Cytotoxic and Proinflammatory CD8+ T Lymphocytes Promote Development of Vulnerable Atherosclerotic Plaques in ApoE-/- Mice

**Running title:** *Kyaw et al.; CD8+ T cells promote atherosclerosis*

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**Journal Subject Codes:** Atherosclerosis:[134] Pathophysiology, Vascular biology:[96] Mechanism of atherosclerosis/growth factors, Basic science research:[131] Apoptosis
Abstract:

**Background**—Heart attacks and strokes, leading causes of deaths globally, arise from thrombotic occlusion of ruptured vulnerable atherosclerotic plaques characterized by abundant apoptosis, large necrotic cores derived from inefficient apoptotic cell clearance, thin fibrous caps and focal inflammation. The genesis of apoptosis and necrotic cores in these vulnerable atherosclerotic plaques remains unknown. Cytotoxic CD8+ T lymphocytes represent up to 50% of leucocytes in advanced human plaques and dominate early immune responses in mouse lesions yet their role in atherosclerosis also remains unresolved.

**Methods and Results**—CD8+ T lymphocyte depletion by CD8α or CD8β monoclonal antibody in Apolipoprotein E-deficient (ApoE−/−) mice fed a high fat diet ameliorated atherosclerosis by reducing lipid and macrophage accumulation, apoptosis, necrotic cores, and MCP1, IL1β, IFNγ and VCAM-1. Transfer of CD8+ T cells into lymphocyte-deficient ApoE−/− mice partially reconstituted CD8+ T cells in lymphoid compartments and was associated with CD8+ T cell infiltration in lesions, increased lipid and macrophage accumulation, apoptotic cells, necrotic cores and IL1β in atherosclerotic lesions. Transfer of CD8+ T cells deficient in perforin, granzyme-B or TNFα but not IFNγ failed to increase atherosclerotic lesions despite partial reconstitution in the lymphoid system and presence in atherosclerotic lesions. Macrophages, smooth muscle cells and endothelial cells were identified as apoptotic targets.

**Conclusions**—We conclude that CD8+ T lymphocytes promote development of vulnerable atherosclerotic plaques by perforin- and granzyme B-mediated apoptosis of macrophages, smooth muscle cells and endothelial cells that in turn leads to necrotic core formation and further augment inflammation by TNFα secretion.

**Key words:** CD8+ T cells, perforin, granzyme B, vulnerable atherosclerotic plaque
Atherosclerosis is a chronic inflammatory disease of the arterial wall and its development and progression is driven by systemic hypercholesterolemia and local accelerated immune responses at atherosclerotic lesions. Current atherosclerosis management to reduce cardiovascular risks depends primarily on therapeutic application of statins to lower plasma lipids. However, despite the introduction of statins, atherosclerotic complications of heart attacks and strokes remain leading causes of morbidity and mortality globally. These lethal clinical syndromes are caused by compromise of blood flow in coronary and cerebral arteries by rupture-initiated thrombus.

Vulnerable atherosclerotic plaques are rupture-prone because of focal inflammation, abundant apoptotic cells, large, soft necrotic cores arising from inefficient apoptotic cell clearance and thin fibrous caps. The genesis of the apoptotic cells and necrotic cores in atherosclerotic lesions remains unknown. As CD8+ T cells are known to target vascular endothelial and smooth muscle cells, we hypothesize they are likely candidates to contribute to the generation of these apopto-necrotic products and thin fibrous caps that promote vulnerable plaque development. Cytotoxic CD8+ T lymphocytes are the dominant leukocyte infiltrate in advanced human atherosclerotic lesions. Intimal CD8+ T cells represent 29% of all leucocytes in early human lesions increasing to 50% in advanced plaques. In mouse lesions, CD8+ T cells predominate early immune responses and accumulate with CD4+ T cells in all stages of plaque development. And yet despite reports of their presence and predominance in human and in early mouse lesions, the definitive role of CD8+ T cells in atherosclerosis and their molecular mechanisms of action remains unresolved.

Previous reports have yielded contradictory reports. Female MHC I-deficient C57BL/6 mice generated by disruption of β-2 microglobulin (β2m) gene showed increased atherosclerosis by ~150% compared to wild-type C57BL/6 mice after 15 week-High Fat Diet (HFD). Since...
β2m−/− mice decreased CD8+ T cells12, the data indirectly suggested that cytotoxic T cells are protective in atherosclerosis. However disruption of the β2m gene affects development of CD8α/α TCR-α/β cells only, while development of CD8α/β TCR-α/β cells is mainly dependent on Kb and Db proteins and CD8α/α TCR-γ/δ cells develop independently of MHC class I molecules13. Indeed peptide-specific CD8+ CTL clones have been reported in β2m-deficient mice14. Also, β2m-deficient mice exhibit iron overload15 that may aggravate atherosclerosis16. In contrast, MHC class I deficiency arising from Tap-1 deficiency does not alter atherosclerosis development in ApoE−/− mice17.

Using knockout of the CD8 gene in ApoE−/− mice, atherosclerotic lesions remain unchanged between female CD8-competent and -deficient ApoE−/− mice fed a normal chow diet18. The study suggested that CD8+ T cells do not have a significant role in atherosclerosis. On the other hand, other reports have provided indirect support for a damaging role of CD8+ T cells in atherosclerosis in that CD8+ T cells are increased in atherosclerotic lesions aggravated by treatment with CD137 agonist19, infection with microbes20, PDL1/L2 deficiency21, and in a transgenic mouse model of immune-mediated arterial inflammation and hypercholesterolemia22.

Here, we provide compelling direct evidence for an atherogenic role for cytotoxic CD8+ T lymphocytes in atherosclerosis. We show that depletion of CD8+ T cells using depleting monoclonal antibodies to CD8α or to CD8β in ApoE−/− mice ameliorates atherosclerosis. Conversely, transfer of CD8+ T cells to lymphocyte-deficient mice aggravates atherosclerosis. Transfer studies using CD8+ T cells deficient in perforin (pfp), granzyme B (GZMB) or TNFα suggest that CD8+ T cells promote atherosclerosis by pfp- and GZMB-mediated cytotoxicity as well as TNFα-mediated inflammation. Double immunostaining analysis showed that apoptotic cells in atherosclerotic lesions were identified as macrophages, endothelial and smooth muscle
cells. The data suggest that CD8⁺ T cells contribute to the development of apoptosis and necrotic cores that are characteristic features of vulnerable atherosclerotic plaques.

