Dynamic Changes in Regulatory T Cells Are Linked to Levels of Diet-Induced Hypercholesterolemia

Elena Maganto-García, PhD; Margarite L. Tarrio; Nir Grabie, PhD; De-xiu Bu, MD, PhD; Andrew H. Lichtman, MD, PhD

**Background**—Regulatory T cells (Treg) are present in atherosclerotic lesions and can modulate disease. In this study we characterized changes in Treg responses associated with prolonged hypercholesterolemia and lesion progression.

**Methods and Results**—Low-density lipoprotein receptor null mice in which Treg express green fluorescent protein were fed a control or cholesterol-rich diet, and green fluorescent protein–positive cells were enumerated in lymphoid tissues and in aorta. Splenic Treg numbers increased after 4, 8, and 20 weeks in cholesterol-diet–fed mice. However, the number of circulating and lesional Treg peaked at 4 weeks and decreased significantly at 8 and 20 weeks, concomitant with increased numbers of CD4+ effector T cells and increased lesion size over this period. Treg expression of selectin ligands and their ability to bind to aortic endothelium decreased after prolonged hypercholesterolemia, and apoptosis of lesional Treg increased. After 4 weeks of cholesterol-rich diet, a switch to a control diet for 4 weeks reduced serum cholesterol and stopped lesion growth, and the high aortic Treg content was maintained, compared with mice fed a cholesterol diet for 8 weeks. After the diet reversal, the splenic Treg retained the phenotype of Treg after 4 weeks of cholesterol diet.

**Conclusions**—Prolonged hypercholesterolemia impairs Treg but not effector T cell accumulation in lesions, but reversal of hypercholesterolemia can prevent loss of lesional Treg. Therefore, cholesterol-lowering therapies may induce dynamic and beneficial changes in Treg:effector T cell ratios in atherosclerotic lesions. (Circulation. 2011;124:185-195.)

**Key Words:** atherosclerosis ■ diet ■ hypercholesterolemia ■ T cells ■ regulatory T cells

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**Clinical Perspective on p 195**

Regulatory T cells (Treg) are essential for regulating T cell immune responses to prevent autoimmunity and possibly to prevent excessive responses to microbial pathogens.11 Regulatory T cells can be identified by cell surface markers (CD3+CD4+CD25highCD127lo), as well as expression of glucocorticoid-induced tumor necrosis factor receptor (GITR) and cytotoxic T-lymphocyte antigen 4.12 The stable intracellular expression of the Foxp3 transcription factor is a defining feature of Treg.13 The influence of Treg on atherosclerosis is of interest because these cells can block Th1 differentiation and effector function and are of potential therapeutic importance.14 Regulatory T cells may influence proatherogenic T cell responses in the lymphoid tissues and/or human and mouse atherosclerotic lesions,15–17 modulating the local proinflammatory processes. For example, reduced or functionally impaired Treg leads to increased atherosclerosis,15,18 and adoptive transfer of Treg into hypercholesterolemic mice reduces lesion development.19,20

Immunotherapy of atherosclerotic disease might be directed toward increasing Treg suppression of proatherogenic cell responses.19–21 Because the ratio of regulatory to effector T cells (Teff) is critical in determining outcomes of T cell responses,22–25 we reasoned that therapeutic interventions aimed at increasing Treg suppression of proatherogenic T cell responses would require induced changes in Treg numbers.
within the arterial wall. In this study, we addressed the question of whether or not numbers of Treg within atherosclerotic aortas are dynamically regulated. We found that numbers of Treg and the Treg:Teff ratio within the atherosclerotic aorta progressively decreased during prolonged hypercholesterolemia, but the hypercholesterolemia-induced lesional Treg response could be maintained by dietary reversal of hypercholesterolemia.

**Methods**

**Mice**

Foxp3−enhanced green fluorescent protein (eGFP) knock-in mice on a C57Bl/6 background,26 were crossed with Ldlr−/− mice on a C57Bl/6 background (Jackson Laboratories) to obtain Foxp3-eGFP+/Ldlr−/− mice. Regulatory T cells in the Foxp3-eGFP mouse have functional Foxp3 and are identifiable by green fluorescence.26 Female and male Foxp3-eGFP/Ldlr−/− mice (all 8 weeks old at the start of experiments) were assigned to different groups in each experiment, and each group was fed a control or cholesterol-rich diet27 for different time periods, depending on the experiments. A total of 208 mice were used in this study. Exact numbers of mice per experimental group are indicated in the Figure legends. All mice were housed and bred in a pathogen-free facility at the New Research Building (Harvard Medical School, Boston, MA) in accordance with the guidelines of the Committee of Institutional Animal Care and the National Animal Research Guidelines. Additional Methods can be found in the online-only Data Supplement.

