SUPPLEMENTAL MATERIAL

Supplemental Methods

Serum Cholesterol Analysis

Blood from individual hypercholesterolemic and control mice was collected by retro-orbital venous plexus sampling after 4, 8 and 20 weeks of diet. Serum fractions were micro-centrifuged and stored at -20°C until the assay was performed. Total serum cholesterol levels were analyzed by Cholesterol Rapid Liquid Reagent (Raichem).

Aortic atherosclerotic lesion quantification

Analysis of the aortic sinuses was performed to quantify atherosclerotic lesion formation, as described [1]. Briefly, alternate cryosections (7 μm thick) throughout the aortic sinus (total ~ 200 μm) were cut, air-dried for 30 min and fixed in 10% Buffered Formalin for 10 min at RT. From the area in which three aortic valve cusps are clearly seen, alternate sections were stained with Oil Red O (ORO) as described [2]. The slides were rinsed in distilled water for 5 min, place in 100% Propylene Glycol for 2 min to eliminate water and stained with Oil Red-O solution for 1 hour at RT. Samples then were differentiated in fresh 85% Propylene Glycol in distilled water for 1 min and stained with Hematoxylin for 2 min to stain the nuclei. Coverslips were mounted using pre-warmed glycerol/gelatin mounting medium. Section images were captured digitally (SV Micro, Zeiss, Germany) and quantified using IMAGEPRO PLUS software (Media Cybernetics). Plaque lesion was quantified and the results were expressed as the average of 6 sections per mouse. All the measurements and evaluation of the atherosclerotic lesions were done in a blinded fashion. The intra-observer and inter-observer variation of the specimens was less than 2%.

Immunohistochemistry of aortic lesions

Serial longitudinal cryostat sections of aortic-sinus adjacent to the ORO-stained sections were stained by standard immunohistochemical technique, using acetone fixation, as described [3], with mouse-specific antibodies against: Rat anti-mouse CD4 clone RM4-5 (Pharmingen), monoclonal mouse anti-actin smooth muscle-alkaline phosphatase clone IA4 (Sigma) and anti-mouse F4/80 clone BM8 for macrophages (Biolegend). Quantitative analysis of SMC was determined by computer-assisted image analysis [4] and expressed as percentage of intimal area to normalize for overall differences between the study groups. Quantification of macrophages was also determined by computer-assisted image analysis and expressed as addition of positive areas. Quantification of $CD4^+$ staining was performed by counting individual positively stained lesional cells because the total area stained by this antibody is too small to permit meaningful computer-assisted image analysis.

Flow cytometry

Three color flow cytometry was performed by standard protocol. Briefly, 0.5–1x10^6 cells from spleens or peripheral blood were incubated in 150 µl staining buffer (PBS with 1% BSA) and Fcγ III/II Receptor blocking antibody (2.4G2, BD Pharmingen) for 10 min. The cells were then stained with mouse-specific antibodies CD4-PE clone RM4-5, CD3-APC clone 145-2C11 (BD Bioscience) and CD62L clone MEL-14 (Biolegend), and purified Rat anti-mouse antibodies GITR clone YGITR765 (BioLegend), PSGL1 clone 2PH-1 (Pharmingen) and LFA-1 clone M17/4 (Biolegend) detected with secondary goat anti rat antibody Alexa-647 (Invitrogen). Mouse P selectin and E selectin Fc chimeras

(R&D Systems, Minneapolis, MN) were detected with secondary antibody Human-Fc-PE (Invitrogen). The staining was combined with endogenous Foxp3-eGFP for 20 min, washed twice and fixed in PBS with 1% paraformaldehyde. The analysis was performed on a FACS caliber flow cytometer (BD Biosciences) with CellQuest software (BD Biosciences). All procedures were performed on ice until analysis. The fraction of positive-staining cells was determined relative to isotype-control stained cells. The different surface markers were analyzed gating on the $CD4^+GFP^+$ population.

