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The Pro-inflammatory Functions of Type 1 CD 8+ T Cells and Interleukin-17-producing Cluster of Differentiation 8+ T Cells Are Exhausted by Cholesterol in Atherosclerosis

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ABSTRACT

Background: $CD8^+$ T cells have been found to accumulate in atherosclerotic plaques. However, the specific role of $CD8^+$ T cell subsets in the development of atherosclerosis is still not fully understood.

Objective: To investigate the presence and functions of type 1 $CD8^+$ T (Tc1) cells and interleukin-17 (IL-17)-producing $CD8^+$ T (Tc17) cells.

Methods: Apolipoprotein E-deficient mice were fed a high-fat diet to induce atherosclerosis. Flow cytometry was used to identify and isolate aortic $CD8^+$ T cell subsets, which were then cultured *in vitro* to assess their pro-inflammatory activities. The cholesterol content of the $CD8^+$ T cell subsets was quantified.

Results: T-box expressed in T cells $(T\text{-bet})^+$ Tc1 cells and retinoic acid-related orphan receptor gamma t $(ROR\gamma t)^+$ Tc17 cells were found in the atherosclerotic aorta. Aortic $CD8^+$ T cells showed lower pro-inflammatory activity compared to splenic counterparts, with less interferon-gamma (IFN-γ) ($P<0.01$) and tumor necrosis factor-alpha (TNF-α) production ($P<0.01$). Surprisingly, aortic $CD8^+$ T cells expressed little IL-17 and interleukin-21 (IL-21) despite the presence of Tc17 cells. Aortic Tc1 and Tc17 cells expressed high levels of 2B4 and programmed cell death protein 1 (PD-1). Furthermore, aortic Tc1 and Tc17 cells had higher cholesterol contents than splenic $CD8^+$ T cells ($P<0.05$, respectively). Cholesterol treatment decreased IFN-γ expression in Tc1 cells ($P<0.001$) and reduced IL-17 expression in Tc17 cells ($P<0.001$). Additionally, cholesterol up-regulated 2B4 and PD-1 on Tc1 ($P<0.001$) and Tc17 cells ($P<0.001$).

Conclusion: Aortic $CD8^+$ T cells, particularly aortic Tc17 cells, are functionally exhausted in atherosclerosis, possibly due to the influence of cholesterol.

Keywords: Cytokine, Exhaustion, Inflammation

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INTRODUCTION

Atherosclerosis is characterized by an imbalance in lipid metabolism and pathological inflammation in the endothelial lining of arteries (1). The development of atherosclerotic lesions is mediated by the responses of endothelial cells and immune cells to the accumulation of lipids in the arterial wall (2). Low-density lipoprotein cholesterol, especially its oxidized form, is one of the major forms of lipids that accumulate in the arterial wall. During the development of atherosclerosis, monocytes derived from the blood, $CD4^+$ T cells, $CD8^+$ T cells, and other immune cells are drawn to and reside in the subendothelial space of the arterial wall (3, 4). Monocytes differentiate into macrophages to internalize lipids and lipoproteins, subsequently provoking the inflammatory reaction (5, 6). T cells participate in subendothelial inflammation and play different roles depending on their subset identities. $CD4^+$ T helper 1 cells secrete interferon-gamma (IFN-γ) and T helper 17 cells produce interleukin-17 (IL-17) to exacerbate subendothelial inflammation and aggravate atherosclerosis, while $CD4^+$ regulatory T cells suppress subendothelial inflammation by expressing anti-inflammatory mediators (7). To prevent and treat atherosclerosis effectively, it is crucial to elucidate the roles of each subendothelial immune cell population in atherosclerotic lesions.

The role of $CD8^+$ T cells in the pathophysiology of atherosclerosis remains incompletely understood. Cumulative evidence suggests that similar to $CD4^+$ T cells, $CD8^+$ T cells can differentiate into subsets under the instruction of various stimuli. Upon activation and education by interleukin-2 (IL-2) and interleukin-12 (IL-12), $CD8^+$ T cells polarize towards type 1 $CD8^+$ T (Tc1) cells. These cells express T-box expressed in T cells (T-bet), IFN-γ, tumor necrosis factor-alpha (TNF-α), and cytotoxic mediators (8). Therefore, Tc1 cells are functionally pro-inflammatory and cytotoxic. Additionally, when instructed by interleukin-6 (IL-6), interleukin-21 (IL-21), and transforming growth factor-beta (TGF-β), activated $CD8^+$ T cells differentiate into IL-17-producing $CD8^+$ T (Tc17) cells characterized by the expression of retinoic acid-related orphan receptor gamma t (RORγt), IL-17, IL-21, interleukin-22 (IL-22), and other inflammatory cytokines (9). In this regard, Tc17 cells participate in protective or destructive inflammation under different conditions. The involvement of Tc17 cells in pathological inflammation has been reported in various autoimmune and inflammatory disorders (9). However, the specific roles of Tc1 and Tc17 cells in the development of atherosclerosis have not been well studied in previous research. No direct evidence of Tc1 cell pathogenicity in atherosclerosis has been found. However, since IFN-γ increases circulating monocyte levels which ultimately contribute to plaque macrophage burden (10). It is possible that Tc1 cells could promote atherosclerosis. However, Tc1 cells are not the sole source of IFN-γ as it can also be secreted by activated $CD4^+$ T cells, natural killer cells, innate lymphoid cells, and even macrophages. Therefore, the extent to which Tc1 cells contribute to the development of atherosclerosis remains unknown. Additionally, although RORγt-expressing Tc17 cells accumulate in atherosclerotic lesions of apolipoprotein E-deficient ($ApoE^{-/-}$) mice, these Tc17 cells produce limited IL-17 and do not impact early atherosclerosis development (11).

In this study, we characterized the presence, phenotype, and function of Tc1 and Tc17 cells in atherosclerotic mice. We found that these cells, particularly Tc17 cells, were phenotypically and functionally exhausted, likely as a result of cholesterol-induced exhaustion.

