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BASIC SCIENCE
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Deficiency of the T cell regulator *Casitas B-cell lymphoma-B* aggravates atherosclerosis by inducing CD8$^+$ T cell-mediated macrophage death

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Aims The E3-ligase CBL-B (*Casitas B-cell lymphoma-B*) is an important negative regulator of T cell activation that is also expressed in macrophages. T cells and macrophages mediate atherosclerosis, but their regulation in this disease remains largely unknown; thus, we studied the function of CBL-B in atherogenesis.

Methods and results The expression of CBL-B in human atherosclerotic plaques was lower in advanced lesions compared with initial lesions and correlated inversely with necrotic core area. Twenty weeks old *Cblb*$^{-/-}$*Apoe*$^{-/-}$ mice showed a significant increase in plaque area in the aortic arch, where initial plaques were present. In the aortic root, a site containing advanced plaques, lesion area rose by 40%, accompanied by a dramatic change in plaque phenotype. Plaques contained fewer macrophages due to increased apoptosis, larger necrotic cores, and more CD8$^+$ T cells. *Cblb*$^{-/-}$*Apoe*$^{-/-}$ macrophages exhibited enhanced migration and increased cytokine production and lipid uptake. *Casitas B-cell lymphoma-B* deficiency increased CD8$^+$ T cell numbers, which were protected against apoptosis and regulatory T cell-mediated suppression. IFNγ and granzyme B production was enhanced in *Cblb*$^{-/-}$*Apoe*$^{-/-}$ CD8$^+$ T cells, which provoked macrophage killing. Depletion of CD8$^+$ T cells in *Cblb*$^{-/-}$*Apoe*$^{-/-}$ bone marrow chimeras rescued the phenotype, indicating that CBL-B controls atherosclerosis mainly through its function in CD8$^+$ T cells.

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Conclusion *Casitas B-cell lymphoma-B* expression in human plaques decreases during the progression of atherosclerosis. As an important regulator of immune responses in experimental atherosclerosis, CBL-B hampers macrophage recruitment and activation during initial atherosclerosis and limits $CD8^+$ T cell activation and $CD8^+$ T cell-mediated macrophage death in advanced atherosclerosis, thereby preventing the progression towards high-risk plaques.

Keywords Atherosclerosis • Innate and adaptive immune system • Macrophages • T cells • CBL-B

Translational perspective

In this study, we demonstrate that the E3-ligase *Casitas B-cell lymphoma-B* (CBL-B) is expressed in human atherosclerotic plaques, and that its expression decreases with plaque progression. Using an atherosclerotic mouse model, we found that CBL-B exerts profound anti-atherogenic effects by regulating $CD8^+$ T cell and macrophage activation. Activation of CBL-B, therefore, represents a promising anti-inflammatory therapeutic strategy in atherosclerosis.

Introduction

Atherosclerosis, a lipid-driven inflammatory disease of the large arteries, is the underlying cause of the majority of cardiovascular diseases (CVD).[1] Although primary and secondary preventive strategies have significantly lowered the incidence of CVD, atherosclerosis remains a major cause of morbidity and mortality.[2] Additional therapeutic strategies, which target the residual cardiovascular risk that persists after optimal pharmacological treatment, are therefore required.[2] In addition to dyslipidaemia, immune cell activation and subsequent inflammation drive atherogenesis.[1–3] Inhibition of atherosclerosis-associated inflammation is therefore a strategy with a great therapeutic potential, as highlighted by the CANTOS (Canakinumab Antiinflammatory Thrombosis Outcome Study) trial, in which antibody-mediated inhibition of interleukin (IL)-1β reduced the incidence of recurrent CVD in patients with a previous myocardial infarction and high residual inflammatory risk.[4]

T cells constitute a variable but substantial proportion of the immune cell population in the atherosclerotic plaque and are significant drivers of the inflammatory responses that underlie atherosclerosis.[1,3] $CD4^+$ T cells are the predominant T cell subset in atherosclerotic lesions of Apolipoprotein E-deficient ($Apoe^{-/-}$) mice. However, subsets of $CD4^+$ T cells contribute differently to atherosclerosis.[1] While T helper (Th)1 cells are considered pro-atherosclerotic, Th2 cells are still controversially discussed.[1] Regulatory T cells (Tregs) are considered protective in atherosclerosis through the release of transforming growth factor (TGF)β and IL10.[1] The function of $CD8^+$ cytotoxic T cells in atherosclerosis is incompletely understood; however, they appear proatherogenic and are abundantly present in advanced human atherosclerotic lesions.[3] Transfer of $CD8^+$ T cells accelerates atherosclerosis and leads to a vulnerable plaque phenotype in $Apoe^{-/-}$ mice, whereas antibody-mediated depletion of $CD8^+$ T cells impedes the formation of atherosclerotic lesions.[3,5,6] Despite the well-described functions of T cell subsets in atherosclerosis, the regulatory mechanisms by which they undergo activation and polarization during atherogenesis are less extensively studied.

The *Casitas B-cell lymphoma* (CBL) E3 ubiquitin ligases—comprising CBL-B, C-CBL, and CBL-C—form one of the protein families that modulate T cell activation and polarization.[7] *Casitas B-cell lymphoma-B* promotes T cell tolerance through ubiquitination and degradation of downstream effectors, such as phosphoinositide phospholipase Cγ and phosphoinositide 3-kinase, and thus is a negative regulator of T cell activation.[7,8] *Casitas B-cell lymphoma-B*-deficient T cells have a hyper responsive phenotype that is accompanied by CD28-independent activation, due to their lower threshold for T cell receptor-mediated responses.[9] Further, these T cells mount delayed responses to anergic signals, contributing to a state of hyper responsiveness.[10]

Notably, macrophages, an important cell type that abounds in atherosclerotic plaques, also expresses CBL-B, the function of which in this cell type remains incompletely described.[7] *Casitas B-cell lymphoma-B* deficiency is linked to enhanced toll-like receptor (TLR)4 signalling and increased macrophage activation and migration in diet-induced obesity[11] and lung inflammation models,[12] processes that are also relevant for the atherosclerosis.

Considering the significant regulatory activity of CBL-B in T cell and macrophage biology, we evaluated the expression pattern of CBL-B in human atherosclerotic lesions and investigated the function of CBL-B in experimental atherosclerosis.

Methods

Human studies

Coronary artery specimens were obtained from autopsy from the Department of Pathology of the Amsterdam UMC and immediately fixed in 10% formalin and processed for paraffin embedding. All use of tissue was in agreement with the 'Code for Proper Secondary Use of Human Tissue in the Netherlands'. CBL-B expression was analysed by immunohistochemistry, as described in the Supplementary material online. Gene expression of CBL-B in human atherosclerosis was examined by microarray-based transcriptional profiling of carotid endarterectomy specimens (BiKE dataset[13,14]).

Animal studies

Male $Cblb^{-/-}Apoe^{-/-}$ and $Apoe^{-/-}$ mice were bred and housed at the animal facility of the University of Amsterdam and kept on a normal chow diet. All mice were treated according to the study protocol (permit nos. 102601 and 102869) that were approved by the Committee for Animal Welfare of the University of Amsterdam, the Netherlands. Detailed methods are provided in the Supplementary material online.

Results

Casitas B-cell lymphoma-B co-localizes with macrophages and T cells in human atherosclerotic plaques

Human coronary atherosclerotic plaques, histologically classified as intimal xanthomas or pathological intimal thickenings (initial/intermediate atherosclerosis) expressed higher levels of CBL-B^{+} cells when compared with fibrous cap atheromata (advanced atherosclerosis) (*Figure 1A–C*). A negative correlation between plaque area and CBL-B expression (*Figure 1D*), and necrotic core area and CBL-B was observed (*Figure 1E*), indicating that CBL-B expression in the plaque decreased during the progression of atherosclerosis. The majority of CBL-B^{+} cells were CD68^{+} macrophages (*Figure 1F*) and CD3^{+} T cells (*Figure 1G*), whereas only few intraplaque vascular smooth muscle cells (VSMCs) and endothelial cells expressed CBL-B (data not shown).