Flow cytometric analysis of immune cells within murine aorta

Mice were perfused via the left ventricle with ice-cold PBS+Heparin (2000U/ml). Aorta were harvested and 2-4 aortas were pooled and digested with 125 U/ml collagenase type XI, 600 U/ml Hyaluronidase type I-s, 60 U/ml DNAse1 and 450 U/ml collagenase type I (all enzymes were from Sigma, St Louis, Mo) in PBS containing 20 mmol/l HEPES at 37°C for 1 hour [5]. The aorta was then mashed through a 70-μm strainer and the cell suspension was incubated in FACS buffer (PBS with 1% BSA) for Foxp3-GFP^+ cells analysis. Apoptotic cells were stained with Annexin V conjugate (Molecular Probes) diluted in annexin-binding buffer (10 mM HEPES, 140 mM NaCl and 2.5 mM $CaCl_2$, pH 7.4), permeabilized with BD Perm/Warm Buffer (Fetal Bovine Serum and Saponin) and intracellular stained with rat anti mouse antibodies CD4-Percp clone RM4-5 and Foxp3-APC clone FJK-16s (Pharmingen). The analysis was performed on a FACS caliber flow cytometer (BD Biosciences) with CellQuest software (BD Biosciences).

Mouse aorta isolation and ex vivo adhesion assay

Assay was performed as described [6]. Aortas were isolated from 8 weeks old wild-type mice, and incubated for 6 h in RPMI-1640 media containing 10% FBS with or without 20ng/ml of mouse IL1β (R&D Systems) and 100ng/ml mouse TNFα (Peprotech) at 37°C. Next, aortas were opened longitudinally and pinned to sterile agar. Splenic $CD4^+$ T cells were isolated from Foxp3-eGFP/*Ldlr*$^{-/-}$ mice by anti-CD4 magnetic beads (Miltenyi Biotec, Auburn, Calif). GFP^+ Treg were isolated from $CD4^+$ cells by green-fluorescent using high speed FACS and then were labeled with Hoechst33342 for 15 min at 37°C (Invitrogen). Treg were incubated with the pinned aortas at 37° C, and 45 min later, unbound cells were washed away with PBS. Treg adherent to the aorta were scanned with Olympus spinning disk microscopy using 10x objective and UV laser. The analysis was performed with Methamorph software taking consecutive images of the whole aorta. Images were deconvoluted to remove noise and the total number of Treg bound to the aorta was counted.

Regulatory T cells binding to E selectin under flow conditions

Interactions of splenic Foxp3-GFP^+ Treg from mice, fed a cholesterol diet for 4 and 8 weeks, with recombinant mouse E-selectin Fc chimera-coated coverslips (R&D Systems, Minneapolis, MN) was examined under defined laminar flow conditions in a parallel plate flow chamber as described previously [7,8]. T cells are resuspended in Dulbecco's phosphate-buffered saline containing 0.1% (v/v) BSA and 20 mM HEPES, pH 7.4, at 37°C (5 x10^5 cells/ml) and perfused over the coated coverslips. T cell interactions are recorded with a phase contrast objective (20x) and a video microscope connected to

Videolab software (Ed Marcus Laboratories, Boston, MA) to record cell behavior (shear 0.8 and 1 dynes/cm^2). Accumulation of the cells is determined after the initial minute of each flow rate by counting cells in five different fields.

