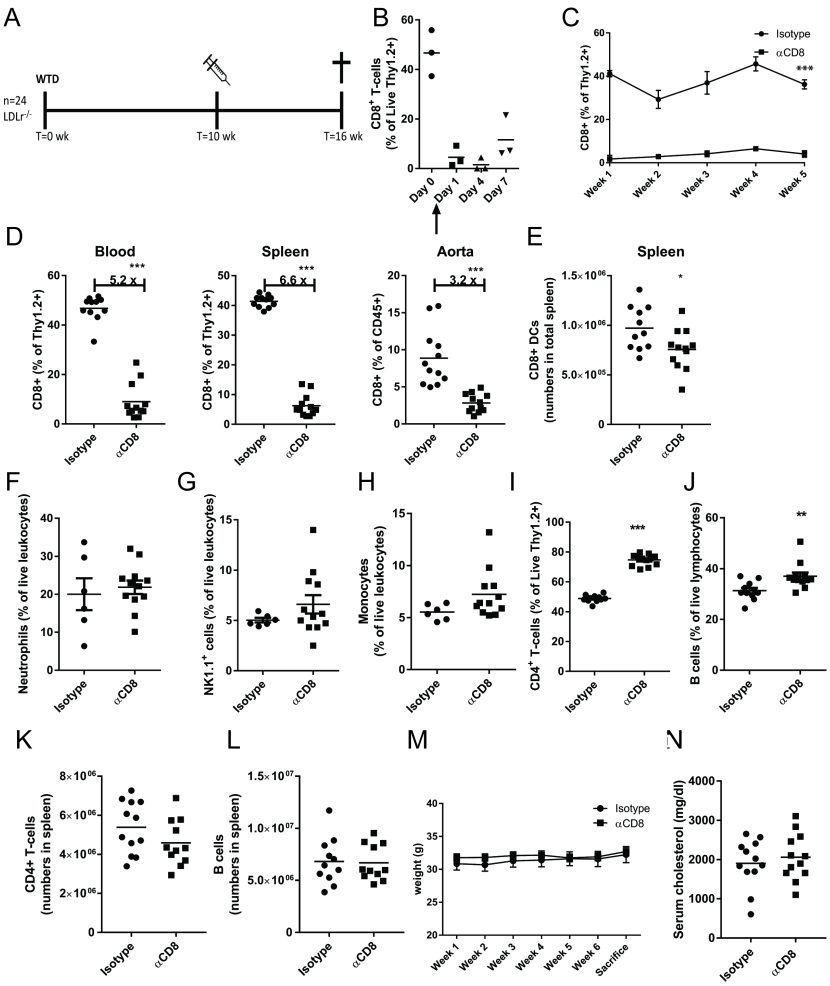

Figure S2: Treatment with CD8α-depleting antibody results in a significant reduction in the number of CD8$^+$ T-cells in various tissues (A) Experimental setup of the CD8 depletion study. 24 male LDLr$^{-/-}$ mice were fed a Western-type diet for 10 weeks to establish lesions, followed by 6 weeks of diet combined with twice weekly i.p. injections of 50 µg isotype (n = 12) or anti-CD8 (n = 12) antibody. (B) Percentage of CD8$^+$ T-cells in the blood of 3 LDLr$^{-/-}$ mice injected with 50 µg of CD8-depleting antibody as measured in the blood over 7 days, arrow indicates injection time point. (C) Analysis of percentages of CD8$^+$ T-cells in blood lysates obtained each week during the injection period. n = 6 mice per group were sampled at each time point. Cells were pregated on Live Thy1.2$^+$ cells. Significance was determined using a two-way ANOVA. (D) Analysis of percentages of CD8$^+$ T-cells in single-cell suspensions obtained from the indicated organs at the time of sacrifice, and fold decrease between the groups, pregated on Live Thy1.2$^+$ cells. Significance was determined using unpaired T-tests. (E) Total number of CD8$^+$ dendritic cells in the spleen at the time of sacrifice. Significance was determined using an unpaired T-test. (F-G) frequencies of NK1.1$^+$ cells (F), neutrophils (G, gated as NK1.1$^-$Ly6G$^+$) and monocytes (H, gated as NK1.1$^-$Ly6G$^-$CD11b$^+$) in the blood of the LDLr$^{-/-}$ mice at the time of sacrifice. Significance was determined using unpaired T-tests. (I-L) Percentages and numbers of CD4$^+$ T-cells (I,K gated as % of Thy1.2$^+$ live lymphocytes) and B-cells (J,L gated as percentage of live lymphocytes) in the spleens of the LDLr$^{-/-}$ mice at the time of sacrifice. Significance was determined using unpaired T-tests. (M) Body weights over time during the period of injection with CD8-depleting antibody or isotype control. Significance was determined using a two-way ANOVA. (N) Serum cholesterol levels at the time of sacrifice. Significance was determined using an unpaired T-test. Data are presented as mean ± SEM or as individual dot plots with bars denoting the mean. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

