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Originally published at:
Intranasal immunization with an apoB-100 fusion protein induces antigen-specific regulatory T cells and reduces atherosclerosis

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Short title: Nasal apoB immunization against atherosclerosis

Word count: 4,710 (total no of words including cover page, abstract, figure legends etc)
ABSTRACT

OBJECTIVE:

Atherosclerosis is an inflammatory disease. Autoimmune responses to low-density lipoproteins (LDL) contribute to its progression, while 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 (apoB100) to the nasal mucosa to induce a protective mucosal immune response.

METHODS AND RESULTS:

A peptide comprising amino acids 3136-3155 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 of Apoe^{-/-} mice. This effect was accompanied by induction regulatory T cells that markedly suppressed effector T cells re-challenged with apoB-100 and increased numbers of 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-beta receptors on T cells.

CONCLUSIONS:

Nasal administration of an apoB100 peptide fused to CTB attenuates atherosclerosis and induces regulatory Tr1 cells that inhibit T effector responses to apoB-100.
CONDENSED ABSTRACT

A novel strategy for atheroprotective immunization has been developed. A peptide sequence from apolipoprotein B-100 of low-density lipoprotein was fused with the B subunit of cholera toxin and used for intranasal immunization of Apoe<sup>−/−</sup> mice. This induced antigen-specific regulatory T cells and led to a 35% reduction of atherosclerosis.
INTRODUCTION:

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). While 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 pathologic 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, beta2-glycoprotein-1b, or heat-shock protein 60/65, and parenteral (7-10) as well as oral (11-14) immunization reduced atherosclerotic disease in hyperlipidemic animals. However, progress has been hampered by 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 upon 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 for 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 that have been identified in the protein moiety of LDL, 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 anti-inflammatory, immunosuppressive responses (19). Distinct immunological features of the respiratory and intestinal mucosa lead to partly different types of protective immunity upon 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 2 trial in Behcet’s disease (22).

In this study we present a novel strategy for immunoprotection against atherosclerosis by nasal administration of an apoB100 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 methods description please see Supplemental Material at http://atvb.ahajournals.org.

A recombinant protein, p210-CTB, was made from amino acids 3136-3155 of human apoB-100 (p210) (17) 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 323-339 of ovalbumin were fused to CTB (OVA-CTB). 8-week-old female apo\(\text{e}^{\text{/-}}\) 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, apo\(\text{e}^{\text{/-}}\) x CD4dnTGFbRII mice (23) 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: apo\(\text{e}^{\text{/-}}\) 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 apo\(\text{e}^{\text{/-}}\) mice immunized intra-nasally 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.
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 or untreated controls, respectively (Fig 1A-D and Online Supplement, Fig I, please see http://atvb.ahajournals.org). Atherosclerosis was not attenuated by administration of OVA-CTB compared with untreated controls indicating an apoB-100 peptide-specific effect (Fig 1A-D). 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-Aᵇ (major histocompatibility complex class II protein) and the vascular cell adhesion molecule-1 (Supplement, Table 1).

Nasal administration of p210-CTB does not affect plasma lipids

Immunization did not significantly affect body weight, serum cholesterol or triglycerides (Table 2, Supplement). Plasma lipoprotein profiles were similar in mice immunized with p210-CTB or OVA-CTB, respectively (Supplement, Fig II).

CTB fusion protein immunization increases aortic FoxP3 and IL-10 mRNA levels

Real-time reverse transcription-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 or OVA-CTB, respectively. Furthermore, numbers of FoxP3⁺ cells in aortic lesions did not differ between the groups ( (Figure III; Supplement). IL-10 was elevated to the same extent in p210-CTB and OVA-CTB groups, pointing to a possible adjuvant effect of CTB. Trends towards
increased 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 anti-p210 was observed in OVA-CTB immunized \textit{apoe}^{-/-} mice. The IgG1/IgG2a ratio of anti-p210 antibodies did not change, implying that there was no Th1/Th2 shift in T helper activity to B cell activation (Figure IV + V, Supplement). Total IgG levels were not influenced by either treatment (Figure VI, Supplement). p210-specific IgM titers did not differ between p210-CTB and OVA-CTB treated groups, however, they were significantly elevated compared to untreated animals (Figure 2B). Total IgM was not influenced by either treatment (Figure VII, Supplement).