Ex vivo functional Treg assay

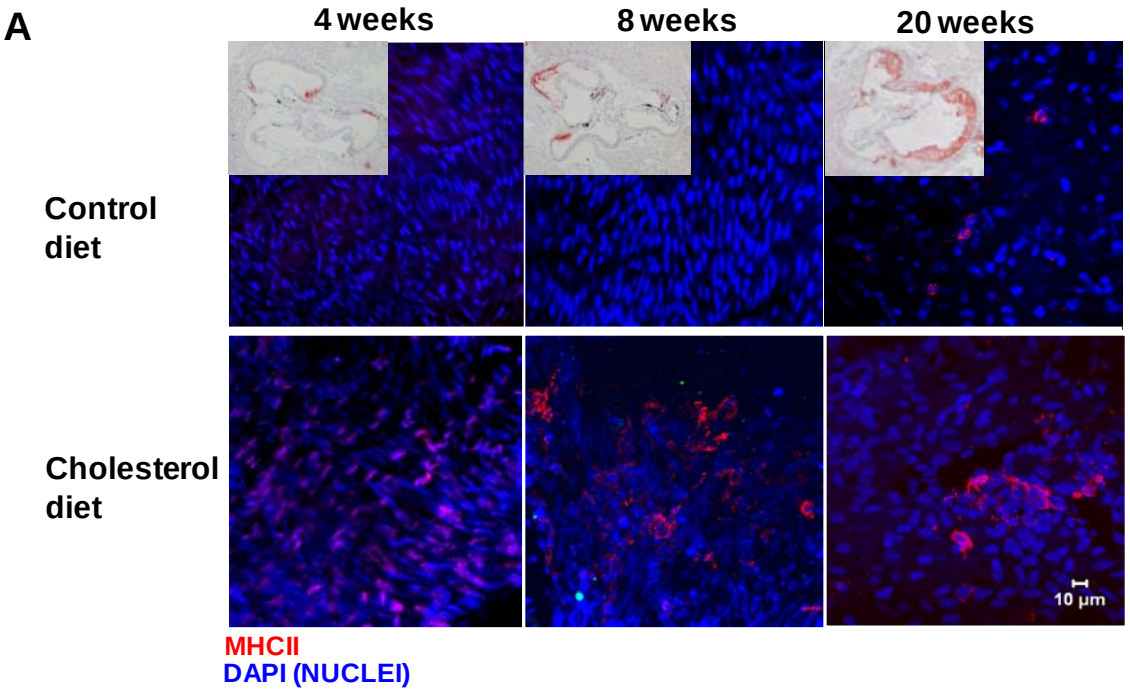
After 4 and 8 weeks of cholesterol diet, splenic CD4$^+$ T cells were isolated from Foxp3-eGFP/*Ldlr*$^{-/-}$ mice by anti-CD4 microbeads (Miltenyi Biotec, Auburn, Calif). GFP$^+$ Treg were isolated from CD4$^+$ cells by green-fluorescent using high speed FACS, available as a core service in our institution. Splenic CD4$^+$CD25$^-$ (responder) cells were purified from wild-type C57Bl/6 mice using mouse anti-C25-PE and anti-CD4 microbeads (Miltenyi Biotec, Auburn, Calif). CD4$^+$CD25$^-$ responder cells (2 x 10^4/well) were cultured in 96-well plates (0.25 ml) with 1μg/ml soluble anti-CD3ε ((BD Bioscience), APCs from whole spleen cells mitomycin C treated of C57BL/6 wild-type mice (10^5/well) and CD4$^+$Foxp3-GFP$^+$ suppressor cells (2 x 10^4/well ;responder to suppressor ratio of 1:1) for 72 h. For the suppression assay with DCs the following we used: CD4$^+$CD25$^-$ responder cells (10^5/well), DCs isolated from spleens of hypercholesterolemic mice (5x10^4/well) and CD4$^+$Foxp3-GFP$^+$ suppressor cells (5x10^4/well-responder to suppressor ratio of 1:1). Cultures were pulsed with ^{3}[H]-thymidine (1μCi/well) for the last 16 h of culture. Data is expressed as mean proliferation indices of triplicates calculated from the ratios of incorporated radioactive counts per minute in the presence or absence of anti-CD3.

Immunofluorescence and confocal microscopy

Mice were perfused via the left ventricle with ice-cold PBS+Heparin (2000U/ml). Aortic arch was harvested, opened in a highly reproducible manner and fix in 4% paraformaldehyde overnight at 4°C [9]. Arch was permeabilized with 0.1% Triton X-100 in PBS for 10 min at 22°C and blocked with 5% Goat serum in PBS, corresponding to the specie of the secondary antibody, for 1 h at 22ºC. Primary antibodies were incubated overnight at 4°C (rat anti-mouse CD4 clone RM4-5 or rat anti-mouse I^A/I^E clone 2G9 from Invitrogen), followed by secondary antibody goat anti-rat Alexa-555 (Invitrogen) for 45 min at 22°C. The arch was washed and mounted with mounting medium with DAPI (Vectashield, Vector Laboratories) which is a nuclei marker (blue color). En face immunofluorescence images were obtained with confocal microscope LSM510 META (Zeiss) using 40x oil-immersion objective. Images were acquired and analyzed with LSM Image Browser software. Number of Regulatory T cells Foxp3-GFP^+ per field was calculated counting 6 different fields per mouse and was related to the total number of $CD4^+$ cells.

Supplemental Figures

Figure S1.