MATERIALS AND METHODS

Animal Model

The animal research was inspected and approved by the Wuhan University Animal

Care and Use Committee. Animals were treated according to the Wuhan University Guidelines for the Use of Animals. Eight-week-old ApoE$^{-/-}$ mice were purchased from Beijing Biocytogen Co., Ltd. The mice were fed a diet supplemented with 0.2% cholesterol and 21% fat by weight for sixteen weeks and atherosclerosis formation was confirmed in the aortas.

Isolation of Immune Cells from Aorta and Spleen

Isolation of immune cells from the aortas was conducted according to previously published reports (12, 13). The reagents were purchased from Sigma-Aldrich. Mice were euthanized and perfused with 2 ml of ice-cold phosphate-buffered saline (PBS) through cardiac puncture. The aorta and associated branches were carefully isolated, minced into pieces, and incubated in 200 µl of digestion buffer (Roswell Park Memorial Institute (RPMI) 1640 containing 10% fetal calf serum, 50 U/ml deoxyribonuclease I, 50 U/ml hyaluronidase I-s, 150 U/ml collagenase XI, and 400 U/ml collagenase I) for 30 minutes in a 37 ˚C water bath, with agitation every 10 minutes. The tissue was dissociated by gently pipetting five times, then passed through a 40-µm cell strainer to prepare a single-cell suspension. The single-cell suspension was centrifuged at 250×g for 5 minutes and the cell pellet was suspended in 200 µl of PBS for further experiments. To acquire sufficient number of cells for analysis, cells from 2 to 5 mice were pooled together.

Splenocytes were isolated by pressing a spleen with a 5-ml syringe plunger through a 40-µm cell strainer containing 1 ml of PBS. Red blood cells (RBC) were lysed by incubating the splenocytes in RBC lysis buffer (Beyotime Inc). The cells were washed with PBS once before analysis.

Flow Cytometry

The following fluorophore-labeled antibodies were purchased from BioLegend: R-phycoerythrin/cyanine7 (PE/Cy7) anti-CD3 (17A2), R-phycoerythrin/cyanine5 (PE/Cy5) anti-CD8 (53-6.7), allophycocyanin (APC) anti-T-bet (4B10), R-phycoerythrin (PE) anti-RORγt (Q31-378), PE anti-IFN-γ (XMG1.2), APC anti-IL-17 (TC11-18H10.1), APC anti-IL-21 (FFA21), allophycocyanin/cyanine7 (APC/Cy7) anti-TNF-α (MP6-XT22), fluorescein isothiocyanate (FITC) anti-2B4 (m2B4 (B6) 458.1), and APC/Cy7 anti- PD-1 (29F.1A12). Cells were incubated with the LIVE/DEAD™ fixable violet dead cell stain kit (Invitrogen) and the mouse Fc receptor blocking reagent (Novobiotec) according to the manufacturers' instructions. After that, cell surface proteins were stained by incubating 1×10^6/ml cells in PBS with 5 µg/ml antibody for 30 minutes on ice. To stain intracellular proteins, cells were fixed and permeabilized using the fixation/permeabilization kit (BD Biosciences) following the manufacturer's instructions. Subsequently, cells were incubated with the permeabilization buffer (BD Biosciences) containing 5 µg/ml antibody for 30 minutes on ice. Cells were then washed with PBS twice and analyzed using a BD LSRII flow cytometer or sorted using a BD FACSAria cell sorter (both from BD Biosciences).

In vitro Stimulation and Differentiation of CD8+ T Cells

CD8$^+$ T cells were isolated using the EasySep mouse CD8$^+$ T cell isolation kit (STEMCELL Technologies) following the vendor's manual. The cells were then incubated at a density of 2×10^5 cells/ml in RPMI 1640 supplemented with 10% fetal calf serum, phorbol 12-myristate 13-acetate (PMA, 50 ng/ml, Sigma–Aldrich), ionomycin (500 ng/ml, Sigma–Aldrich), and 1× brefeldin A working solution (Bio-Rad) for 3.5 hours. Following incubation, flow cytometry was performed to analyze the intracellular production of IFN-γ, IL-17, TNF-α, and IL-21.

To induce Tc1 cell differentiation, 2×10^5 cells were seeded in each well of a 96-well microplate in a total volume of 200 µl of supplemented RPMI 1640. The following

reagents were added to the culture: 1 µg/ml soluble anti-CD3 antibody (MAB484, R&D Systems), 1 µg/ml soluble anti-cluster of differentiation 28 (CD28) antibody (MAB4832, R&D Systems), and 20 U/ml IL-2 (402-ML-020/CF, R&D Systems). Cholesterol (C8667-5G, Sigma-Aldrich) was added at a final concentration of 0.5 mg/ml. The cells were cultured for 2 days at 37 ˚C, after which the medium was refreshed with the same stimuli. The cells were incubated for an additional two days. Three hours before the end of the culture, cells were treated with 50 ng/ml PMA, 500 ng/ml ionomycin, and 1× brefeldin A working solution for 3 hours. Finally, the cells were subjected to flow cytometry to measure the expression of cytokines, 2B4, and PD-1.

Tc17 induction was performed similarly, with the medium containing 1 µg/mL soluble anti-CD3 (MAB484, R&D Systems), 1 µg/ml soluble anti-CD28 (MAB4832, R&D Systems), 1 ng/ml interleukin-7 (IL-7, 407-ML-005/CF, R&D Systems), 20 U/ml IL-2 (402-ML-020/CF, R&D Systems), 20 ng/ml IL-6 (406-ML-005/CF, R&D Systems), 5 ng/ml TGF-β (7666-MB-005/CF, R&D Systems), 20 ng/ml interleukin-1 beta (IL-1β, 401-ML-005/CF, R&D Systems), 20 ng/ml interleukin-23 (IL-23, 1887-ML-010/CF, R&D Systems), 10 µg/ml interleukin-4 (IL-4) antibody (MAB404-SP, R&D Systems l), and 10 µg/ml IFN-γ antibody (MAB4851-SP, R&D Systems). All other procedures were the same as for Tc1 differentiation.

