Disruption of TGF-β signaling in T cells accelerates atherosclerosis

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Increasing evidence suggests that atherosclerosis is an inflammatory disease promoted by hypercholesterolemia. The role of adaptive immunity has been controversial, however. We hypothesized that proatherogenic T cells are controlled by immunoregulatory cytokines. Among them, TGF-β has been implied in atherosclerosis, but its mechanism of action remains unclear. We crossed atherosclerosis-prone ApoE-knockout mice with transgenic mice carrying a dominant negative TGF-β receptor II in T cells. The ApoE-knockout mice with disrupted TGF-β signaling in T cells exhibited a sixfold increase in aortic lesion surface area, a threefold increase in aortic root lesion size, and a 125-fold increase in aortic IFN-γ mRNA when compared with age-matched ApoE-knockout littermates. When comparing size-matched lesions, those of mice with T cell–specific blockade of TGF-β signaling displayed increased T cells, activated macrophages, and reduced collagen, consistent with a more vulnerable phenotype. Ab's to oxidized LDL, circulating T cell cytokines, and spleen T cell activity were all increased in ApoE-knockout mice with dominant negative TGF-β receptors in T cells. Taken together, these results show that abrogation of TGF-β signaling in T cells increases atherosclerosis and suggest that TGF-β reduces atherosclerosis by dampening T cell activation. Inhibition of T cell activation may therefore represent a strategy for antiatherosclerotic therapy.

Introduction

Atherosclerosis is an inflammatory disease in which blood-borne immune cells infiltrate the artery wall. While monocyte macrophages and macrophage-derived foam cells constitute the major cellular component of early atherosclerotic lesions, T cells represent an important additional component. Activation of both cell types is observed at all stages of atherosclerosis and, in particular, at sites of plaque activation and rupture in patients with unstable angina and myocardial infarction (1–4). Cell culture studies show that activated macrophages and T cells secrete factors that may destabilize plaques (5, 6). These factors include proinflammatory cytokines that inhibit smooth muscle cell proliferation, differentiation, and collagen secretion

and promote endothelial expression of tissue factor and adhesion molecules. Activated macrophages may also secrete MMPs, which can directly degrade collagen of the plaque's fibrous cap and may release reactive oxygen and nitrogen species, which can damage cells and modify extracellular components of the lesion.

T cells are key inducers and regulators of immune activity. They recognize antigens in a clonally specific manner, provide help for B cell activation, and activate macrophages by expressing cytokines and costimulatory molecules. Candidate antigens involved in atherosclerosis include oxidized LDL, heat shock protein-60, and microbial components (5). T cells specific for these antigens have been isolated from human atherosclerotic lesions (7–9). The role of T cells has been addressed in several experimental models of atherosclerosis. SCID × *ApoE*⁻/⁻ (E⁰) mice, which lack T and B cells, develop less atherosclerosis than immunocompetent E⁰ mice (10). Similarly, E⁰ mice deficient in IFN-γ signaling, IL-18, or CD40L (CD154) exhibit reduced disease development (11–13). Surprisingly, data from RAG⁻/⁻ × E⁰ mice have been contradictory (14–16), and immunization with candidate antigens may result in protection as well as increased disease, depending on the experimental conditions (for review see refs. 4, 5). This suggests that immunoregulatory mechanisms may counteract the disease-aggravating ones.

T cell activation is controlled by cytokines produced by neighboring cells. IL-10, secreted by macrophages and the Th2 subset of T cells, inhibits secretion of several proinflammatory T cell cytokines by T cells. Another immunoregulatory cytokine, TGF-β, is produced by several different cell types, including endothelial and smooth muscle cells, macrophages, platelets, and regulatory T cells ($T_{reg's}$) (17). Both IL-10 and TGF-β appear to dampen atherosclerosis in mouse models (18–22), and the latter has also been implicated in human disease (23–25). Since TGF-β has multiple targets, its mode of action, however, is not known. For instance, it modulates smooth muscle proliferation and collagen formation, endothelial proliferation, and macrophage activity (26). It has therefore been proposed that the main target for the antiatherosclerotic action of TGF-β is the vascular smooth muscle cell or the macrophage.

We hypothesized that proatherogenic T cells are controlled by immunoregulatory TGF-β, which maintains the T cell population in an inhibited state. To test this, we crossed atherosclerosis-prone E^0 mice with mice carrying a dominant negative TGF-β receptor II in T cells. Our data show that E^0 mice with abrogated TGF-β signaling in T cells develop dramatically increased atherosclerosis. This indicates that TGF-β exerts important antiatherosclerotic activity by inhibiting T cell activation.

Methods

Animals. E^0 mice were obtained from M&B Breeding and Research Center A/S (Ry, Denmark). Transgenic CD4-dnTGFβRIITg+ (CD4dnTβRII) mice were described elsewhere (27). Both strains had been backcrossed ten times to a C57BL/6 background. Genetic screening was performed by PCR using *ApoE* primers oIMR180, oIMR181, and oIMR182, recommended by The Jackson Laboratory (Bar Harbor, Maine, USA), and dnTGFβRII primers 5′-CCCAACCAACAAGAGCTCAAG-3′ and 5′-ACTTGACTGCACCGTTGTTGT-3′. The two strains were crossed and the second generation offspring screened for CD4dnTGβRII+ mice homozygous for E^0. Female E^0 and male $E^0 \times$ CD4dnTβRII+ mice were bred to obtain littermates that were $E^0 \times$ CD4dnTβRII+ and E^0 mice lacking the dominant negative transgene. All mice were fed standard mouse chow and sacrificed under anesthesia. Groups of age-matched, female mice were compared, except when otherwise stated. All studies were approved by the regional ethical committee for animal experiments.

