Role of Naturally Occurring CD4\(^+\)CD25\(^+\) Regulatory T Cells in Experimental Atherosclerosis

Adi Mor, David Planer, Galia Luboshits, Arnon Afek, Shula Metzger, Tova Chajek-Shaul, Gad Keren, Jacob George

Objective—Naturally occurring CD4\(^+\)CD25\(^+\) regulatory T cells (Tregs) exert suppressive effects on effector CD4 cells and downregulate experimental autoimmune disorders. We investigated the importance and potential role of Tregs in murine atherogenesis.

Methods and Results—Tregs were investigated comparatively between aged and young apolipoprotein E–knockout (ApoE-KO) mice and age-matched C57BL/6 littermates. The effect of oxidized LDL (oxLDL) was tested on the functional suppressive properties of Tregs from ApoE-KO and C57BL/6 mice. Tregs, CD4\(^+\)CD25\(^+\) cells, and saline were infused into ApoE-KO mice to study their effects on atherogenesis. Treg numbers were reduced in atherosclerotic compared with nonatherosclerotic ApoE-KO mice. The functional suppressive properties of Tregs from ApoE-KO mice were compromised in comparison with those from their C57BL/6 littermates. Thus, oxLDL attenuated the suppressive properties of Tregs from C57BL/6 mice and more so in ApoE-KO mice. Transfer of Tregs from age-matched ApoE-KO mice resulted in significant attenuation of atherosclerosis compared with that after delivery of CD4\(^+\)CD25\(^-\) T cells or phosphate-buffered saline.

Conclusions—CD4\(^+\)CD25\(^+\) Tregs may play a protective role in the progression of atherosclerosis and could be considered a therapeutic tool if results from human studies can solidify observations in murine models. (Arterioscler Thromb Vasc Biol. 2007;27:000-000.)

Key Words: atherosclerosis ■ T cells ■ immune response ■ adoptive transfer ■ foxp3

Atherosclerosis is a multifactorial process that involves interactions among endothelial cells, macrophages, smooth muscle cells, and lymphocytes.\(^1\)–\(^3\) The outcome of growth of the atherosclerotic plaque is a result of its final influence on organ perfusion, resulting in cerebrovascular events and acute coronary syndromes. The role of the immune system in atherosclerosis has received considerable interest in recent years; however, sufficient knowledge to justify the development of targeted immunomodulatory strategies has not yet been obtained.\(^4\)–\(^6\) Cumulative data based on atherosclerotic experimental models suggest that CD4 lymphocytes are present within murine plaques from initial stages of the disease,\(^4\) and adoptive transfer of these cells is potentially proatherogenic.\(^5\)–\(^7\) Moreover, it has been shown that cytokine products of CD4 cells (interferon-\(\gamma\), interleukin [IL]-10, IL-4, IL-18, etc) also influence the extent and nature of the atherosclerotic plaque (reviewed in Hansson,\(^1\) Libby,\(^2\) and Binder et al\(^\text{a}\)). These collective findings support the contention that antigen-specific responses may be operable in the evolving atheromatous plaque.

In recent years, several T-cell populations have been described that possess predominant regulatory effects (Tregs) on T cells’ responding to infection-driven and self-antigens (reviewed in Sakaguchi \(^8\) and O’Garra and Vieira\(^9\)). Initial studies addressing these regulatory T-cell populations were reported in experimental animal models, and some of these findings were reproduced subsequently in humans. Accordingly, a compromised number or function of Tregs has been associated, with immune system–mediated disorders in humans, such as diabetes,\(^10\) rheumatoid arthritis,\(^11\) and multiple sclerosis.\(^12\)

In this context, 2 populations of T cells were described: the first appears to respond to antigen-specific, major histocompatibility complex–restricted triggers with secretion of anti-inflammatory cytokines,\(^13\)\(^,\)\(^14\) and the second population of regulatory cells comprises the CD4\(^+\)CD25\(^+\) Tregs.\(^15\)\(^,\)\(^16\) It was recently found that the transcriptional regulator forkhead/winged helix transcription factor foxp3 governs mouse CD4\(^+\)CD25\(^+\) Treg function.\(^17\)\(^,\)\(^18\) This finding was strongly supported by the observation that patients with immune system dysregulation, polyendocrinopathy, enteropathy, and X-linked inheritance have a severe inflammatory disease accompanied by a mutation in the FOXP3 gene.\(^19\)
In a recent study by Ait-Oufella et al., it was suggested for the first time that Tregs are protective in murine atherogenesis. Because atherosclerosis is influenced by innate and adaptive immune system responses, we set out to investigate the hypothesis that naturally occurring Tregs are capable of influencing the size and composition of atherosclerotic lesions. Employing the apolipoprotein E–knockout (ApoE-KO) mouse model, we found that Tregs are compromised in atherosclerotic animals and that their delivery attenuates plaque progression.

