Intranasal Immunization With an Apolipoprotein B-100 Fusion Protein Induces Antigen-Specific Regulatory T Cells and Reduces Atherosclerosis

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Objective—Atherosclerosis is an inflammatory disease. Autoimmune responses to low-density lipoproteins (LDL) contribute to its progression, whereas immunization with LDL may induce atheroprotective or proatherogenic responses. The objective of this study was to develop an atheroprotective vaccine by targeting a peptide of the LDL protein constituent apolipoprotein B-100 (apoB-100) to the nasal mucosa to induce a protective mucosal immune response.

Methods and Results—A peptide comprising amino acids 3136 to 3155 of apoB-100 (p210) was fused to the B subunit of cholera toxin (CTB), which binds to a ganglioside on mucosal epithelia. The effect of nasal administration of the p210-CTB fusion protein on atherogenesis was compared with that of an ovalbumin peptide fused to CTB and with untreated controls. Immunization with p210-CTB for 12 weeks caused a 35% reduction in aortic lesion size in Apoe−/− mice. This effect was accompanied by induction of regulatory T cells that markedly suppressed effector T cells rechallenged with apoB-100 and increased numbers of interleukin (IL)-10+ CD4+ T cells. Furthermore, a peptide-specific antibody response was observed. Atheroprotection was also documented in Apoe−/− mice lacking functional transforming growth factor-β receptors on T cells.

Conclusion—Nasal administration of an apoB-100 peptide fused to CTB attenuates atherosclerosis and induces regulatory T1 cells that inhibit T effector responses to apoB-100. (Arterioscler Thromb Vas Biol. 2010;30:946-952.)

Key Words: atherosclerosis ■ immune system ■ lipoproteins ■ regulatory T cells ■ vaccination

Preventing the clinical manifestations of atherosclerosis remains a major challenge despite the success of current statin therapy.1 Lipid retention and modification in the arterial intima elicit a chronic inflammatory process with autoimmune responses and the development of atherosclerotic lesions.2 Both adaptive and innate immune mechanisms contribute to this process.3–6 Although pattern recognition receptors of innate immunity account for cholesterol uptake and contribute to activation of macrophages and endothelial cells, antigen-specific T cells recognizing low-density lipoprotein (LDL) particles in the intima provide strong proinflammatory stimuli that accelerate atherogenesis. Accordingly, immunomodulatory strategies have shown remarkable effects in animal models of atherosclerosis.

Antigen-specific immunomodulation by vaccination is an attractive approach to prevent or treat chronic inflammatory diseases. By mobilizing protective immune responses in an antigen-specific manner, side effects due to hampered host defense against infections are avoided. Therefore, antigen-specific suppression of pathological autoimmunity is of interest in chronic inflammatory diseases such as atherosclerosis. Indeed, several studies have demonstrated beneficial effects on atherosclerosis in mice and rabbits immunized with LDL, β2-glycoprotein-1b, or heat-shock protein 60/65, and parenteral7–10 as well as oral11–14 immunization reduced atherosclerotic disease in hyperlipidemic animals. However, progress has been hampered by a limited understanding of the mechanisms through which immunization inhibits the disease process.

Antigen-specific immunoprotection can be achieved through several different mechanisms, such as production of protective antibodies, deletion or inactivation (anergy) of pathogenic T-cell clones, or induction of suppressive cellular immunity mediated by the family of regulatory T cells (Treg).15,16 Although antibodies to LDL components are formed on immunization, their titers or isotypes correlate
only partially to the protective effect. Cellular immunity appears also to be targeted, but it remains unclear whether it is specific to LDL antigens. Finally, several LDL components are bioactive and proinflammatory and may, when injected, cause undesirable local or systemic effects. Therefore, immunization with immunodominant peptide sequences derived from apolipoprotein B-100 (apoB-100) should be an attractive alternative to immunization with LDL particles.17,18

An immunization protocol that facilitates selective targeting of antigen-specific Treg would constitute a major step forward in the development of a vaccine against atherosclerosis. The type of immune response triggered is largely determined by the route of immunization. Subcutaneous antigen administration often leads to inflammatory responses. In contrast, the mucosal linings of airways and intestines contain lymphatic tissue that, when exposed to antigen, elicits antiinflammatory, immunosuppressive responses.19 Distinct immunologic features of the respiratory and intestinal mucosa lead to partly different types of protective immunity on antigen exposure by the nasal or oral route.20 The B subunit of cholera toxin (CTB) promotes uptake of antigen via the nasal and oral mucosa and induction of protective immunity.21 Therefore, immunization with CTB-antigen conjugates has evolved as a promising therapeutic strategy in a variety of autoimmune diseases, including a first human phase II trial in Behcet disease.22

In this study, we present a novel strategy for immunoprotection against atherosclerosis by nasal administration of an apoB-100 peptide-CTB fusion protein (p210-CTB). This treatment significantly reduced atherosclerosis in apoe−/− mice and was associated with induction of antigen-specific Treg activity.

Methods

For a detailed description of the methods please see the supplemental material, available online at http://atvb.ahajournals.org.