Cholesterol Content Analysis

Cellular cholesterol content was quantified using the cholesterol cell-based detection assay kit (Cat#10009779, Cayman) following the vendor's protocol. Briefly, cells were stained with Filipin III and then analyzed by flow cytometry.

Statistics

The minimum number of animals required for statistical significance of $P<0.05$ with an 80% probability was determined using the free Sample Size & Power Calculator provided by the Chinese University of Hong Kong (http://www.lasec.cuhk.edu.hk/sample-size-calculation.html). All experiments were repeated 2 or 3 times. The data were shown as means±standard deviations. Statistical analysis was conducted using Student's *t*-test or one-way analysis of variance (ANOVA) with post-hoc Tukey's Honest Significant Difference test. A P value<0.05 was considered significant.

RESULTS

T-bet$^+$CD8$^+$ T Cells and RORγt$^+$CD8$^+$ T Cells Are Present in Atherosclerotic Aortas

We induced atherosclerosis in ApoE$^{-/-}$ mice and extracted the aorta from each mouse to isolate aortic immune cells. Splenocytes were also harvested as acontrol. As illustrated in Figure 1a, single cells, lymphocytes, and live cells were sequentially selected. Within the live cells, CD3$^+$CD8$^+$ T cells were gated for further study (Figure 1a). In splenic CD8$^+$ T cells, three subsets were observed: T-bet$^-$ RORγt$^-$ (i.e. double negative, DN), T-bet$^+$, and RORγt$^+$ cells. Approximately 45% of splenic CD8$^+$ T cells were T-bet$^+$, while less than 3% were RORγt$^+$ (Figure 1b to 1d). In aortic CD8$^+$ T cells, about 20% were T-bet$^+$ while more than 25% were RORγt$^+$ (Figure 1b to 1d). Therefore, RORγt$^+$CD8$^+$ T cells accumulated in atherosclerotic aortas ($P<0.01$ aorta vs. spleen).

Aortic CD8$^+$ T Cells Exhibit Weak Pro-inflammatory Activity

We assessed the pro-inflammatory activity of aortic CD8$^+$ T cells by measuring cytokine production after stimulating the cells *in vitro* with PMA and ionomycin. As demonstrated in Figures 2a to 2c, aortic CD8$^+$ T cells produced significantly less IFN-γ compared with splenic CD8$^+$ T cells ($P<0.01$). However, both splenic CD8$^+$ T cells and aortic CD8$^+$ T cells expressed very low levels of IL-17 ($P>0.05$), in contrast to the substantial presence of RORγt$^+$CD8$^+$ T cells in the aorta.

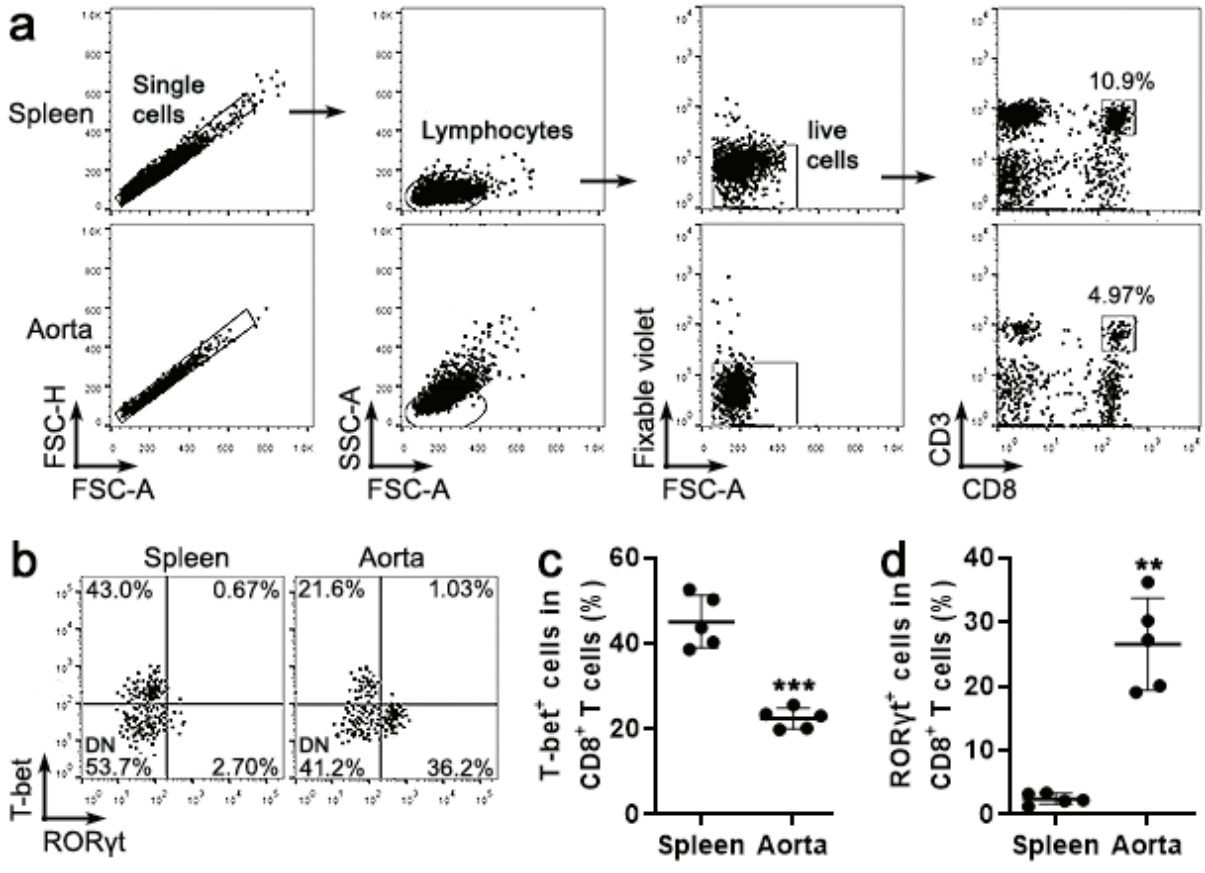

Figure 1. The presence of CD8+ T cell subsets in the spleen and aorta after atherosclerosis induction. (a) The flow cytometry gating strategy for CD8+ T cells. (b) Dot plots illustrate the expression of T-box expressed in T cells (T-bet) and retinoic acid-related orphan receptor gamma t (RORγt) in CD8+ T cells. (c and d) Frequencies of T-bet+ (c) and RORγt+ (d) subsets in total CD8+ T cells. Student's *t*-test. **: P<0.01. ***: P<0.001. N=5 mice per group.