**Methods**

**Animals**

Wild-type and apolipoprotein E (ApoE)-deficient mice were from the Precinct Animal Centre (PAC), the Alfred Medical Research and Education Precinct (AMREP). Pfp⁻, GZMB- and IFNγ⁻ deficient mice were obtained from Mark Smyth, Peter McCallum Cancer Institute, Melbourne. Lymphocyte-deficient atherosclerosis-prone mice (ApoE⁻/⁻, Rag-2⁻/⁻, common cytokine receptor γ-chain⁻/⁻) and TNFα⁻ deficient mice were maintained at the Animal Facilities, Monash Medical Centre. All mice are on a C57BL/6 background. Animal experiments approved by the Animal Ethics Committee of AMREP were conducted at the PAC.

**CD8 Depleting antibodies**

CD8-depleting antibodies, TIB-210 (rat anti-mouse CD8α-IgG2b) and YTS-156.7 (rat anti-mouse CD8β-IgG2b) were used to deplete CD8⁺ T cells in the study. The TIB-210 depleting antibody (ATCC) was purified from hydridoma supernatant by using Protein G Sepharose (Amersham Biosciences) according to manufacturer’s instruction, dialyzed against 1xPBS and determined the antibody concentration. The YTS-156.7 depleting antibody was a gift from Steve Cobbold, Oxford University, UK. Rat IgG (Sigma) was used as control for the depletion studies.

**CD8⁺ T cell isolation**

CD8 T cell isolation kit (Miltenyi Biotec.) was used to isolate CD8⁺ T cells from splenocytes. Detailed procedure is described in supplementary methods. Its purity (>90% by FACS) and viability (>95% by Trypan-Blue exclusion method) were checked before adoptively transferred
FACS-assisted immune cell analysis

All antibodies used for flow cytometry except anti-foxp3 antibody (eBioscience) were from BD Pharmingen. CD4⁺ and CD8⁺ T cells were determined by pacific blue (PB) anti-CD4 and peridinin chlorophyll protein (perCP) anti-CD8α antibodies respectively. In some experiments, fluorescein isothiocyanate (FITC) anti-CD8β antibody was used. B cells were determined by phycoerythrin (PE) anti-CD22 antibody. Regulatory T cells were determined by PB anti-CD4, allophycocyanin-Cy7 (APC-Cy7) anti-CD25 and PE anti-foxp3 antibodies. NK and NKT cells were determined by PE-Cy7 anti-NK1.1 and FITC anti-TCR-β antibodies. Fluorescence-labeled cells were analyzed by using FACS Canto II flow cytometry system (BD Bioscience) and FACSDiva software (BD Bioscience).

Plasma lipid profiles

Plasma total cholesterol, high-density lipoprotein cholesterol, very-low-density lipoprotein/LDL cholesterol, and triglycerides were determined as described previously²³.

Histological analysis of atherosclerotic lesions

Oil red-O, picrosirius red, hematoxylin and eosin (H&E) and terminal dUTP nick end-labeling (TUNEL) were used to assess atherosclerotic lesion size, collagen content, necrotic core area and apoptotic cells as previously described²⁴. Detailed procedure is described in supplementary methods.

Immunohistochemical analysis of atherosclerotic lesions

Immunohistochemical analyses were performed at aortic root atherosclerotic lesions as described²³. Detailed procedure is described in supplementary methods.

Identification of targeted apoptotic cells in atherosclerotic lesions
Co-localization of apoptotic cells with macrophages, smooth muscle cells or endothelial cells were done using double immunofluorescence or immunoenzymatic staining. Detailed procedure is described in supplementary methods.

Real-time PCR analysis

Gene expressions in aortic arches were determined. Detailed procedure is described in supplementary methods.

Statistical analysis

GraphPad Prism 4 software was used for statistical analyses. Comparisons between groups were carried out using Mann-Whitney U test. For multiple comparisons, results were analyzed using Kruskal Wallis test with Dunn’s multiple comparison post-test to determine significant differences. P < 0.05 was considered significant.

Results

CD8α and CD8β depleting antibodies deplete CD8+ T cells

CD8+ T cells express a dimeric CD8 co-receptor consisting of α/α or α/β chains with the vast majority expressing α/β hetero-dimers. In this study, we used two different CD8 depleting antibodies, TIB-210 and YTS-156.7 targeting CD8α and D8β isoforms respectively to deplete CD8+ T cells. To test the efficiency of CD8α-depleting antibody, we injected male 6-8 week old ApoE−/− mice (n=3) 0.3 mg of anti-CD8α antibody twice a week intraperitoneally and assessed the degree of CD8+ T cell depletion in peripheral blood at days 3 and 7 after injection. We found that test mice showed CD8+ T cell depletion of approximately 97% compared to control mice (Supplementary Figure 2A). CD8β-depleting antibody showed similar (about ~99%) depletion until day 7 after a single dose of 0.5 mg anti-CD8β antibody given intraperitoneally to male 6-8
week old ApoE<sup>−/−</sup> mice (n=8) (Supplementary Figure 2B). Based on pilot studies, CD8α-depleting antibody was given twice a week to deplete CD8 T cells whilst CD8β-depleting antibody was injected once a week (Supplementary Figure 2C-D).

To examine the role of CD8 T cells in atherosclerosis, we fed 6-8 week-old male ApoE<sup>−/−</sup> mice (n=8) a HFD for 8 weeks while depleting CD8<sup>+</sup> T cells with CD8α antibody as described earlier. At the end of the experiment, we found that CD8<sup>+</sup> T cells were depleted by ~86% in the spleen (Figure 1A), ~86% in peripheral blood and ~88% in peripheral lymph nodes (Supplementary Figure 3A). In another depletion study, we used anti-CD8β antibody to deplete CD8<sup>+</sup> T cells in male 6-8 week-old ApoE<sup>−/−</sup> mice (n=8) while feeding a HFD for 8 weeks. CD8β-depleting antibody also depleted CD8<sup>+</sup> T cells by approximately 89%, 72% and 58% and in spleen (Figure 1B), peripheral blood and lymph nodes respectively (Supplementary Figure 3B). However both CD8α- and CD8β-depleting antibodies did not affect other lymphocytes in spleen (Figures 1A-B), peripheral blood and lymph nodes (Data not shown). Dendritic cell expressing CD11c and CD8α were 80% depleted in lymph nodes and spleen by CD8α-, but not by CD8β-depleting antibody (Figure 1C).

**CD8<sup>+</sup> T cell-depletion ameliorates atherosclerosis**

Next, we examined whether CD8<sup>+</sup> T cell depletion affects atherosclerosis in ApoE<sup>−/−</sup> mice. CD8α antibody-treated ApoE<sup>−/−</sup> mice showed a 34% reduction in total lesion area, a 30% reduction in lipid accumulation as assessed by oil red-O and a 33% reduction in CD68<sup>+</sup> macrophage accumulation at aortic root atherosclerotic lesions (Figures 2A-B). An atherogenic role conferred by CD8<sup>+</sup> T cells was confirmed by CD8β-depleting antibody which showed that CD8<sup>+</sup> T cell depletion reduced atherosclerotic total lesion area by 44%, oil red-O by 52% and CD68 macrophage accumulation by 47% (Figures 2C-D). These findings were further substantiated
by 72% reduction in lipid accumulation assessed by Oil-red O en face staining of the entire aorta (Figure 2E).

