Attenuated Atherosclerotic Lesions in apoE-Fcγ-Chain-Deficient Hyperlipidemic Mouse Model Is Associated with Inhibition of Th17 Cells and Promotion of Regulatory T Cells

Hang Pong Ng, 1 Ramona L. Burris, and Shanmugam Nagarajan

Though the presence of antioxidized low-density lipoprotein IgG is well documented in clinical and animal studies, the role for FcγRs to the progression of atherosclerosis has not been studied in detail. In the current study, we investigated the role for activating FcγR in the progression of atherosclerosis using apolipoprotein E (apoE)-Fcγ-chain double-knockout (DKO) mice. Relative to apoE knockout (KO) mice, arterial lesion formation was significantly decreased in apoE-Fcγ-chain DKO mice. Bone marrow chimera studies showed reduced lesions in apoE KO mice receiving the bone marrow of apoE-Fcγ-chain DKO mice. Compared to apoE KO mice, antioxidized low-density lipoprotein IgG1 (Th2) and IgG2a (Th1), IL-10, and IFN-γ secretion by activated T cells was increased in apoE-Fcγ-chain DKO mice. These findings suggest that reduced atherosclerotic lesion in apoE-Fcγ-chain DKO mice is not due to a Th1/Th2 imbalance. Interestingly, the number of Th17 cells and the secretion of IL-17 by activated CD4+ cells were decreased in apoE-Fcγ-chain DKO mice. Notably, the number of regulatory T cells, expression of mRNA, and secretion of TGF-β and IL-10 were increased in apoE-Fcγ-chain DKO mice. Furthermore, secretions of IL-6 and STAT-3 phosphorylation essential for Th17 cell genesis were reduced in apoE-Fcγ-chain DKO mice. Importantly, decrease in Th17 cells in apoE-Fcγ-chain DKO mice was due to reduced IL-6 release by APC of apoE-Fcγ-chain DKO mice. Collectively, our data suggest that activating FcγR promotes atherosclerosis by inducing a Th17 response in the hyperlipidemic apoE KO mouse model. The Journal of Immunology, 2011, 187: 6082–6093.

One of the risk factors implicated in the pathogenesis of atherosclerosis is an elevated level of low-density lipoprotein (LDL) that leads to the generation of oxidized LDL (oxLDL) (1). OxLDL induces an autoimmune response as evidenced by the presence of anti-oxLDL IgG in atherosclerotic lesions in the hyperlipidemic mouse model (2, 3) and in humans (4–6). These studies have suggested that the titer of autoantibodies against oxLDL correlates with the progression of atherosclerosis. Epidemiological studies have shown that plasma CRP, another FcγR ligand (7), is a marker of progression of atherosclerosis (8, 9). However, recent studies using human CRP overexpression in hyperlipidemic mouse model showed there was no difference in atherosclerotic lesions. Very recent studies using mouse CRP deficiency in atherosclerosis-susceptible hyperlipidemic mouse models showed no reduction in atherosclerosis in mice (10), suggesting there is no direct link between CRP levels and progression of atherosclerosis.

FcγR plays an important role in inflammatory cell activation, clearance, and presentation of Ag and also in maintaining Ig homeostasis (11–13). In mice, four different classes of FcγRs have been recognized: FcγRI, FcγRII, FcγRIII, and FcγRIIV (11–13). Functionally, FcγRs can be classified into the activating (FcγRI, III, and IV) and inhibitory (FcγRII) receptors (11–13). Fcγ-chain is the signaling subunit that coassociates with the activating FcγRs, and assembly and cell-surface expression of the activating FcγRs (FcγRI, III, and IV) require the coexpression of Fcγ-chain (14, 15). Immune complex (IC) binding to the extracellular domain of the ligand binding subunit of the activating FcγRs results in phosphorylation of the ITAM motifs resides in the cytoplasmic domain of Fcγ-chain subunit (11–13). On the contrary, FcγRII, an inhibitory FcγR, is a single subunit protein, and IC binding to FcγRII induces a negative signal through its ITIM in the cytoplasmic domain (11–13). Earlier studies have presented evidence that mice deficient in Fcγ-chain are resistant to the onset of IC-mediated chronic inflammatory diseases (16, 17).

Activated T cells specific for oxLDL are present in human atherosclerotic plaques, suggesting the involvement of adaptive immune response (18) in the initiation and progression of atherosclerosis. Elevated levels of anti-oxLDL IgG, particularly IgG1 and IgG2a, have been observed in apolipoprotein E single knockout (ApoE KO) mice fed a hyperlipidemic diet (19). The binding of anti-oxLDL IgG to oxLDL can result in the formation of soluble oxLDL IC (oxLDL-IC). Using an in vitro cell-culture...
model, we have shown that monocytes adhere to oxLDL-IC deposited on vascular endothelial cells in vitro via FcγR, and this interaction leads to induction of proinflammatory cytokines and chemokines involved in monocyte recruitment (20). Moreover, recent human genetic study identified an association between the activating FcγRIIA polymorphism to the occurrence of acute coronary syndrome (21), suggesting activating FcγR may be an important contributor to atherosclerosis. These results suggested that FcγR interaction with ox-LDL-IC could contribute to the progression of atherosclerosis. Deletion of the Fcγ-chain in apoE KO mice (22) and FcγRIII in LDL receptor single KO (LDLR KO) background (23) decreased atherosclerosis, whereas deficiency of FcγRIIB, an inhibitory FcγR, in LDLR and apoE KO showed exacerbated lesions (24, 25). The reduced lesions observed in apoE-Fcγ-chain double-knockout (DKO) mice were attributed to the decreased ratio of activating versus inhibitory FcγRs in vascular smooth muscle. Adoptive transfer of CD4+ T cells specific to ox-LDL has been shown to promote atherosclerotic lesions by increasing Th1 cell responses (26, 27), suggesting that CD4+ T cells, specifically Th1 cells, play an important role in promoting atherosclerosis. Though these studies have provided the importance of activating FcγR in the progression of atherosclerosis, the relationship between FcγR expressed on inflammatory cells, including APC and its effect on CD4+ cells, particularly Th1/Th2 responses, in the development of atherosclerosis remains unexplored.

In this report, we tested the hypothesis that ox-LDL-IC binding to activating FcγRs may promote the initiation and progression of atherosclerosis using apoE-Fcγ-chain DKO mice by altering CD4+ T cell responses. We show that apoE-Fcγ-chain DKO mice developed fewer atherosclerotic lesions. Moreover, a bone marrow chimera approach showed attenuated lesions in apoE KO receiving bone marrow from apoE-Fcγ-chain DKO mice. We demonstrated that apoE-Fcγ-chain DKO mice revealed a significant decrease in secretion of Th17-related cytokine (IL-17 and IL-6). In contrast, the number of regulatory T cells (Tregs) and expression of TGF-β and Foxp3, Treg-related cytokine and transcription factor, respectively, was increased in apoE-Fcγ-chain DKO mice as compared with apoE KO mice. These findings suggest a potential role for the activating FcγRs in generation and differentiation of Th17 response, which has been suggested to contribute to the pathogenesis of atherosclerosis.

