Apolipoprotein B-100 Peptide p210 Inhibits Proliferation of Naïve T Effector Cells and Promotes Induction of Tolerogenic Antigen Presenting Cells and Regulatory T Cells in Vitro

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Abstract

Objectives: Modulation of immune responses against LDL antigens through therapeutic vaccines represents a possible new approach for prevention of cardiovascular disease. The mode of action of these vaccines remains to be fully characterized but the protective effect of immunization with the apolipoprotein B-100 (apoB-100) derived peptide p210 has in several studies been associated with activation of regulatory T cells. The present study used an in vitro model to study the effect of p210 on immune cells.

Methods and results: CD11c+ antigen presenting cells, CD25+CD4+ naïve T effector cells and CD25+CD4+ T regulatory cells were isolated from mouse spleens using antibody-coated magnetic beads. Pre-incubation of antigen presenting cells with p210 conjugated to cationized bovine serum albumin (p210-cBSA) down-regulated the expression of CD86 and MHC class II molecules, inhibited proliferation of pre-activated naïve T effector cells and stimulated conversion of these cells into regulatory T cells. These effects were shown to partly be mediated through a suppression of the release of IL-12 from antigen presenting cells.

Conclusions: The present findings demonstrate that p210-cBSA inhibits proliferation of naïve T effector cells and promotes their conversion into regulatory T cells and this is suggested to be associated with a reduced activation status of antigen presenting cells. Taken together these findings suggest that immunization with p210-based vaccines have the capability of inducing tolerogenic APCs that in turn generate regulatory T cells suppressing T effector cell functions.

Keywords: Vaccine; Regulatory T cells; Antigen presenting cells; Immunomodulation

Introduction

Atherosclerosis is characterized by lipid accumulation and chronic inflammation of large and medium-sized arteries. There is accumulating evidence that autoimmune responses against self-antigens play an important role in disease development [1]. Immune responses against low density lipoprotein (LDL) antigens, including apolipoprotein B-100 (apoB-100), are believed to be of particular importance for disease progression [2,3]. Accumulating evidence suggest a hazardous role for T helper 1 (Th1) responses in atherosclerosis development. Th1 cell related cytokines, such as IL-12 and IFN-γ, have been found in atherosclerotic plaques contributing to the local inflammatory environment [4,5]. We have previously identified certain peptide sequences in apoB-100 as targets for autoimmune responses and shown that modulation of these immune responses through immunization with corresponding apoB-100 peptides reduces atherosclerosis development [6,7]. In particular, the 20 amino acids long apoB-100 peptide p210 have been used in a number of different immunization strategies to reduce atherosclerosis development in hypercholesterolemic mice [7,8].

Athero-protection induced by immunizations with p210-cationized BSA (cBSA) and alum has been shown to be associated with an increase in CD4+CD25+Foxp3+ regulatory T cells (Tregs) and the protective effect of p210-cBSA was abolished when Tregs were depleted by a CD25 blocking antibody [9]. Intranasal immunization with p210 fused to the cholera toxin B subunit (CTB) has also been shown to reduce atherosclerosis development. This was shown to be accompanied by an increase in IL-10 expressing Tregs and an antigen-specific inhibition of apoB-100 specific T effector cells [10]. Moreover, p210 has been used together with other apoB-100 peptides and without a carrier in low dose continuous subcutaneous administration resulting in reduced lesion development and an increased Treg population [11]. This strategy for antigen administration has previously been shown to induce antigen specific Tregs with the potential to suppress immune responses [12]. Tregs have immunosuppressive activity and their major function is to maintain self-tolerance and immune homeostasis. Activated Tregs secrete the inhibitory cytokines IL-10 and TGF-b and inhibit T effector cell activation. There are two major subtypes of Tregs, natural Tregs generated in the thymus, and induced Tregs which are generated in the periphery [13]. Dendritic cells (DCs) are professional antigen presenting cells required to induce T cell activation. T cells require two signals from the DCs to become activated. The first is through the T cell receptor and the second through co-stimulatory molecules. If the DC does not become fully activated when it encounters its antigen or is exposed to IL-10, it may acquire a tolerogenic phenotype [14]. Tolerogenic DCs are characterized by a down regulation of co-
stimulatory molecules such as CD80 and CD86, opposed to fully mature DCs that upregulate these markers. Fully mature DCs will activate conventional T effector cells in contrast to the tolerogenic DCs that can induce T_{regs} or directly inhibit the T effector cell differentiation and activation [15]. Furthermore, tolerogenic DCs have an impaired ability to produce Th1 cell specific cytokines, such as IL-12 [16]. It has previously been shown that transfer of DCs made tolerogenic by treatment with IL-10 has the potential to increase the T_{regs} population in spleen and decrease lesion development in an experimental model of atherosclerosis, highlighting the importance of the DC phenotype in the T cell response [17].

