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# 4

## **CD39 identifies a microenvironment-specific anti-inflammatory CD8<sup>+</sup> T-cell population in atherosclerotic lesions**

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## ABSTRACT

**Background and aims** CD8<sup>+</sup> T-cells have been attributed both atherogenic and atheroprotective properties, but analysis of CD8<sup>+</sup> T-cells has mostly been restricted to the circulation and secondary lymphoid organs. The atherosclerotic lesion however, is a complex microenvironment containing a plethora of inflammatory signals, which may affect CD8<sup>+</sup> T-cell activation. Here, we address how this environment affects the functionality of CD8<sup>+</sup> T-cells.

**Methods and Results** We compared the cytokine production of CD8<sup>+</sup> T-cells derived from spleens and enzymatically digested aortas of apoE<sup>-/-</sup> mice with advanced atherosclerosis by flow cytometry. Aortic CD8<sup>+</sup> T-cells produced decreased amounts of IFN- $\gamma$  and TNF- $\alpha$  compared to their systemic counterparts. The observed dysfunctional phenotype of the lesion-derived CD8<sup>+</sup> T-cells was not associated with classical exhaustion markers, but with increased expression of the ectonucleotidase CD39. Indeed, pharmacological inhibition of CD39 in apoE<sup>-/-</sup> mice partly restored cytokine production by CD8<sup>+</sup> T-cells. Using a bone-marrow transplantation approach, we show that TCR signaling is required to induce CD39 expression on CD8<sup>+</sup> T-cells in atherosclerotic lesions. Importantly, analysis of human endarterectomy samples showed a strong microenvironment-specific upregulation of CD39 on CD8<sup>+</sup> T-cells in the plaques of human patients compared to matched blood samples.

**Conclusion** Our results suggest that the continuous TCR signaling in the atherosclerotic environment in the vessel wall induces an immune regulatory CD8<sup>+</sup> T-cell phenotype that is associated with decreased cytokine production through increased CD39 expression in both a murine atherosclerotic model and in atherosclerosis patients. This provides a new understanding of immune regulation by CD8<sup>+</sup> T-cells in atherosclerosis.

## 1. Introduction

Atherosclerosis is a chronic inflammatory disease, characterized by the buildup of arterial plaques that contain both lipids and inflammatory cells. Among these cells are the CD8<sup>+</sup> T-cells, whose numbers have been shown to increase as the lesions progress towards more advanced stages [1]. However, the exact function of CD8<sup>+</sup> T-cells in atherosclerosis remains debated [2]. On the one hand, CD8<sup>+</sup> T-cells can contribute to monocyte recruitment [3] and increased plaque vulnerability [4], suggesting a pro-atherogenic role for these cells. On the other hand, CD8<sup>+</sup> T-cells can mount a cytolytic response against pro-atherogenic dendritic cells [5] and follicular helper T-cells [6], thereby limiting atherosclerosis development. Recently, we have shown a protective role for CD8<sup>+</sup> T-cells in the clinically relevant advanced stages of atherosclerosis [7]. Interestingly, we observed a microenvironment-specific skewing of CD4<sup>+</sup> T-cells upon CD8 depletion, suggesting that there is an interaction between CD8<sup>+</sup> T-cells and the local environment in the plaque.

The atherosclerotic lesion comprises a complex immunological environment. Cholesterol accumulates in the arterial intima in the form of low-density lipoproteins (LDLs). The excess of cholesterol cannot be cleared and becomes oxidized by various enzymes, forming oxidized LDL (oxLDL). This, in turn, is able to activate endothelial cells and drive the recruitment of inflammatory cells into the lesion. Many chemokines have been associated with atherosclerosis, among which are CCL2, CX3CL1, and CCL5 [8, 9]. The immune cells thus recruited to the plaque by these chemokines release an array of cytokines such as IFN- $\gamma$ , TNF- $\alpha$ , and interleukin (IL)-12, which drives atherogenesis, as well as atheroprotective cytokines such as IL-10, IL-13 and transforming growth factor  $\beta$  [10]. Furthermore, antigen presenting cells (APCs) are able to process the intraplaque oxLDL and present oxLDL-derived antigens to induce adaptive immunity. Under the hyperlipidemic conditions in the lesion, APC migration to peripheral tissue is reduced, resulting in systemic as well as local T-cell activation [11]. Furthermore, cell death within the lesion due to the ongoing inflammation results in the release of damage-associated molecular patterns, which further drive the inflammatory response [12].

In this study, we aimed to determine how the aforementioned complex microenvironment in the atherosclerotic lesion affects local CD8<sup>+</sup> T-cells. We used the apolipoprotein E (apoE) knockout mouse model, which spontaneously develops atherosclerosis on a chow diet in response to increased plasma cholesterol levels. In these mice, we demonstrate that CD8<sup>+</sup> T-cells derived from aortic lesions show a dysfunctional phenotype, characterized by impaired cytokine production when compared to their counterparts in the spleen. This CD8<sup>+</sup> T-cell dysfunction was associated with an increased expression of the ectonucleotidase CD39. We further show that CD39 expression was induced by TCR signaling, and that pharmacological inhibition of CD39 could partially reverse the observed phenotypical changes of CD8<sup>+</sup> T-cells in the atherosclerotic microenvironment. Finally, we observed microenvironment-specific CD39 expression on CD8<sup>+</sup> T-cells derived from human atherosclerosis patients, indicating that the murine results may be translated to a clinical setting.

## 2. Materials and methods

### 2.1. Human studies

Plaques from the carotid artery were obtained after endarterectomy from 22 anonymous individuals. Matched blood samples were obtained before surgery from 12 of these patients. The patients underwent endarterectomy surgery between December 2017 and April 2018 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 are in accordance with the declaration of Helsinki regarding ethical principles for medical research involving human subjects (METC registration number 17-046). Single-cell suspensions were obtained from human plaques as described previously [13] (see supplementary data for a full description).

### 2.2. Animals

C57Bl/6, LDL receptor (LDLr)<sup>-/-</sup> and apoE<sup>-/-</sup> 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*. In order to develop advanced atherosclerotic lesions in apoE<sup>-/-</sup> mice, animals were kept on a chow diet for 30-49 weeks before analysis of T-cell content in the lesion. Upon sacrifice, mice were subcutaneously anesthetized with an injection mix of ketamine (100mg/mL), sedazine (25mg/mL) and atropine (0.5mg/mL). 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. Bone marrow transplantation experiment

Bone marrow transplantation was performed as detailed in the supplementary data.