CD8α- and CD8β-depleting antibodies did not alter the hyperlipidemia and body weight in mice fed a HFD for 8 weeks (Figures 2F-G, Supplementary Figures 3C-D). These results confirmed that CD8+ T cell depletion reduced atherosclerosis in hyperlipidemic ApoE−/− mice.

**Reduced atherosclerosis is associated with reduced CD8+ T cells, apoptosis and necrotic cores in lesions**

Next, we investigated if global depletion of CD8+ T cells in hyperlipidemic ApoE−/− mice affects their accumulation in atherosclerotic lesions. We found that the lesion CD8+ T cells were decreased by 84% in ApoE−/− mice treated with CD8α-depleting antibody (Figure 3A). In contrast, CD4+ T cells in atherosclerotic lesions were unaffected (Figure 3A). Mature dendritic cells are essential for dendritic cell-mediated T-cell activation and expansion via interaction with CD83L present on T cells. CD83 immunoperoxidase staining revealed that CD83+ mature dendritic cells were unaffected by the CD8α-depleting antibody (Figure 3A).

After establishing that global and local CD8+ T cell depletion reduced atherosclerosis in hyperlipidemic ApoE−/− mice, we set out to investigate the mechanism by which CD8+ T cells promote atherosclerosis. CD8+ T cells, upon activation, become cytotoxic T cells and cause target cell lysis via secreting pfp and GZMB25, resulting in promotion of apoptosis-induced inflammation and necrosis. Apoptotic cells as assessed by TUNEL assay and necrotic core area as assessed by H&E stain were decreased by 64% and 49% (Figures 3C-D) respectively in ApoE−/− mice treated with CD8α-depleting antibody. Our finding that CD8+ T cells are responsible for apoptosis-induced necrosis is in accordance with literature and provides a mechanism by which CD8+ T cells promote atherosclerosis.
We examined if smooth muscle and collagen contents which are important in plaque stability are also affected by CD8\(^+\) T cell depletion. Contents of smooth muscle assessed by \(\alpha\)-SMactin antibody and collagens assessed by picrosirius red (Figures 3E-F) showed no difference in ApoE\(^{-/-}\) mice treated with CD8\(\alpha\)-depleting antibody. These results indicated that CD8\(^+\) T cells do not play a role in plaque stability in 8 week-HFD fed ApoE\(^{-/-}\) mice.

**Adoptively transferred CD8\(^+\) T cells repopulated systemically and locally in atherosclerotic lesions**

In addition to pfp and GZMB, cytotoxic CD8\(^+\) T cells also produce cytokines and CD8\(^+\) T cell-produced TNF\(\alpha\) and IFN\(\gamma\) are important in modulation of inflammation\(^{26}\). To determine whether CD8\(^+\) T cells promote atherosclerosis via pfp and GZMB and to investigate the roles of CD8\(^+\) T cell-produced TNF\(\alpha\) and IFN\(\gamma\) in atherosclerosis, a total of 6\(\times\)10\(^6\) CD8\(^+\) T cells from different donors were adoptively transferred into lymphocyte-deficient ApoE\(^{-/-}\) mice (Supplementary Figure 2E). At the end of 8 week-HFD, CD8\(^+\) T cells had repopulated in spleen of recipient mice at about 14-25\% of that population in ApoE\(^{-/-}\) mice (Figure 4A) and were also identified in atherosclerotic lesions (Figure 4B).

**CD8 T cells deficient in pfp, GZMB or TNF\(\alpha\) fail to increase atherosclerotic lesions after their transfer into lymphocyte-deficient ApoE\(^{-/-}\) mice**

There were no differences in body weight and plasma lipids in all recipient mice transferred with CD8\(^+\) T cells compared to transfer of saline buffer (data not shown). Atherosclerotic lesion assessment at the end of 8 week-HFD showed that CD8\(^+\) T cells deficient in pfp, GZMB or TNF\(\alpha\) did not augment atherosclerosis development when compared to saline buffer group despite their systemic and local presence in atherosclerotic lesions after successful reconstitution. Only wild-type and IFN\(\gamma\)-deficient CD8\(^+\) T cells increased total lesion area by 120\% and 169\%. 

DOI: 10.1161/CIRCULATIONAHA.112.001347
oil red-O-stained lipid accumulation by 173% and 146% and CD68+ macrophage accumulation by 195% and 155% respectively compared to saline transfer group (Figures 4C-D). These findings were substantiated by augmented oil-red O en face lipid staining of the entire aorta by 33% (Figure 4E).

**Apoptosis and necrotic core are directly related to pfp and GZMB-competent CD8+ T cells**

Cytolytic CD8+ T cells mediate cytotoxic activities via secreted pfp and GZMB. Apoptotic cell analysis by TUNEL assay provided compelling evidence of pfp- and GZMB-mediated apoptosis. TUNEL+ cells from both pfp- and GZMB-deficient CD8+ T cell transfer groups did not differ from those from saline transfer group. Pfp- and GZMB-competent wild-type, IFNg- and TNFα-deficient CD8+ T cells showed increased TUNEL+ cells at 15%, 10% and 13% respectively compared to saline transfer group at 1% (Figure 5A).

Next, we examined necrotic core areas following adoptive transfer of CD8+ T cells to lymphocyte-deficient mice. Compared to the saline transfer group, we found that transfer of wild-type or IFNg-deficient CD8+ T cells into lymphocyte-deficient ApoE-/- respectively increased necrotic core areas by 286% and 335%. In contrast, while transfer of pfp- and GZMB-deficient CD8+ T cells failed to increase necrotic areas to the same extent as those of wild-type CD8+ T cell transfer, their necrotic core areas were still increased by 151% and 171% compared to the saline buffer group. TNFα-deficient CD8+ T cells also showed an increase in necrotic core area by 294% compared to the saline transfer group (Figure 5B). These results suggest that formation of necrotic cores is not only apoptosis-induced but also inflammation-mediated.

**TUNEL+ cells co-localize with macrophages, endothelial and smooth muscle cells in atherosclerotic lesions**

We next investigated the cellular targets of CD8+ T cell mediated apoptosis in atherosclerotic...
lesions by co-localization of TUNEL+ cells with immunological markers of macrophages, smooth muscle cells and endothelial cells. In the depletion experiment, atherosclerotic lesions that developed in the control antibody-treated group showed co-localization of TUNEL+ cells with markers of macrophages, endothelial and smooth muscle cells by double immunofluorescence staining (Figures 6A-C). In the adoptive transfer study using immunoenzymatic co-localization, we also found that these same 3 cell types co-localized with TUNEL+ cells in atherosclerotic lesions that developed following the transfer of wild-type CD8+ cells into lymphocyte-deficient mice (Figure 6D). Together, these findings provide an evidence for macrophages, endothelial and smooth muscle cells as cellular targets of CD8+ T cell-mediated apoptosis in atherosclerotic lesions. Identification of apoptotic endothelial cells as well as apoptotic smooth muscle cells in the atherosclerotic cap support a role for CD8+ T cells in the development of vulnerable, rupture-prone atherosclerotic plaques.