We have previously shown that splenocytes from mice immunized with p210-cBSA in alum have a reduced capacity to proliferate in response to polyclonal activation [9]. This finding was interpreted as an increased inhibitory capacity of the cells in cell cultures from the immunized mice as the fraction of T_{regs} were increased in the spleen of these mice. The aim of the present study was to investigate if p210-cBSA has immune regulatory properties in an in vitro assay that was primarily developed to study antigen-specificity of T_{regs} and their effects on T effector cell proliferation. A p210 based vaccine will likely be taken into clinical phase 1 trials in a close future. An increased understanding of the mechanism of action of p210 immunization is of importance both from a safety perspective and to achieve an optimal administration of the antigen.

Material and methods

Animals and antigen preparation

Male wild type C57Bl/6 and OTII mice from Jackson and MyD88 deficient mice (a kind gift from M. Freeman, Massachusetts General Hospital) were used in this study. Food and tap water were administered ad libitum. The local Animal Care and Use Committee approved the experimental protocols used in this study. ApoB peptide 210 (p210, amino acids 3136-3155) was conjugated to cationized bovine serum albumin (cBSA, Pierce) as described previously [7]. p210-cBSA, p210 alone or only cBSA served as antigen. For the in vivo experiment wild type mice were immunized at 12 to 15 weeks of age with 50 mg p210-cBSA together with Alum as adjuvant. Fully mature DCs will up regulate these markers. For the in vivo experiment wild type mice were immunized at 12 to 15 weeks of age with 50 mg p210-cBSA together with Alum as adjuvant two times with 4 weeks in between. PBS immunized mice served as controls. Mice were killed one week after last immunization and spleens were harvested.

Cell isolation

Spleens were homogenized using spleen dissociation medium (StemCell Technologies) and filtered through a 70 µm mesh (BD). CD11c+ dendritic cells, CD25 CD4+ T effector cells and CD25+ CD4+ T regulatory cells were immunomagnetically sorted according to manufacturer’s protocol. Briefly, CD11c+ dendritic cells were isolated using CD11c positive selection kit and EasySep magnetic beads (StemCell Technologies). CD4+ T cells were then isolated from the CD11c fraction using CD4 negative selection kit (StemCell Technologies) and finally, from the CD4+ cells, CD25+ CD4+ and CD25+ CD4+ cells were isolated using CD25 positive selection kit (StemCell Technologies). Cells were cultured in complete RPMI (RPMI-1640 medium containing 10% heat-inactivated fetal calf serum (FCS), 1 mmol/L sodium pyruvate, 10 mmol/L Hepes, 50 U penicillin, 50 µg/ml streptomycin, 0.05 mmol/L l-mercaptoethanol and 2 mmol/L L-glutamine; all from GIBCO) for all experiments.