A recombinant protein, p210-CTB, was made from amino acids 3136 to 3155 of human apoB-100 (p210) fused with CTB. This sequence is identical to the corresponding murine sequence with the exception of a 2-residue insert at the C-terminal end in the mouse (see online supplement). As a control, amino acids 323 to 339 of ovalbumin were fused to CTB (OVA-CTB). Eight-week-old female apoe−/− mice received a nasal spray with 15 µg (in 15 µL) p210-CTB or OVA-CTB twice weekly. Lesions and immune parameters were analyzed 12 weeks later. In another set of experiments, apoe−/− × CD4dnTGFbRII mice23 were immunized using the same protocol. All experiments were approved by the Stockholm regional ethical board. Atherosclerotic lesions were analyzed in cryostat sections of the aortic root using a standardized protocol.24 Antibodies to p210 and to mouse LDL particles were analyzed by immunometric ELISA.17 Other assays were performed as described in the online supplement.

Antigen-specific Treg activity was analyzed in the following way: apoe−/− mice were immunized subcutaneously with apoB-100 to generate effector T cells. CD4+ T cells from these mice were exposed to antigen and activation recorded as DNA synthesis. CD4+ T cells from apoe−/− mice immunized intranasally with p210-CTB were added to effector T-cell preparations, and Treg activity was recorded as inhibition of DNA synthesis (see online supplement). Intracellular staining was performed on CD4+ T cells to characterize cytokine production and T-cell subtype.

Figure 1. Intranasal p210-CTB administered twice weekly for 12 weeks reduced atherosclerotic lesion size in the aortic root of female apoe−/− mice. A, Data from the 3 groups are shown: black circles represent animals from the p210-CTB group, gray circles represent animals from the OVA-CTB group, and white circles represent animals from the control group. *P<0.05. B through D, Representative photomicrographs show oil red O-stained aortic root sections from each group (magnification ×50); E, Nasal immunization with CTB fusion proteins increased FoxP3 and IL-10 mRNA levels in thoracic aorta from apoe−/− mice after 12 weeks of treatment. mRNA transcript ratios based on hypoxanthine phosphoribosyltransferase expression are shown for each gene of interest for all 3 groups. *P<0.05.

Results

Nasal Administration of p210-CTB Inhibits Atherosclerosis

Nasal immunization with p210-CTB caused a significant 35% reduction in atherosclerotic lesion size (P=0.015; P=0.039) and fractional lesion area (P=0.012; P=0.007) in the aortic root as compared with OVA-CTB and untreated controls, respectively (Figure 1A through 1D; Supplemental Figure I). Atherosclerosis was not attenuated by administration of OVA-CTB compared with untreated controls, indicating an apoB-100 peptide-specific effect (Figure 1A through 1D). The composition of the lesions was not significantly altered by p210-CTB immunization, as indicated by quantitative immunohistochemical analysis of markers for CD4+ T cells, macrophages (CD68), or the inducible surface proteins I-Aβ (major histocompatibility complex class II protein) and the vascular cell adhesion molecule-1 (Supplemental Table I).
Nasal Administration of p210-CTB Does Not Affect Plasma Lipids
Immunization did not significantly affect body weight, serum cholesterol, or triglycerides (Supplemental Table II). Plasma lipoprotein profiles were similar in mice immunized with p210-CTB or OVA-CTB, respectively (Supplemental Figure II).

CTB Fusion Protein Immunization Increases Aortic FoxP3 and IL-10 mRNA Levels
Real-time RT-PCR analysis of the thoracic aorta of apoe−/− mice showed significant increases in FoxP3 and IL-10 mRNA levels in both CTB vaccine groups (p210-CTB and OVA-CTB) (Figure 1E). No statistically relevant differences in FoxP3 or IL-10 mRNA were detected when comparing mice that had received p210-CTB with those that had received OVA-CTB, respectively. Furthermore, the numbers of FoxP3+ cells in aortic lesions did not differ between the groups (Supplemental Figure III). IL-10 was elevated to the same extent in the p210-CTB and OVA-CTB groups, pointing to a possible adjuvant effect of CTB. Trends toward increased transforming growth factor-β (TGF-β) and decreased interferon (IFN)-γ mRNA in vaccinated mice were not significant.

Nasal Vaccination Induces Mucosal and Systemic Humoral and Cellular Immune Responses
P210-CTB immunization induced significantly elevated titers of IgG antibodies to the p210 peptide of apoB-100 (Figure 2A). Modestly increased IgG antip210 was observed in OVA-CTB-immunized apoe−/− mice. The IgG1/IgG2a ratio of antip210 antibodies did not change, implying that there was no Th1/Th2 shift in T helper activity to B cell activation (Supplemental Figures IV and V). Total IgG levels were not influenced by either treatment (Supplemental Figure VI). p210-specific IgM titers did not differ between p210-CTB and OVA-CTB treated groups; however, they were significantly elevated compared with untreated animals (Figure 2B). Total IgM was not influenced by either treatment (Supplemental Figure VII).

Sera of immunized mice were tested for antibodies to mouse LDL particles; however, ELISA did not show any such titers (data not shown). Therefore, antibodies induced to human p210 did not recognize intact, endogenous LDL particles in the immunized mice. Apoe−/− mice immunized with OVA-CTB showed modestly increased titers to p210 (Figure 2). To rule out the possibility of a cross-reaction between the OVA peptide and p210, we performed ELISA analysis of anti-LDL reactivity in wild-type C57BL/6 mice.
immunized with OVA-CTB. It showed no significant titers to mouse LDL (Supplemental Figure XIII).