Furthermore, we quantified the expression of TNF-α and IL-21 (another Tc17-related cytokine). We found that aortic CD8+ T cells produced less TNF-α than splenic CD8+ T cells (P<0.01) (Figure 2d and 2e). Both splenic and aortic CD8+ T cells expressed very low levels of IL-21 (P>0.05) (Figure 2f and 2g). Therefore, aortic CD8+ T cells were less pro-inflammatory than splenic CD8+ T cells when activated.

Aortic CD8+ T Cells Expressed Higher Levels of 2B4 and PD-1

Due to significantly low expression of the above mentioned cytokines, we questioned whether aortic CD8+ T cells were exhausted. We first examined the expression of T cell exhaustion markers 2B4 and PD-1 (14, 15) on total CD8+ T cells. As shown in Figures 3a and 3b, approximately 8% of splenic CD8+ T cells express moderate levels of 2B4 and PD-1, constituting a $2B4^{medium}PD\text{-}1^{medium}$ population. In contrast, less than 5% of aortic CD8+ T cells were $2B4^{medium}PD\text{-}1^{medium}$ (P<0.05). Notably, about 18% of aortic CD8+ T cells expressed high 2B4 and PD-1, representing a $2B4^{high}PD\text{-}1^{high}$ population (P<0.001 aorta vs. spleen). Next, we assessed the expression of 2B4 and PD-1 on $T\text{-}bet^{-}ROR\gamma t^{-}$ (DN), T-bet+, and $ROR\gamma t^{+}CD8^{+}$ T cells, respectively. In the spleen, less than 3% of DN cells were $2B4^{medium}PD\text{-}1^{medium}$ while 13% of $T\text{-}bet^{+}CD8^{+}$ T cells were $2B4^{medium}PD\text{-}1^{medium}$ (P<0.001) (Figure 3c and 3d). In the aorta, approximately 4% of DN cells were $2B4^{medium}PD\text{-}1^{medium}$ and 13% of $T\text{-}bet^{+}CD8^{+}$ T cells were $2B4^{medium}PD\text{-}1^{medium}$ (P<0.01) (Figure 3c and 3e). $2B4^{high}PD\text{-}1^{high}$ cells were rare in aortic DN cells but accounted