**Inflammatory responses mediated by CD8+ T cells in atherosclerotic lesions**

Immunohistochemical and real-time analyses in CD8+ T cell-depleted aortic arches showed reduced MCP-1 (CCR2) and VCAM-1 (Figures 7A-C), suggesting that CD8+ T cell-depletion reduced monocyte/macrophage accumulation by reducing the expression of the monocyte-recruiting chemokine MCP-1 and the adhesion molecule VCAM-1. Real-time analysis of proinflammatory cytokines in CD8+ T cell-depleted aortic arches showed reduced IFNγ and IL1β (Figures 7C) indicating an overall reduced local inflammatory milieu within atherosclerotic lesions. However we did not find any significant changes in TNFα expression in the CD8+ T cell-depleted mice (Figures 7C). We found a similar pattern of increased IFNγ and IL1β proinflammatory cytokine expression with the transfer of wild-type CD8+ T cells compared to pfp-, GZMB- or TNFα-deficient CD8+ T cells into lymphocyte-deficient ApoE−/− mice (Figures 7D).
8A-E). TNFα expression was however unaffected. The findings suggest that while TNFα produced by CD8+ T cells has a role in their proinflammatory response, TNFα may also be produced by other inflammatory cells at the local inflammatory site of atherosclerotic lesions; these cells likely include macrophages as they are known to produce TNFα.

Discussion

Here, we investigated the atherogenic role of CD8+ T lymphocytes in the immunopathogenesis of atherosclerosis and their molecular mechanisms of action.

Selective CD8 T cell depletion in the peripheral blood and lymphoid tissues using two monoclonal antibodies, i.e. CD8α- and CD8β-depleting antibodies, significantly reduced CD8+ T cells within aortic atherosclerotic lesions and reduced atherosclerosis development in hypercholesterolemic ApoE-/- mice. Although CD8α antibody also depleted dendritic cells, this is unlikely to have influenced the outcome given that CD8β antibody that spares dendritic cells also reduced atherosclerosis to the same extent. These results suggest that CD8+ T cells promote atherosclerosis by their infiltration into atherosclerotic lesions. Our identification of about 160 cells/mm² of CD8+ T cells in atherosclerotic lesions (Figure 3A) is comparable to a density of 80-100 cells/mm² reported in human AHA type 5 and 6 atherosclerotic lesions, as defined by a fibrous cap and necrotic core.

Our findings of ameliorated atherosclerosis accompanying CD8+ T cell depletion by CD8+ T cell depleting monoclonal antibodies stand in contrast with previous gene knockout studies of CD8+ T cells. These studies showed no change in atherosclerotic lesions using genetic knockouts of the CD8 gene 18 and of the Tap1 gene that resulted in MHC class 1 and CD8 T cell deficiency17. These genetic knock-out studies are difficult to evaluate because genetic deletion of
single immune factors may produce compensatory effects throughout the immune system. As CD4+ T cells promote atherosclerosis development, thymic selection and expansion of developing CD4+ T cells in CD8 or MHC class I knockout mice may have compensated for the atheroprotective effects of CD8+ T cell deficiency27. This suggestion is supported by the increase in CD4+ T cells in the Tap1 deficient mice17. Tap deficiency may be also be further complicated by activation of NK and γδT cells as has been reported in humans with Tap deficiency28. The present study using antibody-depletion of CD8+ T cells may explain the difference in outcome given that this approach deletes a fully developed and mature CD8+ T cell population but leaving the other lymphocyte populations unchanged. Immunization with ApoB-100 peptide reduced atherosclerotic lesions by generating cytotoxic CD8+ T cells directed to dendritic cells that resulted in their reduction at immunization sites and in lesions29. ApoB-100 peptide immunization also induced CD8+ CD25+ IL10+ T cells29, a potent immunosuppressive regulatory T cell population30.

The atherogenicity of CD8+ T cells was confirmed by their adoptive transfer into lymphocyte-deficient mice23. Despite only partial reconstitution of about 15% of the CD8+ T cell population in the spleen, the transferred CD8+ T cells were found in atherosclerotic lesions that accompanied the augmented atherosclerosis.

The amelioration of atherosclerosis with depleted CD8+ T cells is accompanied by reduced apoptotic cells and necrotic cores in lesions. Conversely, transfer of CD8+ T cells to lymphocyte deficient mice increased apoptotic cells and necrotic cores. As necrotic cores likely represent secondary necrosis arising from inefficient clearance of apoptotic cells4, the findings suggest that cytotoxic CD8+ T cells contribute to the genesis of apoptotic cells and necrotic cores in atherosclerotic lesions. Our findings are in accord with reports that CD8+ T cell
infiltrates are directly correlated with TUNEL-positive apoptotic events in human colorectal cancers\textsuperscript{31} and that CD8\textsuperscript{+} T cells are responsible for the apoptosis-induced necrosis in human chronic inflammatory diseases\textsuperscript{32}.

Transfer of pfp- or GZMB-deficient CD8\textsuperscript{+} T cells into lymphocyte-deficient ApoE\textsuperscript{-/-} mice partially reconstituted CD8\textsuperscript{+} T cells in lymphoid compartments and were found in aortic lesions to a level similar to those found with transfer of wild-type CD8\textsuperscript{+} T cells. However, in striking contrast to the augmented atherosclerosis seen with the transfer of wild-type CD8\textsuperscript{+} T cells, the transferred pfp- or GZMB-deficient CD8\textsuperscript{+} T cells failed to augment atherosclerosis and increase apoptotic cells or necrotic cores. This data indicates that pfp and GZMB secretion by CD8 T cells is a major mechanism of action by which cytotoxic T cells mediate apoptosis followed by secondary necrosis leading to necrotic core formation in atherosclerotic lesions. The data is consistent with the release of cytotoxic granules containing pfp and GZMB by activated cytotoxic CD8\textsuperscript{+} T cells to lyse target cells presenting antigenic peptides in the context of MHC class I molecules to the CD8 T cell receptor\textsuperscript{25}. Pfp is the first and limiting step in immune-mediated cytolysis by cytotoxic CD8\textsuperscript{+} T cells and its absence renders CD8\textsuperscript{+} T cells unable to exert their cytotoxic effector function\textsuperscript{25}