Proliferation assay

The assay was adapted and modified from Bonertz et al. [18]. After cell isolation, 50 000 CD25 CD4+ T effector cells were pre-activated using 10 µg/ml plate-bound anti-mouse CD3 antibody (Biolegend) overnight in a flat bottom 96 well plate (Sarstedt) at 37°C and 5% CO2. In parallel, 100 000 CD11c+ dendritic cells plus 50 000 CD25+ CD4+ Tregs were co-cultured together with the indicated antigen overnight in a 96 well round bottom plate (Sarstedt) at 37°C and 5% CO2. The pre-activated T effector cells were then transferred to the DC/T_{reg} co-culture and incubated for another 72 hours. In some experiments T effector cells were activated by micro beads coated with anti-CD3 and anti-CD28 (Invitrogen) in the absence of DCs. To measure DNA synthesis, the cells were pulsed with 1 µCi (methyl-3H) (Amersham) for an additional 16-18 h. Macromolecular material were then harvested on glass fiber filters using a FilterMate harvester (PerkinElmer) and analyzed using a liquid scintillation counter (Wallac). In some experiments, IL-12 (Peprotech) and MHC class II or IL-10 blocking antibody (Biolegend) were added to selected wells.

Induction of T_{regs}

CD11c+ cells were pulsed with 25 mg/ml p210-cBSA for two hours at 37°C and cells were thereafter washed three times in PBS. 100 000 CD11c+ cells were then cocultured with 100 000 CD4+ CD25- T effector cells in complete RPMI for 72 hours at a plate coated with anti-CD3 antibody (1 mg/ml). IL-2 and TGF-b (Peprotech) were added to selected wells at 25 U/ml and 10 ng/ml respectively, to induce T_{regs} [19]. Additionally, IL-12 (Peprotech) was added to selected wells at 100 pg/ml. T_{reg} induction was verified by flow cytometry.

Cytokine analysis

Cytokine concentrations in cell supernatants were analyzed using a mouse Th1/Th2 9-Plex (IFN-γ, IL-1β, TNF-α, IL-2, IL-12, IL-4, IL-5, IL-10 and KC) Ultra-Sensitive Kit (Meso Scale Discovery), following the instructions of the manufacturer. The lower detection limit for all cytokines in this assay is ~0.5pg/ml.

Flow cytometry

Cells were stained with fluorochrome-conjugated antibodies and acquired on a CyAn ADP flow cytometer (Beckman Coulter). The following antibodies were used MHCI-APC/Cy7, CD86-PB, FoxP3-FITC, CD25-APC, CD4-PB and CD3-PE/Cy7 (all from Biolegend or eBioscience). Data were analyzed with FlowJo software (Tree Star).

FITC labeled p210-cBSA

FITC labeled p210 was used for conjugation with cBSA as previously described [7]. A suspension of total splenocytes was incubated with p210-cBSA-FITC (50 mg/ml) for two hours at 37°C before the cells were studied with immunofluorescence confocal microscopy (Zeiss LSM). For flow cytometric analysis with p210-cBSA-FITC, splenocytes were incubated with increasing (0-300 mg/ml) or a fixed concentration (25 mg/ml) of the conjugate for 2 hours. Cells were thereafter stained with fluorochrome-conjugated antibodies. The following antibodies were used in these experiments MHCI-APC Cy7, CD11c-PE/Cy7 CD45-R-PB (all from Biolegend).
Cytotoxicity and apoptosis assay

Cytotoxicity was measured using LDH assay kit (Roche) according to protocol. Triton-X lysed cells were used as a positive control. Apoptosis was measured by a Caspase 3 assay kit (BD Bioscience) measuring total Caspase 3 activity by fluorescence, according to the manufacturer’s protocol. Camptothecin treated cells served as assay control for this assay.

Statistics

Statistical analysis was performed with GraphPad Prism 5 (Graphpad Software) using unpaired t test or Mann Whitney test for skewed data. Data are presented as mean ± standard deviation and P<0.05 was considered significant.