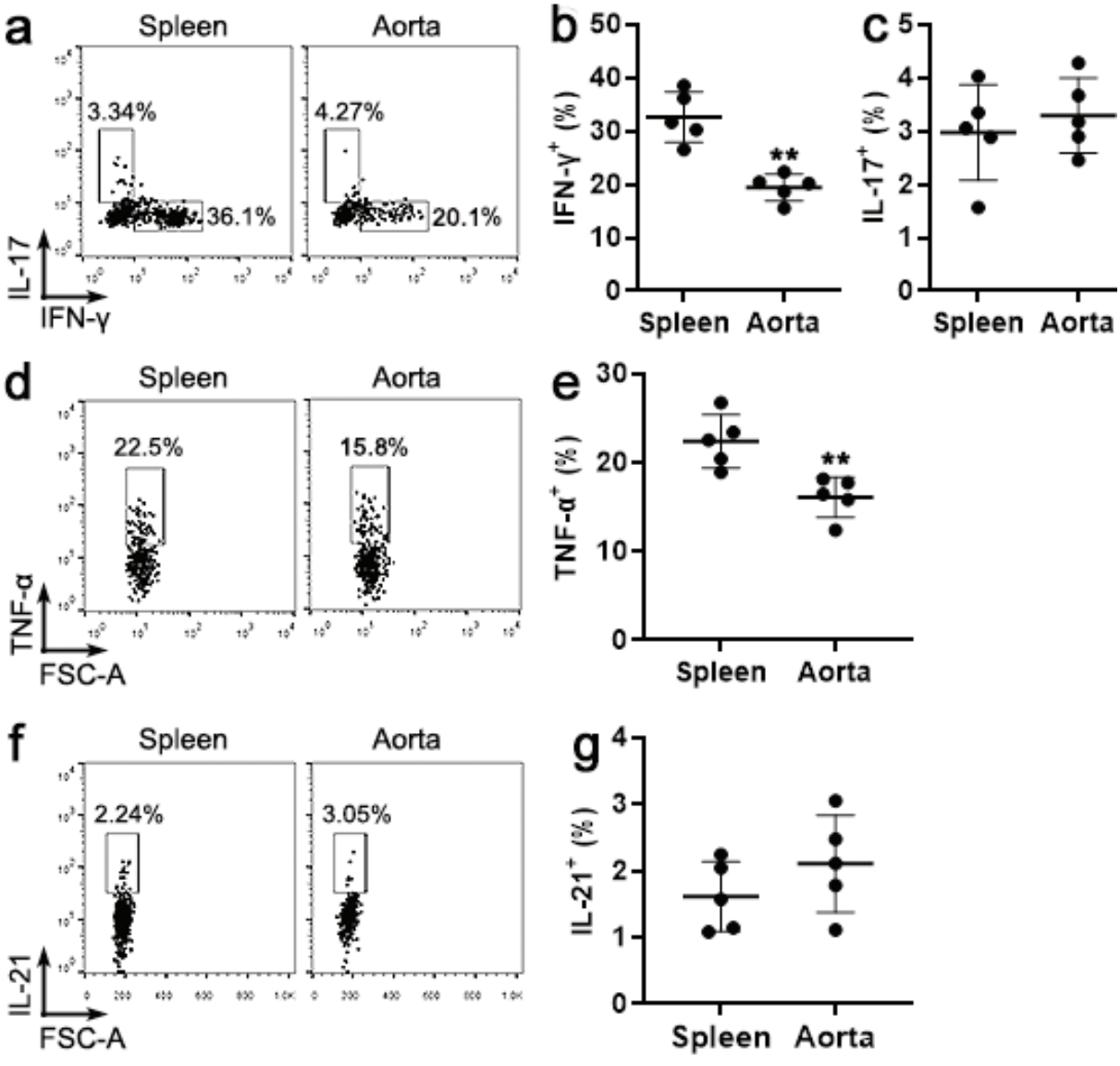

Figure 2. The expression of indicated cytokines in total CD8$^+$ T cells after sorting and *in vitro* stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin for 3.5 hours. (a to c) The expression of interleukin-17 (IL-17) and interferon-gamma (IFN-γ). Representative dot plots are shown in (a) and statistics are shown in (b and c). (d and e) Tumor necrosis factor-alpha (TNF-α) expression. Representative dot plots are shown in (d) and statistics are shown in (e). (f and g) Interleukin-21 (IL-21) expression. Representative dot plots are shown in (f) and statistics are shown in (g). Student's *t*-test. **: P<0.01. N=5 samples per group. Each sample contained cells pooled from 5 mice.

for 9% of aortic T-bet$^+$CD8$^+$ T cells (P<0.01) (Figure 3c and 3e). Remarkably, up to 50% of RORγt$^+$CD8$^+$ T cells were 2B4^{high}PD-1^{high} (Figure 3c and 3e). Therefore, aortic CD8$^+$ T cells, particularly RORγt$^+$CD8$^+$ T cells, express high levels of 2B4 and PD-1, which are indicative of T cell exhaustion.

Aortic CD8$^+$ T Cell Subsets Have Higher Cholesterol Contents

It has been reported that cholesterol induces CD8$^+$ T cell exhaustion (16). To ascertain whether this is the case for aortic CD8$^+$ T cells, we stained CD8$^+$ T cells with Filipin III to quantify cellular cholesterol. As shown in Figure 4, all aortic CD8$^+$ T cell subsets, including aortic DN cells, aortic T-bet$^+$CD8$^+$ T cells, and aortic RORγt$^+$CD8$^+$ T cells, exhibit higher Filipin III signals than splenic CD8$^+$ T cells (P<0.05, respectively). Therefore, aortic CD8$^+$ T cells possess higher cholesterol contents.

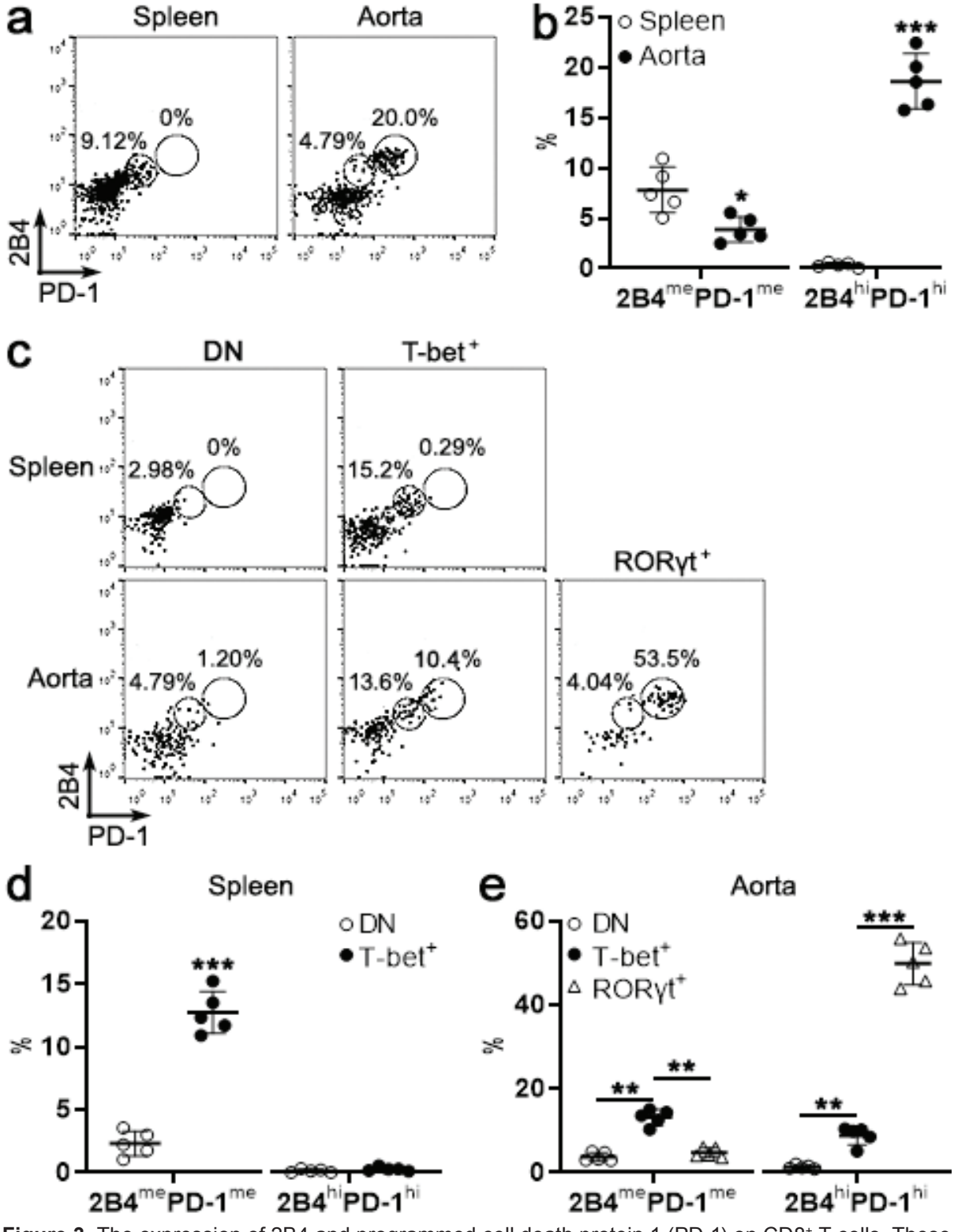

Figure 3. The expression of 2B4 and programmed cell death protein 1 (PD-1) on CD8+ T cells. These cells were not stimulated *in vitro*. (a and b) 2B4 and PD-1 on total CD8+ T cells. Representative dot plots are shown in (a) and statistics of the proportions of gated populations are shown in (b). (c to e) 2B4 and PD-1 on splenic or aortic CD8+ T cell subsets. Representative dot plots are shown in (c) and statistics are shown in (d and e). DN: T-box expressed in T cells (T-bet)-negative-and-retinoic acid-related orphan receptor gamma t (RORγt)-negative cells. Please note that splenic RORγt+CD8+ T cells were not measured due to the extremely low cellularity. Student's *t*-test for (b). One-way ANOVA for (d and e). *: P<0.05. **: P<0.01. ***: P<0.001. N=5 samples per group. Each sample contained cells pooled from 2 mice.