Co-localization studies with TUNEL positive apoptotic cells identified macrophages as TUNEL\textsuperscript{+} apoptotic cells in atherosclerotic lesions. The findings are consistent with the report that impaired clearance of apoptotic macrophages is a characteristic feature of atherosclerotic lesions and that this leads to secondary necrosis, a key element in the genesis of necrotic cores in atherosclerotic lesions\textsuperscript{33}. Monocyte-derived macrophages appear early in the intimal layer and are the most abundant inflammatory cell type in atherosclerotic lesions. Our co-localization studies with TUNEL\textsuperscript{+} cells suggest that macrophages are target cells for pfp- and GZMB-
mediated cytolysis by CD8+ T cells in atherosclerosis. Our previous observation that MHC Class I molecules are dramatically up-regulated on macrophages within atherosclerotic lesions, as shown by double immunostaining of aortic root sections with MHC class I and macrophage markers34 is also consistent with this suggestion. Our co-localization studies also identified TUNEL+ apoptotic endothelial cells and smooth muscle cells including those in the fibrous cap. CD8+ T lymphocytes are cytolytic to human endothelia cells5 and smooth muscle cells in injured arteries6 and also cytotoxic to these cells in vivo35. In programmed cell death-1 (PD-1)-deficient Ldr−/− mice, increased CD8+ T cells in the lesions were also associated with more apoptotic cells comprised of both smooth muscle cells and non-smooth muscle35. As oxLDL is taken up by scavenger receptors on macrophages, endothelial and smooth muscle cells36, these cells may utilize MHC class I molecules to present oxLDL-derived peptides to CD8+ T cells. The suggestion is supported by reports that human T lymphocytes are activated by oxLDL37 and oxLDL induces human cytotoxic T lymphocytes in vitro38. Thus OxLDL is a likely antigen driving the human CTL response. Identification of TUNEL+ apoptotic cells in macrophages, endothelial and smooth muscle cells suggests that cytolysis of these cells may contribute to development of rupture-prone atherosclerotic lesions.

Compared to the transfer of wild-type CD8 T cells into lymphocyte deficient mice, atherosclerotic lesions assessed by lipid stain and macrophage accumulation was reduced with the transfer of CD8 T cells deficient in TNFα but not in IFNγ. The data suggest that TNFα not IFNγ secreted by CD8 T cells contributes to the inflammatory responses in atherosclerosis. This suggestion is supported by the observation that compared to transfer of wild-type CD8+ T cells, transfer of TNFα-deficient CD8+ T cells reduced the expression of MCP-1, IFNγ and IL1β but did not affect the generation of TUNEL+ apoptotic cells nor necrotic cores in atherosclerotic
lesions. Our findings are consistent with the established role of TNFα as a potent proinflammatory cytokine\textsuperscript{39} and with the report that it promotes the development of atherosclerosis\textsuperscript{40}. Our studies indicate the important role of CD8\textsuperscript{T} cell-derived TNFα in atherosclerosis. Compared to the transfer of PBS, transfer of wild-type CD8\textsuperscript{+} T cells into lymphocyte deficient mice increased IL1β. The data is consistent with the report that IL1β promotes atherosclerosis development\textsuperscript{41}. Compared to transfer of wild-type CD8\textsuperscript{+} T cells, transfer of pfp- or GZMB-deficient CD8\textsuperscript{+} T cells also reduced the expression of the chemokine MCP-1(CCR2) and the inflammatory cytokines IFNγ and IL1β. The data suggest that the reduction of cytotoxic activity itself can lead to reduced inflammatory activity in atherosclerotic lesions. The suggestion is consistent with the ability of dying cells to generate a sterile inflammatory response\textsuperscript{42}.

Cytotoxic CD8\textsuperscript{+} T lymphocytes are our major defence against a variety of viral and some bacterial infections\textsuperscript{43}. CD8\textsuperscript{+} T cells are pathogenic in a variety of autoimmune diseases. For instance, adoptive transfer of CD8\textsuperscript{+} T cell clones from the SKG mouse model of rheumatoid arthritis, to athymic nude mice resulted in bone and cartilage destruction\textsuperscript{44} while CD8-deficient mice had a lower incidence of collagen induced arthritis\textsuperscript{45}. Activated CD8\textsuperscript{+} T cells are directly related to disease activity in systemic lupus erythematosus patients\textsuperscript{46}. Myelin basic protein-specific CD8\textsuperscript{+} T cells induce severe CNS disease in mice that is similar to multiple sclerosis\textsuperscript{47} and CD8\textsuperscript{+} T cells destroy insulin-secreting pancreatic islet β cells leading to diabetes\textsuperscript{48}. An approach to target cytotoxic CD8\textsuperscript{+} T cells has been investigated in experimental autoimmune diseases. CD8\textsuperscript{+} T cell depletion by antibody delayed the onset and decreased/reversed the severity of experimental arthritis\textsuperscript{49}. A therapeutic approach targeting insulin-reactive CD8\textsuperscript{+} T cells prevented spontaneous diabetes in NOD mice\textsuperscript{50}.
In summary, we provide direct evidence for an atherogenic role for cytotoxic CD8+ T cells and that these cells promote atherosclerosis by the molecular mechanisms of pfp- and GZMB-dependent cytotoxicity as well as TNFα-induced inflammatory mechanisms. Our findings suggest that CD8+ T cells promote the development of vulnerable atherosclerotic plaques through the development of apoptosis, necrotic cores and possible thinning of atherosclerotic caps. Further our findings provide the first insight into the genesis of apoptotic cells and necrotic cores in these vulnerable atherosclerotic lesions and identify lesion macrophages, endothelial and smooth muscle cells as cellular targets of cytotoxic CD8+ T cells. Our findings are entirely consistent with CD8+ T cells as professional cytotoxic T cells that lyse cells infected with intracellular pathogens such as viruses. Targeting CD8+ T cells together with lipid-lowering agents may have the potential to prevent the development of these vulnerable plaques and reduce cardiovascular risks.

**Funding Sources:** This study is supported by the National Health and Medical Research Council of Australia and the National Heart Foundation of Australia and supported in part by the Victorian Government’s Operational Infrastructure Support Program.