### 2.4. *In vitro* CD39 blockade

Splenocytes were derived from a 31 week-old male apoE<sup>-/-</sup> mouse. To obtain WBCs, splenocytes were lysed for 1 minute at room temperature in lysis buffer (0.15 M NH<sub>4</sub>Cl, 1 mM KHCO<sub>3</sub>, 0.1 mM Na<sub>2</sub>EDTA; pH 7.3). CD8<sup>+</sup> T-cells were isolated using the CD8<sup>+</sup> T-cell isolation kit (Miltenyi Biotec) according to the manufacturer's protocol. Cells were cultured for 24 hours in the presence of 0, 25, 50 or 100 μM of the CD39 inhibitor sodium metatungstate (POM-1, Tocris) in RPMI supplemented with fetal calf serum (10%, PAA), L-glutamine (2%, Lonza), penicillin/streptomycin (1%, PAA), sodium pyruvate (1%, Sigma-Aldrich) and β-mercaptoethanol (60 μM, Sigma-Aldrich) at 37 °C and 5% CO<sub>2</sub>.



## 2.5. *In vivo* CD39 blockade

To study the effect of CD39 blockade *in vivo*, apoE<sup>-/-</sup> mice with advanced atherosclerotic lesions (38-47 weeks old) were injected i.p. with 10mg/kg of POM-1 (Tocris) or with sterile PBS as a control (n = 5/group). After 24 hours, mice were sacrificed as described above.

## 2.6. Flow cytometry

Flow cytometry was performed as detailed in the supplementary data.

## 2.7. Statistical analysis

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

# 3. Results

## 3.1. Decreased cytokine production by CD8<sup>+</sup> T-cells in atherosclerotic lesions is associated with an upregulation of CD39 expression

Although the presence of CD8<sup>+</sup> T-cells in atherosclerosis has been established, the exact phenotype of these cells within the lesional microenvironment is as of yet unknown. Here, we examined the difference in phenotype between CD8<sup>+</sup> T-cells derived from aortic lesions and their counterparts in the spleen in advanced atherosclerosis using the murine apoE<sup>-/-</sup> model. Upon 2 hour stimulation of these cells with PMA and ionomycin, we observed a striking 46% decrease in the percentage of IFN- $\gamma$  producing CD8<sup>+</sup> T-cells (Fig. 1A,C) and a 37% decrease in the percentage of TNF- $\alpha$  producing CD8<sup>+</sup> T-cells (Fig. 1B,D) in the lesion compared to the spleen. This decreased ability to produce cytokines could indicate that the CD8<sup>+</sup> T-cells derived from the lesion are exhausted. Nonetheless, we found no difference in the expression of the classical exhaustion markers PD-1 (Fig. 1E) and CTLA4 (Fig. 1F). However, using flow cytometry, we did observe a 9-fold increased expression of the ectonucleotidase CD39 on lesion-derived compared to splenic CD8<sup>+</sup> T-cells (Fig. 1G, 1H), which could not be explained by a different ratio of effector and central memory populations in aorta and spleen (Fig. S1A,B). This enzyme hydrolyzes extracellular ATP into ADP, which can further be converted into adenosine by the ectonucleotidase CD73. Interestingly, aortic CD8<sup>+</sup> T-cells show a slightly lower expression of CD73 compared to splenic CD8<sup>+</sup> T-cells (Fig. S1C), which may reflect their activation status [14]. Interestingly, CD39 has previously been reported to characterize exhausted CD8<sup>+</sup> T-cells during chronic infection [15]. We next set out to investigate whether CD39-expressing CD8<sup>+</sup> T-cells produce fewer cytokines. Unexpected-

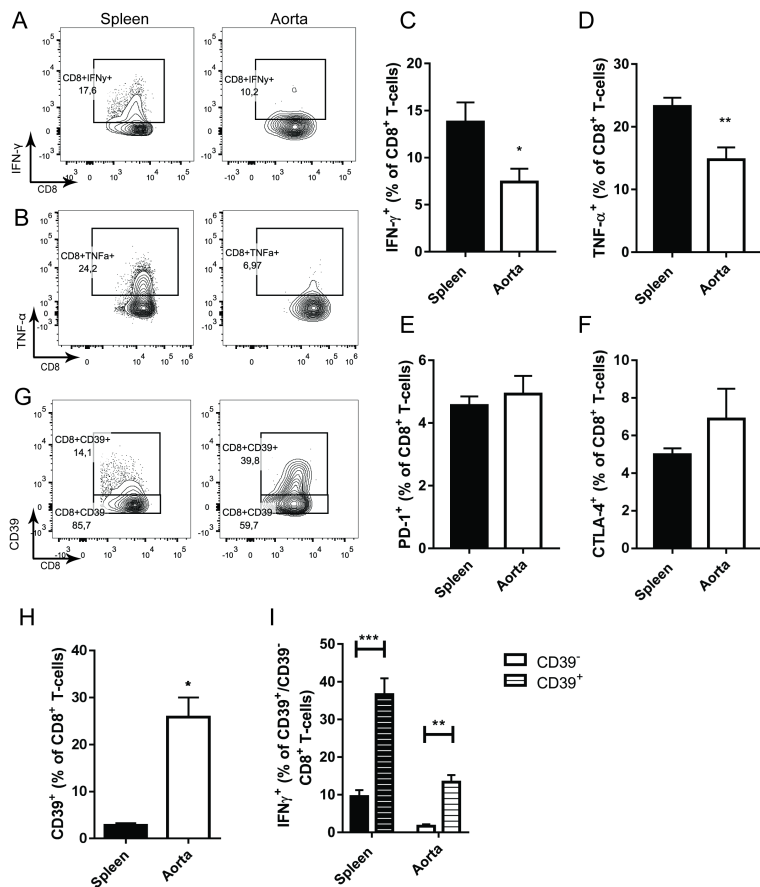
edly, we observed that CD8<sup>+</sup>CD39<sup>+</sup> T-cells are able to produce more IFN- $\gamma$  compared to CD8<sup>+</sup>CD39<sup>-</sup> T-cells in both aorta (13.3% vs. 1.7%, respectively) and spleen (35.6% vs. 9.5%, respectively, Fig. 1I), suggesting CD39 expression does not indicate exhaustion, but rather reflects the most activated CD8<sup>+</sup> T-cells in the plaque. Notably, although CD39<sup>+</sup>CD8<sup>+</sup> T-cells produce more IFN- $\gamma$  than CD39<sup>-</sup>CD8<sup>+</sup> T-cells in the plaque, the production of IFN- $\gamma$  by both of these subsets in the aorta is markedly lower compared to their counterparts in the spleen (Fig. 1I).