Methods

Animals
ApoE-KO mice on a C57BL/6 background and their wild-type littersmates were purchased from Jackson Laboratories (Bar Harbor, Me) and maintained at the local animal facility. Mice were fed a normal chow diet containing 4.5% fat by weight (0.02% cholesterol).

Preparation of Copper-Oxidized LDL
Blood for lipoprotein isolation was collected in EDTA (1 mg/mL) from non–coronary atherosclerotic subjects after a 12-hour fast. LDL (density=1.019 to 1.063 g/L) was isolated from plasma after density adjustment with KBr and preparative ultracentrifugation at 50 000 rpm for 22 hours with a type 50 rotor. LDL preparations were washed by ultracentrifugation and dialyzed against 0.15 mol/L EDTA (pH 7.4), passed through an Acrodisc filter (0.22-μm pore size) to remove aggregates, and stored under N2 in the dark. Copper oxidation of LDL was performed by incubation of postdialyzed LDL (1 mg protein per mL in EDTA-free phosphate-buffered saline (PBS)) with copper sulfate (10 μmol/L) for 24 hours at 37°C. Lipoprotein oxidation was confirmed by analysis of thiobarbituric acid–reactive substances.

Cell Separation and Flow Cytometry
Splenocytes were costained with the following monoclonal antibodies: fluorescein isothiocyanate–labeled anti-CD4 (7D4; Miltenyi Biotec, Bergisch Gladbach, Germany), phycoerythrin-labeled anti-CD25 (GK1.5, Miltenyi Biotec), fluorescein isothiocyanate–labeled mouse IgG2b isotypic control (KLH/G2b-1-2, Southern Biotech), and phycoerythrin-labeled mouse IgMx isotypic control (RTK2118, BioLegend). For fluorescence-activated cell sorting (FACS) analysis of foxp3 intracellular expression, we used the fluorescein isothiocyanate–anti-mouse/rat foxp3 staining set (clone FJK-16s, eBioscience). Stained cells were analyzed on a FACScan flow cytometer with CellQuest software (Becton-Dickinson).

CD4+CD25+ T cells were isolated from total splenocytes by a first step of negative sorting with a cocktail of hapten-conjugated CD8, CD11b, CD16, CD19, CD36, and CD56 antibodies and microbeads coupled to an anti-hapten monoclonal antibody (CD4+ T-cell isolation kit; Miltenyi Biotec). This was followed by positive selection of CD25+ cells by microbead separation (CD25 microbeads; Miltenyi Biotec), providing >95% purity as assessed by FACS analysis.

Isolation, Activation, and Transfer of CD4+CD25+ T-Cell Subsets
Purified CD4+ cells were isolated from spleens of ApoE-KO mice by first staining non–CD4+ T cells with a biotin antibody cocktail (10 μL/107 total cells), incubation for 10 minutes at 4°C, magnetic labeling with antibiotin microbeads (20 μL/107 total cells), incubation for 15 minutes at 4°C, 2 washes, and depletion over a magnetically activated cell sorting liquid desorption column. CD4+CD25+ T cells were directly labeled with anti-CD25 microbeads (10 μL/107 CD4+ cells), incubated for 15 minutes at 4°C, and positively selected with magnetically activated cell sorting mass spectrometry columns according to the manufacturer’s instructions (Miltenyi Biotec). The purity of all cell subsets processed with this method was >95%, as determined by FACS analysis (data not shown). For intravenous injection, 105 cells were suspended in PBS, and a total volume of 450 μL per mouse was injected.