**Serum Cholesterol Analysis**

Blood from individual mice was collected by retro-orbital venous plexus sampling after 4, 8, and 20 weeks of diet, serum samples were prepared, and cholesterol was analyzed (see the online-only Data Supplement).

**Aortic Atherosclerotic Lesion Quantification**

Atherosclerotic lesion formation in aortic sinuses was analyzed in Oil Red O (ORO) stained cryosections as described previously.15,17 See the online-only Data Supplement for a detailed description of the methods of lesion analyses.

**Immunohistochemistry of Aortic Lesions**

Serial fixed cryostat sections of aortic sinus adjacent to the ORO-stained sections were stained by standard immunohistochemical techniques, as described,28 with antibodies specific for CD4, smooth muscle cell (SMC) actin and F4/80. Quantification of SMC and macrophage staining was performed by digital image analysis4 and expressed as percentage of intimal area. See the online-only Data Supplement for a detailed description of antibodies and immunohistochemical techniques.

**Flow Cytometry**

Multicolor flow cytometry was performed by standard protocols as described17 to quantify Treg and Teff cells in spleen, lymph nodes, and collagenase digests of aortic walls from mice after various dietary regimens and to characterize expression of adhesion molecules, chemokine receptors, activation molecules, and apoptosis markers on Treg and Teff cells. See the online-only Data Supplement for a detailed description of cell preparations, antibodies, and flow cytometric analyses.

**Immunofluorescence and Confocal Microscopy**

Lesional GFP+ Treg were counted in confocal microscopic images of en face preparations of the aortic arches from Foxp3-eGFP+/Ldlr−/− mice prepared as described,29 stained with primary antibodies specific for CD4 or class II major histocompatibility complex (IγM),30 followed by Alexa-555-labeled secondary antibody and nuclear DAPI stain. Staining was analyzed by confocal microscopy.

See the online-only Data Supplement for a detailed description of the tissue preparation and confocal microscopic analyses.

**Mouse Aorta Isolation and Ex Vivo Adhesion Assay**

The ability of fluorescently-labeled Treg from hypercholesterolemic mice to adhere to the luminal surface of interleukin1β plus tumor necrosis factor α-treated mouse aortas ex vivo was tested as described.30 Adherent fluorescently labeled Treg were detected by confocal microscopy. See the online-only Data Supplement for a detailed description of this ex vivo adhesion assay.

**Regulatory T Cells Binding to E-Selectin Under Flow Conditions**

Binding of splenic Foxp3-eGFP+ Treg from cholesterol-fed mice to recombinant mouse E-selectin was examined under laminar flow conditions in a flow chamber as described.31,32 See the online-only Data Supplement for a detailed description of the in vitro flow chamber assays.

**Statistical Analysis**

For practical reasons, some experiments with multiple mice per group had to be performed in a 2-part staggered fashion, in order to be able to complete all the technical aspects in a way that did not vary from mouse to mouse. When conducting each of the 2 chronologically different parts, all the different groups of mice were included in each part, with equal numbers in each group. Statistical analyses included the Student t test for experiments with 2 groups, 1-way ANOVA for experiments that included cholesterol diet for 3 different durations, and 2-way ANOVA for experiments that included 2 diets (control and cholesterol) for different durations (4, 8, and 20 weeks). Multiple pairwise comparisons were handled by the Tukey multiple comparison post test or Bonferroni post test. A value of P<0.05 was considered to be significant.