### 3.2. CD39 inhibition can increase cytokine production by CD8<sup>+</sup> T-cells in the lesions

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Although CD39 does not appear to mark exhausted CD8<sup>+</sup> T-cells in the atherosclerotic plaque, we did observe decreased cytokine production in the aortic compared to the splenic CD8<sup>+</sup> T-cells. We, therefore, hypothesized that the role of CD39 in the stepwise conversion of ATP into adenosine may result in high adenosine levels specifically in the lesion, which has been reported to reduce cytokine production [16]. This may allow CD39<sup>+</sup>CD8<sup>+</sup> T-cells to affect cytokine production of CD39<sup>-</sup> T-cells in a paracrine manner as well as CD39<sup>+</sup>CD8<sup>+</sup> T-cells in an autocrine manner. To test this hypothesis, CD39 was inhibited by using the small molecule inhibitor sodium metatungstate (POM-1). *Ex vivo* stimulation of apoE<sup>-/-</sup> derived splenic CD8<sup>+</sup> T-cells with increasing concentrations of POM-1 resulted in a dose-dependent increase in IFN- $\gamma$  production by CD8<sup>+</sup> T-cells (Fig. 2A). The expression levels of CD39 were also slightly increased upon treatment with POM-1 (Fig. 2B). Upon stratification of IFN- $\gamma$  production by each subset, it appeared that CD39<sup>-</sup>CD8<sup>+</sup> T-cells (which made up over 80% of all CD8<sup>+</sup> T-cells in this experiment) were for the most part responsible for this marked increase in IFN- $\gamma$  production, as they showed a 3.9-fold increase in cytokine production upon treatment with the highest concentration of POM-1 (Fig. 2C). Within the CD39<sup>+</sup>CD8<sup>+</sup> T-cell subset we only observed a modest 1.7-fold increase in IFN- $\gamma$  production. This suggests indeed that CD39 expression may regulate the cytokine responses of both CD39<sup>-</sup> and CD39<sup>+</sup> cells by affecting paracrine and autocrine adenosine signaling.

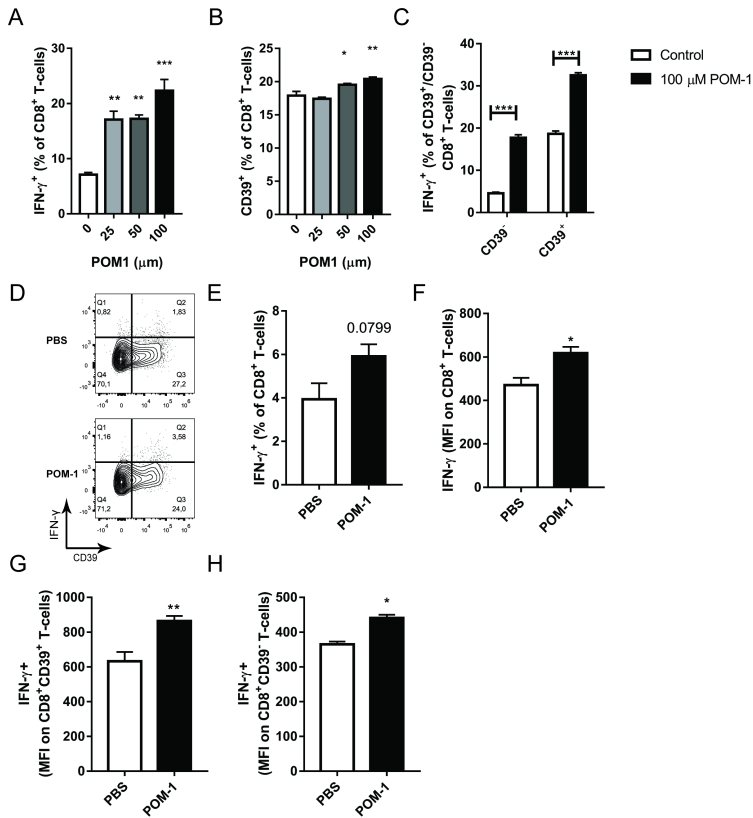
To examine the effects of this inhibitor *in vivo*, apoE<sup>-/-</sup> mice were injected i.p. with 10 mg/kg POM-1 or PBS as a control and sacrificed 24 hours after treatment. Analysis of the aortic phenotype by flow cytometry revealed a trend towards an increased IFN- $\gamma$  production by CD8<sup>+</sup> T-cells after POM-1 treatment in percentages (3.9% vs. 5.9%,  $p = 0.079$  Fig. 2D,E) and a significant increase in mean fluorescent intensity (470 vs. 617 MFI, Fig. 2F). However, the levels are not returned to those observed in the spleen. Concomitantly, we observed an increased percentage of inflammatory mononuclear cells (CD11b<sup>+</sup>/Ly6C<sup>hi</sup>/Ly6G<sup>-</sup>) in the aorta (Fig. S2), suggesting the increased IFN- $\gamma$  production affected mononuclear cell recruitment and/or activation. Interestingly, POM-1 treatment increased the production of IFN- $\gamma$  by both CD39<sup>+</sup> and CD39<sup>-</sup> CD8<sup>+</sup> T-cells. The pro-inflammatory cytokine production increased by 21% in the CD39<sup>-</sup>CD8<sup>+</sup> subpopulation upon POM-1 treatment (Fig. 2D,H) and even more prominently by 37% in CD39<sup>+</sup>CD8<sup>+</sup> T-cells (Fig. 2D,G). This suggests that CD39<sup>+</sup>CD8<sup>+</sup> T-cells in the atherosclerotic lesion, besides inhibiting CD39<sup>-</sup>CD8<sup>+</sup> T-cells in a paracrine fashion,