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. \textit{Apoe}^{-/-} mice immunized with OVA-CTB showed modestly increased titers to p210 (Fig 2). To rule out the possibility of a crossreaction between the OVA peptide and p210, we performed ELISA analysis of anti-LDL reactivity in wildtype C57BL/6 mice immunized with OVA-CTB. It showed no significant titers to mouse LDL (Fig. XIII, Supplement).

Analysis of the cellular immune response in the lung, the major organ targeted after nasal vaccination, showed a significant decrease in CD4^{+} T cells expressing IFN-γ (characteristic of Th1 cells) and IL-17 (characteristic of Th17 cells), respectively, in mice treated with p210-CTB (Figure 3 A,B). In contrast, no such change was recorded for IL-4^{+} CD4^{+} T cells or for FoxP3^{+} CD4^{+} T cells (Fig 3 C,D). 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<sup>+</sup> T cells expressing the anti-inflammatory cytokine IL-10 (Figure 4 A and B). Unlike the situation in the lung, no significant differences were detected in the distribution of the remaining CD4<sup>+</sup> T cell subsets in the spleen, as characterized by intracellular staining for interferon-γ, IL-17, IL-4 and FoxP3 (Figure VIII, Supplement).

**p210-CTB treatment induces apoB-100-specific Treg activity**

To assess whether functional Treg were induced by immunization, we exposed spleen CD4<sup>+</sup> T cells from *apoe<sup>−/−</sup>* mice immunized subcutaneously with human apoB-100 (effector T cells), to CD4<sup>+</sup> T cells from mice immunized nasally with either p210-CTB, OVA-CTB, or no antigen (Fig. 4C). A marked dose-dependent inhibition of effector T cell proliferation was observed in the presence of CD4<sup>+</sup> T cells from p210-CTB immunized mice. No such inhibition was observed when T cells from OVA-CTB- or non-immunized 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 (Figure IX, Supplement). Levels of IL-10 and TGF-β in culture supernatants did not differ between groups (Figure X, Supplement).

**The atheroprotective effect of nasal p210-CTB vaccination is independent of TGF-β signaling in T cells**

To determine whether the atheroprotective effect of nasal vaccination with p210-CTB depended on TGF-β signaling in T cells, we immunized *apoe<sup>−/−</sup>* mice lacking functional TGF-β receptors on T cells (Apoe<sup>−/−</sup> x CD4dnTGFβRII mice). Nasal immunization with p210-CTB significantly reduced atherosclerotic lesion size by 30% in Apoé<sup>−/−</sup> x CD4dnTGFβRII mice
mice, as compared with littermates immunized with OVA-CTB (Fig 5A and Supplement, Figure XI). This indicates that TGFβR 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 TGFβ for their function. IgG but not IgM antibodies directed against the apoB100-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,C). Analysis of mRNA expression in aortas showed no differences in mRNA for, IL-10, TGF-β or IFNγ between p210-CTB- and OVA-CTB-vaccinated mice (Figure XII, Supplement). Surprisingly, FoxP3 mRNA was elevated in Apoe−/− x CD4dnTGFβRII mice compared to Apoe−/− mice, possibly reflecting the presence of immature Treg in the former mice (Figure XII, Supplement and Fig. 1E). Furthermore, quantitative analysis of immunohistochemical staining showed no differences in the cellular composition of lesions between treatment groups (Table 3, Supplement).

**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+ 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−/− 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 pre-existing lesions but differs from that in $ldlr^{-/-}$ 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$^{+}$ CD4$^{+}$ T cells. Therefore, our data suggest that nasal immunization with p210-CTB protects against atherosclerosis by inducing antigen-specific, IL-10$^{+}$ regulatory Tr1 cells. It is unlikely that atheroprotection involved the immunosuppressive cytokine TGF-β since nasal immunization with p210-CTB reduced atherosclerosis also in mice lacking functional TGF-β receptor on T cells.