To further evaluate the expression of CBL-B in human atherosclerosis, gene expression of CBL-B in carotid endarterectomy specimens was examined by microarray-based transcriptional profiling (BiKE dataset[13,14]) Carotid atherosclerotic lesions had a tendency to express less CBL-B mRNA when compared with non-atherosclerotic arteries ($P = 0.056$) (*Figure 1H*). *Casitas B-cell lymphoma-B* was not differentially expressed between atherosclerotic plaques from symptomatic and asymptomatic patients (data not shown), indicating that CBL-B predominantly affects plaque development and not plaque rupture.

Casitas B-cell lymphoma-B deficiency aggravates atherosclerosis in Apoe$^{-/-}$ mice

Casitas B-cell lymphoma-B is expressed in CD68^{+} macrophages and CD3^{+} T cells in murine atherosclerotic plaques (Supplementary material online, *Figure S1*). To study the function of CBL-B in atherosclerosis, *Cblb*$^{-/-}$*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ mice were generated and fed a normal chow diet for 20 weeks. The extent and phenotype of atherosclerosis was determined in the aortic arch and the aortic root (*Figure 2A*). Body weight or basic haematologic parameters did not differ between genotypes (Supplementary material online, *Table S1*). Histological analysis of over 20 organs revealed no abnormalities, particularly no signs of autoimmunity, in *Cblb*$^{-/-}$*Apoe*$^{-/-}$ or *Apoe*$^{-/-}$ mice.

Deficiency of CBL-B increased atherosclerotic plaque area in the aortic arch and its main branch points by 1.8-fold (*Figure 2B* and *C*). Most plaques in the aortic arch were early, macrophage rich lesions (*Figure 2C*). Immunohistochemistry demonstrated that the plaques of *Cblb*$^{-/-}$*Apoe*$^{-/-}$ mice contained significantly more CD45^{+} cells (*Figure 2D*), reflecting a more inflammatory plaque phenotype.

Plaques in the aortic roots of *Cblb*$^{-/-}$*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ mice were not only larger (*Figure 2E*), but also displayed hallmarks of advanced stages of atherosclerosis, especially necrotic core formation (*Figure 2F*). Deficiency of CBL-B resulted in a 1.4-fold increase in atherosclerotic plaque area. Plaques in the aortic root of *Cblb*$^{-/-}$*Apoe*$^{-/-}$ mice contained fewer CD68^{+} macrophages when compared with *Apoe*$^{-/-}$ mice (*Figure 2G* and *H*), which resulted from increased macrophage apoptosis (*Figure 2I*) and a subsequent increase in necrotic core area (*Figure 2J* and *K*). In line with the more advanced plaque phenotype, collagen content increased in plaques of *Cblb*$^{-/-}$*Apoe*$^{-/-}$ mice (30.4 ± 2.6% *Apoe*$^{-/-}$ vs. 45.0 ± 3.8% *Cblb*$^{-/-}$*Apoe*$^{-/-}$), whereas plaque VSMC content (2.1 ± 0.3 *Apoe*$^{-/-}$ vs. 2.0 ± 0.1% *Cblb*$^{-/-}$*Apoe*$^{-/-}$) did not differ. Thus, deficiency of CBL-B increased plaque inflammation and macrophage death, thereby accelerating the progression of atherosclerosis.

Casitas B-cell lymphoma-B deficiency induces an atherogenic phenotype in macrophages

Considering the profound increase in early, macrophage-rich lesions observed in the aortic arch and incremented necrotic core formation in the more advanced stages of atherosclerosis in *Cblb*$^{-/-}$*Apoe*$^{-/-}$ mice, we analysed the effects of CBL-B on monocytes and macrophages.

Deficiency of CBL-B increased the expression of the chemokine receptors *CCR1*, *CCR2*, and *CCR5*, all of which mediate leucocyte recruitment into the arterial wall, in primary monocytes and bone marrow-derived macrophages (BMDMs) (*Figure 3A* and *B*). Transcript levels of *CCR7*, a chemokine receptor that governs macrophage egress in atherosclerosis,[15] also increased (*Figure 3A* and *B*). Consistent with these findings, *Cblb*$^{-/-}$*Apoe*$^{-/-}$ monocytes and BMDMs exhibited an increased migratory capacity towards CCL2 (*Figure 3C* and *D*).

Lipopolysaccharide stimulated *Cblb*$^{-/-}$*Apoe*$^{-/-}$ BMDMs produced significantly more reactive oxygen species (ROS) (*Figure 3E* and *F*), TNF (*Figure 3G* and *H*), and IL6 (*Figure 3I*), whereas IL10 expression was reduced (*Figure 3J*). Moreover, CBL-B-deficient BMDMs expressed significantly more MHC-II, pointing towards increased antigen presenting capacity of these cells (*Figure 3K*). Expression of the M1 macrophage marker *iNOS* was increased in aortic arch lysates of *Cblb*$^{-/-}$*Apoe*$^{-/-}$ mice, the M2 markers *arginase 1* and *CD206* were not affected (Supplementary material online, *Figure S2A*).

Upon phagocytosis and cytoplasmic storage of lipoproteins, macrophages evolve into foam cells, the predominant constituent of atherosclerotic plaques. Notably, CBL-B transcript levels decreased during foam cell formation (Supplementary material online, *Figure S2B*). *Casitas B-cell lymphoma-B*-deficient BMDMs expressed higher protein levels of the scavenger receptor CD36 (*Figure 3L*) and ingested significantly more oxLDL (*Figure 3M*), whereas the cholesterol efflux genes *ABCA1* and *ABCG1* were not affected (Supplementary material online, *Figure S2C*). Thus, deficiency of CBL-B enhanced the migratory potential of macrophages, promoted the expression of inflammatory mediators and increased lipid uptake, resulting in an atherogenic macrophage phenotype.

Casitas B-cell lymphoma-B deficiency increases the abundance of CD8^{+} T cells by reducing apoptosis and regulatory T-cells-mediated suppression

As CBL E3 ubiquitin ligases modulate T cell activation and polarization, we investigated T cell appearance in plaques of *Cblb*$^{-/-}$*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ mice. Immunohistochemistry demonstrated a trend towards increased CD3^{+} T cell abundance in the advanced plaques of