Quantitation of atherosclerosis. The heart and ascending aorta were snap-frozen and cryosections cut from the aortic root using a standardized protocol (28). Eight 10-μm sections were collected at 100-μm intervals starting at a 100-μm distance from the appearance of the aortic valves. Formaldehyde-fixed sections were stained with hematoxylin and oil red-O, and lesion size was analyzed using Leica Q500MC image analysis software. For each mouse, a mean lesion area was calculated from eight sections, reflecting the cross-section area covered by atherosclerosis. Additional sections from the proximal aorta were stained with Masson's trichrome. The descending aorta from arch to trifurcation was fixed in 4% formaldehyde, opened longitudinally, pinned onto black wax plates, and stained with Sudan IV (Merck AG, Darmstadt, Germany). Aortic lesion area and total aortic area were calculated using Leica Q500MC software.

Immunohistochemistry. Acetone-fixed sections of the ascending aorta were stained using rat anti-mouse CD68 (Serotec Ltd., Oxford, United Kingdom), rat anti-mouse VCAM-1, biotinylated mouse anti-mouse I-A^b (both from PharMingen, San Diego, California, USA), rat anti-mouse CD3 (Southern Biotechnology Associates, Birmingham, Alabama, USA), or alkaline phosphatase–conjugated anti-α-smooth muscle actin (Sigma-Aldrich, St. Louis, Missouri, USA). For CD68 and CD3 staining, primary Ab's were followed by biotinylated anti-rat IgG (Vector Laboratories, Burlingame, California, USA). Staining was visualized using biotin-avidin-peroxidase complexes (Vector Laboratories) and diaminobenzidine or a Vector blue alkaline phosphatase substrate kit (Vector Laboratories). For CD3 staining, 0.1% saponin in PBS was used to unmask epitopes. Controls included staining with irrelevant Ab's and omission of primary Ab. Cells were counted at ×400 magnification. For comparative analysis of lesion composition from 12-week-old $E^0 \times$ CD4dnTβRII and 18-week-old E^0 mice, individual lesions between 80,000 and 150,000 μm² were carefully matched for size between groups and compared for histochemical and immunostaining by image analysis using Leica QWin. The difference in mean lesion area between the two groups was never more than 9,000 μm², and sections used for this analysis was selected within plus or minus 100 μm from maximal lesion thickness. Collagen content was assessed by measuring green surface area after Masson's trichrome staining, while cells positive for CD3 and I-A^b were recorded per unit of surface area. Staining with the macrophage marker CD68 was registered as stained surface area rather than number of positive cells, since borders between individual cells could not be identified.

Quantitation of mRNA. Total aortic RNA was isolated from 15-week-old $E^0 \times$ CD4dnTβRII and E^0 mice by using RNeasy (QIAGEN Inc., Valencia, California, USA), reverse-transcribed with Superscript-II (Life Technologies Inc., Rockville, Maryland, USA) and random hexamers, and amplified by real-time PCR using primers and probes for IFN-γ and hypoxanthine guanidine ribonucleosyltransferase (HPRT; sequences available on request) in an ABI 7700 Sequence Detector (Perkin-Elmer Applied Biosystems, Foster City, California, USA). Total RNA was analyzed by BioAnalyzer (Agilent Technologies Deutschland GmbH, Waldbronn, Germany) capillary electrophoresis. Data were expressed as arbitrary units obtained by comparing the threshold cycle value of the test sample with that of a standard curve prepared from spleen mRNA. Data were normalized to the median value for E^0 mice.

Lipids and lipoproteins. Total cholesterol and triglycerides were determined by standard enzymatic meth-

ods (Roche Molecular Biosystems, Indianapolis, Indiana, USA). Size fractionation of lipoproteins was performed by fast-performance liquid chromatography (FPLC) using a 30×0.32 cm Superose 6B micro-FPLC column (Amersham Pharmacia Biotech AB, Uppsala, Sweden) coupled to online cholesterol detection (29). Cholesterol-7α-hydroxylase (CYP7A1) activity was analyzed in liver preparations as described (29).

Serum cytokines. The concentrations of IFN-γ, TNF-α, IL-2, IL-4, and IL-5 were determined by cytofluorometric bead assay (PharMingen) using a FACScalibur flow cytometer as described by the manufacturer. For TGF-β analysis, blood was drawn into tubes with Na-EDTA, theophylline, and PGE$_1$ to prevent platelet degranulation, and plasma concentrations of TGF-β were determined by ELISA (R&D Systems Inc., Minneapolis, Minnesota, USA). β-Thromboglobulin was assessed by ELISA (Roche Diagnostica Stago, Mannheim, Germany) to monitor platelet degranulation and was always less than 25 IU/ml.

FACS analysis. Fresh spleen cells were stained for 30 minutes on ice with FITC-anti-CD4 or FITC-anti-CD8 together with phycoerythrin-anti-CD69 or with appropriate isotype controls (all from PharMingen). An Fc block (PharMingen) was applied before staining. Cells were analyzed with a FACScalibur.