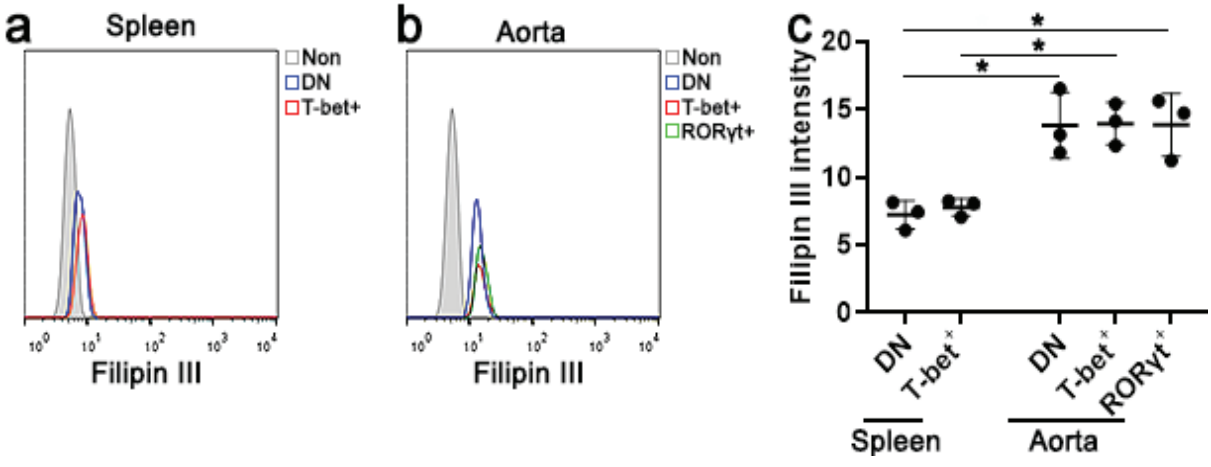

Figure 4. Cellular cholesterol content measured using Filipin III staining of splenic or aortic CD8$^+$ T cells. (a and b) The representative histograms show the Filipin III fluorescence in splenic (a) or aortic (b) CD8$^+$ T cell subsets. Non: Non-staining control. DN: T-box expressed in T cells (T-bet)-negative-and-retinoic acid-related orphan receptor gamma t (RORγt)-negative cells. It is important to note that splenic RORγt$^+$CD8$^+$ T cells were not measured due to the extremely low cellularity. (c) Statistics of Filipin III fluorescence intensities. Student's *t*-test. *: P<0.05. N=3 samples per group. Each sample contained cells pooled from 3 mice.

Cholesterol Induces Exhaustion inTc1 and Tc17 Cells

To test whether cholesterol induces CD8$^+$ T cell exhaustion, splenic CD8$^+$ T cells were enriched and cultured *in vitro* under Tc1 or Tc17 differentiation conditions for 4 days, with or without of 0.5 mg/ml cholesterol. On day 4, the expression of IFN-γ and IL-17 was evaluated. As shown in Figures 5a and 5b, under the Tc1 differentiation condition, CD8$^+$ T cells primarily expressed IFN-γ but not IL-17. Cholesterol moderately reduced IFN-γ expression in CD8$^+$ T cells (P<0.001). Under the Tc17 differentiation condition, CD8$^+$ T cells expressed substantial IL-17 and low IFN-γ. Cholesterol mildly alleviated IFN-γ expression (P<0.05) but significantly down-regulated IL-17 expression (P<0.001) in CD8$^+$ T cells (Figures 5c and 5d). Furthermore, cholesterol significantly up-regulated the expression of 2B4 and PD-1 on both Tc1 (P<0.001) and Tc17 cells (P<0.001) (Figure 5e and 5f). Notably, cholesterol-treated Tc17 cells exhibited the highest expression of 2B4 and PD-1 (Figures 5e and 5f). Therefore, RORγt$^+$CD8$^+$ T cells seemed to be more susceptible to cholesterol-induced exhaustion.

DISCUSSION

The current study reveals the exhaustion state of Tc1 and Tc17 cells in atherosclerotic lesions. To the best of our knowledge, this is the first investigation reporting the exhaustion of CD8$^+$ T cell subsets in atherosclerotic lesions. We first analyzed the presence of Tc1 and Tc17 cells in atherosclerotic aortas by staining T-bet and RORγt in CD8$^+$ T cells. Consistent with recent research (11), we found that splenic CD8$^+$ T cells are mainly T-bet-expressing Tc1 cells whereas aortic CD8$^+$ T cells consist of substantial RORγt-expressing Tc17 cells and minor Tc1 cells. The causes of Tc17 cell accumulation in the atherosclerotic aorta remain unidentified. Because the atherosclerotic lesion is rich in inflammatory mediators, Tc17 cells are likely generated in the aorta under the influences of cytokines such as IL-1β, IL-6, IL-23, and others. However, we should not exclude the possibility that more Tc17 cells than other subsets transmigrate into the lesion due to selective chemotaxis. To identify the sources of Tc17 cell accumulation, an adoptive transfer assay would be conducted to compare the migrating capabilities and