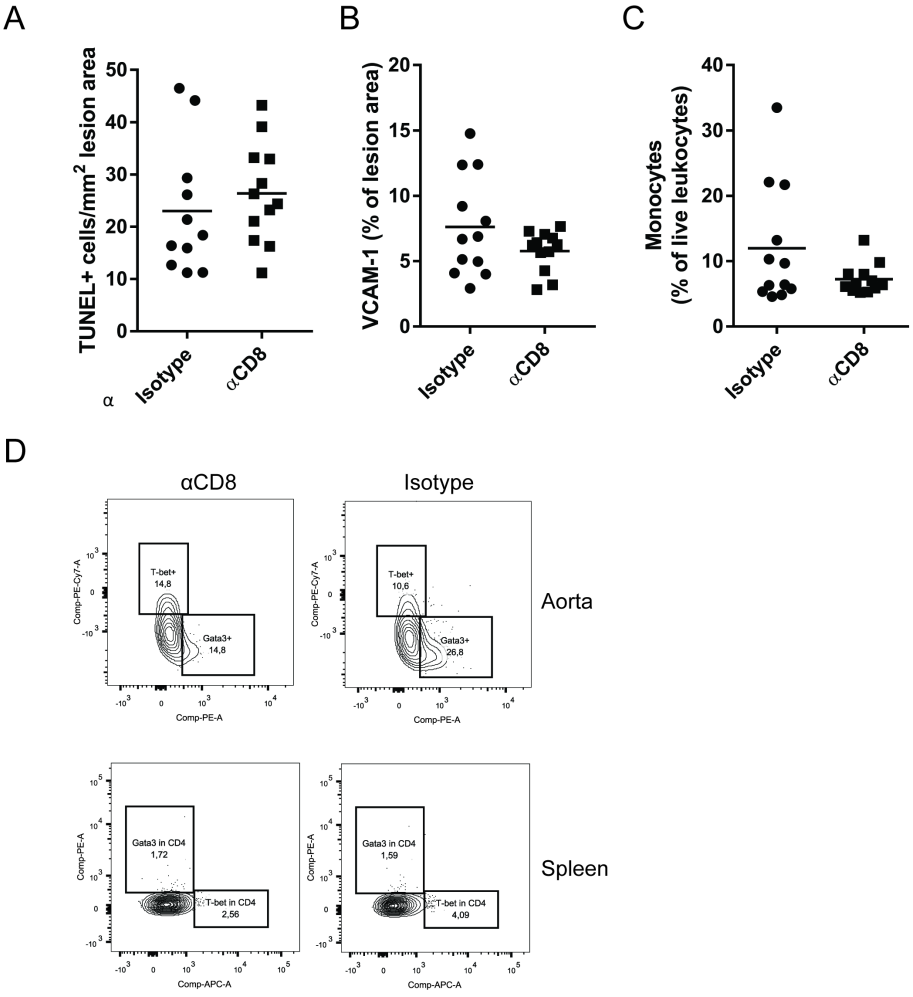

Figure S3: CD8 depletion in advanced lesions does not affect TUNEL staining, VCAM-1 expression or monocytes in the blood. (A) Quantification of TUNEL positive cells per mm^2 of lesion in the aortic roots at the time of sacrifice. (B) Quantification of VCAM-1 positive area in the endothelium of the aortic roots of $LDLr^{-/-}$ mice at the time of sacrifice. (C) Percentage of monocytes in the blood at the time of sacrifice. Monocytes were gated as Viable, $NK1.1^{-}Ly6G^{-}CD11b^{+}$ cells. Data are presented as individual dot plots with bars denoting the mean. n = 12 mice per group. All significance was determined using unpaired T-tests. (D) Representative flow cytometry plots of GATA3 and T-bet staining on aortic and splenic samples of anti-CD8 or isotype antibody-treated $LDLr^{-/-}$ mice. Cells were pregated on Live $Thy1.2^{+}CD4^{+}$ cells. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