Materials and Methods

Mice

ApoE KO mice on a C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). ApoE KO and Fcγ-chain KO (The Jackson Laboratory), both in the C57BL/6 background, were mated, and F1 progeny (apoE−/− Fcγ-chainwt/heterozygous) were mated to generate apoE-Fcγ-chain DKO mice. ApoE KO and apoE-Fcγ-chain DKO mice (5 wk of age) were fed a high-fat Western diet (TD 05576; Harlan Laboratories, Madison, WI) or normal chow diet (TD06715; Harlan Laboratories) for 10 or 20 wk, respectively. This study was reviewed and approved by the Institutional Animal Care and Use Committee at University of Arkansas for Medical Sciences.

Tissue preparation and morphometric determination of atherosclerosis

Mice were anesthetized with isoflurane before euthanization. Animals were sacrificed at 15 (high-fat diet) or 25 wk (chow diet) of age, and blood was collected by the cardiac puncture into heparin-coated tubes. Plasma was collected by the cardiac puncture into heparin-coated tubes. Plasma was separated and stored at −80°C until further analysis. The heart and descending aorta were excised and fixed in glutaraldehyde in 30% sucrose overnight before mounted in OCT medium and frozen at −70°C. Aortic sinus cryosections (10 μm) were stained with Oil Red O (28). For quantitative analysis of atherosclerosis, the percent lesion area in each of five sections from each mouse was measured. En face preparations of the descending aorta were washed in distilled water; en face analysis of the descending aorta was performed after staining descending aorta with Sudan IV as previously described (28).

Bone marrow chimera

The recipient apoE KO mice (male, 8 wk) received bone marrow from either apoE KO or apoE-Fcγ-chain DKO mice. After bone marrow transplantation (BMT), mice were fed a chow diet for 4 wk. Four weeks after BMT, mice were fed a high-fat diet (TD05576) for another 10 wk. Genomic DNA was obtained from circulating WBCs in all mice 4 wk after BMT. PCR was performed to confirm absence of Fcγ-chain using primers specific for Fcγ-chain wild type and mutant. The following primers were used: oLMR0618, 5′-CTCTGCCGTTTACCGTATCGCC-3′ (mutant); oLMR0621, 5′-ACCCTACTCTACTGTCAGCATACG-3′ (common); and oLMR0622, 5′-CTCAGCGTGCTGATCGCTGCTTC-3′ (wild type). The PCR-amplified products 224 bp (wild type) and 260 bp (mutant) were visualized on 2% agarose gels.

Immunohistochemistry

Serial aortic sinus cryosections (10 μm) were stained with anti-mouse monocoyte/macrophage mAb (MOMA-2, 1:25 dilution; AbD Serotec, Raleigh, NC) followed by Vectastain ABC reagent (Vector Laboratories, Burlingame, CA). The sections were developed with DAB and counterstained with Mayer’s hematoxylin. Images were captured using a Carl Zeiss inverted microscope (Carl Zeiss).

Plasma lipid analyses

Concentrations of plasma total and high-density lipoprotein (HDL) cholesterol were determined by enzymatic methods using kits from BioVision (Mountain View, CA) as described earlier (28).

Stimulation of CD4+ T cells

CD4+ T lymphocytes were purified from splenocytes by positive selection using anti-CD4 microbeads (Miltenyi Biotec, Auburn, CA), as recommended by the manufacturer. The purity of CD4+ cells was ~99% as determined by FACS analysis using anti-CD4–APC. Purified CD4+ cells (1 × 106) were stimulated with 5 μg/ml plate-bound (pb) anti-CD3 mAb (hamster IgG1, clone 145-2C11; BD Biosciences, San Diego, CA) in presence of 1 μg/ml soluble anti-CD28 mAb (hamster IgG2, clone 37.51; BD Biosciences) for 48 h. Supernatants were collected to determine cytokines secreted by activated T cells.

Preparation of pb-IC

Plate-bound OVA-IC and malondialdehyde-modified LDL IC (MDALDL-IC) were prepared incubating OVA or MDALDL with a saturating concentration of affinity-purified rabbit anti-OVA IgG and rabbit anti-malondialdehyde (MDA) IgG, respectively. Plate-bound IC were prepared by coating OVA or MDALDL at 15 μg/ml in PBS for 1 h at 37°C, followed by blocking with 10% FCS (Hyclone) in PBS for 1 h and a further 1-h incubation with saturating concentration of rabbit anti-OVA or rabbit anti-MDA IgG (10 μg/ml) in PBS/10% FCS. Parallel wells prepared with OVA/anti-MDA IgG and MDALDL/anti-OVA IgG were used as controls. Formation of pb-OVA-IC or pb-MDALDL-IC was confirmed by ELISA using HRP-conjugated anti-rabbit IgG.

Bone marrow-derived macrophage and bone marrow-derived dendritic cell preparation and IC activation

Bone marrow-derived dendritic cells (BMDC) were prepared from bone marrow cells (2 × 106 cells/100-mm dish) after incubation with RPMI 1640/10% FBS/murine GM-CSF (20 ng/ml; PeproTech) for 6 d (29). Fresh medium with GM-CSF was replaced after 3 d. After 6 d, nonadherent BMDC was collected and used for IC-mediated activation. Bone marrow-derived macrophages (BMDM) were prepared by incubating bone marrow cells in RPMI 1640/10% FBS supplemented with 30% L929 culture supernatant (source of M-CSF) for 6 d (30). BMDM were gently detached using ice-cold PBS. For IC activation, BMDM or BMDM was added to pb-IC and incubated for 24 h. Supernatant was collected to determine IC-mediated secretion of TNF-α, IL-6, IL-12, and IFN-γ. Cells added to wells with Ag alone or Ag with control rabbit IgG were used as controls.

Cytokine analysis

Cytokine secretion in supernatants from anti-CD3/CD28-stimulated CD4+ cells was analyzed using mouse Th1/Th2/Th17 (IL-2, IL-4, IL-6, IFN-γ, IL-17, and IL-12); IL-6; and IL-10. Cells were cultured with Ag alone or Ag with control rabbit IgG were used as controls.
Tnf-α, II-17A, and II-10) cytokine bead array according to the manufacturer’s instructions (BD Biosciences). Beads were analyzed using an FACSCalibur flow cytometer and FCAP array CBA software (BD Biosciences). Murine TGF-β levels in the supernatant from anti-CD3/CD28-stimulated CD4⁺ cells, II-12, and II-17 secretion by activated BMDC and BMDE were determined by corresponding Duoset ELISA kit (R&D Systems, Minneapolis, MN).

Quantitative RT-PCR analysis

The aorta was perfused with nuclease-free PBS. Total RNA was isolated from a proximal portion of the descending aorta (aortic arch and aorta proximal to the subclavian artery) and spleen using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. RNA was quantified using Nanodrop spectrophotometer (Nanodrop Products, Wilmington, DE), and its integrity was determined using Bio-Rad Experion RNA analyzer (Bio-Rad, Hercules, CA). Gene expression was measured by real-time RT-PCR after reverse transcription of total RNA (0.5 µg) as described earlier (28). PCR primer pairs were purchased from SABC Biosciences (Frederick, MD). Two-step PCR with denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min for 40 cycles was conducted in an iCycler (Bio-Rad). Expression of target genes was calculated by the 2⁻¹ΔΔCt method using threshold cycles for β-actin as a normalization control. All real-time PCR reactions were carried out at least twice from independent CDNA preparations. RNA without reverse transcriptase served as a negative control.