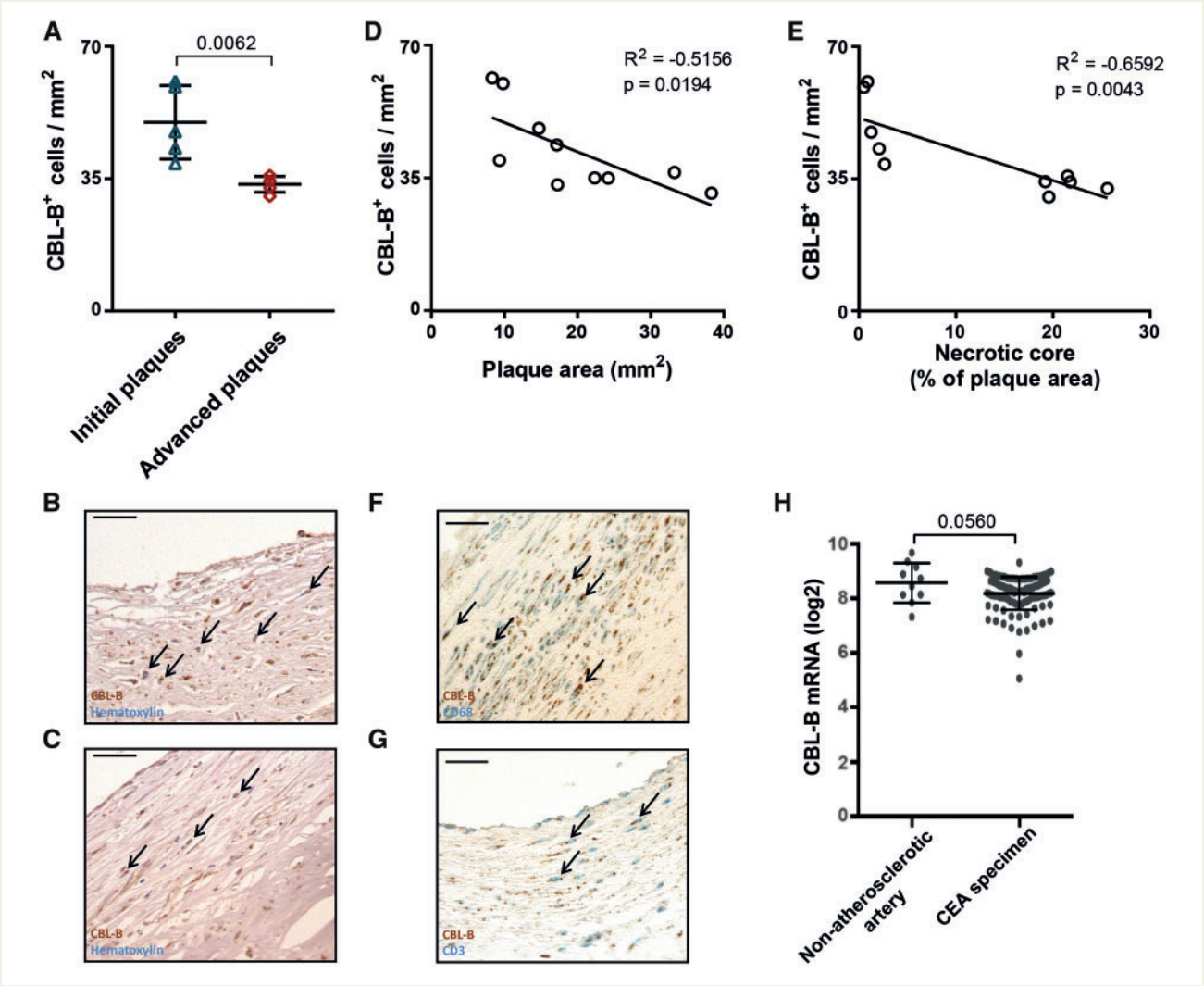


Figure 1 *Casitas B-cell lymphoma-B* is expressed in human atherosclerotic lesions and co-localizes with macrophages and T cells. (*A*) Immunohistochemical analysis of CBL-B expression in initial/intermediate and advanced human coronary atherosclerotic lesions. The percentage of CBL-B$^+$ cells in the lesion decreased in the advanced atherosclerotic plaques ($n = 5$ per plaque phenotype). Representative pictures of (*B*) CBL-B expression in initial/intermediate and (*C*) advanced lesions are shown. Arrows indicate CBL-B$^+$ cells. A negative correlation between (*D*) CBL-B expression and plaque area and (*E*) CBL-B and necrotic core area was observed. CBL-B expression co-localized with CD68$^+$ cells (*F*) and CD3$^+$ cells (*G*), arrows indicate CBL-B$^+$CD68$^+$ or CBL-B$^+$CD3$^+$ cells, respectively. Scale bar 25 μm for all pictures. (*H*) BiKE database: CBL-B mRNA expression in carotid endarterectomy specimens ($n = 127$) when compared with non-atherosclerotic arteries ($n = 10$). Data are presented as mean ± standard deviation.

the aortic roots of *Cblb*$^{-/-}$*Apoe*$^{-/-}$ mice (8.0 ± 3.2% *Apoe*$^{-/-}$ vs. 12.0 ± 3.2% *Cblb*$^{-/-}$*Apoe*$^{-/-}$; $P = 0.08$), specifically due to a significant increase in cytotoxic CD8$^+$ T cells (2.05 ± 1.41% *Apoe*$^{-/-}$ vs. 5.00 ± 2.05% *Cblb*$^{-/-}$*Apoe*$^{-/-}$; $P = 0.003$) (*Figure 4A* and *B*). These findings were supported by flow cytometry, verifying skewing towards more CD8$^+$ T cells in the aortic arch, blood and spleen (*Figure 4C*). In the absence of CBL-B, CD8$^+$ T cells shifted from naïve (CD44$^-$CD62L$^+$) to central memory T cells (CD44$^+$CD62L$^+$) with no differences in the effector memory T cell compartment (CD62L$^-$CD44$^+$) (*Figure 4D*), suggesting enhancement of their activation status.

Next, we studied the potential mechanism underlying the increased abundance of CD8$^+$ T cells and found that CBL-B deficiency enhanced the production of IL2 (*Figure 4E* and *F*), a potent growth factor for T cells. *Casitas B-cell lymphoma-B*-deficient CD8$^+$ T cells were also protected against TNF-induced apoptosis as demonstrated by lower expression of annexin V (*Figure 4G*). Corroborating this finding, more *Cblb*$^{-/-}$*Apoe*$^{-/-}$ CD8$^+$ T cells contained the anti-apoptotic B cell lymphoma 2 (*Bcl2*) protein (*Figure 4H*). Moreover, *Cblb*$^{-/-}$*Apoe*$^{-/-}$ CD8$^+$ T cells were more resistant to Treg-mediated suppression than *Apoe*$^{-/-}$ CD8$^+$ T cells and underwent more vigorous proliferation at various CD8$^+$ T cell:Treg ratios (*Figure 4I*).