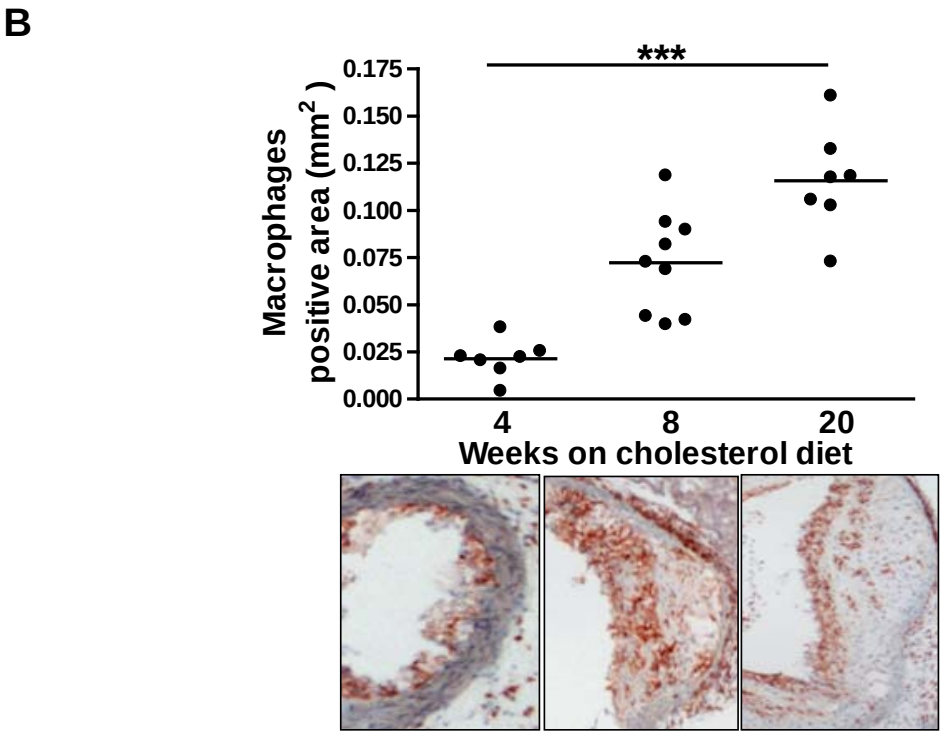


Figure S2.