**Conflict of Interest Disclosures:** None.

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**Figure Legends:**

**Figure 1.** CD8-depleting antibodies selectively deplete CD8+ T lymphocytes. CD8α-monoclonal antibody, TIB-210 (A) and CD8β monoclonal antibody YTS-156.7 (B) selectively deplete CD8+ T cells without affecting CD4+ T cells, CD22+ B cells, NK1.1+ NK cells and
TCRβ⁺ NK1.1⁺ NKT cells in the spleen. CD11c⁺ CD8α⁺ dendritic cells are depleted by CD8α⁻, but not CD8β-depleting antibodies in spleen (C). White- Control Ig, Black- depleting antibody. n=6-8 in each group. Horizontal bar indicates mean value. * p<0.05

**Figure 2.** CD8⁺ T cell-depletion meliorates atherosclerosis in hyperlipidemic ApoE-deficient mice. Aortic atherosclerotic lesions at the aortic root assessed by total lesion areas, oil red-O stained lipid accumulation (A and C) and CD68 immunohistochemical staining for macrophage accumulation (B and D) were reduced in mice treated with CD8α⁻ and CD8β⁻-depleting monoclonal antibodies respectively. Lipid accumulation in the entire aorta assessed by en face oil red-O stain was reduced in mice treated with CD8β-depleting antibody (E). Body weight (F) and plasma lipid profiles (G) were not altered by CD8α-depleting antibody. White- Control Ig, Black- depleting antibody. n=6-10 in each group. Bars – 100 μm. Horizontal bar indicates mean value. * p<0.05

**Figure 3.** CD8⁺ T lymphocytes, TUNEL⁺ apoptotic cells and necrotic cores in atherosclerotic lesions are reduced in CD8 T lymphocyte-depleted ApoE⁻/⁻ mice. In aortic atherosclerotic lesions of ApoE⁻/⁻ mice treated with CD8 depleting antibody or with control Ig, CD8⁺ T cell depletion by CD8α monoclonal antibody reduced (A) CD8⁺ T cell infiltrates in without affecting CD4⁺ T cells nor CD83⁺ mature dendritic cells. (B) Representative micro- image for IgG control used in all immunohistochemical staining. (C) Apoptotic cells identified by TUNEL assay and (D) necrotic cores assessed by H&E stain were also reduced in CD8⁺ T cell-depleted ApoE⁻/⁻ mice. Smooth muscle (E) and collagen content (F) were not affected. White- Control Ig, Black-depleting antibody. n=8 in each group. Bars – 100 μm Horizontal bar indicates mean value. *
Figure 4. Transfer of CD8⁺ T lymphocytes deficient in pfp, GZMB or TNFα but not IFNγ reduces their capacity to augment atherosclerosis in lymphocyte-deficient ApoE⁻/⁻ mice. Partial reconstitution of CD8⁺ T lymphocytes from different donors in spleen (A) promotes their infiltration into atherosclerotic lesions (B) in lymphocyte-deficient ApoE⁺⁺ mice. Atherosclerotic lesions assessed by total lesion areas, oil red-O stained lipid accumulation (C) and by CD68 immunostained macrophage accumulation (D) in aortic roots. Lipid accumulation of the entire aorta assessed by en face oil red-O stain was augmented in mice transferred with CD8⁺ T cells (E). n=6-8 in each group. Horizontal bar indicates mean value. Bars – 100 μm, * p<0.05 compared to PBS transfer (PBS); # p<0.05 compared to wild-type CD8⁺ T cell transfer (WT).

Figure 5. Pfp- or GZMB-deficient CD8⁺ T lymphocytes failed to augment apoptosis and necrosis in lymphocyte-deficient ApoE⁺⁺ mice. Cytolytic enzymes-competent CD8⁺ T lymphocytes increased apoptotic cells as identified by TUNEL assay (A) and necrotic cores as assessed by H&E stain (B) in adoptive transfer studies. n=6-8 in each group. Bars - 100 μm, Horizontal bar indicates mean value. * p<0.05 compared to PBS transfer (PBS); # p<0.05 compared to wild-type CD8⁺ T cell transfer (WT).

Figure 6. Apoptotic cells are identified as macrophages, endothelial cell and smooth muscle cells. A Confocal immunofluorescence microscopic analysis of frozen sections of control mice from CD8 depletion experiments shows co-localization of TUNEL-positive apoptotic cells (green) with (A) CD68⁺ macrophages (red), (B) CD31⁺ endothelial cells (red) and (C) α-SMA+
smooth muscle cells (red) including those in the atherosclerotic cap. Nuclei (blue) were
counterstained with DAPI. Enzymatic immunohistochemical analysis of frozen sections from
wild-type CD8+ T cell transfer experiments confirms co-localization of apoptotic cells with
macrophages (D-top), endothelial cells (D-middle) and smooth muscle cells (D-bottom).
TUNEL+ nuclei (peroxidase-black) co-localized with CD68+ macrophages (alkaline phosphatase
-red), CD31+ endothelial cells (alkaline phosphatase-red) or α-SMA+ smooth muscle cells
(alkaline phosphatase -red). Yellow arrows- double positive; white arrows- TUNEL-positive.
Sections were counterstained with haematoxylin. Bars – 50 μm

**Figure 7.** MCP-1 (CCL2) chemokine, VCAM-1 adhesion molecule and proinflammatory
cytokines are reduced in CD8 T lymphocyte-depleted ApoE-deficient mice. Reduced MCP-1 (A)
and VCAM-1 (B) as assessed by immunostains in aortic roots lesions of CD8+ T lymphocyte-
depleted ApoE-deficient mice. Reduced mRNA expressions of MCP-1, VCAM-1, IL1β, IFNγ
and TNFα in aortic arches of CD8 T lymphocyte-depleted ApoE-deficient mice (C). White-
Control Ig, Black- CD8α-depleting antibody. n=7-8 in each group. Horizontal bar indicates mean
value. Bars – 100 μm. * p<0.05.

**Figure 8.** Analysis of chemokine, adhesion molecule and proinflammatory cytokines in CD8+ T
cell-transferred groups. Real-time PCR analysis showed differential expression of MCP-1
chemokine (A), VCAM-1 adhesion molecule (E) and proinflammatory cytokines (B-D) in aortic
arches of cytotoxic enzymes-deficient or proinflammatory cytokine-deficient CD8+ T
lymphocyte transfers comparing with PBS-transfer group. n=7-8 in each group. Horizontal bar
indicates mean value. # p<0.05 compared to wild-type CD8+ T cell transfer (WT).
Figure 1

A

CD8α depleting antibody (TIB 210)-Spleen

cell no. (x10⁶)/spleen

CD8+ CD22+ CD4+ T-reg NK NKT

B

CD8β depleting antibody (YTS 156.7)-Spleen

cell no. (x10⁶)/spleen

CD8+ CD22+ CD4+ T-reg NK NKT
Figure 1, cont’d

Absolute cell no. (x10^6)

Spleen

LN

CD8α

depleting ab

Spleen

LN

CD8β

depleting ab

Absolute cell no. (x10^6)
Figure 2
Figure 2, cont’d
Figure 2, cont’d
A  

<table>
<thead>
<tr>
<th>Cells/mm² of lesion area</th>
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<tbody>
<tr>
<td><strong>CD8+ T cells</strong></td>
</tr>
<tr>
<td><strong>CD4+ T cells</strong></td>
</tr>
<tr>
<td><strong>CD83+ Dendritic Cells</strong></td>
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</table>