**Figure 1: Murine atherosclerotic lesion-derived CD8<sup>+</sup> T-cells show a decreased inflammatory phenotype and increased CD39 expression compared to their counterparts in the spleen.** (A-D) Flow cytometric analysis of the percentages of IFN- $\gamma$ <sup>+</sup> (A,C) and TNF- $\alpha$ <sup>+</sup> (B,D) CD8<sup>+</sup> T-cells derived from aortas and spleens of apoE<sup>-/-</sup> mice stimulated for 2 hours with PMA and ionomycin. Cells were pregated on live, Thy1.2<sup>+</sup>CD8<sup>+</sup> T-cells. n = 6 (A,C) or n = 5 (B,D) apoE<sup>-/-</sup> mice of 35-49 weeks old, data are representative of 2 independent experiments. (E-H) Percentages of PD-1<sup>+</sup> (E), CTLA-4<sup>+</sup> (F) and CD39<sup>+</sup> (G,H) CD8<sup>+</sup> T-cells derived from aortas and spleens of apoE<sup>-/-</sup> mice. Cells were pregated on live, Thy1.2<sup>+</sup>CD8<sup>+</sup> T-cells. n = 3-6 apoE<sup>-/-</sup> mice of 30-37 weeks old, data are representative of multiple independent experiments. (I) IFN- $\gamma$  production by CD39<sup>+</sup> and CD39<sup>-</sup> CD8<sup>+</sup> T-cells in the aortas and spleens apoE<sup>-/-</sup> mice stimulated for 2 hours with PMA and ionomycin. Cells were pregated on live, Thy1.2<sup>+</sup>CD8<sup>+</sup>CD39<sup>+</sup> or Thy1.2<sup>+</sup>CD8<sup>+</sup>CD39<sup>-</sup> T-cells. n = 6 apoE<sup>-/-</sup> mice of 41-49 weeks old, data are representative of 2 independent experiments. Mean  $\pm$  SEM, \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.

may maintain a negative feedback loop on their own inflammatory state through the production of adenosine.





**Figure 2: *In vivo* CD39 blockade in atherosclerotic mice partially restores pro-inflammatory cytokine production in both CD39<sup>+</sup>CD8<sup>+</sup> and CD39<sup>-</sup>CD8<sup>+</sup> T-cells.** (A-C) Purified CD8<sup>+</sup> T-cells were derived from the spleen of a 31-week old apoE<sup>-/-</sup> mouse and cultured for 24 hours with the indicated concentrations of POM-1, after which the cells were stained and analyzed by flow cytometry for CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> (A) and CD8<sup>+</sup>CD39<sup>+</sup> (B) T-cells. (C) IFN- $\gamma$  production by CD39<sup>+</sup> and CD39<sup>-</sup> CD8<sup>+</sup> T-cells cultured for 24 hours under control conditions or with 100  $\mu$ M of POM-1. Cells were pre-gated on live, Thy1.2<sup>+</sup>CD8<sup>+</sup> T-cells. (D-G) apoE<sup>-/-</sup> mice of 42-47 weeks of age were treated with 10 mg/kg POM-1 (n = 5) or sterile PBS (n = 5). After 24 hours, aortas were isolated and analyzed by flow cytometry. (D) Representative dot plots of PBS or POM-1 treated mice showing CD39 and IFN- $\gamma$  expression on aortic CD8<sup>+</sup> T-cells. (E) Percentages and (F) MFI values of IFN- $\gamma$  expression by CD8<sup>+</sup> T-cells. Cells were pre-gated on live, CD3<sup>+</sup>CD8<sup>+</sup> T-cells. (G) CD39<sup>+</sup>CD8<sup>+</sup> or (H) CD39<sup>-</sup>CD8<sup>+</sup> T-cells producing IFN- $\gamma$ . Cells were pre-gated live, CD3<sup>+</sup>CD8<sup>+</sup>CD39<sup>+</sup> or CD3<sup>+</sup>CD8<sup>+</sup>CD39<sup>-</sup> T-cells. Mean  $\pm$  SEM, \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.

### 3.3. TCR signaling in the aortic microenvironment induces CD39 expression

Recent work has shown that upon T-cell receptor (TCR) activation, reactive oxygen species are generated that activate signaling cascades resulting in increased CD39 expression [16]. Moreover, CD39 has been reported as a useful marker to discriminate antigen-specific from non-specific bystander CD8<sup>+</sup> T-cells in a tumor environment [17]. As there is persistent antigen presentation to T-cells in atherosclerotic

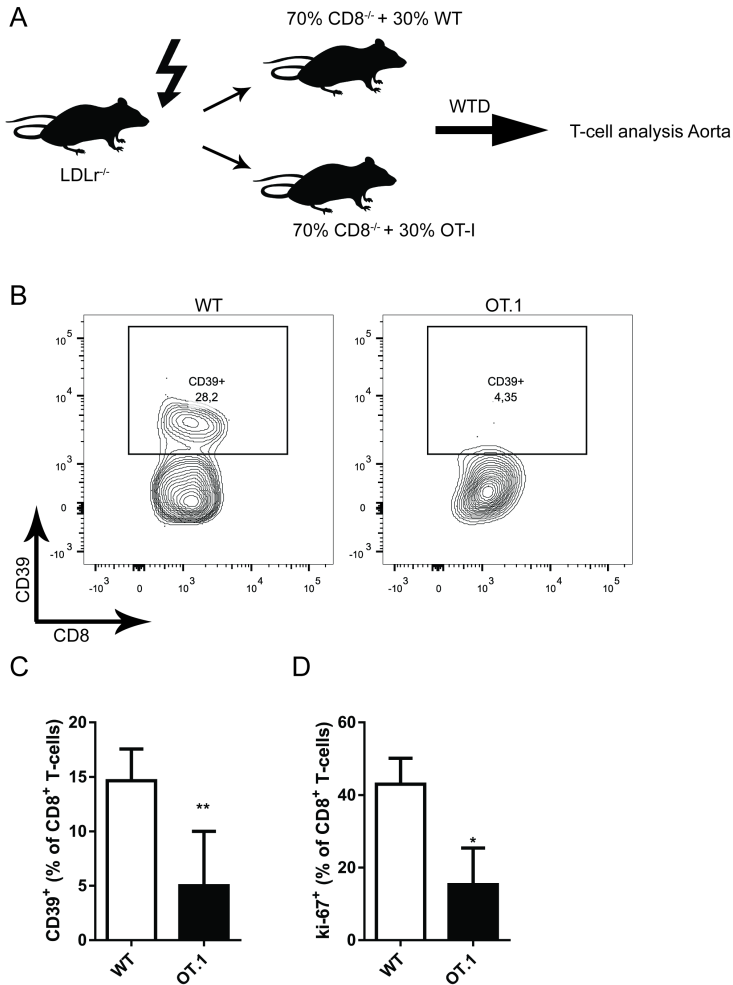
lesions [18], we hypothesized that TCR signaling in the aortic microenvironment may cause the observed increase in CD39 expression on the CD8<sup>+</sup> T-cells within the lesion, whereas non-specific bystander CD8<sup>+</sup> T-cells do not express CD39. To test this, a bone marrow transplantation experiment was performed in which LDLr<sup>-/-</sup> mice received 70% CD8<sup>-/-</sup> bone marrow combined with either 30% WT or OT.1 bone marrow. After a 7-week recovery period, mice were fed a Western-type diet for 8 weeks in order to induce atherosclerosis (Fig. 3A). When using this setup, mice in the OT.1 donor group had only CD8<sup>+</sup> T-cells that are able to recognize ovalbumin through their TCR and were therefore not able to receive any stimulation from atherosclerosis-specific antigens. In contrast, mice in the WT donor group had CD8<sup>+</sup> T-cells that were able to receive TCR stimulation. We opted for this “reverse approach” in which CD8<sup>+</sup> T-cells that recognize an irrelevant antigen are compared to WT cells, as the antigen that activates CD8<sup>+</sup> T-cells in atherosclerosis is as of yet unknown. Strikingly, the number of CD39<sup>+</sup>CD8<sup>+</sup> T-cells in the lesions of the mice transplanted with the OT.1 bone marrow was markedly lower when compared to controls (5% vs. 14.6%, Fig. 3B,C). By extension, the CD8<sup>+</sup> T-cells in the OT.1 group also showed a significantly reduced proliferative capacity, as measured by Ki-67 staining (15.3% vs. 43% Fig. 3D). Thus, TCR signaling is essential for the increased expression of CD39 on CD8<sup>+</sup> T-cells in the atherosclerotic microenvironment, and CD39 may mark antigen-experienced CD8<sup>+</sup> T-cells in the plaque.