Malondialdehyde-LDL Ab's. Specific Ab's to malondialdehyde-modified (MDA-modified) LDL were quantified using ELISA. LDL was isolated from human plasma and modified with MDA as described (30). Ninety-six–well Maxisorp plates (Nunc A/S, Roskilde, Denmark) were coated overnight at +4°C with 5 μg/ml MDA-LDL in PBS. After washing with PBS plus 0.05% Tween, the infranatant from sera centrifuged at 14,000 *g* was diluted 1:100 in PBS with 1% BSA, added to the plates, and incubated for 3 hours in room temperature. Peroxidase-labeled sheep anti-mouse IgM (The Binding Site Ltd., Birmingham, United Kingdom), rat anti-mouse IgG1 peroxidase (Serotec Ltd.), alkaline phosphatase–conjugated rat anti-mouse IgG2a (PharMingen), peroxidase sheep anti-mouse IgG2b (The Binding Site Ltd.), or alkaline phosphatase goat anti-mouse IgG (Southern Biotechnology Associates) were added after washing and incubated for 2 hours at room temperature. ELISAs with alkaline phosphatase–conjugated Ab's were developed with phosphatase substrate (Sigma-Aldrich) in diethanolamine buffer and read at 405 nm. ELISAs with peroxidase-conjugated Ab's were developed with tetramethylbenzidine substrate (PharMingen) and read at 450 nm.

T cell activation. Spleen cells were incubated for 48 hours with 1.5 μg/ml concanavalin A (Sigma-Aldrich), and 1 μCi ^{3}H-thymidine was added 18 hours before harvesting cells onto filter mats. DNA synthesis was determined in a Wallac (Turku, Finland) Microbeta counter. Culture supernatants were analyzed for IFN-γ, IL-4, and IL-10 using ELISA (OptEIA; PharMingen).

Statistics. The nonparametric Mann-Whitney significance test was used for analysis of data.

Results

The T cell response to TGF-β is abrogated in the CD4-dnTGF-βRII transgenic mouse (CD4dnTβRII), which expresses a dominant negative TGF-β receptor under the CD4 promoter (27). TGF-β signaling in all T cells is blocked because the CD8 silencer has been removed from the CD4 promoter. We crossed this mouse with the E^0 mouse, which spontaneously develops hypercholesterolemia and atherosclerosis. At 12 weeks of age, no significant differences were observed between E$^0 \times$ CD4dnTβRII and E^0 mice with regard to body weight (20.1 ± 0.4 versus 20.0 ± 0.4 g). Histopathological examination of the large intestine
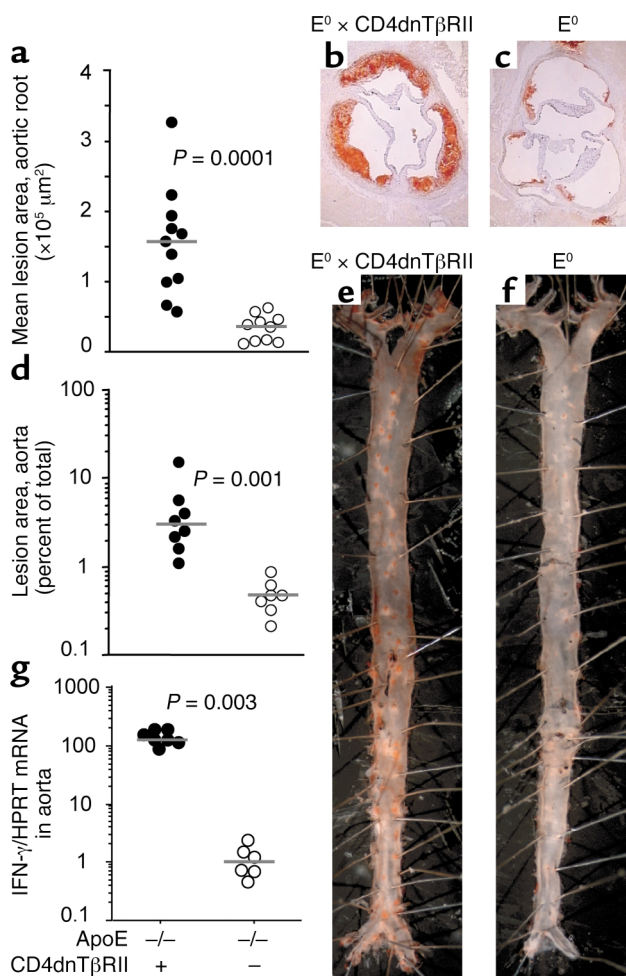