Analysis of the cellular immune response in the lung, the major organ targeted after nasal vaccination, showed a significant decrease in CD4\(^{+}\)/H11001 T cells expressing IFN-\(\gamma\) (characteristic of Th1 cells) and IL-17 (characteristic of Th17 cells), respectively, in mice treated with p210-CTB (Figure 3A and 3B). In contrast, no such change was recorded for IL-4\(^{+}\)/H11001 T cells or for FoxP3\(^{+}\) CD4\(^{+}\) T cells (Figure 3C and 3D). This indicates a shift of the T helper cell balance in the respiratory mucosa, away from the proinflammatory Th1 and Th17 subtypes after nasal immunization with p210-CTB.

Systemic cellular immune responses were monitored in spleen cell preparations. Nasal immunization with p210-CTB significantly increased the proportion of spleen CD4\(^{+}\)/H11001 T cells expressing the antiinflammatory cytokine IL-10 (Figure 4A and 4B). Unlike the situation in the lung, no significant differences were detected in the distribution of the remaining CD4\(^{+}\)/H11001 T-cell subsets in the spleen, as characterized by intracellular staining for IFN-\(\gamma\), IL-17, IL-4, and FoxP3 (Supplemental Figure VIII).

**p210-CTB Treatment Induces ApoB-100-Specific Treg Activity**

To assess whether functional Treg were induced by immunization, we exposed spleen CD4\(^{+}\)/H11001 T cells from apoB\(^{-/-}\)/H11002 mice immunized subcutaneously with human apoB-100 (effector T cells) to CD4\(^{+}\)/H11001 T cells from mice immunized nasally with either p210-CTB, OVA-CTB, or no antigen (Figure 4C). A marked dose-dependent inhibition of effector T-cell proliferation was observed in the presence of CD4\(^{+}\)/H11001 cells from p210-CTB-immunized mice. No such inhibition was observed when T cells from OVA-CTB-immunized or nonimmunized mice were added. The inhibitory effect of T cells from p210-CTB-immunized mice was abolished when these cells were separated from effector T cells by a membrane, indicating that suppression required cell-cell contact (Supplemental Figure IX). Levels of IL-10 and TGF-\(\beta\) in culture supernatants did not differ between groups (Supplemental Figure X).

**The Atheroprotective Effect of Nasal p210-CTB Vaccination Is Independent of TGF-\(\beta\) Signaling in T Cells**

To determine whether the atheroprotective effect of nasal vaccination with p210-CTB depended on TGF-\(\beta\) signaling in T cells, we immunized apoB\(^{-/-}\)/H11002 mice lacking functional TGF-\(\beta\) receptors on T cells (Apoe\(^{-/-}\)/H11002/CD4dnTGF\(\beta\)RII mice). Nasal immunization with p210-CTB significantly reduced atherosclerotic lesion size by 30% in Apoe\(^{-/-}\)/CD4dnTGF\(\beta\)RII mice, as compared with littermates immunized with OVA-CTB (Figure 5A; Supplemental Figure XI). This indicates that TGF\(\beta\)R signaling in T cells is not required for the atheroprotective effect of nasal p210-CTB vaccination. It also argues against a decisive role for FoxP3\(^{+}\) Treg, as these cells are thought to require TGF\(\beta\) for their function.
IgG but not IgM antibodies directed against the apoB-100-peptide were significantly elevated in all groups of mice immunized with p210-CTB, irrespective of whether signaling via TGF-β was blocked during immunization (Figure 5B and 5C). Analysis of mRNA expression in aortas showed no differences in mRNA for IL-10, TGF-β, or IFN-γ between p210-CTB- and OVA-CTB-vaccinated mice (Supplemental Figure XII). Surprisingly, FoxP3 mRNA was elevated in Apoe<sup>−/−</sup>×CD4<sup>d</sup>mTGFβRII mice compared with Apoe<sup>−/−</sup> mice, possibly reflecting the presence of immature Treg in the former mice (Supplemental Figure XII; Supplementary Figure 1E). Furthermore, quantitative analysis of immunohistochemical staining showed no differences in the cellular composition of lesions between treatment groups (Supplemental Table III).

**Discussion**

This study presents a novel strategy for induction of atheroprotective immunity involving antigen-specific Treg. By nasal administration of a fusion protein between an immunodominant peptide of apoB-100 and immunomodulatory CTB, we were able to induce an atheroprotective immune response to apoB-100 that involved expansion of antigen-specific CD4<sup>+</sup> Treg and inhibition of aortic lesion development.

Induction of antigen-specific Treg has not been described in studies of atheroprotection using parenteral or oral routes for LDL immunization. This is also the first study to show that mucosal immunization can induce antigen-specific atheroprotective immunity in apoe<sup>−/−</sup> mice, which spontaneously develop atherosclerosis and are therefore already sensitized to plaque antigens, such as LDL particles, at the time of vaccination. This situation is similar to that in humans with preexisting lesions but differs from that in ldlr<sup>−/−</sup> mice, which do not develop atherosclerosis unless they are fed a high-fat diet.

**Mechanism of Atheroprotection**

The atheroprotective effect paralleled an induction of Treg suppression of apoB-100-specific effector T cells and an increase in IL-10<sup>+</sup> CD4<sup>+</sup> T cells. Therefore, our data suggest that nasal immunization with p210-CTB protects against atherosclerosis by inducing antigen-specific, IL-10<sup>+</sup> regulatory Tr1 cells. It is unlikely that atheroprotection involved the immunosuppressive cytokine TGF-β because nasal immunization with p210-CTB also reduced atherosclerosis in mice lacking a functional TGF-β receptor on T cells.