Results

Previous studies in hypercholesterolemic mice have shown that immunization with the apo B-derived peptide p210 is associated with an expansion of Tregs and inhibition of T effector cell proliferation [9]. To determine if this response is dependent on a previous exposure of the immune system to hypercholesterolemia we immunized wild type C57Bl/6 mice on chow diet with p210 conjugated to cBSA (p210-cBSA). Analysis of spleen cell composition one week after the second immunization demonstrated a 30% increase in CD25 FoxP3+ Tregs (expressed as percent of all CD3+CD4+ T cells) in response to p210-cBSA (Figure 1A). Moreover, spleen cell proliferation in response to CD3/CD28 bead stimulation was reduced by about 25% in immunized mice (Figure 1B). These findings show that the regulatory response to immunization with p210-cBSA does not depend on a previous exposure of immune cells to apo B-100 antigens in the context of hypercholesterolemia.

![Figure 1: p210-cBSA immunization favorably induce a regulatory T cell phenotype in spleen after immunization and in vitro incubation. C57Bl/6 mice were immunized with p210-cBSA and analyzed for Tregs in spleen one week after last immunization using flow cytometry (A) Tregs were gated as CD25 FoxP3+ cells out of CD3+CD4+ T cells. Spleen cell proliferation in response to CD3/CD28 beads activation after p210-cBSA immunization (B). The proliferative capacity of the cells is expressed as counts per minute (CPM) and mice immunized with PBS served as control (*p ≤ 0.05).](image)

To study the mechanisms through which p210 affects T effector cell proliferation we used a modified version of an in vitro model originally developed by Bonertz et al. [18]. In this model antigen loaded DCs are first allowed to interact with CD4+CD25+ Tregs and the cells are subsequently transferred to cultures of CD4+CD25- T effector cells pre-activated with plate-bound CD3. Proliferation of T effector cells was determined after 72 hours of co-culture.

This model shows that T effector cells only proliferate in the presence of DCs and independently of Tregs (Figure 2A). This proliferation was partly inhibited by addition of antibodies against MHC class II suggesting that the activation of T effector cells in this model involves MHC class II - T cell receptor interactions (Figure 2A). To determine if the model could be used to characterize immunemodulatory properties of p210 we pulsed DC/Tregs with p210-cBSA which resulted in a dose-dependent inhibition of T effector cell proliferation. p210 and cBSA given separately also inhibited the T effector cell activation but to a lesser extent than p210-cBSA (Figures 2B and 2C). The effect of cBSA was dependent on the cationization as native BSA did not influence the proliferation of T effector cells (data not shown). To exclude the possibility that the reduction in proliferation was caused by toxicity or induction of apoptosis we determined the release of lactate dehydrogenase (LDH) in the medium as well as cellular caspase 3 activity. However, no increase of LDH release or caspase 3 activity could be observed in cells incubated with the highest concentrations (15 µg/ml) of cBSA, p210 or p210-cBSA (Figures 2D and 2E).

![Figure 2: p210-cBSA dose-dependently inhibits T effector cell proliferation without being cytotoxic in an in vitro model of T cell function. Proliferation of polyclonally activated T effector cells was determined in the presence or absence of DCs, Tregs and/or a neutralizing MHC class II antibody (A). Proliferation of polyclonally activated T effector cells cocultured with DCs and Tregs is determined in the presence of increasing concentrations of p210-cBSA, cBSA or p210 (B and C). The proliferative capacity of the cells is expressed as counts per minute (CPM) and cells without antigen served as control. Cytotoxicity and apoptosis were determined in p210-cBSA treated cells by assessment of LDH (D) and caspase 3 activity (E). Triton-X and camptothecin treated cells served as controls for the LDH and caspase 3 assays, respectively. (*p ≤ 0.05 ***p<0.001)](image)
The binding and uptake of p210-cBSA by splenocytes was studied using FITC-labeled p210-cBSA. Fluorescence microscopy analysis demonstrated that only a part of the splenocyte population could bind and take up p210-cBSA (Figure 3A). This observation was confirmed by flow cytometric analyses demonstrating that FITC-p210-cBSA binding and uptake primarily was restricted to MHCI\(^+\) and CD11c\(^+\) cells (Figure 3B). Saturation of FITC-p210-cBSA binding and uptake was observed only at very high concentrations (>200 µg/ml, Figure 3B) and FITC-p210-cBSA binding and uptake could not be competed by an excess of unlabeled p210-cBSA (Figure 3C) or by incubation on ice (data not shown) suggesting that uptake occurred by unspecific endocytosis rather than by a specific receptor-mediated mechanisms. Furthermore, FITC-p210-cBSA was taken up more effectively than FITC-p210 confirming the highly immunogenic properties of cBSA (Figure 3D).