**Results**

**Hypercholesterolemia Increases the Number of Splenic Regulatory T Cells in Foxp3-eGFP+/Ldlr−/− Mice**

Foxp3-eGFP+/Ldlr−/− mice were fed a control or a cholesterol-rich diet, the latter causing a progressive significant increase of total cholesterol levels in serum between 4 and 20 weeks (Figure 1A). Aortic root atherosclerotic lesions progressively increased in cholesterol diet–fed mice after 4, 8, and 20 weeks (Figure 1B) whereas mice fed control diet had no significant lesions. To determine if hypercholesterolemia had a systemic effect on Treg, we quantified CD4+GFP+ T cells in the spleens of these mice. We observed a progressive increase in the percentage of CD4+ cells that expressed GFP over the 20 weeks of cholesterol-diet feeding and a smaller increase over time in control diet–fed mice whereas the percentage of total CD4+ T cells was not different between the 2 groups of mice at all time points (Figure 1C). The small increase in percentage of splenic Treg in control-diet–fed Ldlr−/− mice likely reflected the mild hypercholesterolemia in these mice (see Figure 1A) because we did not see any increase in splenic Treg in Ldlr−/+ Foxp3-eGFP+ mice fed control diet at 4, 8, and 20 weeks (data not shown). The percentage of CD4+ cells that expressed GFP in blood decreased slightly between 4 and 8 weeks of hypercholesterolemia (Figure 1D) whereas the percentage of blood T cells expressing CD4 did not change (Figure 1E).
Treg Numbers Transiently Increase in the Atherosclerotic Aortas and Then Decrease During Sustained Hypercholesterolemia and Lesion Growth

We examined Treg accumulation in aortas of Foxp3-eGFP/H11001/Ldlr/H11002 mice using 2 different methods. First, we used confocal microscopy of en face samples of the atherosclerosis-prone area of the lesser curvature, as described.29 Prior studies have indicated that the retention of lipoproteins and cholesterol in the arterial intima induces the initial monocyte and dendritic cell homing to the aorta,33 and this process amplifies further leukocyte recruitment during early atherogenesis.27,34 In order to confirm that we were counting Treg in the location of the lesion development and inflammation in the aortic arch, we stained identically prepared samples for

Figure 1. Splenic and circulating Treg numbers change under prolonged hypercholesterolemia. A, Foxp3-eGFP/Ldlr−/− mice (total 63) were fed a control or cholesterol-rich diet and euthanized after 4, 8, and 20 weeks on diet. Total cholesterol levels were measured in serum. B, Aortic root atherosclerotic lesions were quantified by analysis of ORO stained sections. A representative section from each group is also shown. C, Splenocytes were stained for CD4 and the percentage of CD4+ cells that were GFP+ cells was determined by FACS. Horizontal bars and column heights represent the mean for each group. Error bars represent SEM; n=6 to 10 mice per group. Each symbol in A and B represents 1 mouse. *P<0.05, **P<0.01, ***P<0.001, analyzed by 2-way ANOVA with Bonferroni post test (A) or 1-way ANOVA with Tukey post test (B). In a separate experiment, Foxp3-eGFP/Ldlr−/− mice (14 total) were fed a cholesterol-rich diet and euthanized after 4 and 8 weeks, and blood leukocytes were stained for CD4 and analyzed by FACS. D, The percentage of CD4+ cells that were GFP+ is shown. E, The percentage of total lymphocytes by scatter that were CD4+ is shown. Data are mean±SEM; n=6 to 8 mice per group. *P<0.05, analyzed by Student t test. Each symbol in D and E represents 1 mouse. GFP indicates green fluorescent protein.

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class II major histocompatibility complex class II (I\(^{A}/I^{B}\)), which is expressed on macrophages, dendritic cells, endothelial cells, and SMCs in response to interferon \(\gamma\). Consistent with previous studies, after 4 weeks of hypercholesterolemia, we detected I\(^{A}/I^{E+}\) cells in these regions after 8 and 20 weeks of hypercholesterolemia (online-only Data Supplement Figure 1A). In control diet–fed mice, we also observed a small number of I\(^{A}/I^{E+}\) cells in the aortic arch after 20 weeks, likely because these mice are mildly hypercholesterolemic and do develop early lesions by 20 weeks (see insets showing ORO staining of the aortic sinus, online-only Data Supplement Figure 1A). Moreover, we verified the accumulation of F4/80+ macrophages in these samples. This accumulation increased when mice were fed a high cholesterol diet for 20 weeks (online-only Data Supplement Figure 1B).

We quantified GFP\(^+\) Treg, from aortic arches of Foxp3-eGFP\(^+/Ldlr^{−/−}\) mice fed a cholesterol diet for 4, 8, and 20 weeks. The arches were stained with anti-CD4 and DAPI (nuclei) and were examined by confocal microscopy (Figure 2A). Regulatory T cells were present in lesions after 4, 8, and 20 weeks of diet. Remarkably, the number of Treg was maximum at 4 weeks of diet and decreased successively at 8 and 20 weeks, opposite to the increase of total CD4\(^+\) cells from 4 to 20 weeks.