Antigen-specific as well as antigen-independent effects have been reported in studies of Treg. Several studies of autoimmune diseases support the regulation model according to which Treg suppresses conventional effector T cells with the same antigen specificity. Other investigators report that Treg exerts major effects on antigen-presenting cells in an

![Figure 4](http://atvb.ahajournals.org/)

**Figure 4.** Nasal p210-CTB immunization induced IL-10-producing CD4<sup>+</sup> T cells and apoB-100-specific Treg activity in spleen. A, Flow cytometric analysis of cultured spleen cells stained for intracellular IL-10. B, Representative flow cytometric plots. C, Splenic effector cells at 2.5×10<sup>5</sup> cells/well were generated from apoe<sup>−/−</sup> mice that had been immunized with human apoB-100. The stimulation index represents the ratio of [1<sup>H</sup>]thymidine uptake on stimulation with human apoB-100 (20 μg/mL) relative to unstimulated cells. Proliferation of effector cells alone is indicated in the leftmost bar of each group. Addition of purified CD4<sup>+</sup> T cells from nasally immunized animals is indicated at different ratios to effector cells. *P<0.05.

![Figure 5](http://atvb.ahajournals.org/)

**Figure 5.** The protective effect of nasal p210-CTB immunization on atherosclerotic lesion size does not depend on TGF-β signaling in T cells. A, Lesion size in the aortic root of apoe<sup>−/−</sup>×CD4<sup>d</sup>mTGFβRII mice immunized with p210-CTB (black dots) or OVA-CTB (gray dots). B and C, Effect of immunization on p210-specific antibody titers of IgG class (B) and IgM (C). *P<0.05.
antigen-independent manner. Our data clearly show that antigen-specific atheroprotection was paralleled by inhibition of apoB-100-specific effector T cells by Treg specific for p210 but not ovalbumin peptide. These findings support a protective role for autoantigen-specific Tregs in atherosclerosis.

Two major types of Treg induced in the periphery by antigen exposure have been identified: FoxP3

theroprotective effect of nasal immunization.27,28 As atheroprotection was induced by nasal immunization and associated with suppressor T-cell activity and IL-10 producing CD4

T cells, our data are compatible with Tr1 induction by p210-CTB. CD4

T cells with antigen-specific suppressor activity were derived from spleen, a known reservoir of Tr1 cells.26

Because FoxP3 mRNA was increased in the aorta of nasally immunized mice, it cannot be ruled out that FoxP3

Treg may also contribute to atheroprotection in this model. These cells may not only act directly to control proinflammatory effector T cells but also promote the activation of Tr1 cells.19 Furthermore, FoxP3

T reg cells are known to be atheroprotective.29 However, these cells are not likely to mediate atheroprotection induced by nasal immunization because abrogation of TGF-β signaling, which is known to be crucial for the function of natural Treg, did not extinguish atheroprotection. Of note, FoxP3

Treg cells, as well as Tr1 cells, can be detected in mice lacking TGF-β signaling in T cells (reviewed in Lu and Rudensky30). Treg markers were elevated also in OVA-CTB-immunized mice; therefore, antigenically nonspecific effects may have synergized with antigen-specific mechanisms to confer protection.

Adjuvants are components of the vaccine formulation that enhance immunogenicity of the antigen, for instance by promoting their uptake by antigen-presenting cells.19,29 Interestingly, 2 studies documented an atheroprotective effect of complete Freund’s adjuvant in hypercholesterolemic ldlr

and apoe

mice.31,32 In a recent study, subcutaneous administration of alum adjuvant was shown to increase antigen uptake and activation of cellular immune responses in hypercholesterolemic mice.33 Our observation of a specific antibody response against the apoB-100 peptide and an immunomodulatory cytokine profile in the aortas of mice immunized with OVA-CTB corroborates such an adjuvant effect, further underlining the importance of using optimal immunomodulatory components in vaccine preparations.

Antibodies to the apoB-100 peptide sequence were induced by nasal immunization. However, they did not cross-react with native mouse LDL particles. Furthermore, antibody titers were not correlated with lesion size (data not shown), and no difference in lipoprotein profiles was recorded between apoB-100-CTB-immunized and OVA-CTB-immunized mice. Therefore, atheroprotection was likely due to immunomodulation rather than antibody-dependent elimination of LDL particles. When assessing T-cell dependent antibody responses such as antip210 IgG in hypercholesterolemic mice, it should be kept in mind that apoB-100 derived peptides are frequently displayed on major histocompatibility complex class II proteins34 and that ApoE protein modulates the function of antigen-presenting cells.35

**Composition of the “LDL Vaccine”**

Our results confirm and extend previous reports on the atheroprotective effects of immunization with LDL or its components.7–10,14,17,18 The use of complete LDL particles as immunogens is not attractive for clinical vaccination strategies, because these particles may contain multiple proinflammatory and even potentially toxic molecules such as modified lipids and endotoxins. Recent studies have identified a set of apoB-100-derived peptides with significant atheroprotective effects,17,18 enabling development of a structurally defined vaccine candidate. Among them, specific native peptides were immunogenic in humans and mice and correlated with the extent of atherosclerotic disease.36,37 By combining a limited number of peptides in the vaccine, it should be possible to overcome the major histocompatibility complex restriction, if needed. Combining peptide sequences with immunomodulatory components (adjuvants) such as CTB is an attractive approach to selectively induce protective immunity while avoiding side effects caused by nonpeptide components in LDL particles. Unlike LDL, our novel vaccine formulation can be easily manufactured in a reproducible way and under good manufacturing practice conditions.