To determine if the inhibitory effect of p210-cBSA on cell proliferation was due to a direct effect on T effector cells we exposed T effector cells specific for the ovalbumin 323-339 amino acid sequence. Addition of p210-cBSA to DCs pre-loaded with p210-cBSA was determined by flow cytometry and presented as mean fluorescent intensity (MFI) of FITC-p210-cBSA in cells gated as CD11c\(^+\) and MHCI\(^+\) cells (C). Uptake of FITC-p210-cBSA was determined by flow cytometry and presented as mean fluorescent intensity in CD11c\(^+\) and MHCI\(^+\) cells (C). Uptake of FITC-labeled p210-cBSA or FITC-labeled p210 was studied in CD11c\(^+\) cells after in vitro incubation with the respective antigen (D). Uptake was determined as MFI of the CD11c\(^+\) and MHCII\(^+\) cells (C).

Figure 3: p210-cBSA is preferentially taken up by antigen presenting cells in vitro. Uptake of FITC-labeled p210-cBSA by splenocytes was studied by confocal microscopy (A) and flow cytometry (B) after in vitro incubation with 50 µg/ml FITC-p210-cBSA at 37°C. Representative immunofluorescence microscopy picture visualizing cell nucleus in red and FITC-p210-cBSA positive splenocytes in green. Flow cytometry results showing mean fluorescence intensity (MFI) of FITC-p210-cBSA in cells gated as CD11c positive, MHCI\(^+\) positive or MHCI\(^-\) negative. Increasing concentrations unlabeled p210-cBSA was added to in vitro cultures of splenocytes and 25 µg/ml of FITC-labeled p210-cBSA. Uptake of FITC-p210-cBSA was determined by flow cytometry and presented as mean fluorescent intensity in CD11c\(^+\) and MHCI\(^+\) cells (C). Uptake of FITC-labeled p210-cBSA or FITC-labeled p210 was studied in CD11c\(^+\) cells after in vitro incubation with the respective antigen (D). Uptake was determined as MFI of the CD11c\(^+\) cells.

Figure 4: p210-cBSA inhibition of T cell proliferation is dependent on DCs but independent of antigen specific T effector cells and presence of T\(_{regs}\). The direct effect of p210-cBSA on T effector cell proliferation was measured using CD3/CD28 beads in the presence or absence of p210-cBSA (A). Proliferation of polyclonally activated T effector cells was determined using DCs, T\(_{regs}\) and T effector cells from OTII mice after incubation with p210-cBSA (B). T effector cell proliferation with p210-cBSA, cBSA or p210 was assessed in the absence of T\(_{regs}\) and in the presence of DCs (C). The proliferative capacity of the cells is expressed as counts per minute (CPM) and un-stimulated cells served as control. The ability of p210-cBSA to induce conversion of naïve T effector cells into T\(_{regs}\) was studied by pulsing DCs with p210-cBSA (p210DCs) for two hours and then analyzing the frequency of T\(_{regs}\) with flow cytometry after 72 hours coculture. T\(_{regs}\) were induced in the absence (D) and presence (E) of IL-2 and TGF-\(\beta\) and un-stimulated cells served as controls (cDCs). T\(_{regs}\) were gated as CD25\(^+\)FoxP3\(^+\) out of CD3\(^+\)CD4\(^+\) cells. (*\(p \leq 0.05\), **\(p \leq 0.01\), ***\(p<0.001\)).
the effect of p210-cBSA was dependent on an inhibition of this signal.