### 3.4. CD39 expression is increased on CD8<sup>+</sup> T-cells derived from human atherosclerotic lesions compared to their systemic counterparts

We further investigated CD39 expression in human atherosclerotic lesions to determine whether the results described above are relevant to human atherosclerosis. CD8<sup>+</sup> T-cells derived from carotid endarterectomy samples showed a 2.7-fold higher expression of CD39 compared to their counterparts in the blood of these patients (Fig. 4A,B, for gating strategy, see Fig. S3). In agreement with the murine data, CD8<sup>+</sup>CD39<sup>+</sup> T-cells derived from the lesions produced more IFN- $\gamma$  compared to CD8<sup>+</sup>CD39<sup>-</sup> cells (30.6% vs. 20.9%, respectively, Fig. 4C-E). Thus, similar to our findings in apoE<sup>-/-</sup> mice, the percentage of CD39<sup>+</sup>CD8<sup>+</sup> T-cells in human atherosclerotic lesions is strongly increased compared to the blood, and appears to represent the most activated fraction of CD8<sup>+</sup> T-cells in the plaque.

## 4. Discussion

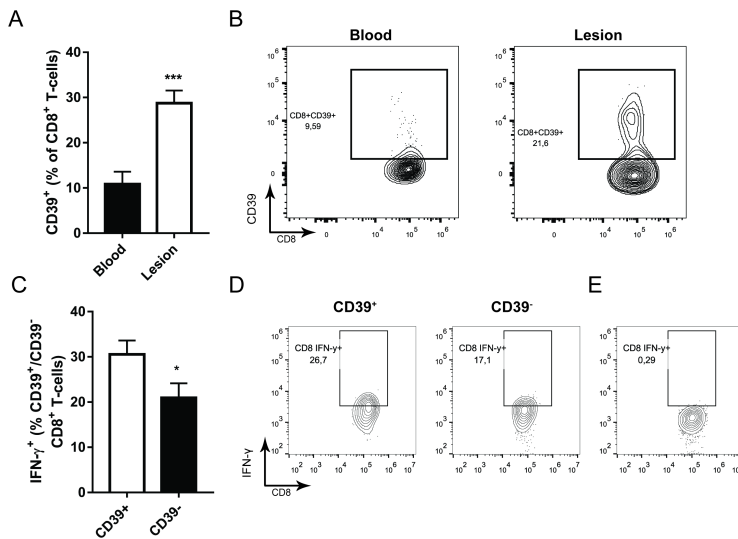
Atherosclerotic lesions are associated with an influx of CD8<sup>+</sup> T-cells as the lesion progresses towards more advanced stages [1]. As the lesion microenvironment is very complex, many factors within the lesion may well affect the phenotype of CD8<sup>+</sup> T-cells. It is of particular interest to understand the function of these cells within the plaque as compared to their circulating counterparts, as these cells can affect the lesion development and composition locally. In this work, we show for the first time that CD8<sup>+</sup> T-cells display an atherosclerotic microenvironment-specific dysfunction, as aortic-derived



**Figure 3: TCR signaling is required to induce CD39 on aortic CD8<sup>+</sup> T-cells.** (A) Schematic overview of the experimental setup. (B) Representative contour plots depicting CD39 expression on aortic CD8<sup>+</sup> T-cells at sacrifice. (C-D) Percentages of CD39<sup>+</sup> (C) and Ki-67<sup>+</sup> (D) CD8<sup>+</sup> T-cells in the aorta of the mice transplanted with WT or OT.1 bone marrow at the time of sacrifice as analyzed by flow cytometry. Cells were pregated on live CD8<sup>+</sup> T-cells. WT (n = 5) and OT.1 (n = 11), unequal distribution due to attrition in the experiment. Mean ± SEM, \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.

CD8<sup>+</sup> T-cells produce lower levels of cytokines compared with their counterparts in the spleen. Chronic antigen stimulation of CD8<sup>+</sup> T-cells is known to result in exhaustion and an associated reduced cytokine production by these cells [19]. Antigen-specific stimulation of T-cells also occurs over prolonged periods of time in atherosclerosis development [20]. We, therefore, hypothesized that CD8<sup>+</sup> T-cells specifically become exhausted in the lesion microenvironment, as there are high numbers of APCs in the plaques. Markedly, we found no differences in the expression of the classical