Coculture of T-Effector and Treg Cells
Ninety-six-well plates (Nunc) were coated with 1 μg/mL anti-CD3 mononclonal antibody (17A2, eBioscience) overnight at 4°C and washed. CD4+ CD25+ (responder) and CD4+CD25+ (suppressor) T cells (105 cells/well) were cultured in RPMI medium supplemented with 10% fetal calf serum at different responder-suppressor ratios (1:1, 1:2, 1:3, and 1:8). All cells were cultured in a final volume of 200 μL in the presence of irradiated 106 T cells–depleted accessory cells per well. After 72 hours, [H]thymidine (1 μCi/well) was added for 16 hours before proliferation was assayed by scintillation counting (beta counter). Percent inhibition of proliferation was determined from the following formula: 1–(median [H]thymidine uptake of 1:1 CD4+CD25+CD4+CD25+ coculture/median [H]thymidine uptake of CD4+CD25+ cells).

Western Blot Analysis of Foxp3
Western blotting was performed with rat serum anti-foxp3 (polyclonal antibody, Bioscience) at a dilution of 1:1000 and a secondary antibody, peroxidase-conjugated AffiniPure donkey anti-rat IgG (H+L. Jackson Laboratories). Actin served as a control protein.

In Vivo Adoptive Transfer Study Design
In a preliminary study, we infused 4 groups of ApoE-KO mice with 105, 103, and 3×105 Tregs and PBS 3 times until sacrifice, 3 months after the initial injection. We found that delivery of 103 or 105 Tregs did not influence aortic sinus plaque size, whereas administration of 3×105 Tregs led to a 30% reduction in plaque size (P=0.08). We thus selected the latter protocol, assuming that doubling the number of animals would produce a statistically significant change in lesion formation.

Three-month-old male ApoE-KO mice were transferred intravenously through the tail vein with PBS (n=10), 3×105 CD4+CD25+ Tregs (n=9), or 3×105 CD4+CD25+ cells (n=10). The same protocol for injection was repeated twice more at 3 weekly intervals. Mice were euthanized 3 months after the initial injection. Plasma was obtained for assessment of lipid profiles, and aortas as well as aortic sinus were frozen for assessment of atherosclerosis and immunohistochemistry.

Lipid Profile
Total plasma cholesterol and triglyceride levels were determined with an automated enzymatic technique (Boehringer Mannheim, Mannheim, Germany).

Assessment of Aortic Sinus Atherosclerosis
Quantification of atherosclerotic lesions was done by calculating the lesion size in the aortic sinus as previously described. The heart and upper section of the aorta were removed from the animals, and the peripheral fat was cleaned away carefully. The upper section was embedded in OCT compound and frozen. Every other section throughout the aortic sinus (400 μm) was taken for analysis. Sections were evaluated for atherosclerosis after being stained with oil red O.

Histochemical Analysis of Aortic Sinus Sections
Immunohistochemical staining was performed with rat anti-CD4 (PharMingen). Staining with Masson’s trichrome was used to delineate the fibrous area and was determined by quantitative morphometry.

Sudan IV Staining of Aortic Lesions
The aortas were dissected from the aortic arch to the iliac bifurcation and washed for 1 hour in PBS, pH 7.4, and 0.5 mmol/L EDTA on a rotating table. The aorta was then fixed with a formal-sucrose solution (4% paraformaldehyde, 5% sucrose, 20 mmol/L butylated hydroxytoluene, and 2 mmol/L EDTA, pH 7.4) overnight. The adventitial fat was trimmed from the aorta while being viewed under...
a microscope, opened longitudinally, rinsed briefly in 70% ethanol, immersed for 6 minutes in a filtered solution of Sudan IV (Sigma Chemical Co) in 35% ethanol and 50% acetone for 10 minutes, and destained in 80% ethanol. Sudan IV-stained aortas were assessed for lesion area by morphometry.

**Reverse Transcription–Polymerase Chain Reaction for Aortic Expression of IL-10**

For reverse transcription—polymerase chain reaction (RT-PCR), aortas from Treg-, CD4^+^CD25^-^ T cell-, and PBS-treated mice were analyzed for expression of mRNA for IL-10. Primers used were 5'-CTGGACAACATCTGCTAACCAGAC-3' (sense) and 5'-ATTCCATCTGCCCATTGTAGACACC-3' (antisense).

**Statistical Analysis**

All parameters were evaluated by 1-way ANOVA. *P*<0.05 was considered statistically significant. Results are expressed as mean±SEM unless specified otherwise in the text.