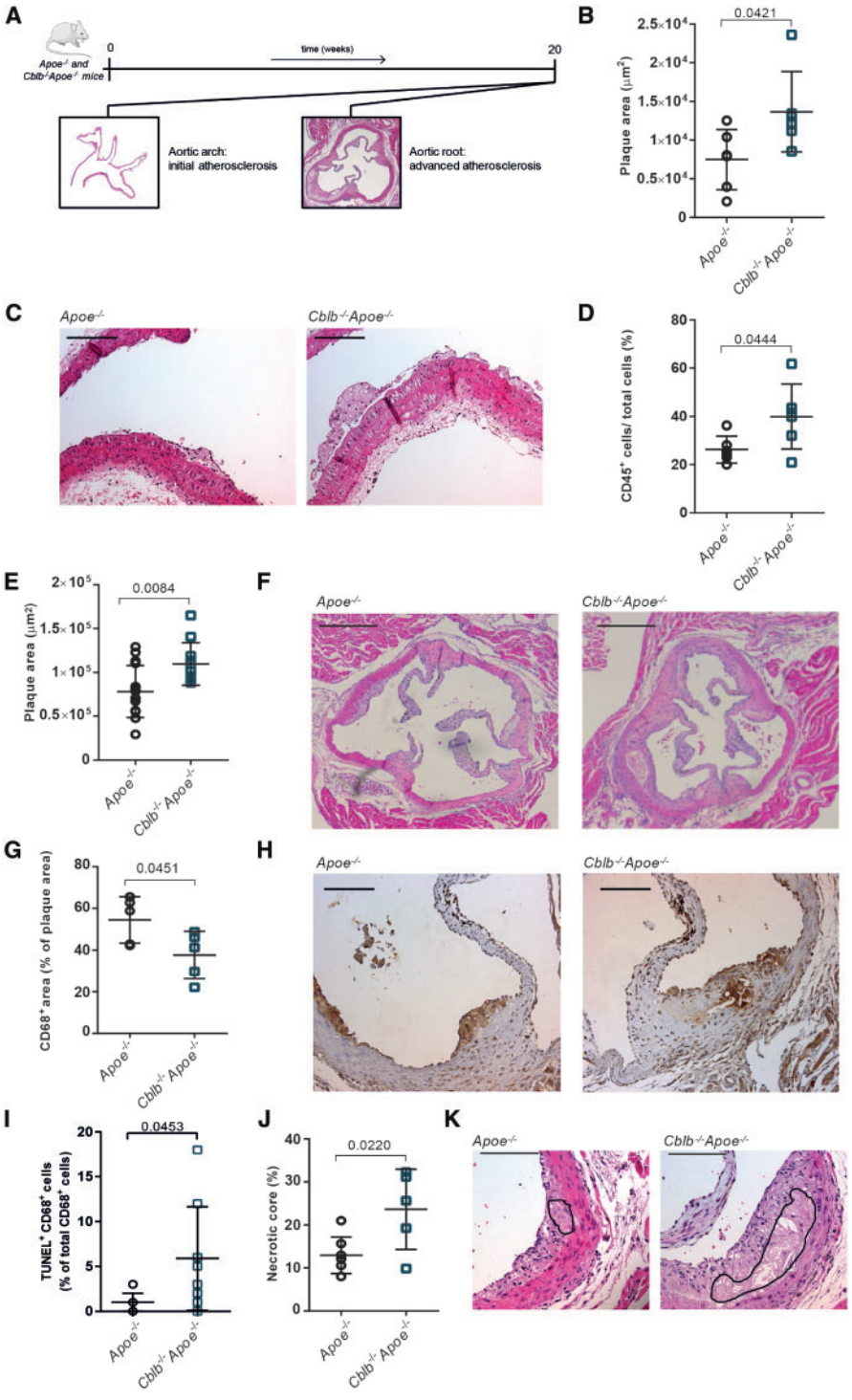


Figure 2 *Casitas B-cell lymphoma-B* deficiency aggravates atherosclerosis in *Apoe*$^{-/-}$ mice. (*A*) Atherosclerosis was analysed in the aortic arch, where initial plaques were present, and the aortic root, which contained advanced atherosclerotic plaques. (*B*) Atherosclerotic plaque area in the aortic arch of 20-week-old *Apoe*$^{-/-}$ ($n = 6$) and *Cblb*$^{-/-}$*Apoe*$^{-/-}$ ($n = 6$) mice. (*C*) Representative longitudinal sections of aortic arches in *Apoe*$^{-/-}$ and *Cblb*$^{-/-}$*Apoe*$^{-/-}$ mice (the brachiocephalic trunk is shown; haematoxylin and eosin staining). Scale bar: 50 μm. (*D*) Immunohistochemical quantification of the relative number of CD45^{+} cells per plaque ($n = 6$ per genotype). (*E*) Aortic roots of 20-week-old *Apoe*$^{-/-}$ ($n = 15$) and *Cblb*$^{-/-}$*Apoe*$^{-/-}$ ($n = 11$) mice were used to analyse the amount of atherosclerosis. (*F*) Representative pictures of haematoxylin and eosin-stained aortic root cross-sections containing advanced atherosclerotic plaques in *Apoe*$^{-/-}$ and *Cblb*$^{-/-}$*Apoe*$^{-/-}$ mice. Scale bar: 500 μm. (*G*, *H*) Plaque macrophage content and representative images of CD68 staining. Scale bar: 200 μm. (*I*) Percentage of apoptotic (TUNEL^{+}CD68^{+}) macrophages in the plaques. (*J*, *K*) Quantification of necrotic core area in plaques of aortic roots. Representative pictures are shown. The black line indicates the necrotic core. Scale bar: 100 μm. Data are presented as mean ± standard deviation.

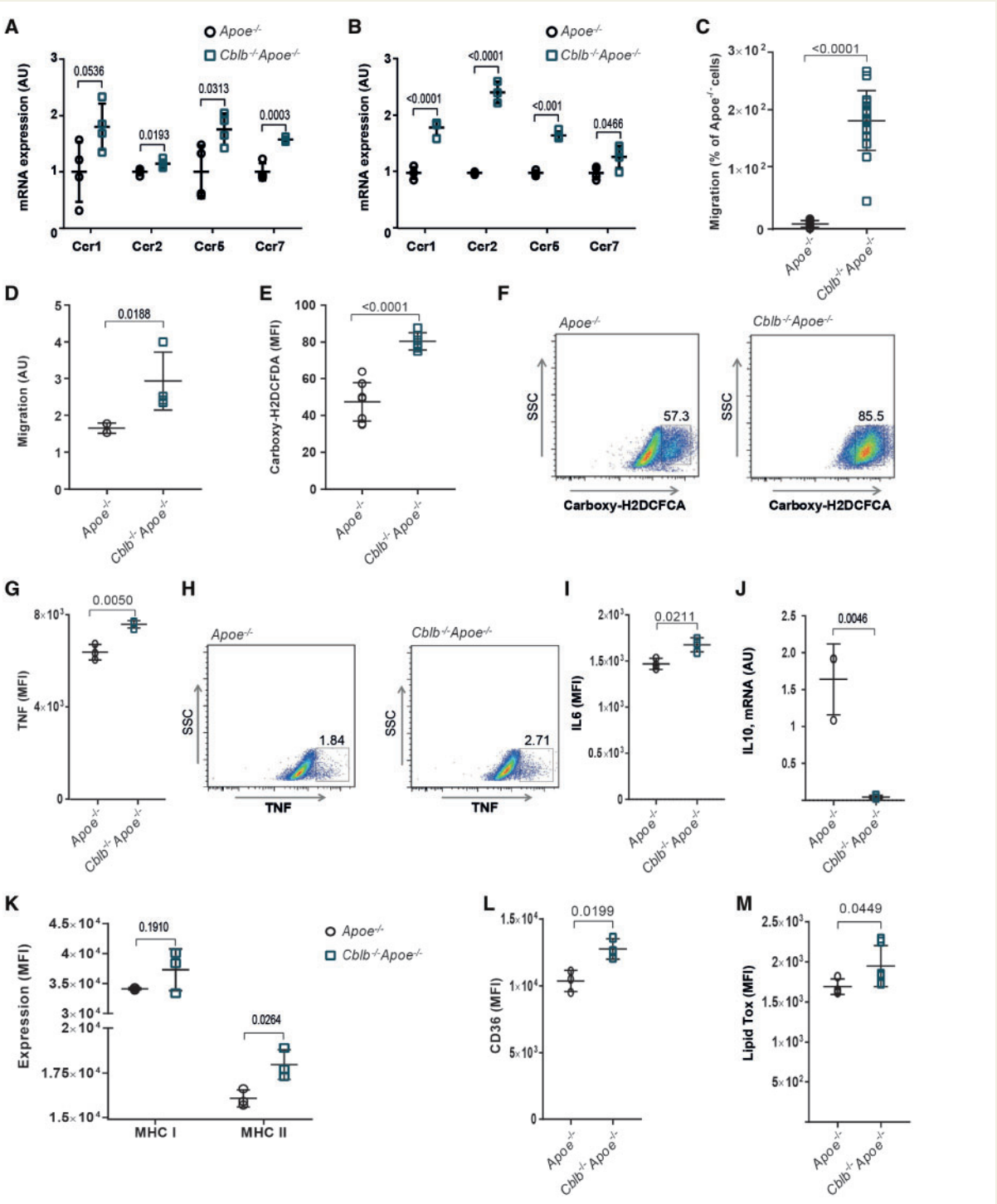


Figure 3 *Casitas B-cell lymphoma-B* deficiency induces an atherogenic phenotype in macrophages. Quantification of mRNA expression of chemokine receptors CCR1, 2, 5, and 7 in monocytes (*A*) and bone marrow-derived macrophages (*B*) of *Apoe*$^{-/-}$ ($n = 4$) and *Cblb*$^{-/-}$*Apoe*$^{-/-}$ ($n = 4$) mice. (*C*) CCL2-induced monocyte migration was increased in *Cblb*$^{-/-}$*Apoe*$^{-/-}$ mice ($n = 16$ per genotype). (*D*) Migration of bone marrow-derived macrophages from *Apoe*$^{-/-}$ and *Cblb*$^{-/-}$*Apoe*$^{-/-}$ mice towards 10 ng/mL MCP-1 by transwell assay ($n = 3$ experiments). (*E*, *F*) Flow cytometric analysis of reactive oxygen species production by *Apoe*$^{-/-}$ ($n = 8$) and *Cblb*$^{-/-}$*Apoe*$^{-/-}$ ($n = 6$) bone marrow-derived macrophages after 48 h LPS stimulation. Representative dot plots; numbers indicate percentage of bone marrow-derived macrophages positive for carboxy-H2DCFCA. Representative dot plot and graph of TNF (*G*, *H*) and interleukin-6 (*I*) production after 24 h exposure to oxLDL ($n = 3$ experiments). (*J*) mRNA expression of interleukin-10 in bone marrow-derived macrophages from *Apoe*$^{-/-}$ and *Cblb*$^{-/-}$*Apoe*$^{-/-}$ mice after 24 h exposure to oxLDL ($n = 3$ experiments). Flow cytometric analysis of MHC-I and MHC-II expression (*K*) and CD36 expression (*L*) of bone marrow-derived macrophages ($n = 3$ experiments). (*M*) Flow cytometric analysis of lipid uptake in bone marrow-derived macrophages ($n = 6$). Data are presented as mean ± standard deviation.