**Figure 4: CD39 expression on human atherosclerotic lesion-derived CD8<sup>+</sup> T-cells is associated with IFN- $\gamma$  production.** (A-B) Flow cytometry analysis and representative images of CD39 expression on CD8<sup>+</sup> T-cells in endarterectomy samples from  $n = 12$  arteria carotis or matched blood. Cells were gated on live CD45<sup>+</sup>CD3<sup>+</sup>CD8<sup>+</sup> cells. (C-D) Flow cytometry analysis and representative images of IFN- $\gamma$  production by CD39<sup>+</sup> and CD39<sup>-</sup>CD8<sup>+</sup> T-cells in endarterectomy samples from  $n = 12$  arteria carotis stimulated for 4 hours with PMA and ionomycin. Cells were pregated on live CD45<sup>+</sup>CD3<sup>+</sup>CD8<sup>+</sup>CD39<sup>+</sup> or live CD45<sup>+</sup>CD3<sup>+</sup>CD8<sup>+</sup>CD39<sup>-</sup> cells. (E) Representative image of IFN- $\gamma$  production by CD45<sup>+</sup>CD3<sup>+</sup>CD8<sup>+</sup> T-cells in an unstimulated sample, used to determine the gating strategy for IFN- $\gamma$ . Mean  $\pm$  SEM, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

exhaustion-associated inhibitory receptors PD-1 and CTLA-4 [21]. However, high expression of the ectonucleotidase CD39 is linked to decreased cytokine production and exhaustion of CD8<sup>+</sup> T-cells [15, 22], and is also associated with regulatory T-cells [23]. This enzyme is involved in hydrolyzing extracellular ATP into ADP which can be further converted into the immunomodulatory adenosine by ecto-5'-nucleotidases such as CD73 [24]. ATP is a known danger-associated molecular pattern which stimulates pro-inflammatory cytokine production and has been linked to atherosclerosis [25]. We demonstrated an increased expression of CD39 on aortic CD8<sup>+</sup> T-cells compared to their splenic counterparts, suggesting a CD39-mediated reduction in cytokine production by lesion-derived CD8<sup>+</sup> T-cells. We found a slight decrease in the expression of CD73 on aortic CD8<sup>+</sup> T-cells. However, the rate-limiting step in the aforementioned cascade is CD39 [26], thus suggesting CD8<sup>+</sup> T-cells in advanced atherosclerotic lesions could have an increased capacity to produce adenosine, although we did not measure this directly. Bai et al. reported that adenosine produced by CD8<sup>+</sup>CD39<sup>+</sup> T-cells can drive the exhaustion of CD8<sup>+</sup>CD39<sup>-</sup> T-cells [16]. In agreement with this, we observed higher IFN- $\gamma$  production by CD8<sup>+</sup>CD39<sup>+</sup> T-cells compared to their CD39<sup>-</sup> counterparts within the aortic microenvironment. As CD39<sup>-</sup> cells produce fewer cytokines than their CD39<sup>+</sup> counterparts, this may suggest a paracrine regulation of cytokine production of CD39<sup>-</sup> cells by CD39<sup>+</sup> cells via increased adenosine-mediated

receptor signaling responses. Besides decreasing cytokine production of CD8<sup>+</sup> T-cells, adenosine-mediated signaling can inhibit Th1 and Th2 CD4<sup>+</sup> T-cell development, as well as their effector functions [27]. Furthermore, adenosine can inhibit the function of type 1 cytotoxic CD8<sup>+</sup> T-cells [28], and induce a tolerogenic APC phenotype [29]. This suggests that the increased CD39 expression on lesion-derived CD8<sup>+</sup> T-cells may have an immune regulatory, and potentially, an atheroprotective function. Indeed, adenosine signaling via type 1 purigenic receptors was shown to be protective against atherosclerosis development [30]. However, as we did not measure adenosine levels *in situ*, we cannot exclude that there could be an increase in extracellular levels of ADP and AMP as well. These nucleotides can induce both atherogenic signaling [30], but may also induce phosphorylation of the AMP-activated protein kinase (AMPK) [31]. This phosphorylation results in functional activation of AMPK, which was shown to reduce atherosclerosis development by inducing autophagy and subsequently promoting cholesterol efflux from macrophages, as well as diminishing inflammatory responses [32].

4

In this study, we show that CD39 expression directly and indirectly affects CD8<sup>+</sup> T-cell functionality, by inhibiting the enzymatic activity of CD39 using POM-1. POM-1 has been shown to effectively inhibit ATP hydrolysis by CD39, and thereby diminish the immunosuppressive function of tumor-associated macrophages in ovarian cancer [33]. Furthermore, it was shown to inhibit adenosine generation by regulatory T-cells in a melanoma mouse model [34]. In agreement with this, we observed an increased IFN- $\gamma$  production by CD8<sup>+</sup> T-cells both *in vitro* on splenic-derived CD8<sup>+</sup> T-cells and *in vivo* in the aortas of apoE<sup>-/-</sup> mice upon treatment with POM-1. It must be noted that some off-target effects of the inhibitor may have occurred *in vivo*, but we show these effects *in vitro* on isolated CD8<sup>+</sup> T-cells as well, which indicates that the increased cytokine production is most likely due to reduced adenosine production. Interestingly, we observed an increase in cytokine production upon POM-1 treatment by both CD39<sup>+</sup> and CD39<sup>-</sup>CD8<sup>+</sup> T-cells, suggesting that the adenosine produced by CD39<sup>+</sup>CD8<sup>+</sup> T-cells may regulate IFN- $\gamma$  production in both a paracrine and autocrine fashion. It must be noted that even though the levels of IFN- $\gamma$  production are increased upon POM-1 treatment, they are still not returned to the levels observed in the spleen. This could be due to their prolonged exposure to higher concentrations of adenosine *in situ*. Alternatively, the short duration of treatment may not be able to completely restore the cytokine production to the levels observed in the spleen, but we cannot exclude other factors that may contribute to the decreased cytokine production of CD8<sup>+</sup> T-cells in the aortic microenvironment. Although we did not investigate this directly, increased CD39 expression on CD8<sup>+</sup> T-cells may affect the phenotype and function of other immune cells present in the lesion. Indeed, it has been shown that CD39-expressing CD8<sup>+</sup> T-cells can suppress CD4<sup>+</sup> T-cell function in a simian immunodeficiency virus infection [35] and suppress the proliferative response of Th1 CD4<sup>+</sup> T-cells in a mycobacterium infection [36]. Full body knockout of CD39 in apoE<sup>-/-</sup> mice results in decreased atherosclerosis [37], suggesting an atherogenic role for this enzyme. However, CD39<sup>-/-</sup>apoE<sup>-/-</sup> mice show impaired platelet activation, enhanced cholesterol efflux and an increase in plasma HDL, which confirms that a full body knockout of CD39 results in a number of complex changes in key factors affecting atherosclerosis development. Similarly, the

blocking of CD39 using POM-1 may affect multiple cell types expressing CD39. Further research that investigates inhibition or knockout of CD39 specifically on CD8<sup>+</sup> T-cells in an atherosclerotic context is needed to shed more light on the exact role of CD8<sup>+</sup> CD39<sup>+</sup> T-cells on lesion initiation and progression. We propose that the increased adenosine produced by these cells functions in an atheroprotective manner, as blocking of CD39 resulted in increased IFN- $\gamma$  production, which is known to drive atherogenesis [38].