The possibility of inducing atheroprotective immunity by nasal administration of an LDL component is attractive for clinical medicine. Therefore, its effect should be tested also in mice with established advanced lesions to mimic the situation when a therapeutic vaccine would be applied. Furthermore, the mucosal immune response to apoB-100-CTB peptides should be studied in other species, and eventually humans, to determine whether it is similar to or different from the one observed in mice. If the results of such studies are encouraging, a vaccination approach in patients likely will have to prove efficacy in individuals who are already sensitized, such as those with ongoing coronary artery disease or those with high risk for future disease.

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**Disclosure**

G.K.H. and J.N. are the inventors of patents regarding immunoprotection against atherosclerosis. J.H. is the inventor of patents on mucosal immunization.

**References**

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SUPPLEMENTAL MATERIAL

MATERIALS AND METHODS:

Cholera toxin B subunit gene fusions for immunization

The gene fusions used in the present study were constructed using a CTB expression vector essentially as described previously (Sadeghi H, Bregenholt S, Wegmann D, Petersen JS, Holmgren J, and Lebens M. Genetic fusion of human insulin B-chain to the B-subunit of cholera toxin enhances in vitro antigen presentation and induction of bystander suppression in vivo. *Immunology*. 2002; 106:237-245). Synthetic oligonucleotides from Innovagen (Lund, Sweden) were used to make double stranded DNA fragments encoding the peptide sequence of interest that could be inserted into the vector such that the added peptide formed a carboxyl extension of mature CTB. The unmodified peptide p210 corresponding to amino acids 3136-3155 of human apoB-100 (KTTKQSFDLSVKAQYKKKNKH) was encoded by the DNA sequence:

\[ 5'\text{CAAAACGACCAAGCAAAGCTTTGATCTGAGCGTGAAAGCGCAGTATAAGAAAAACAAACACTA}^3' \] \[ 3'\text{CATGGTTTTGCTGGTTCGTTTCGAAACTAGACTCGCACCTTTCGCGTCATATTCTTTTTTGTGATTCGA}^5' \]

This sequence is 90.9% identical to amino acids 3157-3185 of the murine apoB100 sequence, the exception being the insertion of a Serine (S) and an Asp (D) residue between N and KH in the C-terminal portion of the peptide:

Human \ KTTKQSFDLSVKAQYKKKH \ murine \ KTTKQSFDLSVKAQYKKNSDKH \n
Oligonucleotides were synthesized that encoded the p210 peptide corresponding to amino acids 3136-3155 of human apoB-100. The coding regions are flanked by sticky ends compatible with
restriction enzymes KpnI and HindIII. Insertion into the expression vector leads to an in-frame extension to the carboxyl terminus of mature CTB.

The synthetic sequence was optimized for expression in E. coli. The single strands were annealed and ligated into the pML-CTB vector digested with KpnI and HindIII. Ligated DNA was used to transform E. coli B strain BL21. Transformants were selected initially on the basis of ampicillin resistance and plasmids were then screened using restriction analysis. Finally the sequence of the insert in selected clones was confirmed by DNA sequencing. Protein expression was induced by addition of IPTG to the growth medium. This resulted in the production of insoluble inclusion bodies containing the recombinant protein. The cells were disrupted by sonication following treatment with lysozyme and DNase. The inclusion bodies could be separated from the soluble cell protein and other insoluble cell debris by low speed centrifugation. LPS was removed by washing the inclusion bodies in three times in 0.1% triton X114 in PBS at 4°C and subsequent extensive washing in PBS in order to remove the detergent. The inclusion bodies were dissolved in 6.5 M urea and reassembled into the biologically active pentameric form by removal of the urea by dialysis against 50 mM sodium carbonate buffer pH 9.0. The assembly and purity of the protein was assessed by SDS-PAGE. Receptor binding activity was confirmed by GM1 ELISA (Svennerholm AM, Holmgren J. Identification of Escherichia coli heat-labile enterotoxin by means of a ganglioside immunosorbent assay (GM1-ELISA) procedure. Curr Microbiol. 1978; 1:19–23). The protein was further partially purified by FPLC gel filtration using a sephadex 200 16/60 column. The OVA-CTB protein used as a control; also a fusion to the carboxyl terminus of mature CTB, was constructed and purified as previously described (George-Chandy A, Eriksson K, Lebens M, Nordström I, Schön E, Holmgren J. Cholera toxin B subunit as a carrier molecule promotes antigen

### Mice and immunization protocols

Female *apoE*-/- mice were obtained from Taconic Europe (Ry, Denmark) and *ApoE*-/- CD4dnTGFβRIItg mice were previously generated in our laboratory (ref. 23). Starting at 8 weeks of age, mice were immunized intranasally twice per week for 12 weeks with either p210-CTB or OVA-CTB, both at 15µg/dose (15 µL volume), or left untreated (PBS). Mice were fed a normal laboratory diet and sacrificed by CO₂ asphyxiation. All experiments were approved by the regional board for animal ethics.