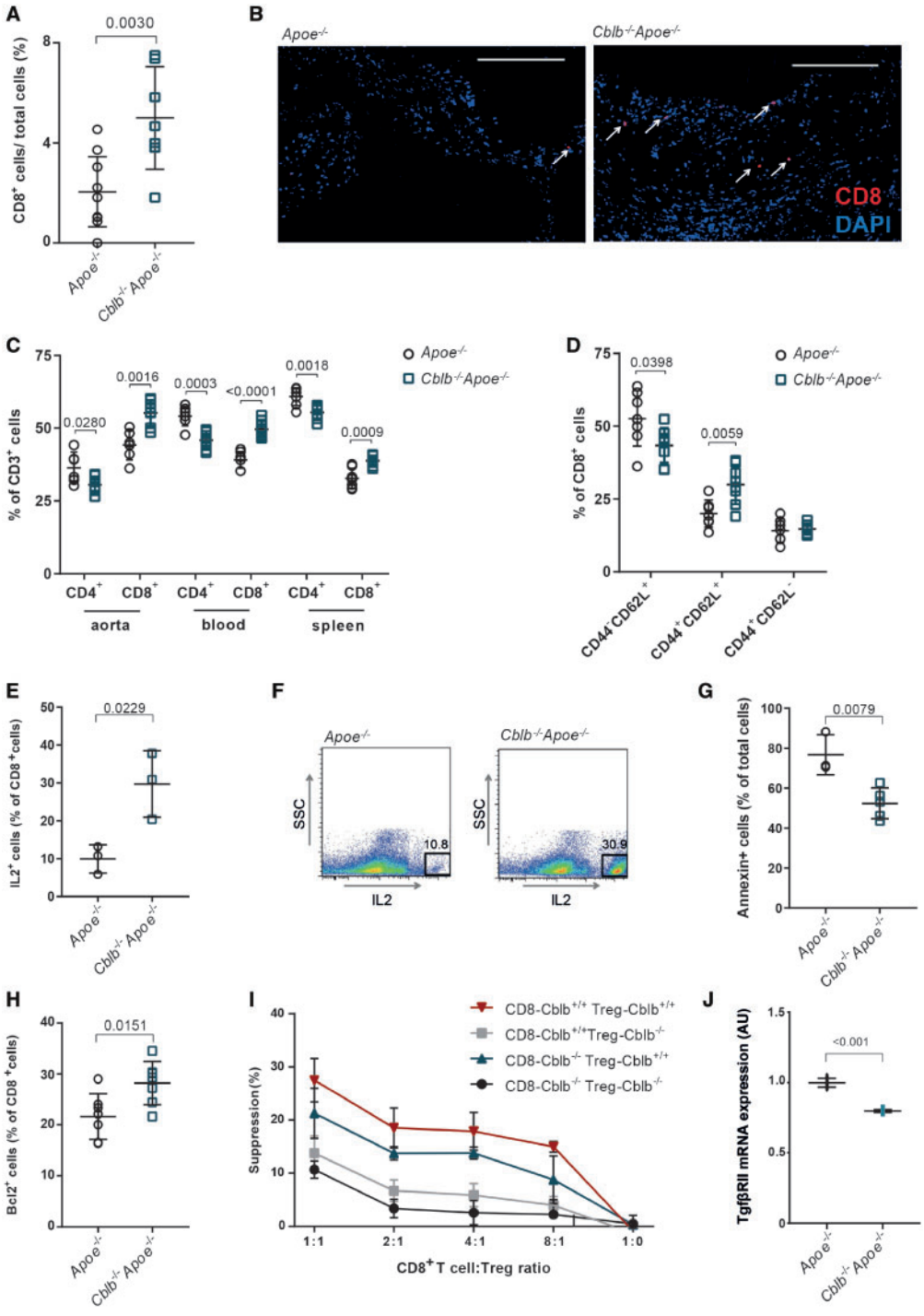


Figure 4 *Casitas B-cell lymphoma-B* deficiency increases CD8$^+$ T cell abundance in *Cblb*$^{-/-}$*Apoe*$^{-/-}$ mice by reducing apoptosis and regulatory T cell-mediated suppression. (*A*) Percentages of CD8$^+$ T cells in advanced atherosclerotic plaques of aortic roots of 20-week-old *Apoe*$^{-/-}$ ($n = 10$) and *Cblb*$^{-/-}$*Apoe*$^{-/-}$ mice ($n = 7$). (*B*) Representative pictures of anti-CD8 (Alexa Fluor 594, red) staining (DAPI staining: blue). White arrows indicate CD8$^+$ T cells. Scale bar: 100 μm. (*C*) Flow cytometric analysis of CD4$^+$ and CD8$^+$ T cells in aortic arch, blood, and spleen of *Apoe*$^{-/-}$ and *Cblb*$^{-/-}$*Apoe*$^{-/-}$ mice ($n = 7$) (*D*) Quantification of naïve (CD44$^-$CD62L$^+$), central memory (CD44$^+$CD62L$^+$), and effector memory (CD44$^+$CD62L$^-$) CD8$^+$ T cells in spleens of *Apoe*$^{-/-}$ and *Cblb*$^{-/-}$*Apoe*$^{-/-}$ mice. (*E*, *F*) interleukin-2 production by CD8$^+$ T cells isolated from *in vitro*-restimulated splenocytes ($n = 3$), Representative dot plots are shown. (*G*) Fraction of apoptotic (Annexin V$^+$) cells of CD3/CD28-activated isolated splenic CD8$^+$ T cells from *Apoe*$^{-/-}$ ($n = 3$) or *Cblb*$^{-/-}$*Apoe*$^{-/-}$ ($n = 5$) mice that were incubated with TNF for 96 h. (*H*) Flow cytometric analysis of BCL2 expression in CD8$^+$ T cells ($n = 7$). (*I*) Regulatory T cell suppression assay using splenic CD8$^+$ T cells and CD4$^+$CD25$^+$ regulatory T cells from *Apoe*$^{-/-}$ and *Cblb*$^{-/-}$*Apoe*$^{-/-}$ mice, co-cultured at various ratios ($n = 3$ experiments). (*J*) TGFβRII mRNA expression in CD8$^+$ T-cells isolated from *Apoe*$^{-/-}$ and *Cblb*$^{-/-}$*Apoe*$^{-/-}$ mice ($n = 3$). Data are presented as mean ± standard deviation.