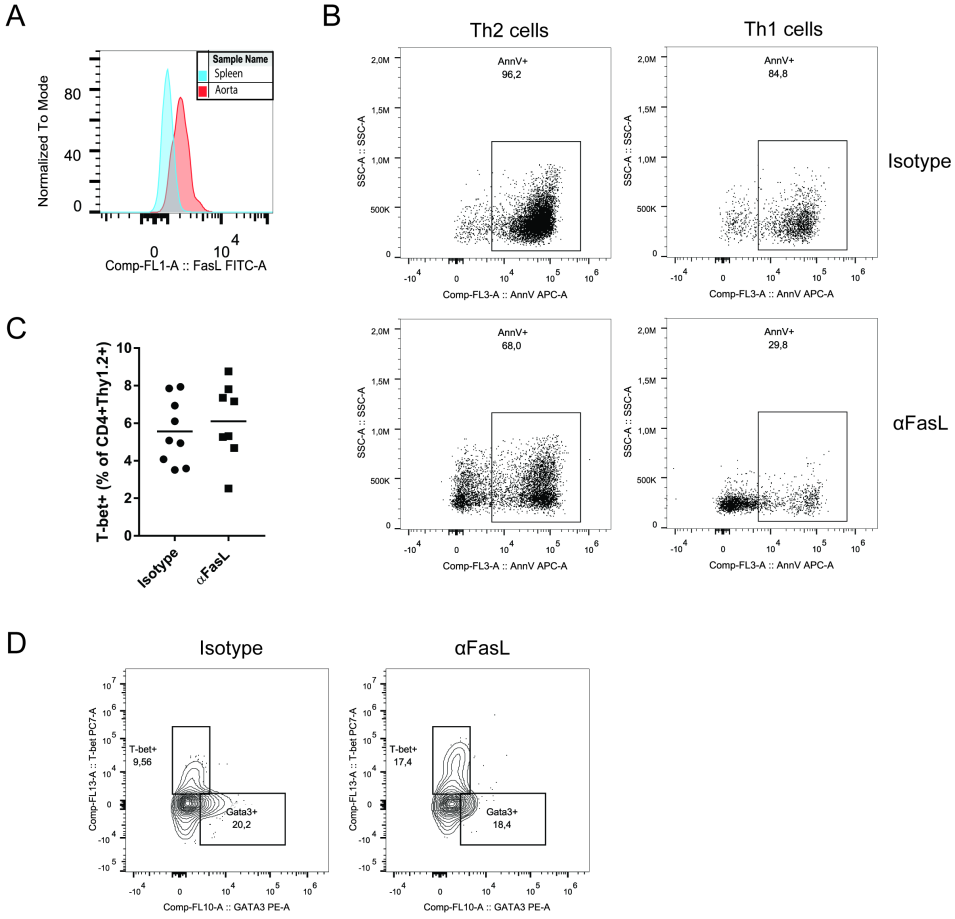

Figure S4: Representative flow cytometric images of FasL expression in aged apoE$^{-/-}$ mice, of annexin V staining on Th1 and Th2 cells and of T-bet and GATA3 staining in the anti-FasL experiment. (A) Representative histogram plots of FasL MFI on CD8^{+} T-cells in single-cell suspensions obtained from the indicated organs of 3 male apoE$^{-/-}$ mice of 65 weeks old. Cells were pregated on Live CD3^{+}CD8^{+} cells (B) LDLr$^{-/-}$ derived CD4^{+} and CD8^{+} T-cells were cultured in a 1:1 ratio for 24h and stimulated with anti-CD3 and anti-CD28 antibodies in the presence of either anti-FasL or isotype antibody. Representative flow cytometry plots of Annexin V expression on Th2 and Th1 cells, pregated on Live Thy1.2^{+}CD4^{+}GATA3^{+} or Live Thy1.2^{+}CD4^{+}T-bet^{+} cells, respectively. (C) Flow cytometry analysis of percentages of splenic CD4^{+} T-cells expressing T-bet upon treatment with anti-FasL antibody or isotype control antibody, cells were pregated on Live Thy1.2^{+}CD4^{+} cells. Significance was determined using an unpaired T-test. (D) Representative flow cytometry plots of GATA3 and T-bet staining on aortic samples of anti-FasL or isotype antibody-treated LDLr$^{-/-}$ mice. Cells were pregated on Live Thy1.2^{+}CD4^{+} cells, n = 9 in αFASL group, n = 8 in isotype group.