Addition of IL-12 increases the expression of CD86 on MHCII in a dose-dependent manner (Figure 5A-5C). Addition of IL-12 (100 pg/ml) to cell cultures of DCs pre-incubated with p210-cBSA and CD4+CD25+ cells in untreated cells and this increase is partly blocked by incubation with p210-cBSA. DC expression of CD86, MHC class II and IL-12 is enhanced by activation of toll-like receptors (TLRs) [21]. To study if the effect of p210-cBSA was dependent on an inhibition of this signal pathway we used cells lacking the TLR signaling protein MyD88 in the coculture assay. However, the inhibitory effects of p210-cBSA remained intact in absence of functional TLR signaling (Figure 6C). We therefore added IL-12 (100 pg/ml) to cell cultures of DCs pre-incubated with p210-cBSA and CD4+CD25+ cells and thereafter determined the frequency of T_{reg}. We found no decreased frequency of induced T_{reg} in the cells cultured in the presence of IL-12 (Figure 6D).

We next investigated the effect of p210-cBSA on DC function. IL-10 and IL-12 released from DCs play important and opposing roles in modulating T effector cell activation [20]. Incubation with p210-cBSA did not influence the release of IL-10 but inhibited the release of IL-12 in a dose-dependent manner (Figure 5A-5C). Addition of IL-12 (100 pg/ml) to the culture medium completely reversed the inhibitory effect of p210-cBSA on T effector cell proliferation (Figure 5D), whereas addition of IL-10 blocking antibodies neutralizes the effect of p210-cBSA (Figure 5E). Neutralizing IL-10 antibody was added to selected wells of DCs, T_{reg} and polyclonally activated T effector cells in the presence or absence of p210-cBSA or cBSA (E). (*p ≤ 0.05, **p ≤ 0.01, ***p<0.001).

**Figure 5:** IL-12 release decreases after incubation with p210-cBSA and addition of IL-12 restores T cell proliferation. Release of IL-10 and IL-12 was determined after incubation of DCs, T_{reg} and polyclonally activated T effector cells with increasing concentrations of p210-cBSA, cBSA or p210 (A and B). IL-12 release was determined after incubation of DCs and polyclonally activated T effector cells with p210-cBSA, cBSA or p210 in the absence of T_{reg} (C). 100 pg/ml of IL-12 was added to selected cocultures of DCs, T_{reg} and polyclonally activated T cells in the presence or absence of p210-cBSA. Proliferation of cells was determined after 72 hours and is expressed as counts per minute (CPM) (D). Neutralizing IL-10 antibody was added to selected wells of DCs, T_{reg} and polyclonally activated T effector cells in the presence or absence of p210-cBSA or cBSA (E). (*p ≤ 0.05, **p ≤ 0.01, ***p<0.001).

**Figure 6:** p210-cBSA decrease the DC activation status and the effect on proliferation is independent of MyD88 signalling. DCs were stimulated with p210-cBSA in the presence or absence of 100 pg/ml IL-12 and the frequency of CD86+ cells out of MHCII+ cells (A) and the expression of MHC class II on all CD11c+ cells (B) were thereafter determined with flow cytometry. DCs, T_{reg} and polyclonally T effector cells from MyD88 deficient mice were used to determine the role of MyD88 signaling in the cocultures. Proliferation was determined after 72 hours and is expressed as counts per minute (CPM) (C). The effect of IL-12 on conversion of naïve T effector cells into T_{reg} was studied by pulsing DCs with p210-cBSA (p210DCs) for two hours and analyzing T effector cell phenotype with flow cytometry after 72 hours coculture with and without 100 pg/ml IL-12 (D). Un-stimulated cells served as controls (cDCs) and T_{reg} were gated as CD25+FoxP3+ out of CD3+CD4+ cells. (*p ≤ 0.05, **p ≤ 0.01, ***p<0.001).