Figure 1
Abrogation of TGF-β signaling in T cells increases atherosclerosis and IFN-γ expression in age-matched mice. (**a**) Lesion size in the aortic root ($\times 10^5$ μm^2) in 12-week-old mice. Individual values are displayed by dots, and medians for each group are indicated by horizontal lines. Representative micrographs showing hematoxylin/oil red-O–stained lesions in E$^0 \times$ CD4dnTβRII (**b**) and E^0 (**c**) mice. Original magnification ×50. (**d**) Lipid lesion area in the entire aorta (percentage of total aortic surface area); dot plots as above. Sudan IV–stained en face preparations of aortas from E$^0 \times$ CD4dnTβRII (**e**) and E^0 (**f**) mice. (**g**) IFN-γ mRNA levels in lesions of E$^0 \times$ CD4dnTβRII and E^0 mice. Data are expressed as arbitrary units as described in Methods; dot plots as above.
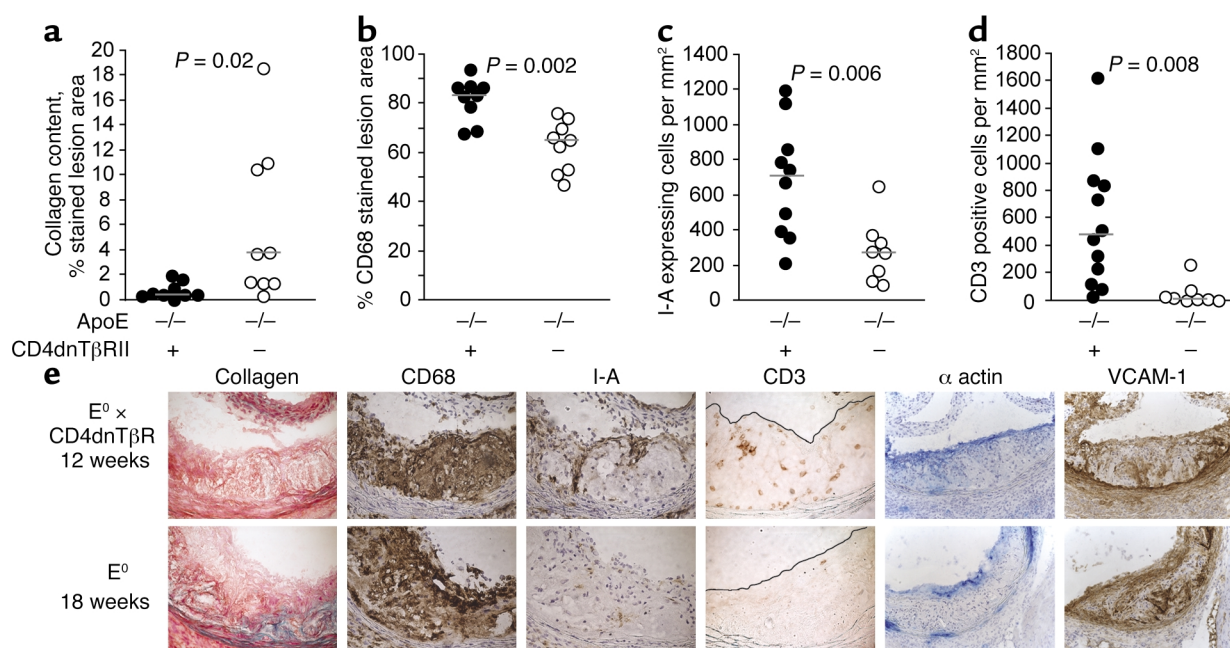
Figure 2
Lesion composition in 12-week-old E^0 mice with abrogated TGF-β signaling in T cells. Comparison with size-matched lesions of 18-week-old E^0 mice. (**a**) Collagen content (collagen-stained cross-section surface/total lesion surface). (**b**) Area occupied by $CD68^+$ macrophages (CD68-stained surface/total lesion surface). (**c**) I-A$^+$ cells per lesion cross-section area. (**d**) $CD3^+$ T cells per lesion cross-section area. (**e**) Representative micrographs showing Masson´s trichrome stain (collagen in green; original magnification ×400), immunohistochemical staining for CD68, I-A, and CD3 (all ×400), and immunohistochemical staining for α-smooth muscle actin (α actin) and VCAM-1 (×200). Lesion contours are outlined in the CD3-stained micrograph.

showed modest infiltration of inflammatory cells in three of nine 12-week-old $E^0 \times CD4dnTβRII$ mice, but there were no clinical signs of inflammatory bowel disease at this age. From 18 weeks of age, body weight was lower than for E^0 mice, and the large intestine showed signs of colitis.

Disrupted TGF-β signaling in T cells leads to increased atherosclerosis. $E^0 \times CD4dnTβRII$ mice exhibited a dramatic increase in atherosclerotic lesion size in the aortic root, visualized by oil red-O staining (Figure 1, a–c). Lesions were substantially thicker and more advanced in 12-week-old $E^0 \times CD4dnTβRII$ mice when compared with E^0 mice of the same age with intact TGF-β signaling in T cells. The extent of atherosclerosis in the entire aorta was quantified in Sudan IV–stained en face preparations of aortas. This analysis revealed a sixfold increase in the lipid lesion area in $E^0 \times CD4dnTβRII$ mice (Figure 1, d–f).

Increased IFN-γ expression in lesions of $E^0 \times CD4dnTβRII$ mice. Activated Th1 cells promote macrophage activation and inflammation by secreting IFN-γ. To directly test the hypothesis that the expression of this cytokine is increased in lesions of $E^0 \times CD4dnTβRII$ mice, we performed real-time RT-PCR on RNA from such atherosclerotic aortas. This analysis revealed a dramatic, 125-fold increase in IFN-γ mRNA in mice with disrupted TGF-β signaling in T cells (Figure 1g).

Vulnerable and inflammatory lesion phenotype in $E^0 \times CD4dnTβRII$ mice. To evaluate whether disrupted TGF-β

signaling in T cells affected the composition as well as size of lesions, lesions from $E^0 \times CD4dnTβRII$ and E^0 mice were characterized. Since lesions were much larger and more advanced in the $E^0 \times CD4dnTβRII$ mice and could not be compared with lesions of the same size in age-matched E^0 mice, we compared lesions of 12-week-old $E^0 \times CD4dnTβRII$ mice with those of 18-week-old E^0 mice. The latter contain aortic root lesions of approximately the same size as those found in 12-week-old $E^0 \times CD4dnTβRII$ mice. Disrupted TGF-β signaling in T cells was associated with reduced collagen staining (Figure 2, a and e). Importantly, reduced collagen in lesions has been linked to plaque vulnerability and a propensity for thrombosis (31). In contrast, the proportion of lesion area stained by the macrophage marker CD68 was higher in $E^0 \times CD4dnTβRII$ mice (Figure 2, b and e). Similarly, the number of $CD3^+$ T cells was increased in lesions of these mice (Figure 2, d and e). The macrophage-activating cytokine IFN-γ is secreted by activated T cells and induces I-A expression in target cells. A higher number of cells expressed I-A in lesions of $E^0 \times CD4dnTβRII$ mice (Figure 2, c and e). Taken together, these data suggest that the lack of TGF-β signaling in T cells leads to accelerated atherosclerosis with the formation of large, vulnerable plaques.