**Control**

**CD8α**

B

Figure 3
Figure 3, cont’d
Figure 4

A

Spleen

Absolute cell no. (×10^3)

WT  Pfp  GZMB  TNFα  IFNγ  ApoE

transferred CD8+ T cells

B

PBS  WT-CD8+ T  Pfp−/− CD8+ T
Figure 4, cont’d
Figure 4, cont’d
Figure 5

A

TUNEL+ cells/total cells in atherosclerotic lesion (%)

WT transferred CD8+ T cells
Pfp GZMB TNF IFN

PBS

B

necrotic core area / lesion area (%)

WT transferred CD8+ T cells
Pfp GZMB TNF IFN

PBS

Figure 5
Figure 6
Figure 6, cont’d
Expression of mRNA normalized to 18S mRNA

MCP-1 expression (% Lesion Area)

IFN-γ

IL1-β

VCAM-1 expression (% Lesion Area)

Figure 7
Expression of mRNA normalized to 18S mRNA

Figure 8
Expression of mRNA normalized to 18S mRNA

VCAM-1

PBS  WT  Pfp  GZMB  TNFα

Figure 8, cont’d
Cytotoxic and Proinflammatory CD8+ T Lymphocytes Promote Development of Vulnerable Atherosclerotic Plaques in ApoE-/- Mice
Tin Kyaw, Amy Winship, Christopher Tay, Peter Kanellakis, Hamid Hosseini, Anh Cao, Priscilla Li, Peter Tipping, Alex Bobik and Ban-Hock Toh

Circulation. published online February 8, 2013;
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/early/2013/02/08/CIRCULATIONAHA.112.001347

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SUPPLEMENTAL MATERIAL

Supplementary Methods

Animal condition, diet and sample processing
All experimental mice were maintained under pathogen-free environment and fed a HFD containing 21% fat and 0.15% cholesterol (Specialty Feeds, Western Australia) for eight weeks. At the end of experiments, mice were killed and peripheral blood, inguinal and axillary lymph nodes (except in lymphocyte-deficient mice) and spleens were collected for analysis. Aortic roots frozen in OCT embedding medium, aortic arches snap-frozen in liquid nitrogen and plasma were kept in -80°C freezer for further analysis. In some experiments, total aorta containing of aortic arch, thoracic and abdominal aortas were dissected for en face Oil Red-O staining.

Assessment of atherosclerotic lesion at aortic root
Frozen sections (6μm) were cut from the aortic sinus, defined as the region where the valve or valve cusps first become visible to where the left and right coronary arteries branch off1. Slides comprised of 6 cross-sectional areas at 30mm intervals from an aortic root were stained with Oil Red O and examined using light microscope. Total cross-sectional area of lipid deposition was quantified using image analysis software (Optimas, Australia) and averaged. Slides assigned with identification numbers were assessed by another member of laboratory blinded to sample identification.

Assessment of atherosclerosis in total aorta
Whole aortas from ascending aorta to abdominal aorta were cut opened and periaortic fat and blood clot were removed, following by staining with Oil Red-O. Images under light
microscope were captured using FVII Olympus camera and lipid deposition was quantified using image analysis software (Optimas, Australia).

**AutoMACS isolation of CD8\(^+\) T cells**

CD8 T cell isolation kit (Miltenyi Biotec.) containing the biotinylated antibodies cocktail (CD4, CD11b, CD45R, DX5 and Ter119a) was used to negatively isolate CD8\(^+\) T cells from splenocytes. Donor spleens were disrupted using frosted ends of glass slides and single suspension was made after lysing red blood cells. After incubation with biotinylated antibody cocktail, anti-biotin magnetic microbeads were added into the cell suspension. Unlabelled CD8\(^+\) T cells were negatively sorted using autoMACS separator and its purity (>90% by FACS) and viability (>95% by Trypan-Blue exclusion method) were checked before adoptively transferred into recipient mice.

**Immunohistochemical analysis**

Rat anti-mouse CD68 (Serotec, Raleigh, NC), rabbit anti-α-actin-smooth muscle (α-SMactin) (Abcam, Cambridge, UK), rat anti-mouse CD4 (BD Pharmingen), rat anti-mouse CD8 (BD Pharmingen), rat anti-mouse CD83 (eBioscience) antibodies were used to detect macrophages, smooth muscle cells, CD4\(^+\) T cells, CD8\(^+\) T cells and mature dendritic cells in atherosclerotic lesions. Host-matched IgG (rat or rabbit IgG) was used as control in immunohistochemical staining procedure.

Frozen sections of the aortic sinus were fixed in ice-cold acetone for 20 minutes and washed with PBS. Quenching of endogenous peroxidase was performed by incubating the slides in 3% hydrogen peroxide in PBS. Normal horse serum (10% in PBS) was used to block non-specific binding sites, followed by incubation with primary antibodies prepared in blocking solution. Biotinylated goat anti-rat or anti-rabbit immunoglobulins prepared in blocking solution were used in secondary antibody incubation. VECTORSTAIN elite Avidin-Biotin
Complex kit (Vector laboratories) and 3,3’-Diaminobenzidine (DAB) were used to generate brown coloration. After counterstaining with haematoxylin and dehydration, slides were mounted with DEPEX.

Slides assigned with identification numbers were assessed by another member of laboratory blinded to sample identification. Quantification of macrophage and smooth muscle accumulation was assessed by CD68-positive or α-SMActin staining area using Optimas software. Cells stained with CD4, CD8 and CD83 were manually counted under light microscopy and corrected to total lesion areas as quantified by Optima software.

**Necrotic core assessment**

Atherosclerotic lesions at aortic roots were stained with haematoxylin and eosin (H&E) stains. Necrotic core area was defined as a cellular area that was not stained by H&E. Atherosclerotic lesion area (total intimal area) and acellular area (H&E negative area in atherosclerotic lesions) were quantified using Optimas software. Necrotic core quantification was done by individual who did not have assessed to identification numbers. Total necrotic core areas were expressed as percentage of total atherosclerotic lesion area.

**Apoptotic cells assessment**

Apoptotic cells were identified by terminal dUTP nick end-labeling (TUNEL) method using In-Situ Cell Death Detection Kit, POD (Roche). Frozen sections of atherosclerotic lesions were fixed in buffered formalin and washed with PBS. After inactivating endogenous peroxidase enzymes, slides were incubated in Triton-X100 permeabilization buffer. Attachment of fluorescein-dUTP to free 3’OH ends in the DNA of apoptotic cells was carried out by incubating with the TUNEL reaction mixture containing TdT and fluorescein-dUTP. Apoptotic cells were developed by using an anti-fluorescein antibody conjugated with peroxidase and
DAB. After counterstaining with haemoxylin and dehydration, slides were mounted with DEPEX.

Slides assigned with identification numbers were assessed by another member of laboratory blinded to sample identification. Apoptotic cells were manually counted under light microscopy and corrected to total lesion areas as quantified by Optima software.