**Discussion**

Immunization with the apoB-100 peptide p210 have been shown to inhibit the development of atherosclerosis when administered as a vaccine conjugated to cBSA or CTB as well as when administered by subcutaneous slow infusion without carrier or adjuvant [7,10,11]. These observations suggest that it could be possible to develop apoB-100 peptide-based vaccines for prevention of cardiovascular disease. Several lines of evidence support the notion that immunization with p210 inhibits atherosclerosis through activation of T_{reg}. Studies based on T_{reg} depletion or T_{reg} transfers have established that T_{reg} inhibits the development of atherosclerosis [22,23]. Moreover, the athero-protective effect of p210 immunization has repeatedly been associated with an activation of T_{reg} and removal of T_{reg} through CD25-blocking antibodies neutralizes the effect of immunization with p210-cBSA [9-11]. However, the mechanisms through which p210 interacts with immune cells have not been previously characterized. The present findings demonstrate that the p210-cBSA conjugate promotes APC-dependent conversion of naïve T
effectors into T<sub>regs</sub> and inhibits the proliferation of pre-activated T
effectors. Exposure of DCs to p210-cBSA also resulted in a down-
regulation of MCH class II and the co-stimulatory molecule CD86.
The inhibition of naïve T cell proliferation was not dependent on
induction of apoptosis and was not explained by a direct effect of
p210-cBSA on T effector cells. These findings provide a possible
mechanistic explanation to the expansion of T<sub>regs</sub> and suppression of T
effector cells observed in previous immunization studies using
the p210 antigen and indicates that p210-cBSA functions by inducing a
tolerogenic APC phenotype.

Since T<sub>regs</sub> are known to be potent suppressors of T effector cells we
investigate if the inhibition of T effector cell proliferation by p210-
cBSA was mediated by T<sub>regs</sub>. However, we unexpectedly observed that
p210-cBSA loaded APCs could suppress the proliferation of naïve T
effector cells also in the absence of T<sub>regs</sub> suggesting the involvement of
a direct inhibitory effect of APCs. This effect could be due to a reduced
release of IL-12 from the APCs. Addition of IL-12 completely restored
T effector cell proliferation in cultures with p210-loaded APCs. IL-12
treatment increased the expression of CD86 on MHCI<sup>+</sup> cells and this
increase could partly be inhibited by p210-cBSA treatment. Since
IL-12 is an important polarizing factor for T effector cells, this suggests
that p210-cBSA suppresses the proliferation of T effector cells by
depletion of an important factor with Th1-polarizing activity. The
down-regulation of MHC class II and CD86 that occurred in APCs as
a result of the p210-cBSA mediated inhibition of IL-12 expression is
also likely to contribute to the reduced activation of T effector cell
proliferation. In the present experiment the down-regulation of MHC
class II is likely to be of particular importance since activation of T
effector cell proliferation in our model was found to be dependent on
an interaction with MHCI class II. Although the suppression of T
effector cells by p210-cBSA was independent of T<sub>regs</sub> in the present
study this does not exclude the possibility that T<sub>regs</sub> mediate suppression of T
effector cells in both our in vitro assay and in p210-
immunized mice. T<sub>regs</sub> are induced to a greater extent by APCs
incubated with p210-cBSA compared to un-stimulated cells indicating that
the tolerogenic phenotype of APCs that are induced by p210-
cBSA have functional properties. Moreover, as addition of IL-12 to the
p210-cBSA incubated APCs did not change the frequency of induced
T<sub>regs</sub> this indicates that additional mechanisms are also responsible for
the changed properties of the APCs. If this also is valid for p210-cBSA
in vivo needs to be further clarified. Experiments in which T<sub>regs</sub>
depletion has been achieved by treatment with CD25 antibodies have
provided evidence for a key role of T<sub>regs</sub> in mediating the athero-
protective effect of p210 immunization. These studies suggest that
T<sub>regs</sub> generated in response to p210 have the ability to suppress
proliferation of T effector cells as well as to inhibit the development of
atherosclerosis. However, it remains to be fully clarified if the athero-
protective action of these T<sub>regs</sub> involves suppression of plaque antigen-
specific T effector cells, a bystander anti-inflammatory effect through
release of IL-10 and TGF-β when encountering the p210 antigen in
plaques or a combination of both.