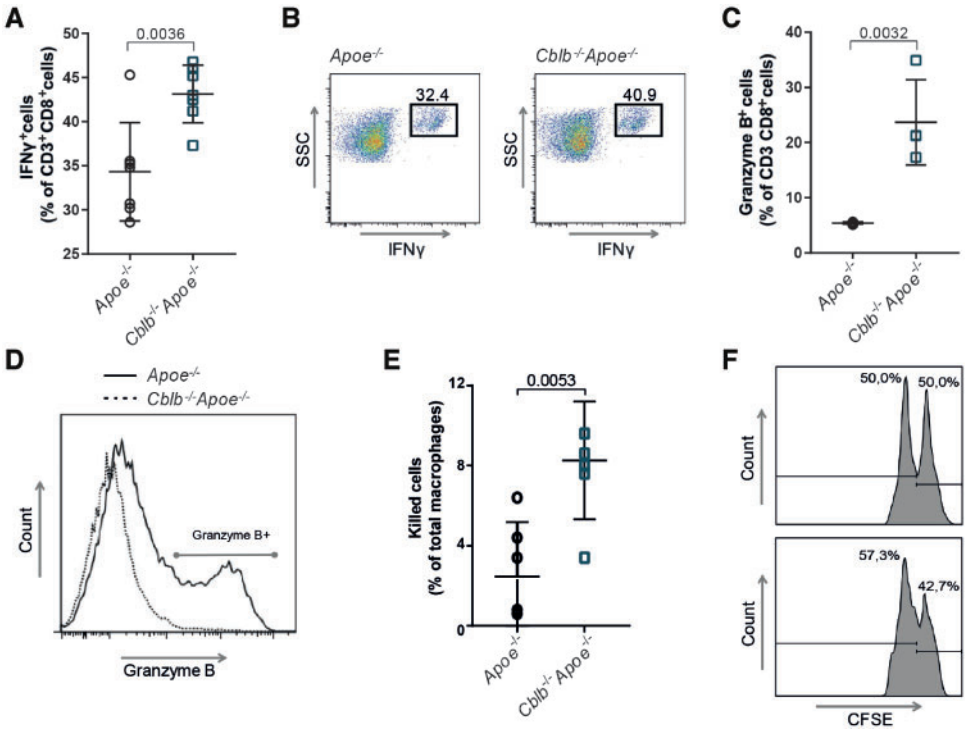


Figure 5 *Casitas B-cell lymphoma-B* deficiency increases the inflammatory and cytotoxic propensity of CD8$^+$ T cells. IFNγ (*A*, *B*) and granzyme B (*C*, *D*) producing CD3$^+$CD8$^+$ cells among *in vitro*-restimulated splenocytes isolated from *Apoe*$^{-/-}$ and *Cblb*$^{-/-}$*Apoe*$^{-/-}$ mice ($n = 7$ for IFNγ; $n = 4$ for Granzyme B). Representative dot plots are shown (dotted line: *Apoe*$^{-/-}$; solid line *Cbl-b*$^{-/-}$*Apoe*$^{-/-}$). (*E*, *F*) *In vitro* macrophage killing assay; *Apoe*$^{-/-}$ and *Cblb*$^{-/-}$*Apoe*$^{-/-}$ splenocytes were cultured in the presence of ovalbumin peptide$^{257–264}$ for 6 days, subsequently CD8$^+$ T cells were isolated and co-cultured with ovalbumin peptide$^{257–264}$-pulsed CFSE^{high} labelled bone marrow-derived macrophages and unpulsed CFSE^{low} labelled bone marrow-derived macrophages ($n = 4$). Representative histograms are shown. Data are presented as mean ± standard deviation.

Additionally, TGFβ receptor II (*TgfβR2*) gene expression was decreased in *Cblb*$^{-/-}$*Apoe*$^{-/-}$ CD8$^+$ T cells, rendering them less sensitive to TGFβ-induced Treg-mediated suppression[16] (*Figure 4J*).

Casitas B-cell lymphoma-B deficiency increases the cytotoxicity of CD8$^+$ T cells and provokes macrophage death

To further characterize, the effects of CBL-B deficiency on cytotoxic T cell function, splenic CD8$^+$ T cells were isolated and the production of effector proteins was analysed. *Cblb*$^{-/-}$*Apoe*$^{-/-}$ CD8$^+$ T cells showed a significant increase in IFNγ protein levels compared with *Apoe*$^{-/-}$ CD8$^+$ T cells (*Figure 5A* and *B*). Moreover, CBL-B-deficient cytotoxic T cells expressed higher levels of granzyme B (*Figure 5C* and *D*), a protein described to promote atherosclerosis by inducing apoptosis in plaque-associated cells.[5] Perforin (*Apoe*$^{-/-}$ 1.0 ± 0.1 vs. *Cbl-b*$^{-/-}$*Apoe*$^{-/-}$ 1.2 ± 0.3) and granzyme A (*Apoe*$^{-/-}$ 0.9 ± 0.1 vs. *Cblb*$^{-/-}$*Apoe*$^{-/-}$ 1.2 ± 0.3) levels remained unchanged.

To investigate whether the increase in effector protein production in CBL-B-deficient CD8$^+$ T cells affected the cytotoxicity of these cells, a macrophage killing assay was performed. Ovalbumin peptide (OVA$^{257–264}$) primed CD8$^+$ T cells were co-cultured with OVA$^{257–264}$-pulsed CFSE^{high}-labelled BMDMs and unpulsed CFSE^{low}-labelled BMDMs. In comparison with OVA$^{257–264}$-primed *Apoe*$^{-/-}$ CD8$^+$ T cells, incubation with OVA$^{257–264}$-primed *Cblb*$^{-/-}$*Apoe*$^{-/-}$ CD8$^+$ T cells significantly reduced the survival of OVA$^{257–264}$-pulsed BMDMs (*Figure 5E* and *F*). These data indicate that the enhanced cytotoxicity of CD8$^+$ T cells, in conjunction with their increased abundance (*Figure 4A–C*), provoked macrophage killing and necrotic core formation in the plaques of *Cblb*$^{-/-}$*Apoe*$^{-/-}$ mice.

CD8$^+$ T cell are the main drivers of atherogenesis in *Cblb*$^{-/-}$*Apoe*$^{-/-}$ mice

To further evaluate the contribution of *Cblb*$^{-/-}$*Apoe*$^{-/-}$ CD8$^+$ T cells to atherosclerosis, *Cblb*$^{-/-}$*Apoe*$^{-/-}$ or *Apoe*$^{-/-}$ bone marrow was transplanted into lethally irradiated *Apoe*$^{-/-}$ recipient mice. Following 6 weeks of recovery, antibody-mediated depletion of CD8$^+$ T cells was initiated and continued for 6 weeks until the assessment of atherosclerosis in the aortic arch and aortic root (*Figure 6A*). Anti-CD8 treatment successfully depleted circulating CD8$^+$ T cells in both *Cblb*$^{-/-}$*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ recipients (*Figure 6B*). CD8$^+$ T cells were also successfully depleted in the lymphoid organs and only a minor increase in CD4$^+$ T cells was observed in *Cblb*$^{-/-}$*Apoe*$^{-/-}$ chimeras (Supplementary material online, *Figure S3*).

Haematopoietic CBL-B deficiency did not affect plaque area in the aortic arch, which contained only very initial plaques (Supplementary material online, *Figure S4*), but markedly increased plaque