**Results**

Initially, we tested the hypothesis that the number and functional properties of Tregs are compromised in ApoE-KO mice compared with those in wild-type C57BL/6 littermates. We found by FACS analysis of spleen cells from 5 mice in each group at 3 months that the number of Tregs (CD4^+^CD25^-^) of the total CD4 population was significantly reduced in ApoE-KO mice (mean of 4.6±0.2%) compared with C57BL/6 mice (8.4±0.4%, *P*<0.0001; Figure 1A). Lymph node (pooled inguinal and para-aortic) Tregs were reduced in ApoE-KO mice (mean of 3.9±1.3%) in comparison with C57BL/6 mice (5.1±1.4%, *P*<0.05). Moreover, thymic Tregs were significantly reduced in ApoE-KO mice (1.1±0.3%) compared with those in C57BL/6 mice (2.4±0.7%, *P*<0.05).

To further comparatively validate the identity of the Tregs in ApoE-KO and C57BL/6 mice, we performed double staining for CD25 and foxp3. We found that in ApoE-KO mice, the number of CD25^+^foxp3^-^ cells was significantly diminished (2.5±0.1%) compared with those from their wild-type littermates (3.1±0.2%, *P*<0.05; Figure 1B). Moreover, we observed differences in the functional properties between both groups. Isolated CD4^+^CD25^-^ Tregs from ApoE-KO mice were significantly less potent than those from their C57BL/6 littermates in the suppression of effector CD4^+^CD25^-^ cells obtained from the latter animals (Figure 1C). Thus, tritiated thymidine uptake by CD4^+^CD25^-^ cells from C57BL/6 mice was significantly higher when they were cocultured in the presence of Tregs from ApoE-KO mice in comparison with Tregs from C57BL/6 mice (at a responder to Treg ratio of 1:1, *P*<0.01; at a 1:2 ratio, *P*<0.05; at a 1:4 ratio, *P*<0.01; and at a 1:8 ratio, *P*<0.01, respectively; probability values indicate the average of all 5 separate experiments). A representative experiment of the 5 that were performed is provided in Figure 1C. No differences were evident between responder cell proliferation in C57BL/6 mice when the assay was conducted in the presence of activated protein C (APC) from ApoE-KO or C57BL/6 mice. We found that when CD4^+^CD25^-^ cells from ApoE-KO mice were used as responders in the same suppressive assay, Tregs from ApoE-KO mice were compromised with regard to their ability to inhibit proliferation compared with Tregs from C57BL/6 mice (a mean reduction of 38.3% over all ratios, *P*<0.01).

To pursue the question of whether the presence of atherosclerosis in ApoE-KO mice is associated with a change in the number and function in these mice at the age of 6 weeks (when mature plaques are not yet evident) with atherosclerotic ApoE-KO mice at an age of 6 months (Figure 1C). We found that the number of spleen cell–derived Tregs was significantly reduced in atherosclerotic (7.2±0.13%) compared with nonatherosclerotic (9.4±0.2%) ApoE-KO mice (*P*<0.01).

OxLDL has been suggested as a leading candidate in triggering atherosclerotic plaque formation.1–3 We hypothesized that oxLDL, known to trigger lymphocyte apoptosis, would exert a more profound depleting effect on Tregs compared with effector CD4^+^CD25^-^ cells. Indeed, incubation of CD4^+^ cells derived from the spleen with oxLDL (1 µg/ml) resulted in a significant (35%) relative reduction in the Treg pool (Figure 2, A and B) compared with a nonsignificant lowering of the numbers of effector CD4^+^CD25^-^ cells (data not shown).

Foxp3 is a key transcriptional regulator of Tregs. We reasoned that the effect of oxLDL on Tregs could be mediated by the effect on foxp3. We found that oxLDL incubated in the presence of murine splenocytes led to a time-dependent downregulation of foxp3 (Figure 2C) compared with no effect on a control protein (β-actin).

We then investigated whether oxLDL is capable of interfering with the suppressor functions of Tregs. For this purpose, different ratios of Tregs were incubated with effector cells in the presence of irradiated APC. OxLDL led to a significant reduction in the suppressive properties of Tregs, evident by enhanced proliferation and thymidine uptake of effector CD4^+^CD25^-^ cells. To study whether a differential response to oxLDL-mediated suppression exists between hyperlipidemic ApoE-KO mice and their wild-type littermates, we performed a comparative coculture assay in the presence of oxLDL (Figure 3). We found that the suppressive properties of Tregs from ApoE-KO mice were significantly more hampered (a mean of 80% inhibition of proliferation at a 1:1 suppressor to responder ratio) compared with sorted Tregs from C57BL/6 mice (Figure 3). Under these conditions, incubation of oxLDL with either Tregs or responder T cells alone did not change their proliferative capacity as measured in (radioactive) counts per minute (data not shown).