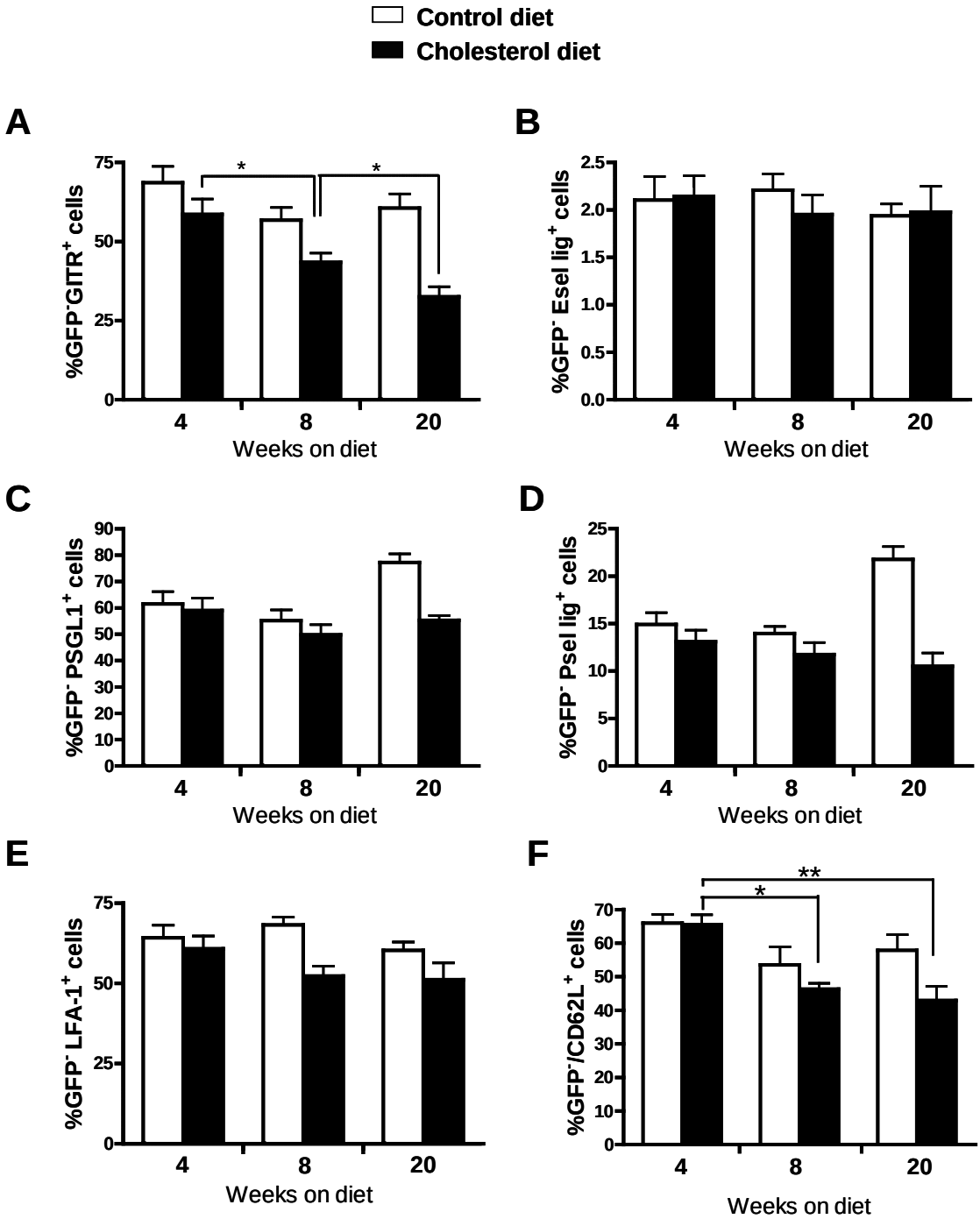

Control diet
Cholesterol diet
A
%GFP-GITR+ cells
Weeks on diet
B
%GFP- Esel lig+ cells
Weeks on diet
C
%GFP- PSGL1+ cells
Weeks on diet
D
%GFP- Psel lig+ cells
Weeks on diet
E
%GFP- LFA-1+ cells
Weeks on diet
F
%GFP-/CD62L+ cells
Weeks on diet

Figure S3.