### Tissue processing and immunohistochemistry

Lesion area per cross-section and fractional area of the lesion in the aortic root were quantified and the results expressed as the mean of 5 sections per mouse (ref. 24). In brief, fractional lesion area is calculated for each section as \( F\% = \left( \frac{100 \times L}{A} \right) \) where \( L \) is lesion area (µm²) and \( A \) is area inside external elastic lamina (µm²). \( F\% \) is averaged over all levels analyzed (200-600 µm² above aortic cusps) and the mean calculated for each treatment group. This method eliminates artifacts caused by oblique sections.

Primary antibodies (CD4, CD68, VCAM-1, I-A\(^b\); all rat anti-mouse from BD Biosciences (Franklin Lakes, NJ, U.S.A.) and FoxP3 by eBioscience (San Diego, CA, U.S.A.) titrated to optimum performance on spleen sections were applied to acetone-fixed cryosections from the aortic root,
followed by detection with the ABC alkaline phosphatase kit from Vector Laboratories (Burlingame, CA, USA). A thresholding technique was implemented using computerized ImagePro analysis (Media Cybernetics, Bethesda, MD, U.S.A.) of immunostained sections. For RNA isolation the thoracic aortic arch distal of the aortic root was dissected and snap-frozen.

**Flow cytometry and Intracellular Cytokine Staining**

Flow cytometry was performed on a CyAn™ (Dako, Glostrup, Denmark) after staining with the appropriate antibodies; data were analyzed using Summit v4.3 software (Dako). Primary labeled antibodies used were from BD Biosciences (anti-CD4) or from eBioscience (anti-FoxP3). To characterize the cytokine expression profiles of CD4+ T cells from lung and spleen of nasally vaccinated mice, cell suspensions were prepared as described before and evaluated by intracellular cytokine staining and FACS analysis. Briefly, lung mononuclear cells were isolated by collagenase Type I digestion (324U/ml; Sigma) for 1h on a shaker and splenocytes were prepared by mechanical disruption followed by incubation in erythrocyte lysis buffer (Qiagen, USA) and extensively washed. CD4+ T cells were purified using MACS magnetic cell separation as described above. 2 x 10^5 spleen or lung cells previously stimulated with 10ug/ml of human apoB-100 for 24 hours, were restimulated for 4h at 37 °C in 7,5% CO₂ with PMA (phorbol 12-myristate 13-acetate; 50 ng/ml), ionomycin (1 ug/ml; Sigma) and GolgiPlug (1ul per 1 ml; BD Bioscience). Alternatively, 2 x 10^5 CD4+ T cells previously stimulated with plate bound anti-CD3 (5 ug/ml) and anti-CD28 (2 ug/ml) for 3 days in culture together with recombinant mouse IL-2 (10 ng/ml; Peprotech) and IL-4 (1 ng/ml; Peprotech) followed by a 3 day incubation with only IL-2 and IL-4, were restimulated with plate bound anti-CD3 (5 ug/ml) and anti-CD28 (2 ug/ml) for 5h in the presence of GolgiPlug. All cells were incubated with FcγR block (BD Bioscience) followed by surface (anti-CD4) and
intracellular staining of IFN\(\gamma\), IL-4, IL-17 or IL-10 (BD Bioscience) and FoxP3 (eBioscience) according to the manufacturer’s instructions. Cells were analyzed on a CyAn™ flow cytometer (Dako).

**Functional immunoassays**

A first group of \(\text{apo}e^{-/-}\) mice were immunized subcutaneously with HPLC-purified human apoB-100 in complete Freund’s adjuvant (CFA) from Pierce (Rockford, IL, U.S.A.) and boosted 4 weeks later with apoB-100 in incomplete Freund’s adjuvant (IFA) from Pierce to generate spleen T cells sensitized to human apoB-100, which were harvested one week later. A second group of mice received the nasal vaccine over 2 weeks (4 doses total/mouse) and CD4\(^+\) T cells were harvested from the spleen 3 days after the last nasal administration of the vaccine. Spleen CD4\(^+\) T cells (> 95% purity) were isolated by negative selection over a magnetic column using MACS microbeads (CD4\(^+\) negative selection kit, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer’s protocol. Splenocytes from apoB100-vaccinated \(\text{apo}e^{-/-}\) mice were cocultured at varying dilution ratios with purified CD4\(^+\) T cells from spleens of mice that had received nasal p210-CTB, OVA-CTB or PBS. To exclude contaminating apoB-100 in cell culture media, FCS-free IMDM from Gibco (Invitrogen, Carlsbad, CA, U.S.A.) was supplemented with ITS™ from BD Biosciences. Cells were incubated for 72 hours in the absence or presence of purified human apoB-100 (20\(\mu\)g/mL) with incorporation of \(^3\)H-thymidine during the last 18 hours. Data are presented as stimulation index (ratio of apoB-100-challenged to unchallenged coculture assay). In a second approach splenocytes and purified CD4\(^+\) T cells were separated in transwell plates from Corning
to analyze whether cell-cell contact inhibition abrogated the suppressive effect of tolerized CD4+ T cells.