In order to confirm the confocal data, we performed flow cytometric analyses of cells collected by collagenase digestion of the aortic walls. This method will detect T cells throughout the aortic wall, including intimal lesions, media, and adventitial cells. Changes in adventitial lymphoid populations are reflective of atherosclerotic disease progression, and studies have demonstrated specific homing of lymphocytes to atherosclerotic aortic adventitia. Before digestion, we thoroughly removed para-aortic connective and adipose tissue, and we have confirmed by histology that there is only a thin adventitia on the outer surface (not \(>30\ \mu m\)), which is devoid of any organized lymphoid tissue. Furthermore, we have never found adventitial or para-aortic tertiary lymphoid tissues on histological sections from aortas of Ldlr\(^{−/−}\) mice fed cholesterol diet up to 20 weeks. Aortas from control or cholesterol-fed Foxp3-eGFP\(^+/Ldlr^{−/−}\) mice were harvested after 4, 8, and 20 weeks, enzymatically digested and GFP expression was analyzed in the final cell suspensions by fluorescence-activated cell-sorting (FACS). We were able to detect GFP\(^+\) Treg by this method, and again we observed an initial increase of Treg number in aortas of cholesterol diet–fed mice compared with control diet–fed mice after 4 weeks, but Treg numbers decreased at 8 and 20 weeks (Figure 2B). Immunohistochemical staining of adventitial sinuses in the same mice indicated that total lesonal CD4\(^+\) T cells increased progressively from 4 to 20 weeks (Figure 2C). We also performed digestion of aortas from different groups of Foxp3-eGFP\(^+/Ldlr^{−/−}\) mice fed cholesterol diet for 4 and 8 weeks, followed by intracellular staining for CD4 and Foxp3. Again, we observed a decrease in Treg from 4 to 8 weeks whereas the total number of CD4\(^+\)Foxp3\(^+\) T cells increased over the same time period (Figure 2D). By quantifying Foxp3\(^+\) and Foxp3 CD4\(^+\) T cells after digestion of the aortas, we established a decreasing ratio between regulatory and effector CD4\(^+\) cells (Treg:Teff) in aortas over a period of sustained hypercholesterolemia and lesion growth (Figure 2E). This result indicates that prolonged hypercholesterolemia inhibits the accumulation of Treg in atherosclerotic arteries whereas Teff accumulation continues.

In order to explain loss of Treg that had already entered the lesion during the initial 4 weeks of cholesterol diet feeding, we explored the possibility that this loss was due to increased cell death by staining aortic digests for Foxp3, CD4, and the apoptosis marker annexin V. The aortic digests were the same as those used to established decreasing Treg:Teff ratios shown in Figure 2D and E. We found that the number of annexin V\(^+\) Foxp3\(^+\) Treg was almost 3 times higher after 8 weeks of cholesterol diet than after 4 weeks (Figure 2F), indicating that prolonged hypercholesterolemia induces Treg apoptosis in atherosclerotic aortas.

**Hypercholesterolemia Induces Changes in Functional Phenotype of Foxp3-eGFP\(^+\) Treg in Ldlr\(^{−/−}\) Mice**

To address the possibility that the reduction of blood and lesional Treg over time in hypercholesterolemic mice reflected a change in migratory function, we analyzed the expression of cell surface molecules on splenic Treg, including chemokine receptors, integrins, and selectin ligands. We also analyzed expression of surface molecules related to function, including GITR, inducible costimulatory molecule, and CTLA4. Interestingly, we found that the expression of GITR, P-selectin glycoprotein ligand-1 (PSGL1), lymphocyte function–associated-1 (LFA-1), L-selectin (CD62L), and binding of P-selectin and E-selectin immunoglobulin G chimeras, all decreased significantly in CD4\(^+\)GFP\(^+\) Treg after 20 weeks of cholesterol diet compared with Treg from control diet–fed mice (Figure 3A through G). In contrast, CD4\(^+\)GFP lymphocytes (Teff) from cholesterol or control diet–fed mice had the same levels of PSGL-1, LFA-1, and binding of P/E selectin immunoglobulin G chimeras, although CD62L and GITR on the Teff did decreased over time (online-only Data Supplement Figure IIA through F). There was also a reduction in Treg expression of CCR6, inducible costimulatory molecule, and CTLA4 from 4 to 8 to 20 weeks, but these changes were comparable in Treg from control and cholesterol diet–fed mice. The expression of glycosylated CD43 or CXCR4 on Treg did not change over time and did not differ in control and hypercholesterolemic mice (data not shown).