To prove that cell viability was not compromised in this assay, representative samples were tested by the trypan blue exclusion test, showing no evident changes.

Aiming to establish the role of Tregs in the progression of experimental atherosclerosis, we performed an in vivo adoptive transfer study. We found that ApoE-KO recipients of Tregs exhibited a significantly reduced extent of atherosclerosis as evident by smaller aortic sinus plaques compared with those in ApoE-KO mice treated with PBS or with effector CD4^+^CD25^-^ T cells (Figures 4A and 5). This finding was validated by aortic en face analysis of atherosclerosis surface coverage area, showing decreased lesions in Treg-transferred ApoE-KO mice compared with effector T cell– and PBS-treated recipients (Figures 4B and 5).
We further explored the nature of plaque development in response to the transfer of Tregs. Whereas PBS and Treg transfer led to a similar distribution of a fibrous cap and lipid core (mean fibrous areas of 61±2% and 65±3%, respectively) as evidenced by Masson’s trichrome staining, infusion of CD4⁺CD25⁺ T cells resulted in lesions that were more unstable, exhibiting larger lipid cores and smaller fibrous caps (mean fibrous area of 44±3%, \(P<0.005\) for both

Figure 1. Comparative analysis of the number and function of Tregs from ApoE-KO and C57BL/6 mice. A, Spleen cells were obtained from 3-month-old ApoE-KO and C57BL/6 littermates and stained with antibodies to CD4 and CD25 as outlined in Methods. Data are presented for 5 mice in each group. B, Staining of splenocytes from ApoE-KO and C57BL/6 mice was performed for detection of CD25⁺Foxp3⁺ cells. C, Different ratios of magnetic bead–sorted Tregs from ApoE-KO and C57BL/6 mice were incubated with effector CD4⁺CD25⁻ T cells from the latter animals in the presence of irradiated APC. Figure shows 1 representative experiment of 4. Thymidine uptake was used for assessment of effector cell proliferative indices. D, The comparative effect of Tregs from ApoE-KO and C57BL/6 mice expressed as percent inhibition of responder T cells. E, Spleen cells from young nonatherosclerotic and older atherosclerotic mice were assayed for assessment of the number of Tregs (\(*P<0.001\)). Results represent data from 5 mice in each group. Representative FACS sheets are provided in E.
comparisons; Figures 4C and 5). We then further characterized the nature of the plaques by staining for CD4$^+$H11001 and found that Treg and PBS transfer led to a similar extent of infiltration of the plaques compared with increased numbers of CD4$^+$H11001 cells observed in plaques from effector T cell–transferred mice (Figures 4D and 5). To exclude the possibility that Treg transfer was associated with a change in lipid profile, we performed a plasma metabolic assessment, which showed that no differences were evident between the groups with respect to total cholesterol and triglyceride levels (data not shown). Importantly, we also analyzed the numbers of Tregs in the spleens of 3 recipient groups and found no differences.

IL-10 expression is considered 1 of the mediators of suppression, either in naturally occurring or in antigen-specific Tregs (reviewed in O’Garra and Vieira$^{9}$). We thus tested the hypothesis that IL-10 expression within atherosclerotic aortas could have been altered by cell transfer. Interestingly, we found that aortas from mice that received Tregs exhibited significantly increased mRNA expression of IL-10 (online Figure I), attesting to a possible mediating effect on plaque progression. No differences in mRNA expression by RT-PCR were evident between aortas of PBS- and Treg-treated ApoE-KO mice.

**Discussion**

In the current study, we investigated the functional role of CD4$^+$CD25$^+$ Tregs in atherosclerosis by using the reference experimental model of the ApoE-KO mouse. Atherosclerosis is a progressive disorder in which the role of regulatory T cells has been suggested by several observations: (1) adoptive transfer studies of lymphocytes, which have been shown to accelerate murine atheromas$^{5–7}$; (2) T-helper 2 cytokines, such as IL-10 that are produced by Tregs, that have been shown to attenuate lesion formation as opposed to T-helper 1 cytokines, which have been demonstrated to enhance lesion formation (reviewed in Hansson,$^{1}$ Libby,$^{2}$ and Binder et al$^{3}$); and (3) induction of oral tolerance with respective generation of antigen-specific Tregs that has been suggested to attenuate experimental atherogenesis.$^{20,21,24}$