**Double immunofluorescence and immunoenzymatic staining of apoptotic cells**

Frozen aortic root sections were stained by TUNEL assay kit (Roche), followed by rat anti-CD68 (Serotec, Raleigh, NC), rat anti-mouse CD31 (BD Pharmigen) or rabbit anti-α-actin-smooth muscle (α-SMactin) (Abcam, Cambridge, UK) for macrophages, endothelial and smooth muscle cells respectively. Goat anti-rat or anti-rabbit secondary antibody conjugated with Alexa-Flor 546 (Molecular Probes) was used to detect target cells in red fluorescence and TUNEL-positive nuclei emitted green fluorescence. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Images were scanned and generated by using Carl Zeiss Laser Scanning System LSM 510 and Zeiss LSM imaging software.

In enzymatic immunohistochemical analysis, TUNEL-stained cells were first detected by peroxidase system (Roche), followed by detection of macrophages, endothelial or smooth muscle cells with anti-CD68, anti-CD31 and anti-α-SMactin antibodies in alkaline phosphatase system (Vector Laboratories). TUNEL+ nuclei (peroxidase-black), CD68+ macrophages (alkaline phosphatase-red), CD31+ endothelial (alkaline phosphatase-red) or α-SMactin+ smooth muscle cells (alkaline phosphatase-red) were examined under light microscope.

**RNA extraction and adhesion molecule and proinflammatory cytokine gene expression**
RNeasy fibrous tissue mini kit (Qiagen) was used to extract total RNA from aortic arches according to manufacturer’s instruction. RNA quantity and integrity were determined using MultiNA electrophoresis system (Shimadzu, Japan). Adhesion and proinflammatory cytokine gene expression was determined using single-step QuantiFast SYBR Green RT-PCR kit (Qiagen) on 7500 Fast Real-Time PCR system (Applied Biosystem). The target gene expression levels were analyzed using comparative cycle threshold method with 18S rRNA primers (Applied Biosystems)\textsuperscript{3,4}. The primers used were as follows:

- IFN\textgamma\textsuperscript{\textgamma} sense (S) 5'-AAGTTTGAGGTCAACAACCCAC-3';
- IFN\textgamma\textsuperscript{\textgamma} antisense (AS) 5'-GCTGGCAGAATTATTCTTATTGGG-3';
- TNF-\alpha (S) 5'-TCTCAGCCTCTTCTTCTCCT-3';
- TNF-\alpha (AS) 5'-ACTTGGTGTTTGTGCTACGAC-3';
- MCP-1 (S) 5'-CTCAGCCAGATGCAGTTAACG-3';
- MCP-1 (AS) 5'-GGGTCAACTTCACATTCAAAGG-3';
- IL1\beta (S) 5'-CCACCTCAATGGACAGAATCTCAA-3';
- IL1\beta (AS) 5'-GTCGTTGCTTGGTTCTCCTTGT-3'
- VCAM-1 (S) 5'-AGAACCAGACAGACAGTCC-3'
- VCAM-1 (AS) 5'-GGATCTTCAGGGAATGAGTAGAC-3'
**Supplementary Table 1. Differential lymphocyte populations in purified CD8+ T cells**

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<th>Cell types</th>
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<td>CD8</td>
<td>CD8^+</td>
<td>90.53%</td>
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<tr>
<td>CD8αβ</td>
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<td>CD8β⁺</td>
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<tr>
<td>CD4</td>
<td>CD4⁺</td>
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<td>NK</td>
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<td>NKT</td>
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<td>DNT</td>
<td>CD4⁺ CD8 NK1.1⁻ TCRβ⁻</td>
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<td>Others</td>
<td>CD4⁻ CD8 NK1.1⁻ TCRβ⁻</td>
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Splenocytes were subjected to magnetic sorting procedures as instructed in CD8 T cell isolation kit (Miltenyi Biotec). Differential analysis of negatively-sorted CD8 cells was performed by flow-cytometry (n=3)
Supplemental Figure 1

- CD8β vs. CD8α
- CD8β vs. CD4
- NK 1.1 vs. TCRβ
- CD19 vs. TCRβ
Supplemental Figure 2

A

Control
CD8α

88% depletion
Day 3

97% depletion
Day 7

CD4

CD8

B

Control
CD8β

99% depletion
Day 3

99% depletion
Day 7

CD4

CD8

C

CD8 T cell depletion
by CD8α antibody

ApoE⁻/⁻ mice

HFD for 8 weeks

CD8 T cell depletion
by CD8β antibody

ApoE⁻/⁻ mice

HFD for 8 weeks

D

E

CD8 T cell transfer
at week 0

CD8 T cell transfer
at week 4

Lymphocyte-deficient ApoE⁻/⁻ mice

HFD for 8 weeks

Kill

Kill

Kill
Supplemental Figure 3

A. CD8α depleting antibody (TIB 210)

B. CD8β depleting antibody (YTS 156.7)

C. Body weight (gm)

CD8β depleting antibody

D. Plasma cholesterol (mmol/L)

CD8β depleting antibody
Figure legends

Supplemental Figure 1. Purity assessment of isolated CD8 T cells: Representative FACS analysis of autoMACS-sorted CD8$^+$ T cells before their adoptive transfer to lymphocyte-deficient ApoE$^{-/-}$ mice.

Supplemental Figure 2. Depletion efficiency of CD8-depleting antibodies and experimental design. Pilot study to test depletion efficiency of CD8$\alpha$-depleting antibody-TIB-210 (A) and CD8$\beta$-depleting antibody-YTS-156.7 antibody (B) in spleen, n=3 in each group. CD8$^+$ T cell depletion in ApoE$^{-/-}$ mice fed a HFD for 8 weeks using (C) CD8$\alpha$-depleting antibody (0.3mg injected intraperitoneally twice a week) or (D) CD8$\beta$-depleting antibody (0.5mg injected intraperitoneally once a week). Rat IgG (Sigma) was used as control for the depletion studies. In the transfer study, a total of 6x10$^6$ CD8$^+$ T cells (3x10$^6$ cells via tail vein at weeks 0 and 4) were adoptively transferred to lymphocyte-deficient ApoE$^{-/-}$ mice fed a high fat diet for 8 weeks (E).

Supplemental Figure 3. CD8$^+$ T cells are depleted without affecting body weight and lipid profiles: At the end of 8 week-HFD, CD8$\alpha$-depleting antibody (A) and CD8$\beta$-depleting antibody (B) depleted CD8$^+$ T cells in peripheral blood and lymph nodes. Body weight (C) and plasma lipid profiles (D) were unaffected by CD8$\beta$-depleting antibody. n=7-9 in each group. White- Control Ig, Black- depleting antibody. Horizontal bar indicates mean value. * p<0.05
References