Determination of MDALDL Ab

MDALDL (Academy Biomedical, Houston, TX) and anti-MDALDL IgG and IgM responses were determined by methods described earlier (26). Briefly, MDALDL (10 µg/ml in PBS/1 mM EDTA) was coated on Nunc Microflow white plates (Thermoscientific, Pittsburgh, PA) overnight at 4°C and blocked with PBS/1% BSA/1 mM EDTA for 2 h at room temperature. Sera (1:50 diluted) was added to the plate and incubated at 4°C for 1 h at room temperature. Plates were washed, and alkaline phosphatase (AP)-conjugated anti-mouse IgG, anti-mouse IgG2a, anti-mouse IgG2b, anti-mouse IgG3, or anti-mouse IgM (Southern Biotechnology Associates, Birmingham, AL) was added and incubated for 1 h at room temperature. The AP enzymatic activity was determined using luminescence substrate Lumi-Phos530 (Lumigen Southfield, MI), and luminescence was read at 530 nm in a Polarstar plate reader (BMG Labtech, Cary, NC).

Flow cytometric analysis of T cell subsets

Splenocytes (3 × 10⁶ cells) were stained with T lymphocyte subset Ab mixture (BD Biosciences), and different T cell subsets were analyzed in an FACSCalibur flow cytometer followed by data analysis using FlowJo analytical software (Tree Star, Ashland, OR). T lymphocyte subset Ab mixture consists of the PE-Cy7–anti-CD3e (clone 145-2C11), PE–anti-CD4 (clone RM4-5), and APC–anti-CD8a (clone 53-6.7). Cells stained with corresponding isotype IgG were used as controls. To determine CD152/CD4⁰ T cells, splenocytes were stained with APC–anti-CD4, PE–Cy5–CD25, and PE-CTLA4 and were analyzed by flow cytometry. For Treg analysis, splenocytes were incubated with APC–anti-CD4 and PE–Cy5–anti-CD25. After surface staining, cells were fixed, permeabilized, and stained with PE–anti-Foxp3 according to the manufacturer’s instructions (BD Biosciences). For Th17 cell analysis, purified CD4⁺ cells (10⁶ cells/ml) were incubated with 50 ng/ml PMA and 1 µM ionomycin for 6 h in the presence of monensin. Then, cells were stained with APC–anti-CD4 at 4°C for 20 min. Cells were fixed, permeabilized, and stained with PE–anti-II-17A mAb. Cells stained with isotype control IgG were used as controls. Cells were analyzed by flow cytometric analysis using an FACSCalibur flow cytometer equipped with CellQuest software (BD Biosciences). Percent positive cells were determined using FlowJo software (Tree Star).

Assay for p-Stat3

CD4⁺ T cells were stimulated with anti-CD3/CD28 mAb as described earlier and lysed in lysis buffer containing protease and phosphatase inhibitors (Cell Signaling Technology, Danvers, MA). To determine IL-6–dependent STAT3 phosphorylation, CD4⁺ T cells were stimulated with IL-6 (20 ng/ml) for 30 min. Levels of p-STAT3 and total STAT3 in the cell lysates were determined using Pathscan ELISA kits specific for each one (Cell Signaling Technology). Color was developed using TMB substrate, and the reaction was stopped with 2 N sulfuric acid. Absorbance was read at 450 nm in a Polarstar multiplate reader (BMG Labtech). Phospho-STAT3 levels were normalized to total STAT3 levels in each sample.

Statistical analyses

Values are expressed as mean ± SD. Differences between the groups were considered significant at p < 0.05 using the two-tailed Student t test. All data were analyzed using GraphPad InStat version 3.1a for Macintosh (GraphPad, San Diego, CA).

Results

ApoE-Fcγ-chain DKO mice show reduced atherosclerotic lesions

To determine the role of activating FcγR in the progression of atherosclerosis, total activating FcγR deficiency in apoE KO background was generated. Genotype analyses showed complete knock-out for Fcγ-chain in the apoE KO background (Fig. 1A). Spontaneous atherosclerotic lesions in apoE KO and apoE-Fcγ-chain DKO mice fed a chow diet (contains 5% fat with no cholesterol) were determined by staining aortic sinus cryosections (10 µm) with Oil Red O (Fig. 1B). Atherosclerotic lesions in the aortic tree were performed by staining descending aorta from the aortic arch to the iliac bifurcation with Sudan IV (Fig. 1C). Both analyses showed an ∼50% reduction in lesions (p < 0.01, Student t test) in apoE-Fcγ-chain DKO mice compared with apoE KO mice (Fig. 1D). We then determined whether Fcγ-chain deficiency also had an effect on accelerated atherosclerosis in mice fed a high-fat diet (21% fat and 0.15% cholesterol). ApoE KO mice showed more lesions in the aortic sinus (Fig. 1E, 1F), where Fcγ-chain deficiency was associated with a significant decrease in lesion area in the aortic root and aortic arch relative to apoE KO male mice (Fig. 1E, 1F). Female apoE-Fcγ-chain DKO mice also showed a similar reduction in lesions, suggesting there is no effect of gender on the extent of lesions (data not shown). Because Fcγ-chain is essential for expression and assembly of FcγRI, FcγRIII, and FcγRIV (activating FcγRs), these findings suggest activating FcγRs contribute to the atherosclerotic lesion formation.

Activating FcγR expressed on hematopoietic cells is sufficient to contribute to the atherosclerotic lesions

Studies using human cell lines have shown the expression of FcγR in vascular endothelial and smooth muscle cells (31, 32). Next, we investigated whether activating FcγRs expressed on cells of hematopoietic origin contribute to the progression of atherosclerosis by bone marrow chimera. Bone marrow cells from apoE KO or apoE-Fcγ-chain DKO mice were transplanted to apoE KO recipient mice. Deletion of the Fcγ-chain in hematopoietic cells was confirmed by the genomic PCR analyses (Fig. 2A). ApoE KO mice transplanted with apoE KO bone marrow cells showed significantly increased atherosclerotic lesions in the aortic root (Fig. 2B, 2D). However, apoE KO mice transplanted with apoE-Fcγ-chain DKO bone marrow cells revealed a reduction of >50% (p < 0.01) in atherosclerotic lesions (Fig. 2B, 2D). Immunohistochemical analyses showed reduced macrophage at the lesions in apoE-Fcγ-chain DKO chimeras than in apoE KO chimera mice, suggesting reduced monocyte migration and subsequent transformation to macrophages at the lesion site. Moreover, analyses of mRNA expression of macrophage FcγRIIB (inhibitory) and the activating FcγR (FcγRII, III, and IV) did not reveal any difference in the ratio between the activating and inhibitory FcγR expression in inflammatory cells (data not shown). These findings suggest that activating FcγR expression on hematopoietic cells is sufficient to contribute the atherosclerotic lesions.

Fcγ-chain deficiency does not alter plasma cholesterol levels

To determine the molecular mechanisms contributing to the decreased number of atherosclerotic lesions in apoE-Fcγ-chain DKO mice, plasma total and HDL cholesterol levels were determined.
There were no differences in plasma lipid profiles in apoE-Fc\textsubscript{g}-chain DKO mice compared with apoE KO controls fed a chow or high-fat diet (Table I). Similar findings were observed in bone marrow chimera mice (Table I). These data suggest that the reduction in lesion formation in apoE-Fc\textsubscript{g}-chain DKO mice compared with apoE KO mice was not due to changes in plasma lipid levels.

