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$CD8^+CD25^+$ T cells reduce atherosclerosis in apoE(−/−) mice

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ABSTRACT

Background: It is increasingly evident that $CD8^+$ T cells are involved in atherosclerosis but the specific subtypes have yet to be defined. $CD8^+CD25^+$ T cells exert suppressive effects on immune signaling and modulate experimental autoimmune disorders but their role in atherosclerosis remains to be determined. The phenotype and functional role of $CD8^+CD25^+$ T cells in experimental atherosclerosis were investigated in this study.

Methods and results: $CD8^+CD25^+$ T cells were observed in atherosclerotic plaques of apoE(−/−) mice fed hypercholesterolemic diet. Characterization by flow cytometric analysis and functional evaluation using a CFSE-based proliferation assays revealed a suppressive phenotype and function of splenic $CD8^+CD25^+$ T cells from apoE(−/−) mice. Depletion of $CD8^+CD25^+$ from total $CD8^+$ T cells rendered higher cytolytic activity of the remaining $CD8^+CD25^-$ T cells. Adoptive transfer of $CD8^+CD25^+$ T cells into apoE(−/−) mice suppressed the proliferation of splenic $CD4^+$ T cells and significantly reduced atherosclerosis in recipient mice.

Conclusions: Our study has identified an athero-protective role for $CD8^+CD25^+$ T cells in experimental atherosclerosis.

1. Introduction

The presence of $CD8^+$ T cells in atherosclerotic lesions is widely demonstrated but studies investigating their role in atherogenesis have yielded contradictory results. It was reported that mice genetically deficient in $CD8^+$ T cells did not have altered atherosclerosis development [1,2]. More recently, apoE(−/−) mice severely depleted of $CD8^+$ T cells using depleting antibodies showed significantly reduced atherosclerosis suggesting a pro-atherogenic role [3]. On the other hand, Fyfe et al. demonstrated that an atherogenic diet induced a three-fold increase in atherosclerotic lesion size in MHC I knockout mice which are $CD8^+$ T cell deficient [4]. These discrepant findings may be attributed to different experimental designs. However, it is also possible that $CD8^+$ T cells have subtype specificity in their functional role as suggested by our recent report wherein reduced neointima formation after vascular injury was rendered by $CD8^+CD28^{hi}$ T cells but not by $CD8^+CD28^+$ T cells [5], and by the reported association between $CD8^+$ T cell subsets and cardiovascular disease [6]. It is notable that $CD8^+$ T cells have historically been referred to as T suppressor cells [7].

Evidence suggests that specific $CD8^+$ T cell phenotypes may be involved in modulating atherosclerosis. It was reported that hypercholesterolemia activated $CD8^+CD28^+$ T cells that preceded $CD4^+$ T cell activation in mice [8], and that residing $CD8^+$ T cells in human atherosclerotic plaques have an activated phenotype with increased CD25 expression [9]. However, it remains unclear whether the observed phenotypes contribute to disease progression or if it operates to down-modulate pro-atherogenic immune signaling. For example, $CD8^+CD25^+$ T cells functioning as suppressors are involved in the modulation of autoimmune disorders with similar function to $CD4^+$ Tregs [7,10–15]. The role of this phenotype in atherogenesis is highlighted by our previous report showing increased $CD8^+CD25^+$ T cells early after immunization of apoE(−/−) mice with an apoB-100 derived peptide vaccine [16].

In this study, we characterized the phenotype and function of $CD8^+CD25^+$ T cells from apoE(−/−) mice and defined their role in experimental atherosclerosis using adoptive cell transfer strategy.

2. Materials and methods

2.1. Animals

Male apoE(−/−) mice on a C57BL/6 background were purchased from Jackson Laboratories (Bar Harbor, Me), housed in a pathogen-free animal and kept on a 12-h day/night cycle with unrestricted access to water and regular mouse chow (5015, PMI Nutrition

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International) unless mentioned otherwise. The Institutional Animal Care and Use Committee of Cedars-Sinai Medical Center approved the experimental protocols.

A group of 7 week-old apoE(−/−) mice were fed regular mouse chow or an atherogenic diet (TD 88137, Harlan-Teklad) for 6 weeks. Mice were euthanized and the peripheral blood mononuclear cells were collected, stained and subjected to flow cytometric analysis. $CD8^+$ T cells in atherosclerotic plaques were visualized by immunohistochemical staining of cryo-sections of aortic sinus of atherogenic diet-fed apoE(−/−) mice euthanized at 25 weeks of age using standard immuno-staining protocol with a monoclonal anti-mouse CD8b antibody (BD Biosciences) as primary antibody. Negative control involved immunostaining with omission of primary antibody. Detection of $CD8^+CD25^+$ T cells in atherosclerotic plaques was assessed by enzyme digestion of plaques of apoE(−/−) mice fed the atherogenic diet and euthanized at 29 weeks of age. Plaques were enzymatically digested (0.25 mg/ml collagenase, 0.125 mg/ml elastase, 60 U/ml hyaluronidase in DMEM/F12 medium at 37 °C for 45 min) and subjected to flow cytometry. Flow cytometric analysis was performed by staining cells with FITC-conjugated CD8b and PE-conjugated CD25 antibodies (eBioscience) and analyzed on an LSR II apparatus (BD Biosciences).

2.2. Phenotypic characterization of $CD8^+CD25^+$ T cells

Splenocytes pooled from two to three 13 week old male apoE(−/−) mice fed regular mouse chow were characterized by flow cytometric analysis using standard protocols with antibodies from eBioscience unless otherwise indicated: anti-CD8b, anti-CD25, anti-CD28, anti-FoxP3, anti-IL-10, anti-TGF-β (R&D Systems), anti-IL-12, anti-IFN-γ, anti-CCR7, anti-GITR (Biolegend), and anti-CTLA-4 (Biolegend). Cell permeabilized for staining intracellular molecules was performed using standard procedure. Gating and cut off were based on isotype controls. Data files were analyzed using Summit V4.3 software (DAKO).