The leukocyte adhesion molecule, VCAM-1, which can mediate T cell recruitment, was expressed in lesions of both $E^0 \times CD4dnTβRII$ and E^0 mice (Figure 2c). Its distribution was compatible with expression by smooth

muscle cells and macrophages, as previously reported (32, 33). This pattern did not differ between lesions of E⁰ × CD4dnTβRII mice and those with similar size from E⁰ mice. Similarly, the distribution of α-smooth muscle actin did not differ between groups (Figure 2c).

Effects on lipids and lipoproteins. To assess whether the effects on atherosclerosis were due to changes in lipid metabolism, we analyzed serum lipids and lipoproteins. Surprisingly, total serum cholesterol was significantly lower in E⁰ × CD4dnTβRII mice (13.0 ± 0.6 versus 17.7 ± 0.9 mmol/l in E⁰ mice), while serum triglycerides did not differ between groups (1.0 ± 0.1 versus 1.1 ± 0.1 mmol/l). Separation of serum lipoproteins by FPLC showed a reduced chylomicron/VLDL fraction, whereas LDL and HDL remained unchanged (Figure 3a). Cholesterol-7α-hydroxylase activity in the liver did not differ between groups (data not shown). It is therefore unlikely that the reduced serum cholesterol levels were due to hepatic or ileal dysfunction with concomitant effects on cholesterol clearance.

Increased Ab response to modified LDL in E⁰ × CD4dnTβRII mice. Atherosclerosis is associated with Ab formation to oxidatively modified LDL. Such modifications include MDA conjugation of the LDL protein (34). Anti–MDA-LDL Ab's of all isotypes were increased in E⁰ × CD4dnTβRII mice (Figure 3b), with no apparent bias toward any T helper–dependent isotype. The finding of elevated anti–MDA-LDL is compatible with an increased antigen-specific immune response.

Increased systemic immune activity in E⁰ × CD4dnTβRII mice. Analysis of spleen cells revealed increased T cell activation and cytokine secretion in E⁰ × CD4dnTβRII mice. Thus, a higher proportion of T cells expressed the activation marker CD69 (Figure 4, a and b), and mRNA was increased for the T cell cytokines, IFN-γ and IL-4 (data not shown). Similarly, larger amounts of IFN-γ, IL-4, and IL-10 were secreted after mitogen stimulation of T cells from E⁰ × CD4dnTβRII compared with E⁰ mice (Figure 4, d–f). In contrast, proliferation and IL-2 secretion of activated T cells was

reduced in the former group, probably because the enhanced activation recruited a larger proportion of T cells from the naive, highly proliferative, and IL-2–secreting population into the memory effector subsets (Figure 4c and data not shown).

Serum levels of cytokines were determined by cytometric bead arrays. As shown in Figure 4, g–i, IFN-γ, TNF-α, and IL-5 were increased in E⁰ × CD4dnTβRII mice when compared with E⁰ mice. This indicates that the increased cytokine expression in immune cells resulted in elevated systemic steady-state levels of these molecules. No significant changes were registered, however, for IL-2 or IL-4 (Figure 4j and data not shown). Similarly, plasma levels of TGF-β did not differ between groups (7.63 ± 1.66 versus 5.62 ± 0.82 ng/ml).

Disruption of the ApoE gene leads to increased disease-related immune response but does not affect cytokine levels in CD4dnTβRII mice. In the CD4dnTβRII mice, T cell activation is antigen driven rather than spontaneous (17). To evaluate the disease-related immune response in E⁰ × CD4dnTβRII mice, Ab titers to MDA-LDL were compared with those in CD4dnTβRII mice with intact *ApoE* genes. Sera were harvested from 24-week-old mice, permitting the development of autoimmune disease and severe atherosclerosis, respectively. Anti–MDA-LDL titers were significantly increased in E⁰ × CD4dnTβRII mice when compared with those in CD4dnTβRII mice with intact *ApoE* genes and with those in E⁰ mice with functional TGF-β receptors in T cells (Figure 5a), implying an increased antigen-specific, disease-related immune response in the E⁰ × CD4dnTβRII mice. It remains to be determined to what extent the effects on lesion formation are due to immune responses to modified lipoproteins.