Anti-oxLDL IgG levels were elevated in apoE-Fc\textsubscript{g}-chain DKO mice. Anti-oxLDL Ab (IgG and IgM) responses were determined for each isotype at 10 wk of the high-fat diet. The apoE KO mice lacking Fc\textsubscript{g}-chain expression had significantly higher levels of oxLDL-specific IgG response (Fig. 3A), whereas no difference was found in anti-oxLDL IgM response (Fig. 3B). Anti-oxLDL IgG isotype analyses showed anti-oxLDL IgG2a Abs were elevated several fold, as well as the anti-oxLDL IgG1, IgG2b, and IgG3 (Fig. 3C). Ratio of IgG2a/IgG1 was not different between apoE KO and apoE-Fc\textsubscript{g}-chain DKO mice (Fig. 3D). Similar results were obtained when oxLDL was used in this assay (data not shown). Total plasma IgG and IgM were not different between apoE KO mice and apoE-Fc\textsubscript{g}-chain DKO mice (data not shown). These findings suggest there was no Th1/Th2 bias in apoE-Fc\textsubscript{g}-chain DKO mice. These findings suggest there is no apparent increase in Th1/Th2 shift in apoE-Fc\textsubscript{g}-chain DKO mice.

Attenuated atherosclerotic lesion in apoE-Fc\textsubscript{g}-chain DKO mice is not due to Th1/Th2 shift

Earlier studies have suggested that CD4\textsuperscript{+} T cells specific to oxLDL promote lesions by increasing Th1 cell responses (26, 27). To investigate if the Th1/Th2 shift could have contributed to the attenuated lesions in the activating Fc\textsubscript{g}R-deficient mice, cytokine response by activated CD4\textsuperscript{+} T cells (from high fat-fed mice) and anti-oxLDL IgG2a (Th1) and IgG1 (Th2) levels were determined. Analysis of CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell populations showed there was no difference in CD4\textsuperscript{+}/CD8\textsuperscript{+} ratio in apoE-Fc\textsubscript{g}-chain DKO mice and apoE KO mice cells (data not shown). Activated CD4\textsuperscript{+} T-cells secreted high levels of IL-10 with no difference in IL-4 levels in apoE-Fc\textsubscript{g}-chain DKO mice compared with apoE KO mice (Fig. 4A, 4B). Surprisingly, a 2-fold increase in the secretion of IFN-\gamma was observed from activated CD4\textsuperscript{+} T cells of apoE-Fc\textsubscript{g}-chain DKO mice relative to apoE KO controls (Fig. 4C). Quantitative RT-PCR analyses of CD4\textsuperscript{+} cells showed mRNA expression of IL-10 and IL-4 was ∼3- and 1.5-fold high in apoE-Fc\textsubscript{g}-chain DKO mice (Fig. 4D). Notably, IFN-\gamma mRNA levels were also ∼3-
fold higher in apoE-Fcγ-chain DKO compared with apoE KO mice (Fig. 4D). Zhou et al. (19) have shown that IFN-γ mRNA expression was high in the arterial lesion site, suggesting that local Th1 response at the lesion site may be contributing to the progression of atherosclerosis. We then investigated whether the change in Th1/Th2 immune responses at the lesion sites may have contributed to the reduction in lesion formation in the apoE-Fcγ-chain DKO mice. Using aorta from high fat-fed mice, we determined the mRNA levels of cytokines in apoE KO and apoE-Fcγ-chain DKO mice. Consistent with the changes in cytokine production by activated CD4+ T lymphocytes, mRNA levels for IL-10 in apoE-Fcγ-chain DKO were significantly higher relative to apoE KO mice (Fig. 4E). These findings suggest that Th1/Th2 imbalance may not be contributing to the attenuated atherosclerotic lesions in apoE-Fcγ-chain DKO mice. To address whether CD4+ T cells from apoE-Fcγ-chain DKO show a bias in Th1/Th2 differentiation intrinsically, basal expression of transcription factors T-bet (Th1) and GATA3 (Th2), which initiate Th1 and Th2 cell development, respectively (33, 34), was analyzed. The mRNA expression of T-bet and GATA-3 in CD4+ T cells of apoE-Fcγ-chain DKO and apoE KO mice was not different (Fig. 4F).

**Th17 cells were reduced in apoE-Fcγ DKO mice**

We then investigated alternative mechanism(s) that may be contributing to the reduced lesions in apoE-Fcγ-chain DKO mice. CD4+ T cells of apoE-Fcγ-chain DKO mice showed ∼50% lower IL-17 mRNA expression compared with apoE KO mice (Fig. 5A).
Moreover, compared with CD4+ cells of apoE KO, IL-17 secretion by CD4+ T cells of apoE-Fcγ-chain DKO mice was significantly reduced after stimulation with anti-CD3 and anti-CD28 (Fig. 5B). Then we determined whether Fcγ-chain deficiency in hyperlipidemic apoE KO mice resulted in a reduced number of Th17 cells. Intracellular staining for IL-17 in PMA/ionomycin-activated CD4+ cells revealed apoE KO mice have ∼2% Th17+ cells (Fig. 5C, 5D). However, the number of Th17+ cells was reduced to <0.5% of CD4+ cells in apoE-Fcγ-chain DKO mice (Fig. 5C, 5D). Then we determined the expression of IL-17 in the aorta samples to address whether IL-17 expression is reduced at the lesion site. IL-17A mRNA expression was lower in aorta sample from apoE-Fcγ-chain DKO mice (Fig. 5E). These findings suggest that decreased generation of Th17 cells and IL-17 expression in apoE-Fcγ-chain DKO mice could in part contribute to the attenuated atherosclerotic lesions in these mice.

More Tregs in apoE-Fcγ-chain DKO-deficient mice

Recent studies using apoE KO mice have shown that there is an inverse relationship to the number of Th17 and CD4+CD25+Foxp3+ Tregs (35–37). Hence, we determined the number of Tregs in apoE KO and apoE-Fcγ-chain DKO mice fed a high-fat diet. The deficiency of Fcγ-chain in hyperlipidemic apoE KO mice was associated with an increase in the number of Tregs (Fig. 6A). Moreover, an ∼2-fold increase in Foxp3 mRNA expression was observed in apoE-Fcγ DKO mice compared with apoE KO mice (Fig. 6B). As CD152 have been shown to be upregulated in Tregs, we determined the mRNA expression of CD152 in the CD4+ T lymphocytes of apoE KO and apoE-Fcγ-chain DKO mice by real-time RT-PCR. Interestingly, CD152 mRNA expression was increased significantly in the spleen of apoE-Fcγ-chain DKO mice compared with apoE KO mice, whereas expression of CD28, another T cell costimulatory molecule, was similar in apoE KO and apoE-Fcγ-chain DKO mice (Fig. 6C). These findings suggest that Fcγ-chain deficiency in a hyperlipidemic mouse background increased the regulatory CD4+ T cell population, causing an imbalance between Th17 and Tregs.