2.3. Functional characterization of $CD8^+CD25^+$ T cells

Pooled splenocytes from 13-week old male apoE(−/−) mice fed regular mouse chow were purified by sorting with ARIA II Cell Sorter (BD Biosciences) for the following cell populations: $CD8^+CD25^+$, $CD8^+CD25^-$ and $CD4^+CD25^-$ T cells. Purified $CD4^+CD25^-$ T cells (responder cells) were labeled with CFSE (Invitrogen, 2.5 μM at 37 °C for 10 min) and then cultured (1×10^5 cells in 100 μl culture medium) in the presence of CD3/CD28 Dynabeads (Invitrogen) at the cell-to-bead ratio of 1:2 with $CD8^+CD25^+$ or $CD8^+CD25^-$ T cells (treatment groups) at a ratio of 1:1. The control was $CD4^+CD25^-$ T cells without $CD8^+$ T cells. Four days after co-culture, cells were stained for CD4 and analyzed by LSR II analyzer. The results are expressed as the CFSE mean fluorescent intensity (MFI).

Cytolytic activity was assayed using bone marrow-derived dendritic cells (BMDCs) as target cells [17]. In brief, bone marrow cells from femurs and tibiae of male apoE(−/−) mice were cultured in complete RPMI-1640 containing 10 ng/ml GM-CSF (R&D Systems) and 10 ng/ml IL-4 (Invitrogen). Immature DCs were harvested on day 8 and subcultured into new culture plates with 2×10^5 DCs in 1.5 ml medium. The negatively isolated $CD8^+$ T cells or $CD8^+$ T cells depleted of $CD8^+CD25^+$ cells were then co-culture with DCs at a CD8:DC ratio of 3:1 for 4 h, cells were then collected and processed for flow cytometric determination of CD11c and 7-AAD (eBioscience) by LSR II analyzer. CD11c positive cells stained with 7-AAD were identified as lysed target cells using target cell death without $CD8^+$ T cells as baseline [17].

2.4. Adoptive transfer experiment

T cells used for adoptive transfer were harvested from male apoE(−/−) mice fed regular mouse chow at 13 weeks of age. $CD8^+$ T cells were negatively isolated from pooled splenocytes using Dynal Mouse CD8 Negative Isolation Kit (Invitrogen) according to the manufacturer's protocols. Purity of the isolated $CD8^+$ T cells was ⩾90%. The selected $CD8^+$ T cells were then stained with PE anti-mouse CD25 antibody (eBioscience), and $CD25^+$ T cells were collected by sorting with a MoFlo cell sorter (BD Biosciences), similar to a previously reported antibody based cell sorting method [18]. Pilot experiments using total $CD8^+$ T cells at doses of 1×10^5 and 1×10^6 showed that the lower dose recipients had a trend for reduced atherosclerosis. We therefore used the 1×10^5 dose to investigate the effect of $CD8^+CD25^+$ subtype on atherosclerosis given their suppressor phenotype as previously reported [7,10–12]. The isolated $CD8^+CD25^+$ T cells or $CD8^+CD25^-$ T cells were then adoptively transferred (1×10^5 cells/mouse) to naïve male apoE(−/−) recipient mice at 7 weeks of age via tail vein injection. Homing experiments were performed using adoptively transferred CFSE-labeled $CD8^+$ T cells [19,20] and mice were euthanized 3 days later. Aortic tissues were enzymatically digested (0.25 mg/ml collagenase, 0.125 mg/ml elastase, 60 U/ml hyaluronidase in DMEM/F12 medium at 37 °C for 45 min) and subjected to flow cytometry. Size gating indicated that 0.49% of collected cells were lymphocytes, 0.2% of which were $CFSE^+$ (Supplemental Fig. 1). Mice injected with PBS served as control.

Recipient mice were fed regular mouse chow until 13 weeks of age when chow was switched to high cholesterol diet (TD 88137, Harlan-Teklad) until euthanasia at 25 weeks of age. Serum was collected to determine total and free serum cholesterol levels with colorimetric assays (Wako Diagnostics). Aortas were cleaned and stained en face with oil-red-O to assess the extent of atherosclerosis with computer-assisted histomorphometry with the samples blinded to the assessor. Hearts were embedded in OCT for sectioning of the aortic sinuses to analyze plaque size, lipid content by oil red-o staining, and T cell and macrophage content by CD3 and MOMA-2 antibody staining, respectively.

2.5. Ex-vivo cell proliferation

Splenocytes from recipient mice were collected and labeled with CFSE. Cells were stimulated with CD3/CD28 Dynabeads (Invitrogen) at the cell-to-bead ratio of 1:1 for 4 days. Cells were then collected, stained with CD4 and CD8 antibodies as indicated and subjected to flow cytometry. The results are expressed as the CFSE mean fluorescent intensity (MFI).

2.6. Statistics

Data are presented as mean ± SD. Number of animals in each group is listed in text or figure legend. Data were analyzed by ANOVA followed by Newman-Keuls multiple group comparison, or by *t*-test when appropriate. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Effect of atherogenic diet on peripheral blood $CD8^+CD25^+$ T cells

Six weeks of high cholesterol diet significantly reduced $CD8^+CD25^+$ T cells in peripheral blood of apoE(−/−) mice (11.2 ± 2.9% vs. 3.8 ± 1.8%, $P = 0.0011$), suggesting a role in