To assess whether the changes in circulating cytokines were related to the increased immune activity due to disruption of TGF-β signaling in T cells or to the atherosclerotic disease, we compared serum levels between 24-week-old E⁰ × CD4dnTβRII mice, CD4dnTβRII mice with intact *ApoE* genes, and E⁰
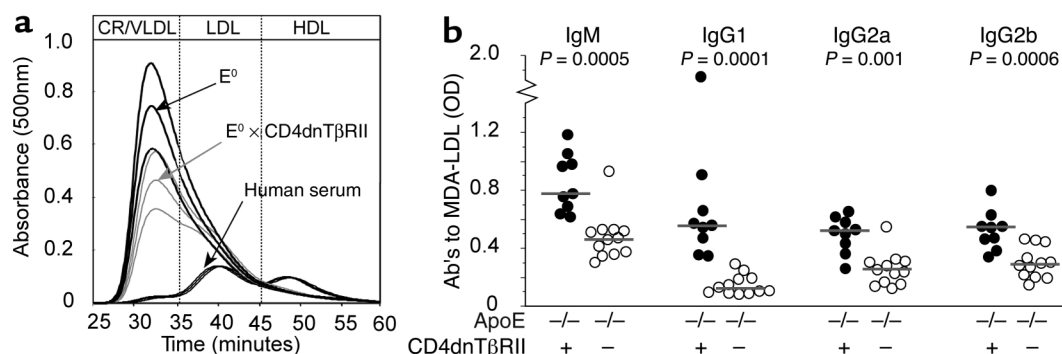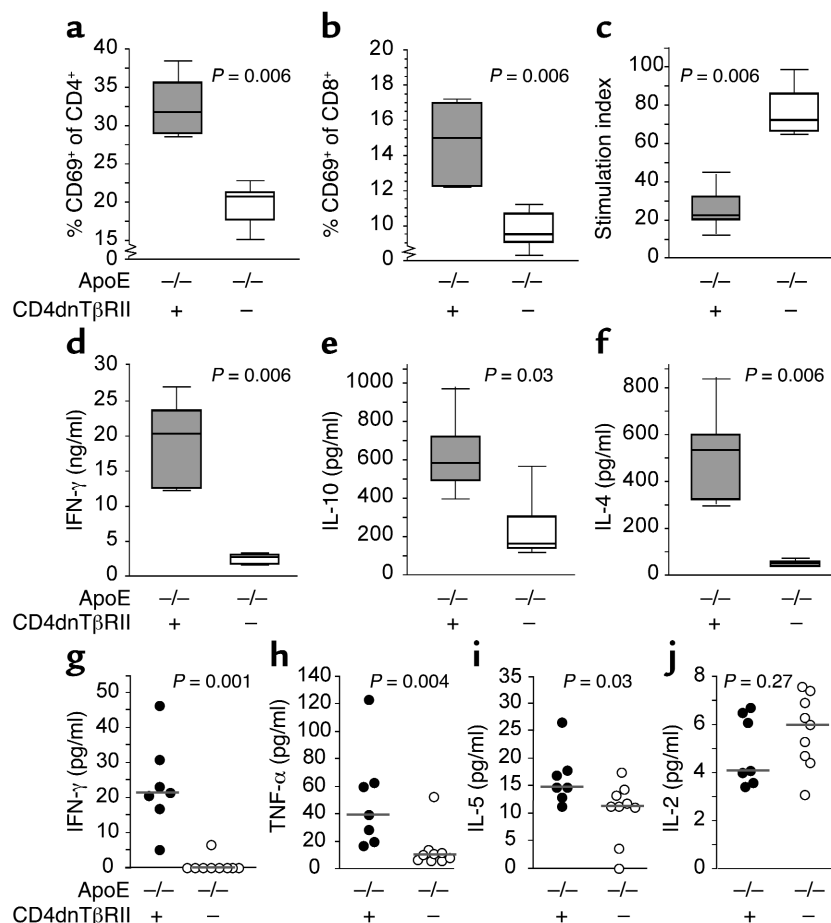

Figure 3
Effects of abrogated TGF-β signaling on serum lipoproteins and Ab's to modified LDL. (**a**) FPLC analysis of lipoprotein profiles from sera of 12-week-old mice. The cholesterol concentration in each fraction (*y* axis) is plotted against retention time (*x* axis); curves show mean ± SD for E⁰ × CD4dnTβRII (gray line) and E⁰ (black line) sera (*n* = 9 per group). The lipoprotein profile of a human serum is plotted for reference. CR, chylomicron remnants. (**b**) Titers of IgM, IgG1, IgG2a, and IgG2b Ab's to MDA-LDL (OD values of ELISA) from 12-week-old E⁰ × CD4dnTβRII and E⁰ mice; individual values for each mouse and medians for each group are displayed.

Figure 4

Effects of abrogated TGF-β signaling on T cell activation and cytokine secretion. (**a**,**b**) FACS analysis of the activation marker CD69 on CD4⁺ (**a**) and CD8⁺ (**b**) spleen T cells from 12-week-old $E^0 \times$ CD4dnTβRII ($n = 6$, gray boxes) and E^0 mice ($n = 5$, white boxes). Box plots (median, quartiles, tenth, and ninetieth percentiles) show percentage of CD69⁺ among all CD4⁺ or CD8⁺ T cells; staining for isotype control is subtracted. Experiments were repeated twice. (**c**–**f**) Cell proliferation and cytokine secretion after in vitro stimulation of spleen cells from 12-week-old $E^0 \times$ CD4dnT ($n = 6$) and E^0 ($n = 5$) mice. (**c**) Stimulation index after ³H-thymidine incorporation. Concentrations of (**d**) IFN-γ, (**e**) IL-10, and (**f**) IL-4 in supernatants 48 hours after stimulation of spleen cells with the T cell mitogen, concavalin A. Box plots as above. (**g**–**j**) Concentrations of IFN-γ (**g**), TNF-α (**h**), IL-5 (**i**), and IL-2 (**j**) were determined in sera from 12-week-old $E^0 \times$ CD4dnTβRII and E^0 mice by using cytofluorometric bead assays. Dot plots show values for individual mice and medians for each group.

mice with functional TGF-β receptors in T cells. While the levels of IFN-γ, TNF-α, and IL-5 were significantly higher in $E^0 \times$ CD4dnTβRII than in E^0 mice, they did not differ between $E^0 \times$ CD4dnTβRII and ApoE-competent CD4dnTβRII mice (Figure 5, b–d). IL-2 did not differ between any of the groups (Figure 5e), and IL-4 was undetectable (data not shown). Therefore, the increase in circulating cytokines was due to the lack of TGF-β regulation of T cells rather than the atherosclerotic process.