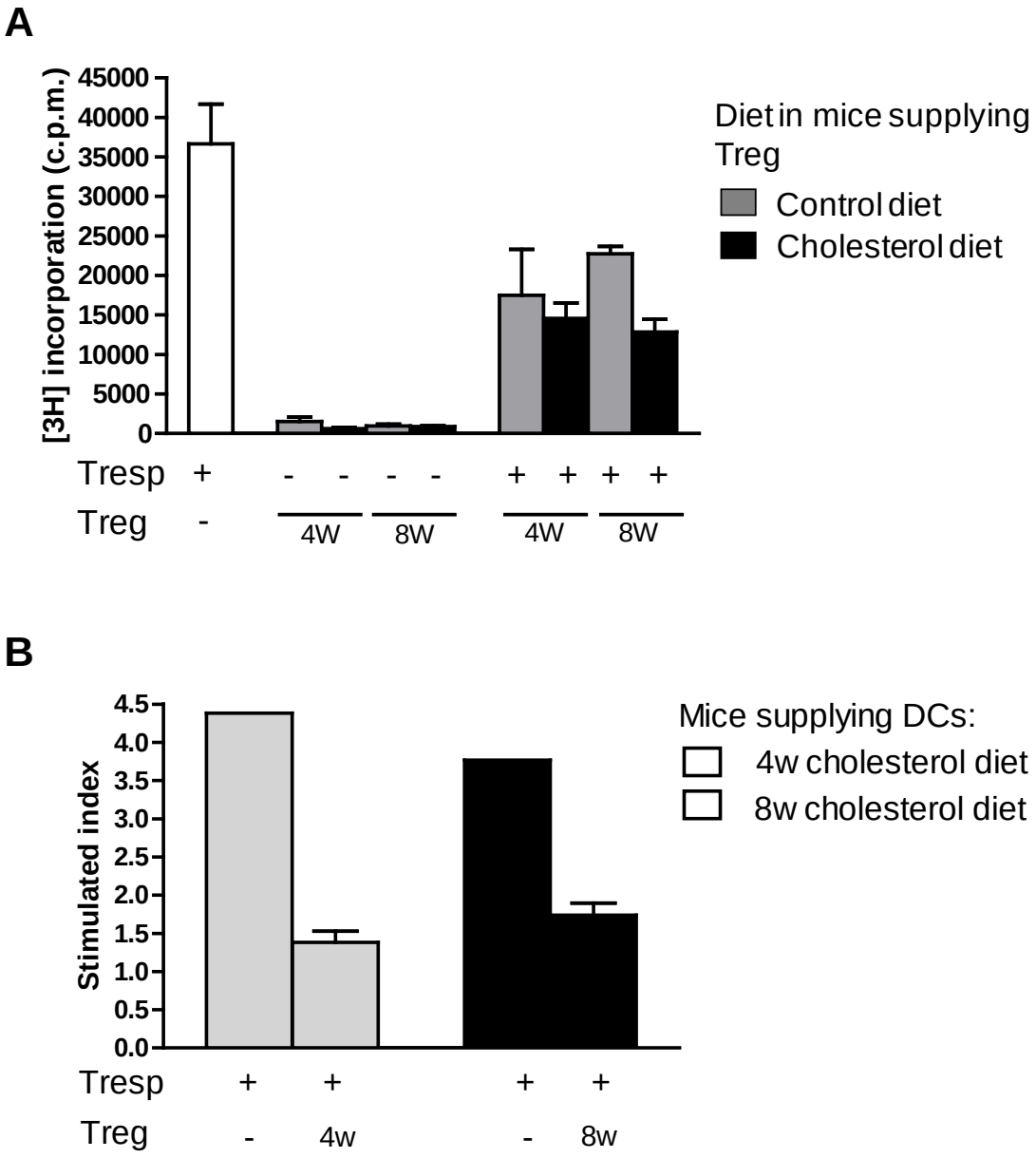

A
[3H] incorporation (c.p.m.)
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
Tresp + - - - - + + + +
Treg - 4W 8W 4W 8W
Diet in mice supplying Treg
Control diet
Cholesterol diet
B
Stimulated index
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Tresp + + + +
Treg - 4w - 8w
Mice supplying DCs:
4w cholesterol diet
8w cholesterol diet

Figure S4.