Using a bone-marrow transplantation setup in which atherosclerotic mice are engrafted with bone marrow that gives rise to either wild-type or ovalbumin-restricted CD8<sup>+</sup> T-cells, we were able to demonstrate the need for TCR signaling for the upregulation of CD39 on lesional CD8<sup>+</sup> T-cells. The aortic microenvironment contains many APCs that are able to locally activate T-cells via their TCR [39]. Upon TCR ligation, reactive oxygen species (ROS) are generated [40], which in turn are able to augment the expression of CD39 [16]. As the atherosclerotic lesion contains many APCs and is characterized by high levels of ROS [41], we suggest that this local microenvironment boosts CD39 expression on the CD8<sup>+</sup> T-cells. In agreement with our findings, previous reports have shown that CD39 expression is upregulated on T-cells upon TCR signaling [14, 16, 22, 23]. Moreover, on CD4 regulatory T-cells, the catalytic activity of CD39 was enhanced by TCR ligation [23]. In support of our findings on CD8<sup>+</sup> T-cell skewing by the local microenvironment, recent work by Simoni et al. has also shown a role for TCR signaling in inducing CD39 expression on CD8<sup>+</sup> T-cells in the tumor microenvironment [17]. Furthermore, Duhon et al. report a unique tumor-microenvironment specific population of CD8<sup>+</sup> T-cells co-expressing CD39 and CD103 [42]. Collectively, this suggests that microenvironment specific TCR-signaling is important in inducing CD39 expression on CD8<sup>+</sup> T-cells.

Importantly, we show that in human atherosclerotic lesions CD39 is upregulated on CD8<sup>+</sup> T-cells as well. Moreover, we observed that the human CD39<sup>+</sup> T-cells produce more IFN- $\gamma$  compared to the CD39<sup>-</sup> T-cells, which is in agreement with the phenotype we observed in our murine model. Thus, it can be presumed that the immunomodulatory role we observe for CD39<sup>+</sup>CD8<sup>+</sup> T-cells in apoE<sup>-/-</sup> mice can be translated to a clinical setting. This may open up new treatment avenues targeting CD39 expression on CD8<sup>+</sup> T-cells in atherosclerosis.

In conclusion, our studies highlight a new role for CD8<sup>+</sup> T-cells in advanced atherosclerosis. We propose that the increased adenosine produced by CD8<sup>+</sup> T-cells inside the lesions acts in an anti-inflammatory manner. These results suggest that boosting CD39<sup>+</sup>CD8<sup>+</sup> T-cell function could be an interesting approach for the treatment of atherosclerosis. As CD39 expression is upregulated in an antigen-specific manner, vaccination strategies boosting CD8<sup>+</sup> T-cell responses, which have been shown to be effective in a murine model [5], may be a promising treatment avenue.

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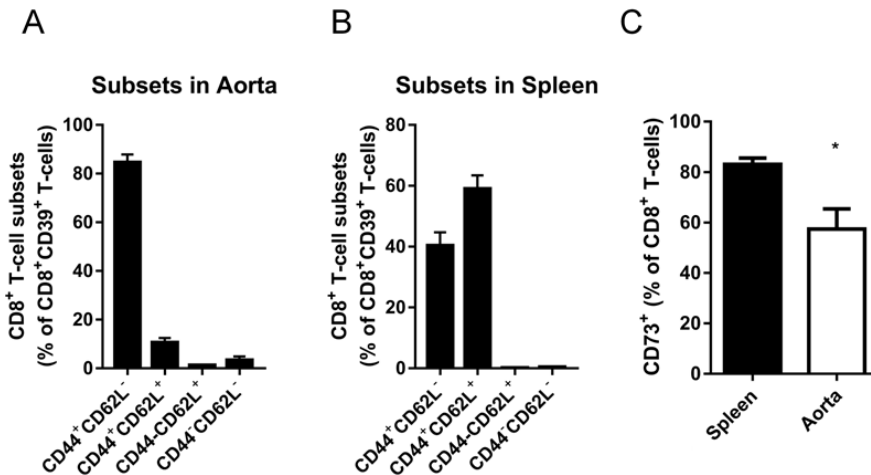
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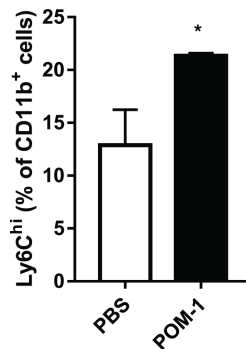


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## Supplementary information