Mechanism contributing to the Th17/Treg shift in apoE-Fcγ-chain DKO mice

We then determined mechanisms contributing to the reduced Th17 cells in apoE-Fcγ-chain DKO mice. Generation of Th17 cells in mice requires the presence of TGF-β and IL-6 (38–40). Hence, the levels of TGF-β and IL-6 in anti-CD3/CD28 activated CD4+ cells. Activated CD4+ cells of the apoE-Fcγ-chain secrete 3-fold higher levels of TGF-β than in activated CD4+ cells of apoE KO mice (Fig. 7A). The decrease in TGF-β secretion also paralleled a 3-fold increase in TGF-β mRNA expression in apoE-Fcγ-chain DKO mice (Fig. 7B). TGF-β can prime the naive CD4+ T cells to differentiate into Tregs or Th17 cells depending on the presence of IL-6 (39). Hence, IL-6 secretion by activated CD4+ cells was determined. IL-6 mRNA (Fig. 7C) and IL-6 secretion by activated CD4+ cells (Fig. 7D) was significantly reduced in apoE-Fcγ-chain DKO mice. Recent studies showed that the IL-6/STAT3 signaling pathway plays a predominant role in the induction of Th17 response in autoimmune disease (41, 42). Because IL-6 and IL-17 secretion was reduced in anti-CD3-stimulated CD4+ cells from apoE-Fcγ-chain DKO mice, we investigated whether STAT3 activation is reduced in apoE-Fcγ-chain DKO mice. Total and phospho-STAT3 levels in activated CD4+ T cells were determined by ELISA-based assay. Total STAT3 levels were not different between the two strains of mice (Fig. 7E). However, less p-STAT3 was detected in CD4+ T cells from apoE-Fcγ-chain DKO mice when compared with that from apoE KO mice (Fig. 7F). IL-21 selectively produced by Th17 cells has been shown to serve as an autocrine factor for promoting and sustaining Th17 lineage commitment and drive IL-17 production in a STAT3-dependent manner (43). Hence, we determined whether lower p-STAT3 in apoE-Fcγ-chain DKO mice affects IL-21 secreted by activated T cells. IL-21 secretion in activated T cells was significantly lower.
in apoE-Fc-chain DKO mice (Fig. 7G) compared with T cells from apoE KO mice. Because we observed both reduced IL-6 and STAT3 phosphorylation in apoE-Fc-chain mice, we then determined whether Fcγ-chain deficiency affects IL-6 signaling pathway, which may lead to an IL-17 response. CD4+ T cells were treated with IL-6, and p-STAT3 was detected. Total STAT3 levels were also not different between both strains (data not shown). Basal and IL-6–mediated p-STAT3 levels were not different between apoE KO and apoE-Fc-chain DKO mice (Fig. 7H), suggesting that Fcγ-chain deficiency does not affect IL-6 signaling pathway. These findings indicate that less Th17 response in apoE-Fc-chain DKO mice may result from lack of optimal cytokine responses, such as IL-6 resulting in less activation of STAT3 signaling pathway.

Reduced IL-6 secretion by BMDM and BMDC of apoE-Fc-chain DKO mice

IL-6 release by APC such as DC has been shown to be critical for the generation and differentiation of Th17 cells (44, 45). Hence, to identify the FcγR-dependent factors for Th17 skewing in vivo, OVA-IC– and MDALDL-IC–mediated IL-6 release by BMDM and BMDC of apoE-Fc-chain DKO mice was determined. Plate-bound OVA-IC or MDALDL-IC was confirmed by ELISA assay prior to adding the cells on IC-coated plates (data not shown). OVA- or MDALDL-treated apoE KO BMDM and BMDC did not induce IL-6 (Fig. 8A, 8B) secretion. However, OVA-IC or MDALDL-IC activation of apoE KO BMDM and BMDC showed very high levels of IL-6 secretion, ~30-fold for BMDM and 8-fold for BMDC (Fig. 8A, 8B). Interestingly, both MDALDL-IC and OVA-IC activation of apoE-Fc-chain–deficient BMDM and BMDC did not induce IL-6 secretion (Fig. 8A, 8B). Similar findings were observed for IC-mediated TNF-α (Fig. 8C, 8D) and IL-12 (Fig. 8E, 8F) secretion by BMDC and BMDC from apoE-Fc-chain DKO mice. Under similar conditions, INF-γ secretion was too low to be detected (data not shown). These findings suggest that more attenuated Th17 cells in apoE-Fc-chain DKO mice may be due to reduced levels of IL-6 secretion by APC deficient in the Fcγ-chain.

Discussion

In the current study, we tested the hypothesis that oxLDL-IC binding to the activating FcγR promotes the progression of atherosclerosis. We showed a significant reduction in the formation of arterial lesions in Fcγ-chain–deficient mice after a high-fat diet. Interestingly, bone marrow chimera studies showed attenuated lesions in apoE KO mice transplanted with bone marrow from apoE-Fc-chain DKO mice. These findings suggest that the activating FcγR expressed on hematopoietic cells is sufficient for the progression of atherosclerosis. Remarkably, the reduction in atherosclerotic lesion progression in the activating FcγR deficiency in hyperlipidemic mouse model was associated with the increased...
generation of CD4+ Tregs with concomitant decrease in CD4+ Th17 cells.

Earlier studies have shown expression of activating FcγRI in vascular endothelial and smooth muscle cells (31, 32). Moreover, studies using apoE-Fcγ-chain DKO mice revealed that expression of the inhibitory FcγRIIB was elevated in vascular smooth muscle cells (19). These studies have suggested that the ratio between inhibitory/activating FcγRs in vascular smooth muscle

FIGURE 5. Th17 CD4+ T cells were reduced in apoE-Fcγ DKO mice. RNA was isolated from purified CD4+ cells of apoE KO and apoE-Fcγ-chain DKO mice fed high-fat diet for 10 wk. IL-17A mRNA levels (A) were determined by quantitative RT-PCR assays and normalized relative to housekeeping gene β-actin transcripts. Each value indicates mean ± SD results from five mice. B, IL-17 secretion is reduced in apoE-Fcγ-chain DKO mice fed high-fat diet. Purified CD4+ lymphocytes from apoE KO and apoE-Fcγ-chain DKO mice were stimulated with pb-anti-CD3 and soluble CD28 for 72 h. The concentration of IL-17 was measured by cytokine bead array. Each value indicates mean ± SD results from five mice. C and D, Th17 cell number was reduced in apoE-Fcγ-chain DKO mice. CD4+ cells were stimulated with PMA/IONOMYCIN, and CD4+IL-17+ cells were determined by intracellular staining for IL-17 as described in Materials and Methods. Representative flow cytometric analysis of Th17 cells (C) was presented. Quantification of number of CD4+IL-17+ cells was determined by analyzing the FACS data using FlowJo software (Tree Star). Each value indicates mean ± SD results from five mice. E, IL-17A mRNA expression in aorta was determined by quantitative RT-PCR analysis. Values are expressed as means ± SD; n = 5/group. ***p < 0.001 compared with apoE KO mice.