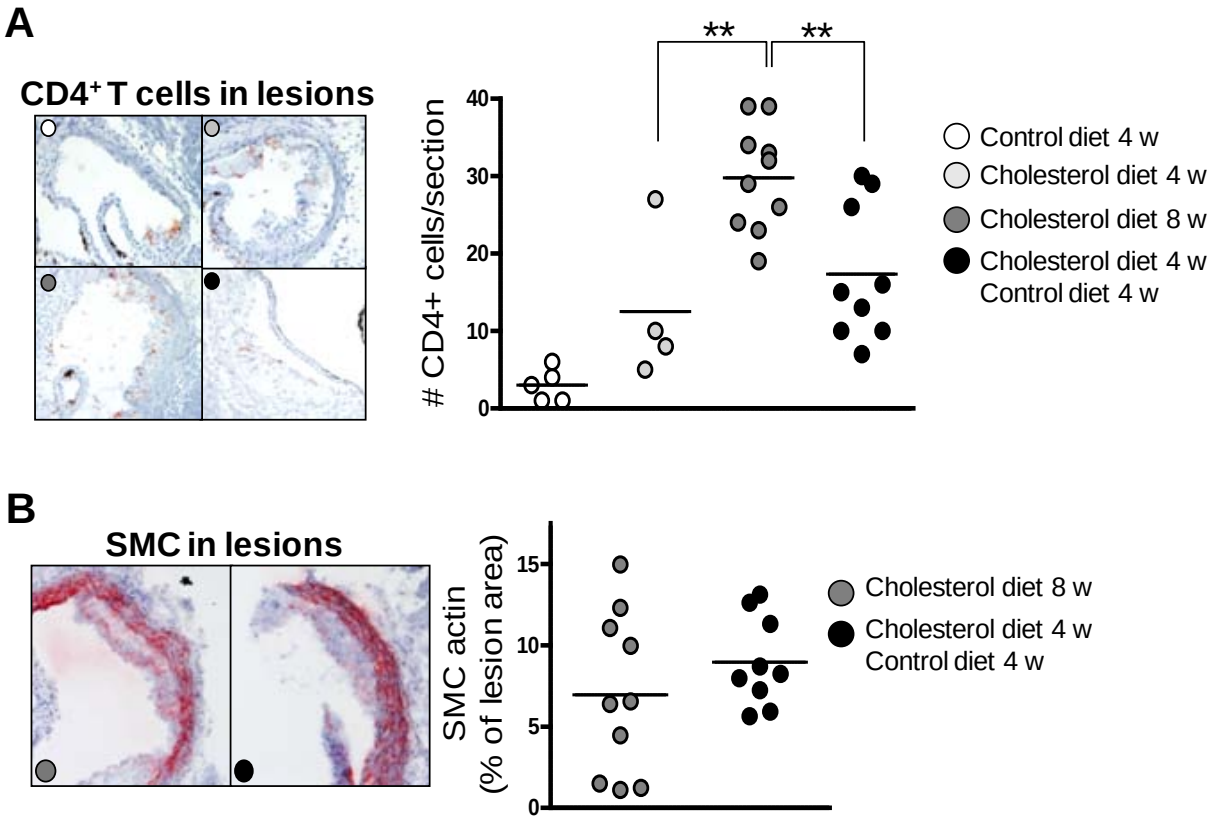
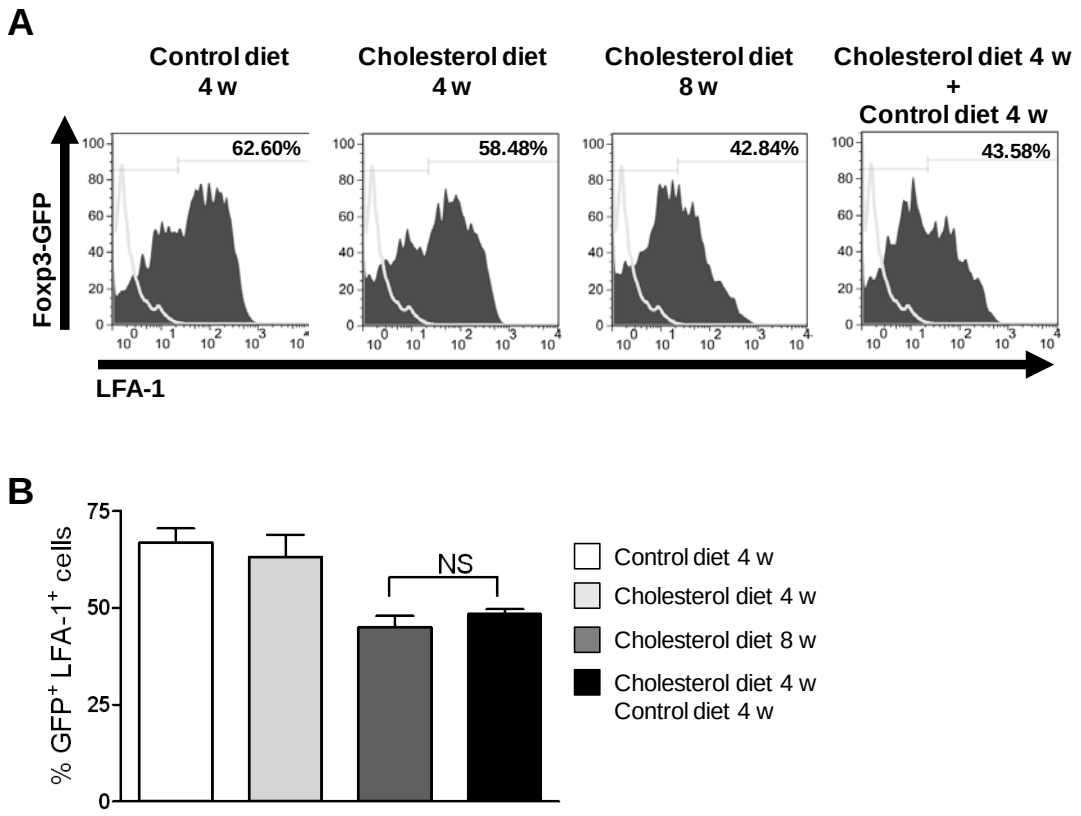

A
CD4+ T cells in lesions
CD4+ cells/section
40
30
20
10
0
**
**
Control diet 4 w
Cholesterol diet 4 w
Cholesterol diet 8 w
Cholesterol diet 4 w
Control diet 4 w
B
SMC in lesions
SMC actin
(% of lesion area)
15
10
5
0
Cholesterol diet 8 w
Cholesterol diet 4 w
Control diet 4 w

Figure S5.