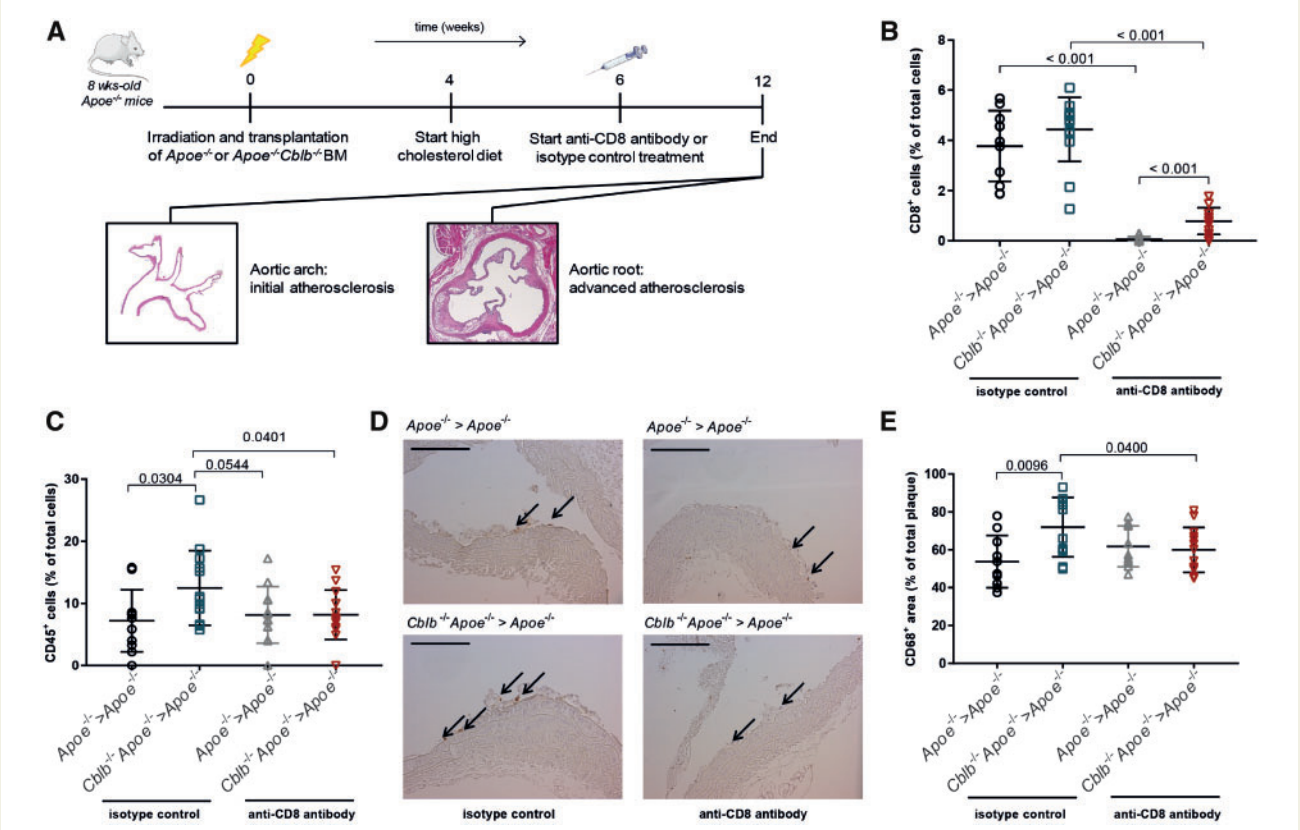


Figure 6 Depletion of *Cblb*$^{-/-}$*Apoe*$^{-/-}$ CD8^{+} T cells reduces inflammation in initial atherosclerotic plaques. (*A*) *Apoe*$^{-/-}$ mice were lethally irradiated and reconstituted with *Apoe*$^{-/-}$ or *Cblb*$^{-/-}$*Apoe*$^{-/-}$ bone marrow and either treated with a CD8^{+} T cell depleting antibody or isotype control for 6 weeks. (*B*) CD8^{+} T cell numbers in the blood of isotype and anti-CD8-treated mice. (*C*, *D*) Immunohistochemical quantification of the relative number of CD45^{+} cells. And representative pictures of CD45-stained aortic arch sections containing initial atherosclerotic plaques. Scale bar: 100 μm. (*E*) Quantification of plaque macrophage content. Data are presented as mean ± standard deviation (n = 11–14).

inflammation as reflected by an increased abundance of CD45^{+} cells (*Figure 6C* and *D*) and MAC3^{+} macrophages (*Figure 6E*). A trend towards increased CD3^{+} T cell content in the plaques of haematopoietic CBL-B-deficient mice was observed (Supplementary material online, *Figure S4*). Depletion of CD8^{+} T cells prevented the increase in CD45^{+} and CD68^{+} cells in the plaques of haematopoietic CBL-B-deficient mice (*Figure 6C–E*), demonstrating that *Cblb*$^{-/-}$*Apoe*$^{-/-}$ cytotoxic T cells drive plaque inflammation in the early stages of atherosclerosis.

In the aortic root, where more advanced plaques were present, haematopoietic deficiency of CBL-B resulted in a 1.8-fold increase in lesion area (*Figure 7A* and *B*). Depletion of *Cblb*$^{-/-}$*Apoe*$^{-/-}$ CD8^{+} T cells prevented this increase (*Figure 7A* and *B*) and ameliorated plaque inflammation, as reflected by the decrease in CD45^{+} cells (*Figure 7C*). Although MAC3^{+} content was not affected by haematopoietic CBL-B deficiency (*Figure 7D*), depletion of CD8^{+} T cells prevented the increase in necrotic core formation that was observed in *Cblb*$^{-/-}$*Apoe*$^{-/-}$ bone marrow chimeras (*Figure 7E* and *F*). This experiment, which demonstrates that depletion of CD8^{+} T cells improves plaque inflammation and halts the progression of atherosclerosis in CBL-B-deficient bone marrow chimeras, indicates that the atheroprotective effect of CBL-B predominantly relies on its function in cytotoxic T cells.

Discussion

Here, we report that CBL-B, the 'natural inhibitor' of T cell activation, has a critical function in atherosclerosis. The expression of CBL-B in human atherosclerotic plaques is lower in advanced lesions when compared with initial lesions and negatively correlated with necrotic core area, indicating that CBL-B expression decreases during the progression of atherosclerosis. Absence of CBL-B aggravates initial atherosclerosis in *Apoe*$^{-/-}$ mice by inducing an atherogenic phenotype in macrophages and accelerates the progression towards advanced atherosclerotic lesions with large necrotic cores. This phenotype results from an increase in CD8^{+} T cell numbers in CBL-B-deficient mice, in conjunction with an enhanced inflammatory and cytotoxic potential of *Cblb*$^{-/-}$*Apoe*$^{-/-}$ CD8^{+} T cells, which provoked macrophage death, as illustrated in our schematic model (*Take home figure*).

Circulating levels of activated CD8^{+} T cells are increased in patients with coronary artery disease and CD8^{+} T cells are abundantly present in human atherosclerotic lesions, where they outnumber CD4^{+} T cells.[3] Experimental studies have attributed a detrimental role to CD8^{+} T cells in atherosclerosis as antibody-mediated depletion of CD8^{+} T cells in *Apoe*$^{-/-}$ mice mitigated atherosclerosis by reducing the number of circulating proinflammatory

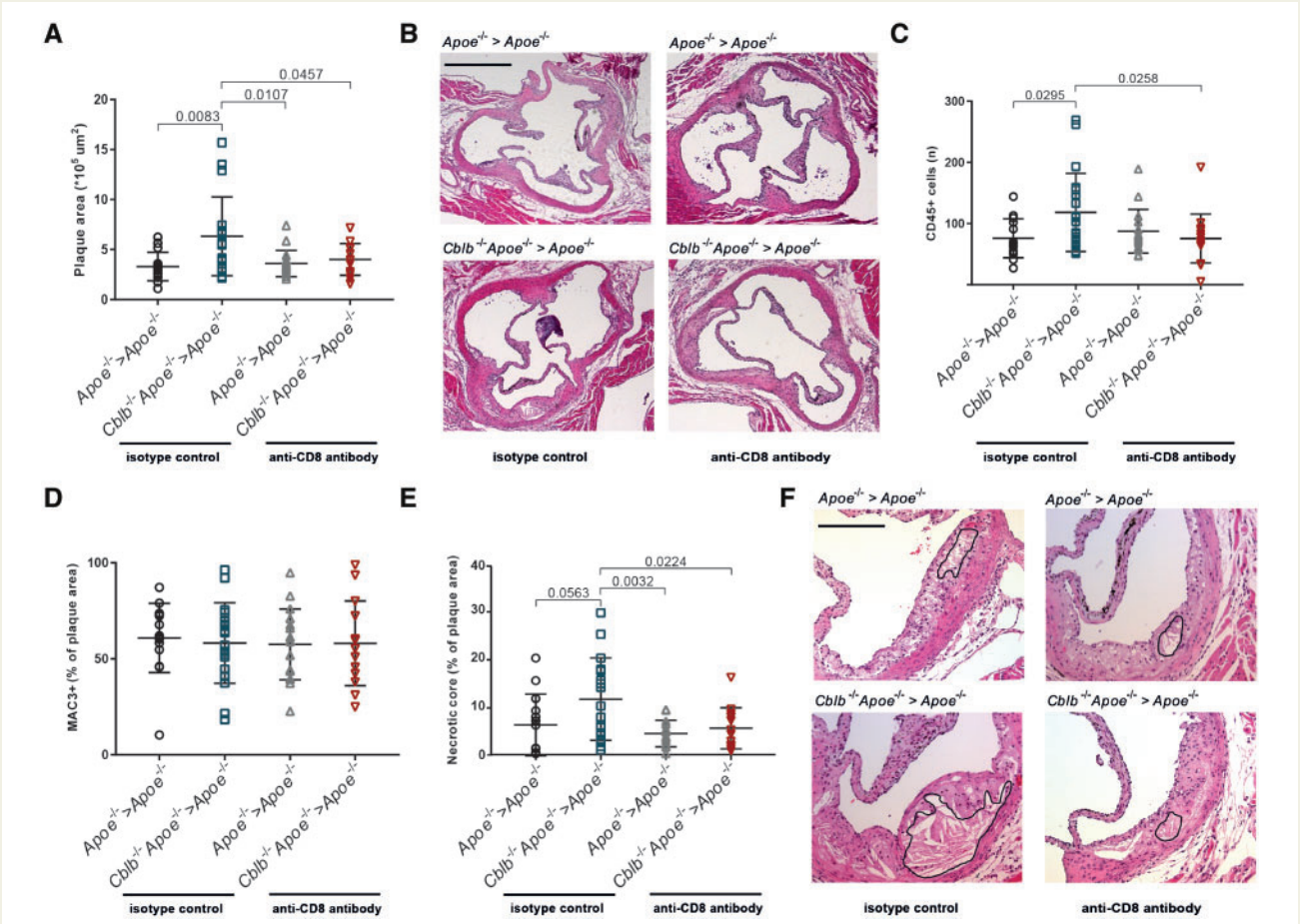
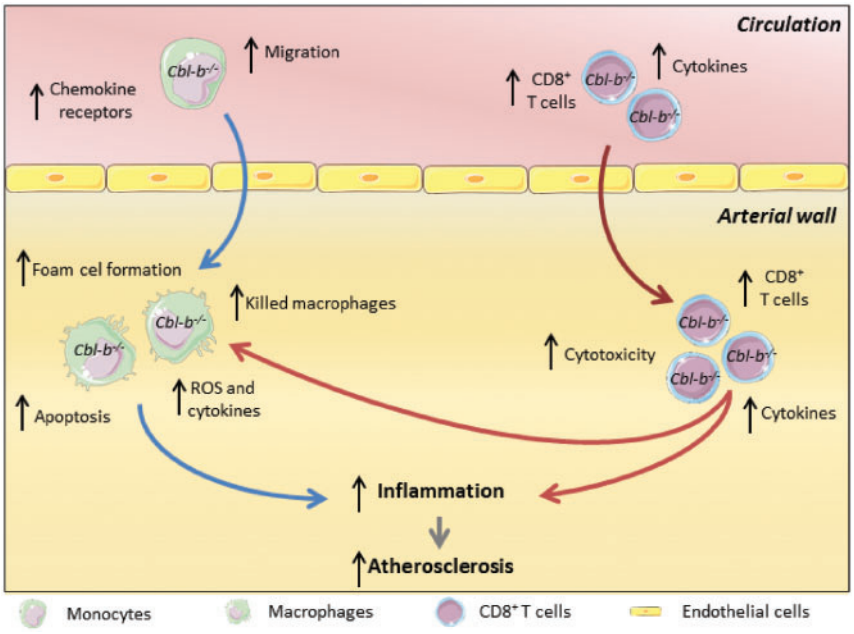