Studies using FITC-labeled p210-cBSA demonstrated that binding
and uptake of p210-cBSA primarily occurs in APCs. Inhibition of
IL-12 release was identified as the key mediator of the suppressive
effects of p210-cBSA. IL-12 is primarily produced by APCs in response
to antigen stimulation and plays an important role in activation of Th1
T cells. It consists of two subunits (p40 and p35) and the expression is
controlled by transcriptional induction of the p40 subunit by NF-κB.
We used splenocytes from MyD88-deficient mice to investigate if
p210-cBSA reduced the production of IL-12 through inhibition of
TLR-mediated activation of NF-κB but found no evidence for
involvement of this pathway. IL-10 is one of the physiologically most
important inhibitor of IL-12 production through suppression of c-rel
[20]. However, we observed no increase in IL-10 release in APCs
exposed to p210-cBSA and treatment with IL-10 blocking antibodies
did not affect the ability of p210-cBSA loaded APCs to inhibit T
effector cell proliferation. Collectively, these observations demonstrate
that the effect of p210-cBSA on APC function is independent of the
NF-κB pathway. The role of the IL-10/c-rel pathway needs to be
further characterized, preferably with an antibody blocking the IL-10
receptor. A tolerogenic phenotype of DCs has been shown to be
associated with an impaired ability of producing IL-12 [16]. The
tolerogenic DCs may then reduce T effector cell activation directly or
induce T<sub>reg</sub> formation, both resulting in a reduced proliferation. IL-12
has also directly been implicated in the progression of atherosclerosis
and blocking IL-12 production can inhibit atherogenesis in LDL
receptor deficient mice [24]. Inhibition of IL-12 expression in
dendritic cells exposed to the p210 antigen could thus play a protective
role in atherosclerosis.

An important question is whether the suppressive effect of p210-
cBSA loaded APC is antigen-specific in the respect that only the
proliferation of p210-specific T effector cell is inhibited. Although
pre-activated naive T effector cell started to proliferate when co-cultured
with APC in absence of p210 it cannot be excluded that these APCs
presented apo B antigens derived from the serum-containing medium.
However, p210-cBSA loaded APCs were equally effective in inhibiting
the proliferation of T effector cells derived from OTII mice
demonstrating that the suppressive effect is not restricted to p210-
specific effector cells.

The present observations are of relevance because they provide a
better understanding of the mechanisms through which apoB-100
peptide-based vaccines activate immune responses that protect against
development of atherosclerosis. The athero-protective potential of
immunization with LDL, oxidized LDL, and apoB-100 peptides is well
documented from a number of different animal models of
atherosclerosis [7,25]. Vaccines based on apoB-100 peptides have the
best clinical potential because they can be synthesized under
conditions that meet regulatory requirements. To make it possible for
this type of therapy to enter into clinical testing an improved
understanding of the mode-of-action will be required in order to
design relevant safety studies, decide on dosing regimens and to
monitor the response to treatment.

In summary, results from the present study suggest that p210-cBSA
inhibits proliferation of naïve T effector cells trough suppression of
IL-12 and a reduced frequency of CD86 on MHCI<sup>+</sup> cells and a
reduced expression of MHCI<sup>+</sup> on CD11c<sup>+</sup> cells. Moreover, p210-
cBSA treated CD11c<sup>+</sup> cells have an increased ability to induce T<sub>regs</sub>
from CD4<sup>+</sup>/CD25<sup>+</sup> T cells. This T<sub>regs</sub> induction can, however, not be
reversed by addition of IL-12. Taken together, these observations
suggest that immunization with p210-based vaccines inhibits
atherosclerosis by inducing tolerogenic APCs that in turn generate
T<sub>regs</sub> that suppress T effector cells and inflammation in atherosclerotic
lesions.

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