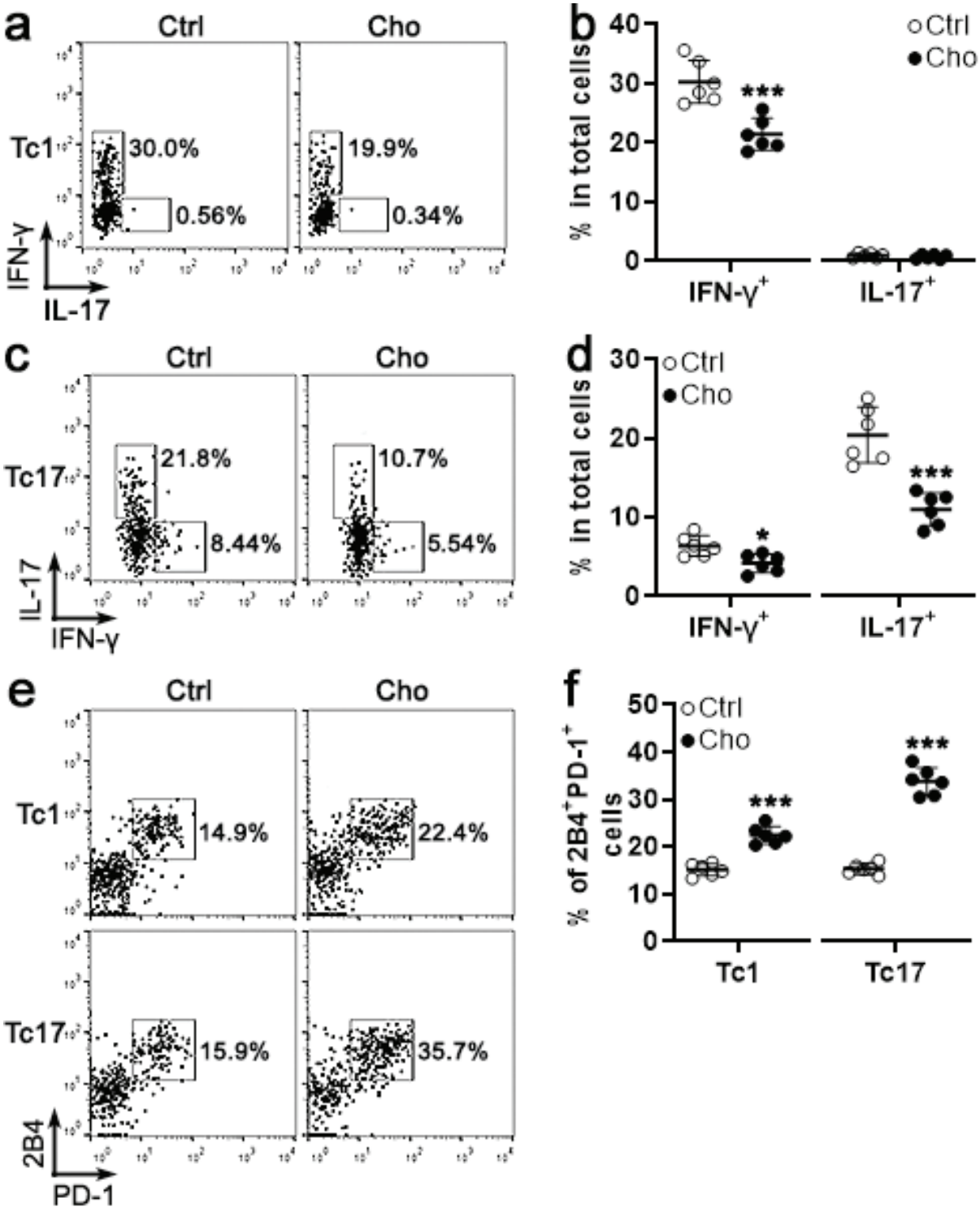

Figure 5. The effect of cholesterol on CD8+ T cell exhaustion under the conditions of type 1 CD8+ T (Tc1) or interleukin-17 (IL-17)-producing CD8+ T (Tc17) differentiation. (a and b) The expression of IL-17 and interferon-gamma (IFN-γ) in CD8+ T cells under the Tc1 differentiation condition. The representative dot plots are shown in (a). The percentages of cytokine-expressing cells are illustrated in (b). Ctrl: vehicle control. Cho: cholesterol. (c and d) The expression of IL-17 and IFN-γ in CD8+ T cells under the Tc17 differentiation condition. The representative dot plots are shown in (c). The percentages of cytokine-expressing cells are presented in (d). (e and f) The expression of 2B4 and programmed cell death protein 1 (PD-1) on CD8+ T cells following Tc1 or Tc17 differentiation. The representative dot plots are shown in (e).The percentages of 2B4+PD-1+ cells are depicted in (f). Student's *t*-test. ***: P<0.001. N=6 samples per group.

on-site differentiation statuses of individual $CD8^+$ T cell subsets in the future.

We found that aortic $CD8^+$ T cells expressed lower levels of IFN-γ and TNF-α compared to their splenic counterparts when exposed to the same stimulation conditions. This finding is align with the observation of fewer Tc1 cells in the aorta compared to the spleen. Surprisingly, despite the presence of aortic Tc17 cells, intracellular expression of IL-17 and IL-21 was scarce in aortic $CD8^+$ T cells, leading to questions about potential dysfunction in aortic Tc17 cell. T cell dysfunction can be attributed to senescence, anergy, or exhaustion according to previous reports (17). T cell senescence seems unlikely because senescent T cells lack PD-1 expression (18). T cell anergy is also less likely because it is caused by excessive T-cell receptor stimulation and restricted concomitant co-stimulation (19). T cell exhaustion occurs when T cells are chronically activated in a chronic inflammatory environment (20). Our results suggest that the atherosclerotic lesion is a site of chronic inflammation, potentially leading to T cell exhaustion. Therefore, we focused on the exhaustion state of Tc1 and Tc17 cells. Evaluation of two exhaustion markers, 2B4 and PD-1, revealed an abundance of $2B4^{high}PD\text{-}1^{high}$ Tc17 cells in the aorta. In contrast, only 9% of Tc1 cells were $2B4^{high}PD\text{-}1^{high}$ while 13% of Tc1 cells were $2B4^{medium}PD\text{-}1^{medium}$. This finding indicates a significantly higher presence of exhausted Tc17 cells compared to exhausted Tc1 cells in the aorta. Although almost half of the aortic Tc17 cells were exhausted, the other 50% of aortic Tc17 cells (i.e. the non-exhausted ones) would express IL-17 and exhibit 10% of IL-17-expressing cells in the whole aortic $CD8^+$ T cells, rather than the observed less than 5%. We speculate that the function of aortic Tc17 cells is further suppressed by other factors such as immunosuppressive regulatory T cells. However, this hypothesis needs to be tested in further investigations.