The changes in Treg but not Teff phenotype that we observed in response to prolonged hypercholesterolemia suggested that Treg might also have a reduced ability to adhere to endothelial adhesion molecules in comparison with Teff. We analyzed the ability of Treg and Teff from Foxp3-eGFP\(^+/Ldlr^{−/−}\) mice to bind to E-selectin under physiological flow conditions in an in vitro flow chamber assay. The results showed that prolonged hypercholesterolemia reduced the ability of Treg to bind to E-selectin under conditions of shear stress (1 and 0.8 dynes/cm\(^2\)) (Figure 4A). In contrast, we did not find significant differences in binding of Teff from 4 versus 8 weeks cholesterol diet–fed mice (Figure 4B). We also tested the effect of prolonged hypercholesterolemia on the ability of Treg and Teff to bind to the endothelial lining
of intact aortas ex vivo. Aortas from C57BL/6 wild-type mice were harvested and stimulated with tumor necrosis factor and interleukin 1 to induce the expression of receptors involved in inflammation and migration. Consistent with the above findings from the flow assays, significantly less splenic Treg from Foxp3-eGFP/Ldlr−/− mice fed a cholesterol diet for 8 weeks bound to the aortas than did Treg from mice fed the diet for 4 weeks (Figure 4C). In contrast, Teff taken after 4 and 8 weeks of cholesterol diet bound equally well (Figure 4D). These results indicate that prolonged hypercholesterolemia...

Figure 2. Aortic Treg in Ldlr−/− mice decreases during prolonged hypercholesterolemia and lesion development. Foxp3-eGFP/Ldlr−/− mice (54 total) were fed a cholesterol diet for 4, 8, or 20 weeks, aortic arches were harvested, and en face preparations were stained with DAPI plus anti-CD4/Alexa-555 for confocal microscopic examination. A, The mean number of total CD4+ cells (white bars) and Treg (black bars) per field among 5 fields was determined in 2 mice per diet/time. Images of 2 Treg in the same field of an atherosclerotic arch, but at 2 different z-stacks, are shown. B, Descending aortas from the same mice described in A were enzymatically digested, and the GFP+ population was analyzed by FACS. Data in A and B represent mean±SEM; n=9 mice per group; *P<0.05, **P<0.01 analyzed by 1-way ANOVA with Tukey post test (A) or 2-way ANOVA with Bonferroni post test (B). C, Frozen sections of aortic sinus from the cholesterol-fed mice described in A were stained for CD4 by immunohistochemistry. Data represent mean±SEM; n=7 to 9 mice per group. Each symbol represents 1 mouse. **P<0.01, analyzed by 1-way ANOVA with Tukey post test. In a separate experiment, Foxp3-eGFP/Ldlr−/− mice (18 total) were fed a cholesterol diet for 4 or 8 weeks. Aortic digest cells were stained for CD4, Foxp3, and annexin V and analyzed by flow cytometry. D, The number of CD4+ Foxp3− (white bars) and CD4+ Foxp3+ cells (black bars) is shown. E, The CD4+ Foxp3−:CD4+ Foxp3+ ratio is shown. F, The percentage of apoptotic Treg is shown. Data represent mean±SEM, from n=9 mice per group; *P<0.05, **P<0.01, analyzed by Student t test. GFP indicates green fluorescent protein; Teff, effector T cells; and Treg, regulatory T cells.
emia significantly reduces Treg but not Teff binding to the aortic endothelium.