Discussion

The present findings point to an important role for TGF-β as an immunomodulating cytokine that dampens atherosclerosis by regulating T cell activation. Abrogation of TGF-β signaling in T cells in E^0 mice led to (a) increased atherosclerosis, (b) increased IFN-γ expression in lesions, (c) reduced plaque collagen and a plaque morphology compatible with increased vulnerability, (d) increased Ab's to oxidized LDL, and (e) increased T cell activation and cytokine secretion. These data lend new support to the notion that activated T cells are proatherogenic and strongly suggest that TGF-β inhibits atherosclerosis by dampening proatherogenic T cells. This hypothesis is illustrated in Figure 6. Downstream pathogenic mechanisms induced by activated T cells may include increased

plaque inflammation, increased lipoprotein uptake via scavenger and Fc receptors, and increased plaque vulnerability due to protease secretion, tissue factor expression, reduced smooth muscle proliferation, and inhibited collagen synthesis (5, 31).

Several studies have provided evidence for a role of TGF-β in atherosclerosis. Plaque-infiltrating lymphocytes display low or undetectable levels of TGF-β receptors (25). Smooth muscle cells derived from human plaques exhibit resistance to the antiproliferative and apoptotic effects of TGF-β and may contain acquired mutations in TGF-β receptor-II (24, 35). Mice heterozygous for a targeted TGFβ1 allele have an increased tendency to fatty streak formation upon challenge with a fatty diet (36). In line with this, the estrogen receptor agonist, tamoxifen, which has TGF-β–inducing effects, suppressed fatty streak formation in fat-fed mice (18). Administration of soluble TGF-β receptors to E^0 mice reduced lesion size and increased the number of inflammatory cells (21) while neutralizing TGF-β Ab's increased plaque size and intraplaque inflammation (22). This was thought to be due to effects on lesion cells by TGF-β, which modulates vascular cell proliferation, stimulates collagen production, suppresses macrophage activation, and modulates scavenger receptor expression (37, 38). While all these effects of TGF-β may be significant, the present results show that inhibition of T cell
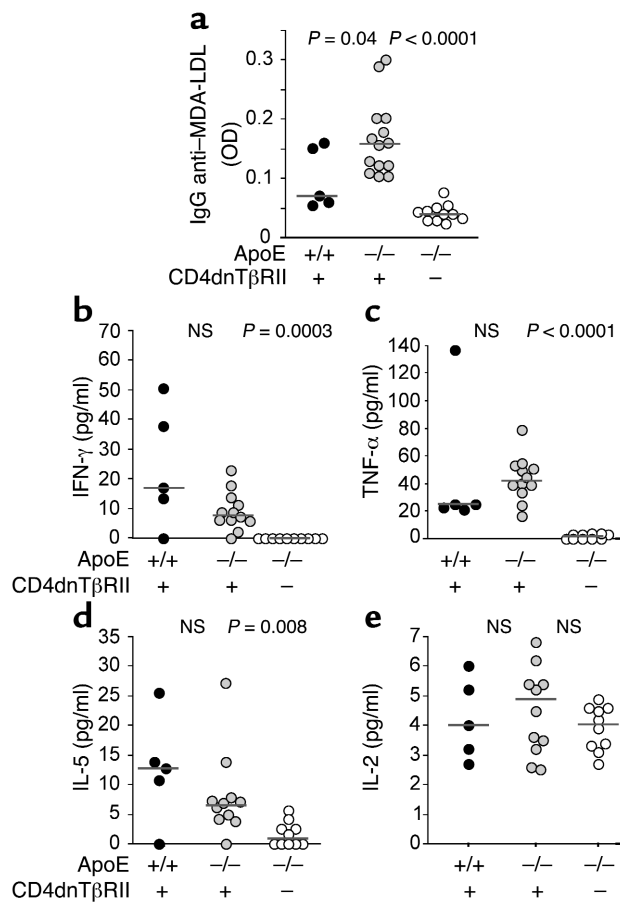
Figure 5
Serum anti–MDA-LDL Ab's and cytokines in mice with ApoE deficiency and/or abrogated TGF-β signaling in T cells. (**a**) Titers of IgG Ab's to MDA-LDL in 24-week-old male mice carrying intact or targeted *ApoE* genes, dominant negative CD4dnTβRII genes, or both. OD values of ELISA, dot plots with values for individual mice, and medians per group. (**b**–**e**) Cytokine concentrations of IFN-γ, TNF-α, IL-5, and IL-2 in sera from mice with intact or targeted *ApoE* genes, dominant negative CD4dnTβRII, or both, as determined by cytofluorometric bead assay. Dot plots as above.

bystander suppression by T$_{reg's}$ specific for other antigens could also play a role, and it is likely that TGF-β production by cells other than T$_{reg's}$ are as important for modulating proatherogenic T cell activation.