Tregs comprise several cell populations. Antigen-specific Tregs are operable via secretion of cytokines such as IL-10 and transforming growth factor-$eta$, whereas naturally occurring Tregs act by cell-to-cell contact but predominantly through secretion of transforming growth factor-$eta$$^{8,9,25–27}$

Despite the lack of a unifying and acceptable membrane antigen that characterizes Tregs, the most likely molecule on CD4 used for phenotyping is CD25. We therefore assayed the number of CD4$^+$CD25$^+$ cells in the spleen of atherosclerotic ApoE-KO mice and compared them with those of control C57BL/6 littermates. Indeed, we found that ApoE-KO mice had a significantly reduced number of splenic Tregs. To further confirm the identity of these Tregs, we made a comparative analysis of CD25$^+$foxp3$^+$ cells in these same mice. We observed a reduction in the number of CD25$^+$foxp3$^+$ cells in ApoE-KO mice that mirrored the differences in CD4$^+$CD25$^+$ cells.

When compared with younger, nonatherosclerotic littermates, 6-month-old ApoE-KO mice exhibited a lower number of CD4$^+$CD25$^+$ Tregs. Collectively, these findings suggest that the development of atheromatous plaques in the mouse could be associated with depletion of a peripheral Treg pool, although no cause-and-effect relation could be inferred. We then compared the functional suppressive properties of Tregs from atherosclerotic ApoE-KO mice with those in C57BL/6 littermates. Interestingly, we found that the suppressive effect of Tregs on the proliferative capacity of the effector CD4$^+$CD25$^+$ population was significantly hampered in ApoE-KO mice compared with C57BL/6 mice. The inhibitory action of Tregs has been shown to be associated with their ability to suppress experimental autoimmune disorders,$^{29}$ and their transfer could correct the respective syndromes.$^{29,30}$ Thus, we hypothesize that Treg dysregulation in the atherosclerotic mouse could promote lesion progression.

**Figure 2.** OxLDL downregulates Tregs and foxp3 protein content in murine splenocytes. OxLDL was incubated in 2 concentrations with spleen cells obtained from ApoE-KO mice. A representative figure of 4 is shown, and a combined analysis of 4 collective separate experiments is displayed in B. P<0.01. C, Protein content of foxp3 by Western blotting compared with a control protein (actin) and densitometric analysis performed with the indicated concentrations of oxLDL.

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There are sufficient data to support the presence and role of oxLDL in the development of the atherosclerotic plaque. We sought to explore the influence of oxLDL on effector and Treg relative numbers in mice. The relative percentage of Tregs of the total CD4 population was significantly reduced by incubation with oxLDL compared with a nonsignificant depleting effect on CD4^+^CD25^-^ cells. Moreover, the compromising effect of oxLDL on the suppressive effects of Tregs from atherosclerotic ApoE-KO mice appeared to be more robust than in their C57BL/6 littermates. The current study design, however, did not allow us to rule out the possibility that ApoE deficiency by itself was causally associated with the differential effect of oxLDL. This hypothesis could be tested by performing the assays in vitro in the presence or absence of ApoE or its derivatives.

**Figure 3.** OxLDL induces a differential inhibitor effect on Tregs from ApoE-KO and C57BL/6 mice. Coculture of Tregs with responder CD4^+^CD25^-^ cells in the presence of irradiated APC was performed in cells isolated and sorted from 3-month-old mice in the presence and absence of 1 μg/mL oxLDL. Thymidine incorporation was assayed to evaluate the proliferative properties of the responder T cells. Figure shows a representative experiment from 4 performed. Lower panel shows the differential effect of oxLDL expressed as percent inhibition of responder T cells.

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**Figure 4.** Adoptive transfer of Tregs influences plaque size and composition in ApoE-KO mice. Recipient ApoE-KO mice received 3 injections of Tregs, CD4^+^CD25^-^ cells, and PBS at 3 weekly intervals. At sacrifice, 4 months after initial cell transfer, the hearts were removed for assessment of aortic sinus atherosclerosis (A), and the aortas were stripped longitudinally for en face evaluation of atherosclerotic surface coverage after Sudan IV staining (B). To study stability indices, morphometric analyses were performed to measure the fractional area occupied by fibrous tissue (C). CD4 infiltration of the plaques was assayed with the appropriate monoclonal antibody (D). Collective results of A–D represent mean ± SEM from sections of mice in each group.
Unanswered questions remain with regard to the cause of depletion and functional compromise of Tregs in ApoE-KO mice. Because some degree of LDL oxidation has been noted in the plasma of subjects with hyperlipidemia and may also be evident in hypercholesterolemic mice, we speculate that the Treg pool may contact modified LDL in the circulation, not just within the atheroma. The effects of oxLDL to which these Tregs were exposed could well have been maintained during the 3-day culture of the functional suppressive assays.