We also tested if hypercholesterolemia can modulate splenic Treg suppressive activity. Using a standard ex vivo Treg suppression assay in which Treg, responder T cells, and splenic antigen presenting cells are cultured with anti-CD3, we observed that Treg isolated from Foxp3-eGFP/Ldlr mice fed a cholesterol diet for 8 weeks were as effective in inhibiting responder T cell (CD4/CD25-) proliferation as Treg isolated after only 4 weeks of cholesterol diet (online-only Data Supplement Figure IIIA). The ability to suppress interferon γ secretion was also not impaired by prolonged hypercholesterolemia (data not shown). In addition, when the assay was run with DCs as the antigen presenting cells recovered from the spleen of the same animals from which CD4+GFP+ Treg were isolated, no effects of prolonged hypercholesterolemia on Treg suppression were found (online-only Data Supplement IIIB). These results support the idea that Treg remain functional in the setting of hypercholesterolemia even if their capacity to bind to aortic endothelium is impaired.

### Reversal of Hypercholesterolemia by Change in Diet Prevents Reduction in the Initial Treg Response and Change in Treg Phenotype

In order to prove that the reduction in the initial Treg response was due to prolonged hypercholesterolemia, we designed a study in which mice were fed a cholesterol diet for 4 weeks,
and then the mice were fed a cholesterol-free diet for an additional 4 weeks in order to reverse the hypercholesterolemia. We reasoned that this would allow the initial Treg response to be maintained. The diet reversal mice were compared with mice fed a cholesterol diet for 8 weeks. We confirmed that the diet switch resulted in significantly reduced serum cholesterol compared with the mice fed cholesterol diet continuously for 8 weeks (Figure 5A). There was significant inhibition of lesion progression between 4 and 8 weeks in mice changed to a control diet compared with mice fed cholesterol diet for 8 weeks (Figure 5B). Flow cytometric analyses of aortic digestions indicated that the diet reversal resulted in sustained peak Treg numbers in the aorta, which were approximately the same numbers observed after 4 weeks of cholesterol diet (Figure 5C). Moreover, staining of CD4 and Foxp3 cells in the aortic digest confirmed that the decrease in dietary and blood cholesterol resulted in a sustained high number of Treg (CD4<sup>+</sup>Foxp3<sup>+</sup>) within the aorta, which was higher than the number of Teff cells (CD4<sup>+</sup>Foxp3<sup>-</sup>) (Figure 5D). Quantification of Treg and Teff showed again a decreasing ratio Treg:Teff in aortas over a period of sustained hypercholesterolemia that was prevented in mice changed to a control diet (Figure 5E). In addition, diet reversal also prevented the increase in Treg apoptosis seen in mice with prolonged hypercholesterolemia (Figure 5F). We also found, by immunohistochemical analysis, a lower number of total CD4<sup>+</sup> cells in aortic sinus lesions in the diet reversal group (online-only Data Supplement Figure IVA). Smooth muscle cell content in the lesion did not show any difference between the groups (online-only Data Supplement Figure IVB), suggesting that changes in SMC content are not as readily altered with changes in diet.

We compared phenotypic markers on GFP<sup>+</sup> lymphocytes from spleens of mice changed to a control diet and mice fed a cholesterol diet continuously. The decreased expression of GITR, PSGL1, and P/E-selectin immunoglobulin G chimera binding that we observed in the GFP<sup>+</sup> population of hypercholesterolemic mice were not seen in the mice changed to control diet at 4 weeks (Figure 5F). We also found, by immunohistochemical analysis, a lower number of total CD4<sup>+</sup> cells in aortic sinus lesions in the diet reversal group (online-only Data Supplement Figure IVA). Smooth muscle cell content in the lesion did not show any difference between the groups (online-only Data Supplement Figure IVB), suggesting that changes in SMC content are not as readily altered with changes in diet.
only Data Supplement Figure VA and B), suggesting that the changes in selectin ligand expression were more important for Treg accumulation. These results show that Treg, which increase in numbers and accumulate in the aortic wall along with CD4/Teff during the first 4 weeks of hypercholesterolemia, selectively undergo phenotypic changes due to prolonged hypercholesterolemia beyond 4 weeks, which results in their absolute reduction in lesions compared with Teff.

**Discussion**

Emerging evidence indicates that Treg can suppress proatherogenic T cell responses, similar to their established function in regulating autoreactive T cells in immune-mediated/inflammatory disease. Studies from mouse and humans show that both induced Treg and Teff are increased in numbers in many immune responses to foreign and self antigens, and the ratio of Treg to Teff cells determines the outcome of these responses.22–24 We reasoned that therapeutic interventions aimed increasing Treg to Teff ratios in atherosclerotic lesions might favor control of or reduction in growth of the lesions and would diminish inflammation. Such a therapeutic approach would be feasible only if lesional Treg content could be dynamically changed by those interventions.