FIGURE 6. Number of Tregs was high in apoE-Fcγ-chain DKO-deficient mice. A, Flow cytometric analysis of CD4+CD25+Foxp3+ cells was determined by staining purified CD4 cells from apoE KO and apoE-Fcγ-chain DKO mice fed high-fat diet for 10 wk. Number of Tregs was determined by analyzing the FACS data using FlowJo software (Tree Star). B, Foxp3 mRNA expression was high in CD4+ cells from apoE-Fcγ-chain DKO mice. RNA was isolated from purified CD4+ cells of apoE KO and apoE-Fcγ-chain DKO mice fed high-fat diet for 10 wk. Foxp3 mRNA levels were determined by quantitative RT-PCR assays and normalized relative to housekeeping gene β-actin transcripts. Each value indicates mean ± SD results from five mice. C, CD152 mRNA expression was high in CD4+ cells from apoE-Fcγ-chain DKO mice. RNA was isolated from purified CD4+ cells of apoE KO and apoE-Fcγ-chain DKO mice fed high-fat diet for 10 wk. CTLA4 and CD28 mRNA levels were determined by quantitative RT-PCR assays and normalized relative to housekeeping gene β-actin transcripts. Each value indicates mean ± SD results from five mice. *p < 0.05, ***p < 0.0001 compared with apoE KO mice.
cells could be responsible for the observed reduced atherosclerotic lesions in apoE-Fcγ-chain DKO mice (22). It is well established that both activating and inhibitory FcγRs are constitutively expressed on cells of hematopoietic origin, which include monocytes/macrophages, neutrophils, and NK cells (11–13). Notably, using the bone marrow chimera approach, we showed that the deficiency of activating FcγR expression on hematopoietic cells is sufficient to inhibit the progression of atherosclerotic lesions. Thus, the findings from the current study indicate the possibility that the activating FcγR expressed on hematopoietic cells is the major contributor in the progression of atherosclerosis in apoE KO mice.

![Graphs](image)

**FIGURE 7.** Reduced Th17 and higher Tregs are due to attenuated IL-6 expression. Purified CD4+ lymphocytes from apoE KO and apoE-Fcγ-chain DKO (fed high-fat diet for 10 wk) were stimulated with pb-anti-CD3 and soluble CD28 for 72 h. The concentration of TGF-β (A) and IL-6 (C) was measured by cytokine bead array. Each value indicates mean ± SD results from five mice. TGF-β (B) and IL-6 (D) mRNA expression in CD4+ cells from apoE-Fcγ-chain DKO mice. RNA was isolated from purified CD4+ cells of apoE KO and apoE-Fcγ-chain DKO mice fed high-fat diet for 10 wk. TGF-β and IL-6 mRNA levels were determined by quantitative RT-PCR assays and normalized relative to housekeeping gene β-actin transcripts. Each value indicates mean ± SD results from five mice. E and F, Reduced STAT3 phosphorylation in apoE-Fcγ-chain DKO mice. Purified CD4 cells were stimulated with pb-CD3, and cell lysates were prepared. Total STAT (E) and p-STAT3 (F) were determined by Pathscan ELISA kit (Cell Signaling Technology) as described in Materials and Methods. G, IL-21 secretion by activated CD4+ cells from apoE KO and apoE-Fcγ-chain DKO mice fed high-fat diet for 10 wk was determined by ELISA. Each value indicates mean ± SD results from five mice. H, IL-6 induced STAT3 phosphorylation in apoE-Fcγ-chain DKO mice. Purified CD4 cells of apoE KO and apoE-Fcγ-chain DKO mice were stimulated without or with IL-6, and phospho-STAT3 was determined by Pathscan ELISA kit as described in Materials and Methods. Values are expressed as means ± SD; n = 5/group. **p < 0.01, ***p < 0.0001 compared with apoE KO mice.

![Graphs](image)

**FIGURE 8.** Reduced IL-6 secretion by BMDM and BMDC of apoE-Fcγ-chain DKO mice. BMDM and BMDC from apoE KO and apoE-Fcγ-chain DKO mice were stimulated with pb-OVA-IC or MDALDL-IC for 24 h. Secretion of IL-6 (A, B), TNF-α (C, D), and IL-12 (E, F) by BMDM and BMDC was measured by corresponding Duoset ELISA kits (R&D Systems). Each value indicates mean ± SD results from triplicates. ***p < 0.0001 compared with apoE KO mice.
Th1 response has long been recognized as having a proatherogenic potential and an important role in the development of atherosclerosis (19, 26). Plasma anti-oxLDL IgG analyses revealed there is no clear distinction of Th1 (IgG2a) and Th2 (IgG1) type of Ab response in apoE-Fcγ-chain DKO mice. Notably, we showed anti-oxLDL IgG1 and IgG2a levels were higher in apoE-Fcγ-chain DKO mice compared with apoE KO mice, despite lesions being lower in apoE-Fcγ-chain DKO mice. Earlier studies have shown that in moderate hypercholesterolemia, anti-oxLDL Ab response is predominantly the IgG2a isotype, correlating with increased IFN-γ-producing T cells (19). However, severe hypercholesterolemia was shown to be associated with a shift from Th1 to Th2 response as evident from elevated anti-oxLDL IgG1 isotype and appearance of IL-4, Th2 cytokine, producing cells in atherosclerotic lesions (19). These findings suggest that antigenic load may determine the Th1/Th2 autoimmune responses in atherosclerosis. Immunization of apoE KO with MADILDL resulting in elevated plasma anti-oxLDL IgG was atheroprotective (46–48). This raises an interesting possibility that higher anti-oxLDL levels in apoE-Fcγ-chain DKO mice may be atheroprotective. However, this possibility needs to be resolved by passive transfer of anti-oxLDL IgG.

Th1 response has long been recognized having a proatherogenic potential and an important role in the development of atherosclerosis (19, 26). Our findings showed no difference in IL-4 levels in apoE-Fcγ-chain DKO compared with apoE KO mice, ruling out a role for IL-4 in reduced lesions in apoE-Fcγ-chain DKO mice. Surprisingly, IFN-γ levels were elevated in activated CD4+ T cells of apoE-Fcγ-chain DKO mice though the lesions were fewer in these mice. This finding is in agreement with earlier report showing elevated IFN-γ production with attenuated lesion in LDLR-FcγRIII DKO mice (23). The mechanism(s) contributing to the elevated IFN-γ in the total activating FcγR deficiency (our report) and FcγRIII deficiency (23) needs to be explored. To address whether Fcγ-chain deficiency inherently influences different T cell subsets, we determined basal level expression of T-bet and GATA-3, transcription factors essential for Th1 and Th2 cell differentiation. We did not detect the difference in T-bet and GATA-3 expression in CD4+ T cells from apoE-Fcγ-chain DKO mice, ruling out that there is an intrinsic effect of Fcγ-chain deficiency on Th1 and Th2 differentiation. Nevertheless, these findings suggest that the imbalance in Th1/Th2 may not be contributing to the attenuated lesions seen in apoE-Fcγ-chain DKO mice. Notably, our findings also showed activated CD4+ T cells produced elevated IL-10 in apoE-Fcγ-chain DKO compared with apoE KO mice, suggesting that IL-10 secreted by other T cell subsets such as Tregs may be contributing to the reduced lesions in apoE-Fcγ-chain DKO mice.

Th17 cells, a subset of CD4 cells secreting IL-17, have been implicated in proinflammatory responses (41), suggesting a role for IL-17 in the progression of atherosclerosis. However, the role of IL-17 and Th17 in atherosclerosis is still evolving. The proatherogenic role of IL-17 was demonstrated in apoE KO mice using anti–IL-17 mAb (35), recombinant soluble IL-17R-A (36), or LDLR KO recipient mice transplanted with IL-17R-deficient bone marrow cells (49) or IL17 KO mice (50). On the contrary, two studies from the Mallat group (51, 52) have suggested that the elevated level of IL-17 is atheroprotective. In the first study, LDLR KO mice received suppressor of cytokine signaling 3-deficient bone marrow cells showing reduced atherosclerosis with elevated IL-17 and IL-10 levels (51). In the second study, the atheroprotective role of IL-17 was suggested in B cell-depleted mice showing elevated IL-17 levels (52). It should be pointed out that in all of the studies examining the direct role for IL-17 using apoE KO, the hyperlipidemic mouse model showed IL-17 is proatherogenic. Our findings showing reduced lesions, number of Th17 cells, and secretion of IL-17 by activated CD4+ lymphocytes in apoE-Fcγ-chain DKO compared with apoE KO mice suggest a proatherogenic role for IL-17. Moreover, IL-21 selectively produced by Th17 cells has been shown to serve as an autocrine factor for promoting and sustaining Th17 lineage commitment and to drive IL-17 production (43). Our findings showing reduced IL-21 secretion by activated T cells further confirmed that Th17 response is lower in apoE-Fcγ DKO mice. However, more studies may be needed to resolve the cloud around the role for IL-17 in atherosclerosis.