Foxp3 has been shown to govern the development and function of Tregs, and evidence supports its presence in both CD25+ Tregs and CD25+ cells with regulatory activity. Loss-of-function mutations of foxp3 eliminate CD25+ Tregs and result in autoimmune disorders in both humans and mice. We thus explored whether the suppressive function of oxLDL was associated with its effect on foxp3. We found that oxLDL triggered a time-dependent downregulatory effect on the expression of foxp3. Collectively, these findings suggest that the oxLDL that is present within the atheroma and possibly in minor concentrations in serum could be causally associated with the compromised population of Tregs in the atherosclerotic mouse.

To pursue a cause-and-effect relation between Tregs and atherosclerosis, we performed adoptive transfer assays, as has been done in models of experimental autoimmune encephalomyelitis and diabetes. We found that transfer of Tregs resulted in a significant reduction in atherosclerotic lesion formation compared with the delivery of either PBS or effector CD4+CD25+ cells. This finding was validated by separately measuring plaque size in the aortic sinus and the entire longitudinally stripped aortas, with essentially similar findings. Transfer of CD4+CD25+ did not significantly influence plaque formation when compared with PBS. It should be taken into consideration that we infused a total of 9×10^5 Tregs and effector T cells divided into 3 injections, yet the relative number of Tregs accounts for a significantly higher proportion of the peripheral population in the mouse, as Tregs represent only 2% to 5% of total lymphocytes. Thus, we cannot exclude the possibility that infusion of effector T cells in larger numbers would have promoted murine atherogenesis, as may be inferred by previous observations. By studying the nature of the plaques induced after cell transfer, we found that delivery of effector CD4+CD25+ cells resulted in lesions that were more unstable, exhibiting a smaller fibrous area and a higher number of inflammatory cells compared with the situation in Treg- or PBS-injected mice.

The exact mechanisms mediating the suppressive properties of naturally occurring Tregs have not yet been elucidated. As mentioned, cell-to-cell contact could be a mechanism, supported by our in vitro coculture experiments. However, there is also evidence to suggest that a population of naturally occurring Tregs are IL-10 producers. This would be consistent with an antiatherogenic effect, as IL-10 is causally associated with attenuation of experimental atherogenesis (reviewed in Hansson, Libby, and Binder et al). We found by RT-PCR that expression of mRNA for IL-10 in aortas of Treg-transferred ApoE-KO mice was significantly enhanced. In view of the strength of the evidence in favor of an antiatherogenic role for IL-10 in experimental atherogenesis, we reason that this is 1 of the plausible mechanisms for the plaque-attenuating effect of Treg transfer. We also analyzed the Treg pool in the 3 recipient groups and found no differences in their numbers. However, despite similar numbers in the 3 groups, a systemic elevation of Tregs as a possible protective mechanism could not be ruled out, because analysis was performed several weeks after the final delivery of Tregs.

It was recently observed by Ait-Oufella et al that Tregs have a protective role in atherogenesis by successfully blocking CD25 monoclonal antibodies in the ApoE-KO mouse model. Our results provide complementary data that further support the potential role of Tregs in the pathogenesis of
atherosclerosis in the mouse. These findings are also consistent with our recent report, showing that patients with acute coronary syndromes exhibit a reduced number and functional compromise of their peripheral Tregs.

In conclusion, we found that atherosclerotic mice exhibit a compromised pool of naturally occurring Tregs and that transfer of these CD4+CD25+ Tregs significantly reduces plaque progression in the ApoE-KO mouse model. These findings suggest a novel role for naturally occurring Tregs in atherogenesis and open new potential therapeutic avenues.

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Disclosures

None.

References

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**Fig. I**

**Fig. I: Assessment of mRNA expression of IL-10.**

Upon sacrifice, 5 aortas were obtained from Treg, CD4⁺CD25⁻ cells and PBS injected mice and studied by RT-PCR for the expression of mRNA for IL-10. Bars display mean±SEM of densitometric expression of IL-10 in each group.* p<0.05.