Mediators produced by Th1 cells are present in human atherosclerosis and exacerbate disease in animal models (5). Gene-targeted deletion of IFN-γ receptors, IL-18, or CD154, as well as blocking Ab's to CD154, attenuate disease, while injection of recombinant IFN-γ or IL-18 aggravates it (11–13, 40–43). Similarly, treatment with pentoxifyllin, which inhibits Th1 development, reduces atherosclerosis in E^0 mice (44). The pathway(s) causing immune-dependent reduction of disease have been unclear, however, and recent studies suggest that the Th2 cytokine, IL-4, may also have proatherogenic properties (45). In the present study, both Th1 and Th2 activity were increased in E^0 cells × CD4dnTβRII mice when compared with E^0 mice with intact TGF-β signaling. The effect on atherosclerosis was therefore not due to a switch of the Th1/Th2 balance. The net effect of the increased T cell activity was a dramatic acceleration of atherosclerosis despite a concomitant reduction of serum cholesterol levels. Switching the balance from Th1 to Th2 activity may therefore not be sufficient to attenuate atherosclerosis. Instead, it may be desirable to suppress T cell activation.

A comparison between atherosclerotic lesions from E^0 × CD4dnTβRII and E^0 mice revealed increased amounts of macrophages and T cells as well as increased I-A expression and reduced collagen content. Furthermore, IFN-γ expression was dramatically

activation is an important pathway through which TGF-β inhibits atherosclerosis.

Immunomodulating TGF-β may be derived from several sources. TGF-β expressed by T$_{reg's}$ is suggested to play an important role in controlling T cell activation, but TGF-β secreted from other cells is also likely to contribute (17, 39). In addition, T$_{reg's}$ produce other immunomodulating cytokines in addition to TGF-β, in particular IL-10, which is also atheroprotective (19–20). Since T$_{reg}$ activation is antigen specific, presentation of disease-associated antigens to T$_{reg's}$ might dampen activation of proinflammatory Th1 cells. In addition,
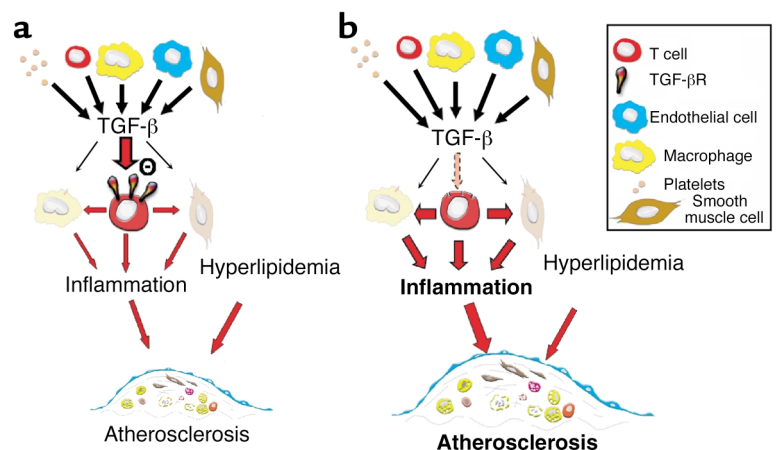

Figure 6
Proposed mechanism by which T cell–dependent inflammation promotes atherosclerosis but is dampened by TGF-β (**a**). The lack of functional TGF-β receptors on T cells abrogates the modulatory action of this cytokine, leading to aggravated atherosclerosis in E^0 × CD4dnTβRII mice (**b**). TGF-βR, TGF-β receptor.

increased in E⁰ × CD4dnTβRII mice. Taken together, these findings point to a more vulnerable lesion phenotype in the absence of TGF-β signaling in T cells. Since IFN-γ is produced by activated T cells, induces I-A expression and macrophage activation, and reduces collagen production, this cytokine may play a key role for the aggravation of atherosclerosis in this model. The dramatic increase in T cells could reflect increased recruitment as well as local clonal expansions, which have been detected in E⁰ mice (46).

The importance of specific antigens in atherosclerosis remains controversial. Although several studies have demonstrated that lesions contain antigen-specific T cells and that immunization with specific antigens can protect against disease (4, 5, 7–9), others have shown that antigen-independent pathways may also cause inflammatory activation in lesions. The latter include signaling through Toll-like receptor ligation (47, 48) and production of inflammatory mediators by nonimmune cells (49, 50). In the CD4dnTβRII model employed in the present study, T cell activation is antigen driven rather than spontaneous, since no activation of T cells takes place in the absence of antigen (17). Therefore, disease-related antigens may be responsible for the increased atherosclerosis seen in the E⁰ × CD4dnTβRII mouse. In support of this, Ab titers to the disease-associated antigen MDA-LDL were significantly increased in the E⁰ × CD4dnTβRII animals when compared with ApoE+/+ CD4dnTβRII mice as well as TGFβRII-competent E⁰ mice. Further studies will be needed to determine the role of antigen-specific T cells and Ab's in this model. Our data imply that loss of TGF-β regulation of T cells, however, aggravates the immune process in atherosclerotic vascular disease.

To summarize, we have shown that abrogation of TGF-β signaling in T cells increases atherosclerosis in hypercholesterolemic E⁰ mice. These findings imply that activated T cells are strongly proatherogenic, but controlled by TGF-β. They point toward a new strategy for development of antiatherosclerotic therapy by targeting TGF-β signaling in T cells.

Note added in proof. At the time that this paper was accepted for publication, a report by Gojova et al. appeared in preliminary form on the webpage of *Blood* (51). The report shows that the transfer of bone marrow containing T cells with defective TGRβ receptors increases lesion vulnerability in irradiated *ldlr⁻/⁻* mice.

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