Another important finding of this research is the impact of cholesterol on aortic $CD8^+$ T cell function. Recently, cholesterol has been found to induce $CD8^+$ T cell dysfunction in the tumor microenvironment through the endoplasmic reticulum stress-X-box binding protein 1 pathway (16). In atherosclerotic lesions, aortic $CD8^+$ T cells may encounter cholesterol aggregates and experience similar effects as they do in tumors. Indeed, we found higher cholesterol contents in aortic DN, Tc1, and Tc17 cells compared with splenic $CD8^+$ T cells, suggesting that aortic $CD8^+$ T cells internalized more cholesterol than their splenic counterparts. However, we saw no differences in the cholesterol content between aortic DN, Tc1, and Tc17 cells, suggesting that the cholesterol uptake abilities of these aortic $CD8^+$ T cell subsets were comparable. It is unclear which receptors or signal pathways are involved in cholesterol uptake by aortic $CD8^+$ T cells. Consistent with a previous report, we also observed cholesterol-induced down-regulation of IFN-γ and up-regulation of inhibitory 2B4 and PD-1 in differentiated Tc1 cells (16), suggesting that cholesterol caused Tc1 exhaustion and dysfunction. Furthermore, the down-regulation of IL-17 and up-regulation of 2B4 and PD-1 in Tc17 cells induced by cholesterol were more pronounced than the changes seen in Tc1 cells. This suggests that Tc17 cells may be more susceptible to exhaustion caused by cholesterol. However, the specific molecular mechanisms underlying this phenomenon are still unknown. In the future, it will be interesting to determine whether cholesterol induces stronger endoplasmic reticulum stress in Tc17 cells than in Tc1 cells. Bulk or single-cell transcriptome sequencing might provide more clues about the differential effect of cholesterol on Tc1 and Tc17 cells. Additionally, the remarkable cholesterol-induced Tc17 cell exhaustion might explain why Tc17 cells could not affect the early development of atherosclerosis in a previous study (11). The suppression of pro-inflammatory cytokine expression makes aortic Tc17 cells less pathogenic. Therefore, although cholesterol is the culprit of atherosclerosis development, it might suppress Tc1 and Tc17 cells to prevent their pathogenicity.

The role of $CD8^+$ T cells in the development of atherosclerosis is still a topic of debate. These cells have been found in the atherosclerotic plaques of both patients and mouse models (21, 22). Interestingly, $CD8^+$ T cells within the plaques express high levels of PD-1 and low levels of perforin, suggesting that they may undergo an exhaustion reprogramming in response to chronic inflammation within the plaque (23). This is consistent with our data showing exhausted aortic Tc1 and Tc17 cells. $CD8^+$ T cell-deficient $ApoE^{-/-}$ mice show no changes in plaque size compared to $ApoE^{-/-}$ control mice fed a normal chow for up to 1 year, suggesting no significant impact of $CD8^+$ T cells on atherosclerosis development in this model. However, antibody-induced $CD8^+$ T cell depletion mitigates atherosclerotic lesions in $ApoE^{-/-}$ or low-density lipoprotein receptor-deficient ($LDLR^{-/-}$) mice fed a high-fat diet (10, 22). Of note, the research also indicates that the cytotoxicity of $CD8^+$ T cells is associated with apoptosis and necrosis in atherosclerotic lesions (22). Since Tc1 cells express abundant cytotoxic and pro-inflammatory mediators, they may play a significant role in promoting atherosclerosis. Additionally, IFN-γ, produced by Tc1 cells and other immune cells, triggers the expression of C-C motif chemokine ligand 2 in macrophages, subsequently recruiting circulating monocytes to early lesions, and accelerating the development of atherosclerosis (10). Interestingly, the transfer of activated undifferentiated $CD8^+$ T cells also increases atherosclerosis in $LDLR^{-/-}CD8^{-/-}$ mice fed a high-fat diet, suggesting that undifferentiated $CD8^+$ T cells exacerbate plaque development (11). However, undifferentiated $CD8^+$ T cells probably differentiate into Tc1 cells in atherosclerotic lesions under the influences of local pro-inflammatory mediators. Importantly, Tc17 cell transfer did not promote atherosclerosis, suggesting that Tc17 cells are not atherogenic (11). Our results imply that cholesterol-induced exhaustion might remarkably down-regulate the pathogenicity of Tc17 cells in atherosclerosis.

In summary, this study confirms the presence of Tc17 cells in atherosclerotic aortas and reveals differential cholesterol-induced exhaustion on Tc1 and Tc17 cells. In this regard, we discovered a novel mechanism by which pathogenic aortic Tc1 and Tc17 cells are suppressed in atherosclerotic lesions.

CONCLUSION

$T\text{-}bet^+$ Tc1 cells and $ROR\gamma t^+$ Tc17 cells are present in the atherosclerotic aorta. They are found to be functionally exhausted possibly due to the effects of cholesterol.

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AVAILABILITY OF DATA AND MATERIAL

The data that support the findings of this study are available from the corresponding author upon reasonable request.

AUTHORS' CONTRIBUTION

All authors contributed to the study. Research design, supervision and manuscript writing were performed by Xiaofang Xiong. The animal model, cell isolation, and flow cytometry were conducted by Guizhen Lin and Lei Zhang. In vitro cell culture and cholesterol content analysis were carried out by Zheng Yan and Wei Jiang. Cell sorting was performed by Beibei Wu.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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