One important finding from our studies is that hypercholesterolemia induces an accumulation of Treg in the atherosclerotic aorta, but the Treg content is not maintained over time under sustained hypercholesterolemic conditions. Significantly, Teff content in the aorta increases while Treg content goes down. Therefore proinflammatory Teff within lesions will be subject to decreased local regulation as the...
A possible mechanism for this decrease over time is the loss of the ability of Treg to migrate into lesions after prolonged hypercholesterolemia. In support of this, we observed an increase in Treg in peripheral lymphoid tissue (spleen) concomitant with the decrease of Treg in lesions. This finding indicates that there is not a failure to generate or expand Treg systemically, nor a systemic decrease in their survival under hypercholesterolemic conditions. However, the expression of functional selectin ligands decreases on splenic Treg between 4 weeks and 20 weeks of hypercholesterolemia. Previous studies have shown that T-cell accumulation in atherosclerotic lesions is selectin dependent\textsuperscript{34–37} and that Treg migration is also selectin dependent.\textsuperscript{38,39} Interestingly, selectin ligands did not decrease on other lymphocytes in the spleens of the hypercholesterolemic mice, which might explain why Teff continue to accumulate in the atherosclerotic aortas of the mice. The basis for the effect of hypercholesterolemia on Treg selectin binding decreases on splenic Treg between 4 weeks and 20 weeks of hypercholesterolemia. Previous studies have shown that T-cell accumulation in atherosclerotic lesions is selectin dependent\textsuperscript{34–37} and that Treg migration is also selectin dependent.\textsuperscript{38,39} Interestingly, selectin ligands did not decrease on other lymphocytes in the spleens of the hypercholesterolemic mice, which might explain why Teff continue to accumulate in the atherosclerotic aortas of the mice. The basis for the effect of hypercholesterolemia on Treg selectin binding...
ability and the selectiveness of the effect for Treg but not Teff requires further investigation. One possibility is that hypercholesterolemia alters dendritic cells in a way that alters Treg differentiation. We have observed that naïve T cells from Foxp3-eGFP" mice fail to bind selectins, but Treg derived in vitro from naïve Foxp3-eGFP" T cells acquire selectin ligand capabilities (Nir Grabie, PhD and Andrew Lichtman, MD, PhD, unpublished data). We have also shown that dendritic cells become foam cells and retain antigen-presenting functions. It is possible that cholesterol-loaded dendritic cells are less capable of inducing expression of the glycosyltransferase enzymes required for functional selectin ligand synthesis.

A loss of ability of Treg to enter lesions after prolonged hypercholesterolemia may explain a lack of continued Treg accumulation but does not explain loss of cells that had already entered the aortic wall at 4 weeks. Increased Treg death after exposure to the increasingly altered microenvironment of the atherosclerotic arterial wall could be the basis of the increased Treg loss at 8 and 20 weeks. Our finding that there were significantly more apoptotic aortic Treg after 8 weeks than 4 weeks supports this explanation.

One limitation of the data shown in Figure 1 and 2 is that they cannot distinguish effects of prolonged hypercholesterolemia from effects of age, given that the longer the mice were kept on the cholesterol diet the older they were at time of sacrifice. However, we did not find any age-related change in selectin binding by Treg from normocholesterolemic C57BL/6 mice at ages that were equivalent to those in our experiments with LDLr−/− mice (data not shown). Furthermore, in the diet reversal study (Figures 5 and 6), the mice were the exact same age at sacrifice, and difference in Treg numbers and phenotype correlated with cholesterol content of diet during the final 4 weeks of life.

An important finding in this study is that changes in induced Treg content in atherosclerotic aortas are linked to hypercholesterolemia and can indeed be prevented by changes in diet-dependent blood cholesterol levels. Moreover, these changes are inversely correlated with total Teff content in lesions and lesion size. Thus, by changing a cholesterol-rich diet to a cholesterol-free diet we were able to maintain high Treg numbers in the aorta and prevent the decrease in Treg binding to selectins and aortic endothelium. Our finding supports the interpretation that therapeutic reversal of hypercholesterolemia would enhance Treg migration into the lesions.