Figure 7 The progression of atherosclerosis is hampered in CD8$^+$ T cell-depleted haematopoietic *Cbl-b*$^{-/-}$*Apoe*$^{-/-}$ chimeras. (*A*) Aortic roots of 20-week-old haematopoietic *Apoe*$^{-/-}$ and *Cblb*$^{-/-}$*Apoe*$^{-/-}$ chimeras analysed for the amount of atherosclerosis. (*B*) Representative pictures of haematoxylin and eosin-stained aortic root cross-sections containing advanced atherosclerotic plaques. Scale bar: 500 μm. (*C–E*) Quantification of plaque CD45$^+$ cells, MAC3$^+$ cells and necrotic core area in plaques of the aortic roots. Representative pictures are shown. The black line indicates the necrotic core. Scale bar: 200 μm. Data are presented as mean ± standard deviation (n = 14–18).

monocytes and hampering macrophage accumulation and apoptosis in the plaque.[6] Accordingly, adoptive transfer of CD8$^+$ T-cells aggravated atherosclerosis and increased necrotic core formation in *Apoe*$^{-/-}$*Rag*$^{-/-}$ mice, due to granzyme B- and perforin-induced macrophage death, resulting in clinically unfavourable high-risk plaques.[5] In the current study, we confirmed that an excess of CD8$^+$ T cells is detrimental in atherosclerosis, particularly due to the increase in CD8$^+$ T cell-mediated macrophage apoptosis and necrotic core formation.

One cause of the increase in CD8$^+$ T cells in *Cblb*$^{-/-}$*Apoe*$^{-/-}$ mice is the lower susceptibility to Treg-mediated suppression. A similar phenotype has been found in *Cblb*$^{-/-}$ CD4$^+$ T cells, which had developed resistance to TGFβ due to SMAD7-mediated down-regulation of TGFβR-II.[16] In our study, CBL-B-deficient CD8$^+$ T cells also expressed less TGFβR-II, rendering them less prone to TGFβ-mediated Treg suppression. Furthermore, Tregs suppress T cell proliferation by capturing IL2, thereby limiting IL2-dependent T-cell proliferation.[17] We found that *Cblb*$^{-/-}$*Apoe*$^{-/-}$ CD8$^+$ T cells secrete more IL2 than *Apoe*$^{-/-}$ CD8$^+$ T cells, lowering their sensitivity to Treg-mediated reductions in IL2. In addition, we demonstrate that CBL-B promotes the suppressive effects of Tregs, which contrasts previous findings that showed no effect of CBL-B ablation on Treg-mediated suppression in *in vitro* polyclonal CD8$^+$ T-cell proliferation assays.[18] This discrepancy might be due to the use of stimulating anti-CD28 and anti-CD3 beads in our study vs. irradiated splenocytes and CD3 stimulation in the earlier reports.[18] In such an experimental setup, Tregs can modulate antigen-presenting cells, interfering with T cell activation, in addition to the suppressive effects on CD8$^+$ T cells.

In addition to the significant effect on CD8$^+$ T cells, CBL-B deficiency also resulted in an atherogenic phenotype in monocytes and macrophages, characterized by an increased migratory potential, increased cytokine production and lipid uptake. Little is known about the function of CBL-B in cells of myeloid origin, but it has been demonstrated that CBL-B mediates TLR4 ubiquitination and impedes the association of the adhesion proteins Lymphocyte Function-associated Antigen 1 (LFA-1) and Intercellular Adhesion Molecule 1 (ICAM-1), thereby inhibiting adhesion and diapedesis.[19] In other disease models, such as diet-induced obesity and sepsis, CBL-B deficiency enhanced the infiltration of macrophages into adipose tissue, causing insulin resistance in obesity, and excessive macrophage infiltration into the lung during sepsis.[11,12] Our study shows that CBL-B deficiency not only increased the migratory potential of monocytes



Take home figure Proposed model of the role of *Casitas B-cell lymphoma-B* in atherosclerosis. During the initial stages of atherosclerosis, *Casitas B-cell lymphoma-B* deficiency increases lesion formation by enhancing monocyte influx into the arterial wall; these monocytes subsequently develop into macrophages with an atherogenic phenotype (indicated by the blue arrows). When atherosclerosis progresses, *Casitas B-cell lymphoma-B* deficiency increases plaque $CD8^+$ T-cell abundance, which aggravates plaque inflammation and provokes macrophage death (indicated by the red arrows), thereby enhancing the progression of plaques towards clinically unfavourable high-risk plaques with large necrotic cores. Agonizing, the function of *Casitas B-cell lymphoma-B* therefore represents a novel therapeutic strategy to target detrimental $CD8^+$ T-cell-driven responses in atherosclerosis.

and macrophages, but also increased the production of inflammatory mediators, which accelerates plaque initiation. Accordingly, we found that depletion of $CD8^+$ T cells in haematopoietic $Cblb^{-/-}Apoe^{-/-}$ mice did not affect lesion formation in the very early stage of atherosclerosis, which is primarily monocyte/macrophage-driven. In the later stages of atherosclerosis, depletion of $CD8^+$ T cells reduced plaque area, plaque inflammation and necrotic core formation, indicating that the progression of atherosclerosis in CBL-B-deficient mice was predominantly driven by $CD8^+$ T cells.

Conclusion

In summary, this study demonstrates that CBL-B puts a brake on $CD8^+$ T cell activation during atherogenesis, thereby inhibiting plaque inflammation and progression towards a clinically unfavourable high-risk plaque phenotype. Although our experimental results should be extrapolated to patients with caution and the effects of targeting ubiquitination in specific immune cells must be scrutinized before being translated into a clinical application, our study attributes a critical role to CBL-B in the regulation of cytotoxic T cell-driven responses in atherosclerosis and provides the basis for novel CBL-B-targeting therapeutic strategies.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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