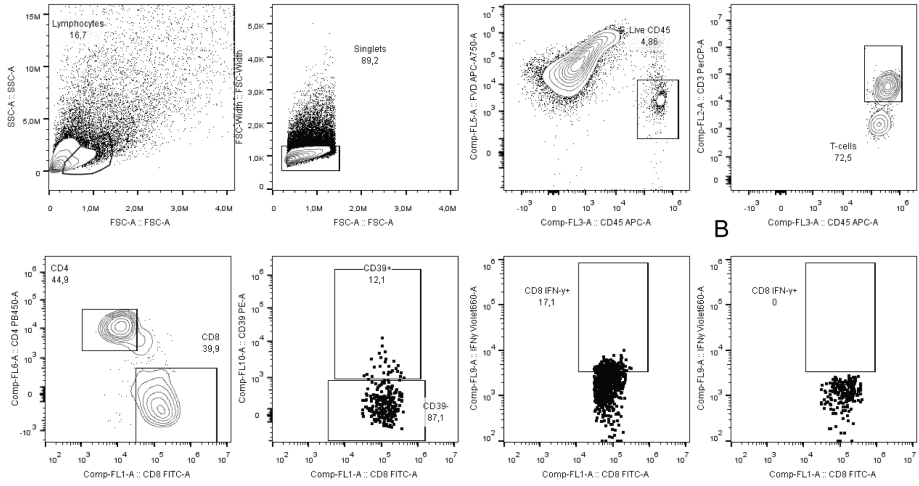


**Figure S1: apoE<sup>-/-</sup>-derived CD8<sup>+</sup> T-cells show CD39 expression within all T cell subsets, and lesion-derived CD8<sup>+</sup> T-cells show a decrease in CD73 expression compared to their counterparts in the spleen.** (A,B) Percentages of different subsets within the CD39<sup>+</sup>CD8<sup>+</sup> T-cells classified by their expression of CD44 and CD62L in aortas (A) and spleens (B) of apoE<sup>-/-</sup> mice. Cells were pregated on live, Thy1.2<sup>+</sup>CD8<sup>+</sup> T-cells. n = 5 apoE<sup>-/-</sup> mice of 37-43 weeks old. (C) Percentages of CD73<sup>+</sup>CD8<sup>+</sup> T-cells derived from aortas and spleens of apoE<sup>-/-</sup> mice analyzed by flow cytometry. Cells were pregated on live, Thy1.2<sup>+</sup>CD8<sup>+</sup> T-cells. n = 6 apoE<sup>-/-</sup> mice of 36 weeks old, data is representative of 2 independent experiments. Mean ± SEM, \*p < 0.05, \*\*p < 0.01.



**Figure S2: In vivo CD39 blockade in atherosclerotic mice results in increased pro-inflammatory mononuclear cell content the lesion.** apoE<sup>-/-</sup> mice of 38-43 weeks of age were treated with 10 mg kg<sup>-1</sup> POM-1 (n = 5) or sterile PBS (n = 5). After 24 hours, aortas and blood were isolated and analyzed by flow cytometry. Percentages of inflammatory Ly6C<sup>hi</sup> mononuclear cells in the aorta are shown, pregated on live, NK1.1<sup>-</sup>Ly6G<sup>-</sup>CD11b<sup>+</sup> cells. Mean ± SEM, \*p < 0.05, \*\*p < 0.01.

A



**Figure S3: Flow cytometry gating strategy for human atherosclerotic lesions.** (A) Flow cytometry gating strategy for determining the expression of CD39 and IFN- $\gamma$  on CD8<sup>+</sup> T-cells in endarterectomy samples stimulated for 4 hours with PMA and ionomycin. Cells were gated on leukocytes, single cells, fixable viability dye-CD45<sup>+</sup>, CD3<sup>+</sup>, CD8<sup>+</sup>, CD39<sup>+</sup> and finally IFN- $\gamma$ <sup>+</sup>. (B) Unstimulated control samples were used to determine the placing of the IFN- $\gamma$  gate.

## Supplementary methods

### Obtaining single cell suspensions from human material

Single cell suspensions were obtained 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), human serum albumine and DNase (both Sigma), as previously described [13]. Blood samples were centrifuged to remove the serum and subsequently lysed twice for 10 minutes in ACK lysis buffer (0.15 M NH<sub>4</sub>Cl, 1 mM KHCO<sub>3</sub>, 0.1 mM Na<sub>2</sub>EDTA; pH 7.3) at room temperature and washed once in PBS in order to obtain white blood cells. All samples were frozen on the day of surgery and stored at -80 °C. Cells were defrosted and stained for flow cytometric analysis at the same time.

### Bone marrow transplantation experiment

30 LDLr<sup>-/-</sup> mice of 13-15 weeks of age were injected with 50  $\mu$ g of an anti-CD8 antibody (clone 2.43, BioXcell) in order to deplete the CD8<sup>+</sup> T-cells. After 24 hours, all mice were exposed to a single dose of 9 Gy (0.19 Gymin<sup>-1</sup> 200 kV, 4 mA) total body irradiation using an Andrex Smart 225 Röntgen source (YXLON International) with a 6 mm aluminum filter to induce bone marrow aplasia. Donor bone marrow was isolated from CD8<sup>-/-</sup> mice (provided by Dr. Oxenius, ETH Zurich, Switzerland), C57BL/6 (WT) or C57BL/6-Tg(TcraTcrb)1100Mjb/J (OT.1) mice (Jackson Laboratory) by flushing the femurs and tibias with PBS. Single-cell suspensions were prepared by passing the cells

through a 70  $\mu\text{m}$  cell strainer (Greiner Bio-One). Donor bone marrow cells were combined to obtain a mixture of 70% CD8<sup>-/-</sup> and 30% WT or OT.1 bone marrow, and  $1.4 \times 10^7$  cells were injected into tail veins of the irradiated LDLr<sup>-/-</sup> mice. Drinking water was supplemented with antibiotics (83 mgL<sup>-1</sup> ciprofloxacin, 67 mgL<sup>-1</sup> polymyxin B sulfate, and 6.5 gL<sup>-1</sup> sucrose). After a recovery period of 7 weeks on a regular chow diet, animals were placed on a Western-type diet containing 0.25% cholesterol and 15% cacao butter (Special Diet Services) for 8 weeks, after which the mice were sacrificed as described above.

### Flow cytometry

Upon sacrifice, spleens and aortas were harvested after in situ perfusion with PBS. Single-cell suspensions of spleens were obtained by using a 70  $\mu\text{m}$  cell strainer (Greiner Bio-One). WBCs from splenocytes were obtained by lysing for 1 min at room temperature in lysis buffer. Aortas were cleaned of perivascular fat, cut up into small pieces, and digested by incubation with digestion mix (collagenase I 450 U ml<sup>-1</sup>, collagenase XI 250 U ml<sup>-1</sup>, DNase 120 U ml<sup>-1</sup> and hyaluronidase 120 U ml<sup>-1</sup>; all Sigma-Aldrich) for 30 min at 37 °C while shaking and subsequently strained over a 70  $\mu\text{m}$  strainer. Approximately 100000 cells (or all available cells from aortic tissue) were stained with the appropriate antibodies (supplementary table I) in PBS containing 2% FCS. For intracellular staining, cells were fixed and permeabilized by using an intracellular staining kit (eBioscience) according to the manufacturer's protocol. To detect cytokine production, cells were stimulated for two or four hours with phorbol 12-myristate 13-acetate (PMA, 50 ngml<sup>-1</sup>, Sigma-Aldrich) and ionomycin (500 ngml<sup>-1</sup>, Sigma-Aldrich) in the presence of brefeldin A (ThermoScientific) in complete RPMI as stated above at 37 °C and 5% CO<sub>2</sub>. Flow cytometry analyses were performed on a Beckman Coulter Cytoflex S or BD Biosciences Canto II and FlowJo software (Treestar).