Finally, we determined the FcγR-dependent factors that might be a possible mechanism for the reduced Th17 cells in apoE-Fcγ-chain DKO mice. IL-6 and IL-6–dependent STAT3 signaling pathways are essential for Th17 generation (42, 44). We showed IL-6 secretion and STAT3 phosphorylation in activated CD4+ cells was reduced in apoE-Fcγ-chain DKO mice, suggesting less IL-6 secretion and STAT3 phosphorylation may relate to reduction of Th17 response in apoE-Fcγ-chain DKO mice. This raises an interesting link between the Fcγ-chain and IL-6 signaling pathway. However, exogenous addition of IL-6 did not show a difference in p-STAT3 levels in CD4 cells of apoE KO and apoE-Fcγ-chain DKO mice (Fig. 7H), ruling out a direct link between the Fcγ-chain and IL-6 signaling pathway. Previous studies showing DC-derived IL-6 is critical for differentiation (45) raise the possibility that lack of IL-6 secretion by APCs from Fcγ-chain–deficient mice may affect Th17 differentiation. We showed IC-mediated IL-6 and TNF-α secretion was impaired in BMDM and BMDC from apoE-Fcγ-chain DKO mice, but not from apoE KO mice. Hence, it is possible that in the absence of IL-6, the Treg differentiation pathway may be activated rather than Th17 differentiation (53, 54). Recent studies have also shown an inverse relationship between Th17 and Tregs in the progression of atherosclerosis in hyperlipidemic mouse models (37) and human studies (55–57). We showed that deficiency of Fcγ-chain resulted in expansion of Tregs producing high levels of IL-10 and TGF-β, which have been implicated in antiatherogenic effects (58–60). Collectively, our findings suggest that an anti-inflammatory response by CD4+ CD25+ Tregs may inhibit Th17 differentiation and inhibit the atherosclerosis in hyperlipidemic mouse models.

In summary, our investigation demonstrated that Fcγ-chain deficiency leading to the impaired functions of the activating FcγR (FcγRI, III, and IV) has resulted in reduced atherosclerotic lesions in hyperlipidemic apoE KO mouse model. The attenuated lesions in apoE-Fcγ-chain DKO mice are not due to the imbalance in Th1/Th2 shift. On the contrary, our findings showed higher Tregs with a concomitant decrease in Th17 cells in part contributed to the abridged lesions in Fcγ-chain–deficient mice in hyperlipidemic conditions. Importantly, reduced FcγR-mediated IL-6 secretion may contribute to attenuated Th17 response and subsequently attenuated atherosclerosis in apoE-Fcγ-chain DKO mice. These studies collectively suggest that the lack of IL-6 secretion in Fcγ-chain–deficient mice may be contributing to the reduced number of Th17 cells in apoE-Fcγ-chain DKO mice.

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Disclosures

The authors have no financial conflicts of interest.
References

1. Steinberg, D. 1997. Low density lipoprotein oxidation and its pathobiological significance. J. Am. Coll. Cardiol. 27: 2066–2072.

2. Yla-Herttuala, S., W. Palinski, S. W. Butler, S. Picard, D. Steinberg, and J. L. Witztum. 1994. Rabbit and human atherosclerotic lesions contain IgG that recognizes epitopes of oxidized LDL. Arterioscler. Thromb. 14: 32–40.

3. Palinski, W., N. K. Tangirala, E. Miller, S. G. Young, and J. L. Witztum. 1995. Increased autobody titers against epitopes of oxidized LDL in LDR receptor-deficient mice with increased atherosclerosis. Arterioscler. Thromb. Vasc. Biol. 15: 1569–1576.

4. Salonen, J. T., S. Yla-Herttuala, R. Yamamoto, S. Butler, H. Korpela, R. Salonen, N. Yamamoto, W. Palinski, and J. L. Witztum. 1992. Autobody against oxidized LDL and progression of carotid atherosclerosis. Lancet 339: 883–887.

5. Bergmark, C., R. Wu, U. de Faire, A. K. Lefvert, and J. Swedinberg. 1995. Patients with early-onset peripheral vascular disease have increased levels of autoantibodies against oxidized LDL. Arterioscler. Thromb. Vasc. Biol. 15: 4: 445.

6. Tsikaakis, S., E. S. Brilakis, R. J. Lemon, E. R. Miller, J. L. Witztum, J. P. McConnell, K. S. Kornman, and P. B. Berger. 2007. Relationship of IgG and IgM autoantibodies to oxidized low density lipoprotein with coronary artery disease and cardiovascular events. J. Lipid Res. 48: 425–433.

7. Bhardwaj, D. M., M. P. Stein, M. Volzer, C. Mold, and T. W. Du Clos. 1999. The major receptor for C-reactive protein on leukocytes is f staggera receptor II. J. Exp. Med. 190: 585–590.

8. Ridker, P. M., N. Rifai, L. Rose, J. E. Buring, and N. R. Cook. 2002. Comparison of C-reactive protein and low-density lipoprotein cholesterol levels in the prediction of first cardiovascular events. N. Engl. J. Med. 347: 1557–1565.

9. Hashimoto, K., K. Kitagawa, H. Hougaku, Y. Shimizu, M. Sakaguchi, Y. Nagai, S. Iyama, H. Yamashini, M. Matsumoto, and M. Horii. 2001. C-reactive protein is an independent predictor of the rate of increase in early carotid atherosclerosis. Circulation 104: 63–67.

10. Teupser, D., O. Weber, T. N. Rao, K. Saks, J. Thiery, and H. J. Fehrs. 2011. No reduction of atherosclerosis in C-reactive protein (CRP)-deficient mice. J. Biol. Chem. 286: 6277–6282.

11. Hulett, M. D., and P. M. Hogarth. 1994. Molecular basis of Fc receptor function. Adv. Immunol. 57: 1–127.

12. Ravetch, J. V., and L. Lanier. 2000. Immune inhibitory receptors. Science 290: 84–89.

13. Ravetch, J. V., and S. Bolland. 2001. IgG Fc receptors. Annu. Rev. Immunol. 19: 275–290.

14. Kurosaki, T., L. Gander, and J. V. Ravetch. 1991. A subunit common to IgG Fc receptor and the T-cell receptor mediates assembly through different intercellular interactions. Proc. Natl. Acad. Sci. USA 88: 3837–3841.

15. Nagarajan, S., S. E. Chesla, L. Cobern, P. Anderson, C. Zhu, and P. Selvaraj. 2006. Adoptive transfer of CD4(+) T cells aggravates atherosclerosis in immunodeficient apolipoprotein E knockout mice. J. Immunol. 177: 1579–1587.