**Antibody assays**

ELISA methods were used to quantitate serum Ig isotypes specific for the apoB-100 peptide as well as total IgG and IgM as previously described (ref. 17). Sera from immunized mice were tested for antibodies to mouse LDL by incubation (1/50, 1/150 and 1/450 dilutions) in plates coated with mouse LDL (10 µg/ml) and using alkaline phosphatase-conjugated anti-mouse-IgG as detector antibody. Sera from C57BL/6 mice immunized with OVA-CTB were assayed for reactivity to mouse LDL, or to apoB100, at dilutions of 1/25, 1/250 and 1/2500.

**Real-Time Polymerase Chain Reaction**

RNA was isolated from the aortic arch using the RNeasy kit from Qiagen (Hilden, Germany). Total RNA was analyzed by BioAnalyzer from Agilent Technologies (Waldborrn, Germany). Reverse transcription was performed with Superscript-II and random hexamers (both from Invitrogen) and cDNA amplified by real-time PCR using primers and probes for FoxP3, IL-10, TGF-β, IFN-γ and hypoxanthine guanine ribonucleosyltransferase (HPRT) in an ABI 7700 Sequence Detector from Applied Biosystems. All primers and probes were obtained as “assays on demand” from Applied Biosystems (Foster City, CA, U.S.A.) Data were analyzed on the basis of the relative expression method with the formula $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_T$ (sample) – $\Delta C_T$ (calibrator = average $C_T$ values of all samples within each group), and $\Delta C_T$ is the $C_T$ of the housekeeping gene (HPRT) subtracted from the $C_T$ of the target gene.
Serum analyses

Total serum triglycerides were determined with an enzymatic assay from Roche Diagnostics (Mannheim, Germany) using a TECAN InfiniTE M200 plate reader (TECAN Nordic, Täby Sweden). Total serum cholesterol and lipoprotein profiles were determined by FPLC separation of 2 μL serum of all individuals using a micro-FPLC column from GE Healthcare coupled to a system for online separation and subsequent detection of cholesterol as described, using human serum as reference (Parini P et al., Lipoprotein profiles in plasma and interstitial fluid analyzed with an automated gel-filtration system. Eur J Clin Invest 2006;36:98-104). IL-10 ELISA from Mabtech (Nacka Strand, Sweden) and TGF-β ELISA from R&D Systems (Minneapolis, MN, U.S.A.) was used to measure cytokine levels in supernatants.

Statistical analysis

Values are expressed as mean ± standard error of the mean (SEM) unless otherwise indicated. Non-parametric Kruskal-Wallis test was used for multiple comparisons, Mann-Whitney U test was used for pairwise comparisons. A p-value of < 0.05 was considered significant.
Table 1:

**Cellular composition and inflammatory markers in aortas of 20 weeks old apoe<sup>−/−</sup> mice**

<table>
<thead>
<tr>
<th></th>
<th>P210-CTB (A)</th>
<th>OVA-CTB (B)</th>
<th>control</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CD4 (cells/mm²)</strong></td>
<td>176 ± 31</td>
<td>151 ± 35</td>
<td>124 ± 21</td>
<td>n.s.</td>
</tr>
<tr>
<td><strong>I-&lt;sub&gt;A&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt; (cells/mm²)</strong></td>
<td>87 ± 25</td>
<td>62 ± 17</td>
<td>112 ± 34</td>
<td>n.s.</td>
</tr>
<tr>
<td><strong>CD68 (%lesion)</strong></td>
<td>15.9 ± 3.6</td>
<td>22.6 ± 7.4</td>
<td>12.6 ± 2.3</td>
<td>n.s.</td>
</tr>
<tr>
<td><strong>VCAM-1 (%lesion)</strong></td>
<td>12.7 ± 4.6</td>
<td>22.3 ± 11.3</td>
<td>10.9 ± 1.0</td>
<td>n.s.</td>
</tr>
<tr>
<td><strong>FoxP3 (cells/mm²)</strong></td>
<td>4.1 ± 0.8</td>
<td>4.2 ± 1.0</td>
<td>6.5 ± 2.6</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Positive cells are calculated per mm² lesion area except for FoxP3 cells, which are presented as cells per total vessel surface area as they were also found in the adventitia. Statistics were performed using the non-parametric Kruskall Wallis test.
### Table 2:

**Weight, cholesterol and triglyceride levels in plasma**

<table>
<thead>
<tr>
<th>group</th>
<th>weight (g)</th>
<th>cholesterol (mg/dL)</th>
<th>triglycerides (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>apoel/~</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p210-CTB</td>
<td>21 ± 1.3</td>
<td>281 ± 102</td>
<td>46 ± 9.1</td>
</tr>
<tr>
<td>OVA-CTB</td>
<td>21.5 ± 0.6</td>
<td>257 ± 51</td>
<td>36 ± 3.9</td>
</tr>
<tr>
<td>control</td>
<td>20.5 ± 1.0</td>
<td>313 ± 59</td>
<td>52 ± 7.9</td>
</tr>
<tr>
<td><em>apoel/~</em> x CD4dn</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p210-CTB</td>
<td>19.1 ± 2.9</td>
<td>254 ± 84</td>
<td>40 ± 8.3</td>
</tr>
<tr>
<td>OVA-CTB</td>
<td>20.8 ± 1.2</td>
<td>250 ± 94</td>
<td>40 ± 6.2</td>
</tr>
<tr>
<td>TbRIItg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVA-CTB</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**p value**

|                       | n.s. | n.s. | n.s.  |

Mean values and standard deviations are shown. Non-parametric group comparisons were performed using the Kruskal-Wallis test.
Table 3:

**Cellular composition and inflammatory markers in aortas of 20 weeks old apoec^−/−CD4dnTGFβRIItg mice**

<table>
<thead>
<tr>
<th></th>
<th>p210-CTB (C)</th>
<th>OVA-CTB (D)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4 (cells/mm²)</td>
<td>120 ± 43</td>
<td>173 ± 55</td>
<td>n.s.</td>
</tr>
<tr>
<td>I-A^b (cells/mm²)</td>
<td>94 ± 16</td>
<td>238 ± 102</td>
<td>n.s.</td>
</tr>
<tr>
<td>CD68 (%lesion)</td>
<td>9.9 ± 2.8</td>
<td>14.5 ± 2.6</td>
<td>n.s.</td>
</tr>
<tr>
<td>VCAM-1 (%lesion)</td>
<td>7.6 ± 2.6</td>
<td>19.3 ± 2.2</td>
<td>n.s.</td>
</tr>
<tr>
<td>FoxP3 (cells/mm²)</td>
<td>19.7 ± 5.2</td>
<td>25.6 ± 3.4</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Positive cells are calculated per mm² lesion area except for FoxP3 cells, which are presented as cells per total vessel surface area as they were also found in the adventitia. Statistics were performed using the non-parametric Kruskall Wallis test.
Figure I. Lesion size in immunized mice.

Fractional area of lesions in aortic root of apoe<sup>-/-</sup> mice treated nasally with p210-CTB (black bar), OVA-CTB (grey bar) and untreated controls (white bar). Mean ± SD values are shown. * indicates p<0.05.
Figure II. Plasma lipoprotein profiles analyzed by FPLC.

Thick lines represent immunized groups: black = p210-CTB, grey = OVA-CTB and white shows the control group. Thin lines represent SEM. CR/VLDL = chylomicrons/very low density lipoproteins; LDL = low density lipoproteins; HDL = high density lipoproteins.
Figure III. T cells in lesions.

Representative immunohistochemical stains of atherosclerotic lesion in the aortic root of *apoE*−/− mice. Arrows indicate FoxP3+ cells; they were CD4+ in directly adjacent sections.
Figure IV. Serum IgG1 antibody titers to apoB-100 peptide.

ELISA analysis of sera from apoe<sup>−/−</sup> mice treated with p210-CTB (black bar), OVA-CTB (grey bar) or controls (white bar). * indicates p<0.05.
Figure V. Serum IgG2a antibody titers to apoB-100 peptide.

Apoe<sup>−/−</sup> mice treated with p210-CTB (black bar), OVA-CTB (grey bar) or controls (white bar). * indicates p<0.05.
Figure VI. Total IgG in sera of immunized mice.

*Apoe*−/− mice treated with p210-CTB (black bar), OVA-CTB (grey bar) or controls (white bar).
Figure VII. Total IgM in sera of immunized mice.

*Apoe*" mice treated with p210-CTB (black bar), OVA-CTB (grey bar) or controls (white bar).
Figure VIII. T cell subsets in spleen after immunization.

Flow cytometric analysis of intracellular cytokine expression is shown as percentage of cytokine-producing cells per CD4\(^+\) T cells for each of the three groups. (A) Interferon-\(\gamma\); (B) IL-17; (C) IL-4; (D) FoxP3.
Figure IX. Abrogation of Treg suppressor effect upon separation of cells.

Cocultures of effector cells and CD4\(^+\) T cells from nasally immunized mice are indicated by plain bars (generated from black = p210-CTB, grey = OVA-CTB and white = control group animals). Abrogated effect of CD4\(^+\) T cell-mediated inhibition of proliferation when effector cells and CD4\(^+\) T cells are cultured separate from each other in a transwell plate (contact inhibition assay).
Figure X. Cytokine levels for TGF-β and IL-10 in the supernatants from the coculture assay.
Striped bars represent effector cells alone. Cocultures of effector cells and CD4+ T cells from nasally immunized mice (ratio 1:1) are indicated by plain bars (generated from black = p210-CTB, grey = OVA-CTB and white = control group animals).
Figure XI. Lesion size in immunized mice with defective TGFβ receptors on T cells.

Fractional area of the lesion in the aortic root of apo<sup>−/−</sup> x CD4dnTGFβRIItg mice treated nasally with p210-CTB (black bar) or OVA-CTB (grey bar). Mean ± SD values are shown. * indicates p<0.05.
Figure XII. mRNA levels in aorta of mice with defective TGFβ receptors on T cells.

Real-time reverse-transcription PCR normalized to HPRT in thoracic aorta of apoe<sup>−/−</sup> x CD4dnTGFβRIItg mice. Of note, the development of functional Foxp3<sup>+</sup> Treg cells depends on TGFβ. Therefore, the relevance of FoxP3-expressing cells in apoe<sup>−/−</sup> x CD4dnTGFβRIItg mice is unclear.
Fig XIII. IgG antibodies to mouse LDL in mice immunized with LDL or OVA.

C57BL/6J mice were immunized with mouse LDL (mLDL) or ovalbumin (OVA). ELISA was performed with serum dilutions in wells coated with mouse LDL, followed by alkaline phosphatase-labeled anti-mouse-IgG. SC, subcutaneous administration, IN, intranasal administration.