Supplemental figure legends

Figure S1. Class II MHC^{+} cells migrate to the lesion in early stages and form foam cells in advance stages of atherosclerosis. Foxp3-eGFP^{+}/*Ldlr*$^{-/-}$ mice were fed a control or cholesterol rich diet for 4, 8 and 20 weeks; these were the same mice as described in Figure 2A-C. Aortic arches were harvested and en face preparations were stained with DAPI (nuclei dye-blue) and α-I^{A}/I^{E}-PE (red) and analyzed by confocal microscopy. These images are a projection of 15-30 z-stacks (20-30 μm). Small insets are pictures of aortic sinus from mice fed a cholesterol diet for 4, 8 and 20 weeks (A). Frozen sections of aortic sinuses from hypercholesterolemic were stained for macrophages (F4/80 antibody)

in the lesion. Data represent mean +/- S.E.M., N=7-9 mice per group. Each symbol represents one mouse. *** P<0.001, analyzed by one way ANOVA with Tukey's post test (B).

Figure S2. Hypercholesterolemia does not cause changes in functional phenotype of Foxp3-eGFP$^-$ cells. Total splenic cells from the same Foxp3-eGFP$^+$/*Ldlr*$^{-/-}$ mice described in Figure 3 were isolated after 4, 8 and 20 weeks of cholesterol or control diet, stained with antibodies against CD4, GITR, PSGL1, LFA-1 and CD62L or with P-selectin and E-selectin IgG chimeras, and stained cells were analyzed by flow cytometry gating on the CD4$^+$ GFP$^-$ cells (A_F). Data represent mean +/- S.E.M, N=6-9 mice per group, analyzed by two way ANOVA with Bonferroni's post test * P <0.05.

Figure S3. Regulatory T cells from hypercholesterolemic mice have the capacity to suppress T cell proliferation in vitro. Responder T cells (CD4$^+$CD25$^-$) were cultured with splenic APCs (A) or DCs (B) and cultured in 96-well plates with suppressor GFP$^+$ Treg (responder to suppressor ratio 1:1) in the presence of anti-CD3ε (1μg/ml) for 72 h. Treg were isolated by sorting CD4$^+$GFP$^+$ from Foxp3-eGFP$^+$/*Ldlr*$^{-/-}$ mice fed a cholesterol diet for 4 and 8 weeks; these were the same mice described in Figure 2A. ^{3}[H]-thymidine was added during the last 16 h of culture and CPMs were measured to follow proliferation.

Figure S4. Reversal from cholesterol to control diet after 4 weeks reduced further accumulation of CD4$^+$ T cells in aortic sinus lesions. Foxp3-eGFP$^+$/*Ldlr*$^{-/-}$ mice were fed control or cholesterol diet for 4 or 8 weeks, or cholesterol diet for 4 weeks and then

control diet for 4 weeks; these were the same mice described in Figures 5 and 6. Frozen sections of aortic sinus were stained for CD4 (A) or SMC α-actin (B). Data represent mean +/- S.E.M., N=5-10 mice per group, ** P<0.01, analyzed by one way ANOVA with Tukey's post test (A) or by Students *t* test (B). Each symbol represents one mouse.

Figure S5. Reversal from cholesterol to control diet after 4 weeks does prevent reduction in Treg LFA-1 expression. Foxp3-eGFP^{+}/*Ldlr*$^{-/-}$ mice were continuously fed control or cholesterol diet for 4 or 8 weeks, or cholesterol diet for 4 weeks and then control diet for 4 weeks; these were the same mice described in Figures 5 and 6. Total splenic cells were stained with CD4 and LFA-1 and analyzed by flow cytometry gating on the CD4^{+}GFP^{+} population. Histograms correspond to one representative mouse (A). Data represent mean +/- S.E.M, N= 5-9 mice per group, analyzed by one way ANOVA with Tukey's post test (B).

Supplement References

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