16. Covarrubias, S. V., C. J. Binder, J. L. Witztum, and A. S. Major. 2011. The integrin αvβ3 is a determinant of immunity and autoimmunity. Immunity 34: 157–168.

17. Zheng, W., and R. A. Flavell. 1997. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. Cell 89: 587–596.

18. Erbel, C., L. Chen, F. Bea, S. Wangerl, S. Celik, F. Laschitschka, Y. Wang, D. Bockerl, H. A. Katus, and T. J. Dengler. 1999. Inhibition of IL-17A attenuates atherosclerosis lesion development in apolipoprotein-deficient mice. J. Immunol. 163: 8167–8175.

19. Smith, E., K. M. Prasad, M. Butcher, A. Dobrian, J. K. Kolls, K. Ley, and E. Galkina. 2010. Blockade of interleukin-17A results in reduced atherosclerosis in apolipoprotein E-deficient mice. Circulation 121: 1746–1755.

20. Xie, J. J., J. Wang, T. T. Tang, J. Chen, X. L. Gao, Y. Juan, Z. H. Zhui, M. Y. Liao, R. Yao, X. Yu, et al. 2010. The Th17/Treg functional balance during atherosclerosis in ApoE-/- mice. Cytokine 49: 185–193.

21. Bettelli, E., Y. Carrier, W. Gao, T. Korn, T. B. Strom, M. Oukka, H. L. Weiner, V. K. Kuchroo. 2006. Reciprocal developmental pathways for the generation of pathogenic effector Th17 and regulatory T cells. Nature 441: 235–238.

22. Veldhoen, M., R. J. Hocking, C. A. Roberts, M. Locksley, and C. Stockinger. 2006. TGFbeta in the context of an inflammatory cytokine milieu supports differentiation of IL-17-producing T helper cells. Nature 441: 231–234.

23. Bettelli, E., M. Oukka, and V. K. Kuchroo. 2007. Th(17)-cell deficiencies in the circle of immunity and autoimmunity. Nat. Immunol. 8: 345–350.

24. Oghara, H., M. Murakami, Y. Okumama, M. Tsuura, K. Katayoshi, K. Kanamoto, M. Nishihara, Y. Iwasaki, and T. Hirano. 2008. Interleukin-17 promotes autoimmune by triggering a positive-feedback loop via interleukin-6 induction. Immunity 29: 628–635.

25. Wei, L., A. Laurence, K. M. Elias, and J. J. O'Shea. 2007. IL-21 is produced by T cells in the context of an inflammatory cytokine milieu and supports de novo differentiation of IL-17-producing T cells. Immunity 24: 179–189.

26. Mangan, P. R., L. E. Harrington, D. B. O’Quinn, W. S. Helms, D. C. Bullard, C. O. Elson, R. D. Hatton, S. M. Wahl, T. R. Schoeb, and T. C. Weaver. 2006. Transforming growth factor-beta induces development of the Th(17) lineage. Nature 441: 231–234.

27. Torelli, J. M., B. A. Martin, A. P. Martin, and J. M. Blander. 2009. Intraoesophageal infection-accelerated atherosclerosis in mice. J. Immunol. 182: 4368–4379.

28. Thampi, P., B. W. Stewart, L. Joseph, S. B. Melnyk, L. J. Henings, and S. Nagarajan. 2008. Dietary homocysteine promotes atherosclerosis in apoE-deficient mice by inducing scavenger receptor expression. Atherosclerosis 197: 620–629.

29. Lutz, M. B., N. Kukathas, A. L. Ogilvie, S. Röösner, F. Koch, N. Roman, and G. P. Hunter. 1999. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. J. Immunol. Methods 223: 77–92.

30. Marim, F. M., T. S. Lima, D. S. Lima, Jr., and D. S. Zamboni. 2010. A method for generation of bone marrow-derived macrophages from cryopreserved mouse bone marrow cells. PLoS ONE 5: e15263.

31. Devaraj, S., T. W. Du Clos, and I. Jialal. 2005. Binding and internalization of C-reactive protein by Fgemma receptors on human aortic endothelial cells increases cellular biological effects of C-reactive protein. Atherosclerosis 179: 367–374.

32. Wu, J., M. J. Stevenson, J. M. Brown, E. A. Grunz, T. L. Strawn, and W. P. Fay. 2008. C-reactive protein enhances tissue factor expression by vascular smooth muscle cells: mechanisms and in vivo significance. Arterioscler. Thromb. Vasc. Biol. 28: 698–704.

33. Szabo, S. J., S. T. Kim, G. L. Costa, X. Zhang, C. G. Fathman, and L. H. Gimlicher. 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell 100: 655–669.

34. Zheng, W., and R. A. Flavell. 1997. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. Cell 89: 587–596.

35. Tsimikas, S., E. S. Brilakis, R. J. Lennon, E. R. Miller, J. L. Witztum, J. P. McConnell, K. S. Kornman, and P. B. Berger. 2007. Relationship of IgG and IgM autoantibodies to oxidized low density lipoprotein with coronary artery disease and cardiovascular events. J. Lipid Res. 48: 425–433.
antagonism and plasticity of regulatory and inflammatory T cell programs. Immunity 29: 44–56.
54. Xu, L., A. Kitani, I. Fuss, and W. Strober. 2007. Cutting edge: regulatory T cells induce CD4+CD25-Foxp3- T cells or are self-induced to become Th17 cells in the absence of exogenous TGF-beta. J. Immunol. 178: 6725–6729.
55. de Boer, O. J., J. J. van der Meer, P. Teeling, C. M. van der Loos, and A. C. van der Wal. 2007. Low numbers of FOXP3 positive regulatory T cells are present in all developmental stages of human atherosclerotic lesions. PLoS ONE 2: e779.
56. Cheng, X., X. Yu, Y. J. Ding, Q. Q. Fu, J. J. Xie, T. T. Tang, R. Yao, Y. Chen, and Y. H. Liao. 2008. The Th17/Treg imbalance in patients with acute coronary syndrome. Clin. Immunol. 127: 89–97.
57. de Boer, O. J., J. J. van der Meer, P. Teeling, C. M. van der Loos, M. M. Idu, F. van Maldegem, J. Aten, and A. C. van der Wal. 2010. Differential expression of interleukin-17 family cytokines in intact and complicated human atherosclerotic plaques. J. Pathol. 220: 499–508.
58. Pinderski Oslund, L. J., C. C. Hedrick, T. Olvera, A. Hagenbaugh, M. Territo, J. A. Berliner, and A. I. Fyfe. 1999. Interleukin-10 blocks atherosclerotic events in vitro and in vivo. Arterioscler. Thromb. Vasc. Biol. 19: 2847–2853.
59. Robertson, A. K., M. Rudling, X. Zhou, L. Gorelik, R. A. Flavell, and G. K. Hansson. 2003. Disruption of TGF-beta signaling in T cells accelerates atherosclerosis. J. Clin. Invest. 112: 1342–1350.
60. Frutkin, A. D., G. Otsuka, A. Stempnien-Otero, C. Sesti, L. Du, M. Jaffe, H. L. Dichek, C. J. Pennington, D. R. Edwards, M. Nieves-Cintorin, et al. 2009. TGF-[beta] limits plaque growth, stabilizes plaque structure, and prevents aortic dilation in apolipoprotein E-null mice. Arterioscler. Thromb. Vasc. Biol. 29: 1251–1257.