Antigen-specific as well as antigen-independent effects have been reported in studies of Treg(25). Several studies of autoimmune diseases support the regulation model according to which Treg suppress conventional effector T cells with the same antigen specificity. Other investigators report that Treg exert major effects on antigen-presenting cells in an antigen-independent manner. Our data clearly show that antigen-specific atheroprotection was paralleled by inhibition of apoB100-specific effector T cells by Treg specific for p210 but not OVA. 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$^{+}$ induced Treg (Th3) (25) and Tr1 cells (26). Tr1 cells are FoxP3 negative, secrete IL-10, and are believed to play an important role when regulatory immunity is induced by 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 is 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).
Since 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+ natural Treg are known to be atheroprotective\(^{(29)}\). However, these cells are not likely to mediate atheroprotection induced by nasal immunization since abrogation of TGF-β signaling known to be crucial for the function of natural Treg did not extinguish atheroprotection. Of note, FoxP3+ natural Treg as well as Tr1 cells can be detected in mice lacking TGF-β signaling in T cells \(\text{(reviewed in}^{(30)})\). 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 \(\text{(19, 29)}\). Interestingly, two studies documented an atheroprotective effect of complete Freund’s adjuvant in hypercholesterolemic \(\text{ldlr}^{-/-}\) and \(\text{apo}e^{-/-}\) mice \(\text{(31-32)}\). In a recent study, subcutaneous administration of alum adjuvant was shown to increase antigen uptake and activation of cellular immune responses in hypercholesterolemic mice \(\text{(33)}\). Our observation of a specific antibody response against the apoB-100 peptide and an immunomodulatory cytokine profile in aortas of mice immunized with OVA-CTB corroborates such an adjuvant effect, further underling the importance of using optimal immunomodulatory components in vaccine preparations.

Antibodies to the apoB100 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 \(\text{(data not shown)}\) 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 anti-p210 IgG in hypercholesterolemic mice, it should be kept in mind that apoB100 derived peptides are frequently displayed on MHC class II proteins(34) and that ApoE protein modulates the function of antigen-presenting cells(35).

Composition of “LDL vaccine”

Our results confirm and extend previous reports on 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 since these particles may contain multiple pro-inflammatory 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 MHC 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 non-peptide components in LDL particles. Unlike LDL, our novel vaccine formulation can be easily manufactured in a reproducible way and under Good Manufacturing Practice (GMP) conditions.

The possibility to induce 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 apoB100-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 already sensitized individuals such as those with ongoing coronary artery disease or those with high risk for future disease.

ACKNOWLEDGEMENTS:

SOURCES OF FUNDING: RK was supported by the German Research Foundation (KL1398/2-1/2-2) and the Swedish Heart-Lung Foundation (SHLF). JN, GNF, MR, NG, JH, and GKH received support from the Swedish Research Council and Vinnova Foundation; additional grants to GKH, JN and MR were from SHLF, to GKH and MR from the Stockholm City Council, and to JH from the Wallenberg Foundation and Sahlgrenska University Hospital. GKH and JN participate in the European Vascular Genomics Network, IMMUNATH and AtheroRemo projects sponsored by the European Commission.

DISCLOSURES: GKH and JN are inventors of patents regarding immunoprotection against atherosclerosis. JH is inventor of patents on mucosal immunization.
FIGURE LEGENDS:

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 three groups are shown: black circles represent animals from the p210-CTB group, grey circles animals from the OVA-CTB group and white circles animals from the control group, respectively. * indicates p<0.05. (B-D) Representative photomicrographs show oil red O stained aortic root sections from each group (50X magnification). (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 HPRT expression are shown for each gene of interest for all three groups. * indicates p<0.05.

Figure 2:
Nasal immunization induced a systemic humoral immune response in apoe\(^{-/-}\) mice. (A) IgG-anti-p210 titers in mouse plasma; titration curves are shown in the inset. (B) p210-specific IgM titers in plasma from the same mice. * indicates p<0.05.

Figure 3:
Nasal immunization altered T cell subset composition in lung mucosa. Flow cytometric analysis of intracellular subset markers, with cytokine-producing CD4\(^{+}\) T cells as percentage of total CD4\(^{+}\) T cells for each of the three groups. (A) IFN-\(\gamma\); (B) IL-17; (C) IL-4; (D) FoxP3. *) indicates p<0.05.
Figure 4:

Nasal p210-CTB immunization induced IL-10-producing CD4+ 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 x 10^5 cells/well were generated from apoe^-/- mice that had been immunized with human apoB100. The stimulation index represents the ratio of ^3H thymidine uptake upon stimulation with human apoB100 (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+ T cells from nasally immunized animals is indicated at different ratios to effector cells. * p<0.05.

Figure 5:

The protective effect of nasal p210-CTB immunization on atherosclerotic lesion size does not depend on TGF-β signalling in T cells. A) Lesion size in the aortic root of apoe^-/- x CD4dnTGFβRIItg mice immunized with p210-CTB (black dots) or OVA-CTB (grey dots). Effect of immunization on p210-specific antibody titers of IgG class (B) and IgM (C). * p<0.05.
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