Acknowledgments

We are grateful to Dr Vijay Kuchroo (Center for Neurological Diseases, Brigham and Women’s Hospital) for providing Foxp3-eGFP knock-in mice. We thank George Stavrakis (Morphology Core, Pathology, Brigham and Women’s Hospital) for technical assistance with immunohistochemistry staining. We thank Pilar Alcaide and Francis Lusciniskas (Brigham and Women’s Hospital) for helping with in vitro flow studies. We also thank the Harvard Neurodiscovery Optical Imaging Program, Harvard Medical School, for the contribution helping with the immunofluorescence images acquisition.

Sources of Funding

This work was supported by National Institutes of Health grant HL087282 and the Foundation Antonio Martín Escudero.

Disclosures

None.

References


CD4\(^+\) effector T cells have multiple proinflammatory properties that contribute to the chronic inflammatory phenotype of evolving atherosclerotic lesions, as well as to the destabilization of plaques associated with acute coronary events. Regulatory T cells (Treg) actively suppress T cell–mediated immune responses, and reduced Treg function or numbers are associated with immune-mediated inflammatory disease. The influence of Treg in atherosclerosis has become a central area of interest because of potential therapeutic implications. This study demonstrates that in a mouse model of atherosclerosis, a Treg response is induced by hypercholesterolemia, but the response declines while the effector T cell response is maintained when hypercholesterolemia is prolonged. The decline in the Treg response is associated with selective decrease in homing properties and increased apoptosis of Treg but not Teff during prolonged hypercholesterolemia. The Treg response is sustained by dietary reversal of hypercholesterolemia early after the initial response is induced. Our data suggest that an important therapeutic goal in atherosclerotic patients is to reestablish favorable lesional Treg:Teff ratios, and this may be one of the mechanisms of benefit of profound cholesterol lowering.
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Circulation. 2011;124:185-195; originally published online June 20, 2011;
doi: 10.1161/CIRCULATIONAHA.110.006411
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the
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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. 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 CaCl₂, pH 7.4), permeabilized with BD Perm/Warm Buffer (Fetal Bovine Serum and Saponin) and intracellular stained with rat anti mouse antibodies CD4-Percep 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. 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. 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 x10⁵ 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/cm²). 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 10⁴/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 (10⁵/well) and CD4⁺Foxp3-GFP⁺ suppressor cells (2 x 10⁴/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 (10⁵/well), DCs isolated from spleens of hypercholesterolemic mice (5x10⁴/well) and CD4⁺Foxp3-GFP⁺ suppressor cells (5x10⁴/well-responder to suppressor ratio of 1:1). Cultures were pulsed with [³H]-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. 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 22°C. 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.

A

4 weeks 8 weeks 20 weeks

Control diet

Cholesterol diet

MHCII
DAPI (NUCLEI)

B

Macrophages positive area (mm²)

4 8 20

Weeks on cholesterol diet

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Figure S2.

**Control diet**

**Cholesterol diet**

![Bar charts showing the percentage of different cell types over weeks on diet for control and cholesterol diets.]

- **A**: %GFP-GITR+ cells
- **B**: %GFP-Esel lig+ cells
- **C**: %GFP-PSGL1+ cells
- **D**: %GFP-Psel lig+ cells
- **E**: %GFP-LFA-1+ cells
- **F**: %GFP-/CD62L+ cells

Weeks on diet: 4, 8, 20

Legend:
- Open bars: Control diet
- Solid bars: Cholesterol diet

Significance levels are indicated for selected data points:
- *p < 0.05
- **p < 0.01
**Figure S3.**

**A**

- **[3H] incorporation (c.p.m.)**
- Diet in mice supplying Treg
  - Control diet
  - Cholesterol diet

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**B**

- **Stimulated index**
- Mice supplying DCs:
  - 4w cholesterol diet
  - 8w cholesterol diet

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Figure S4.

A  
**CD4**^+ T cells in lesions**  

![Image of CD4+ T cells in lesions]

B  
**SMC in lesions**

![Image of SMC in lesions]

**SMC actin (% of lesion area)**

- **Control diet 4 w**
- **Cholesterol diet 4 w**
- **Cholesterol diet 8 w**
- **Cholesterol diet 4 w**
- **Control diet 4 w**
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 $\alpha$-I$^A$/I$^E$-PE (red) and analyzed by confocal microscopy. These images are a projection of 15-30 z-stacks (20-30 $\mu$m). 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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