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+ regulatory T 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 Treg 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 Treg 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-β and inhibit T effector cell activation. There are two major subtypes of Treg, natural Treg generated in the thymus, and induced Treg, 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 up regulates these markers. Fully mature DCs will activate conventional T effector cells in contrast to the tolerogenic DCs that can induce Tregs 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 Treg 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 Treg 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 Treg 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 OliI 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 two times with two 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/Treg 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 Tregs

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 Treg [19]. Additionally, IL-12 (Peprotech) was added to selected wells at 100 pg/ml. Treg 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 MHCII-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 MHCII-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. Camptohecin 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+ 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).

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 immunomodulatory 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 camptohecin treated cells served as controls for the LDH and caspase 3 assays, respectively. (*p ≤ 0.05 ***p<0.001)
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 mg/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 proliferation was due to a direct effect on T effector cells we exposed anti-CD3/CD28 bead-activated effector T cells to p210-cBSA in the absence of both DCs and Tregs. T 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, Tregs and T effector cells from OTII mice after incubation with p210-cBSA (B). T effector cells were sorted out from mouse spleen by immunomagnetic cell isolation. DCs were first pulsed with p210-cBSA for 2 hours and then co-cultured with naïve T effector cells for 72 hours on plate-bound aCD3. DCs pre-loaded with p210-cBSA were found to have an increased ability to convert naïve T effector cells into Tregs both in the absence and presence of IL-2 and TGF-β (Figures 4D and 4E).

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 Tregs. 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, Tregs 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 Tregs 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 Tregs was studied by pulsing DCs with p210-cBSA (p210DCs) for two hours and then analyzing the frequency of Tregs with flow cytometry after 72 hours coculture. Tregs were induced in the absence (D) and presence (E) of IL-2 and TGF-β and un-stimulated cells served as controls (cDCs). Tregs were gated as CD25+FoxP3+ out of CD3+CD4+ cells. (*p ≤ 0.05, **p ≤ 0.01 , ***p<0.001).
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 (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, Tregs, and polyclonally activated T effector cells with increasing concentrations of p210-cBSA, cBSA or p210 in the absence of IL-12 (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 Tregs (C). 100 pg/ml of IL-12 was added to selected cocultures of DCs, Tregs 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, Tregs 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).

Discussion

Immunization with the apoB-100 peptide p210 have been shown to inhibit the development of atherosclerosis when administered as a vaccine conjugated to BSA 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 Tregs. Studies based on Treg depletion or Treg transfers have established that Tregs inhibits the development of atherosclerosis [22,23]. Moreover, the athero-protective effect of p210 immunization has repeatedly been associated with an activation of Tregs and removal of Tregs 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 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 Tregs were gated as CD25+FoxP3+ out of CD3+CD4+ cells. (*p ≤ 0.05, **p ≤ 0.01, ***p<0.001).
effectors into T_{reg} and inhibits the proliferation of pre-activated T effector cells. Exposure of DCs to peptide-BSA also resulted in a down-regulation of MHC 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 peptide-BSA on T effector cells. These findings provide a possible mechanistic explanation to the expansion of T_{reg} and suppression of T effector cells observed in previous immunization studies using the peptide-BSA and indicates that peptide-BSA functions by inducing a tolerogenic APC phenotype.

Since T_{reg} are known to be potent suppressors of T effector cells we investigate if the inhibition of T effector cell proliferation by peptide-BSA was mediated by T_{reg}. However, we unexpectedly observed that peptide-BSA loaded APCs could suppress the proliferation of naïve T effector cells also in the absence of T_{reg} 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 peptide-BSA loaded APCs. IL-12 treatment increased the expression of CD86 on MHCII^{+} cells and this increase could partly be inhibited by peptide-BSA treatment. Since IL-12 is an important polarizing factor for T effector cells, this suggests that peptide-BSA 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 peptide-BSA 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 MHC class II. Although the suppression of T effector cells by peptide-BSA was independent of T_{reg} in the present study this does not exclude the possibility that T_{reg} mediate suppression of T effector cells in both our in vitro assay and in peptide-BSA immunized mice. T_{reg} are induced to a greater extent by APCs incubated with peptide-BSA compared to un-stimulated cells indicating that the tolerogenic phenotype of APCs that are induced by peptide-BSA have functional properties. Moreover, as addition of IL-12 to the peptide-BSA incubated APCs did not change the frequency of induced T_{reg} this indicates that additional mechanisms are also responsible for the changed properties of the APCs. If this also is valid for peptide-BSA in vivo needs to be further clarified. Experiments in which T_{reg} depletion has been achieved by treatment with CD25 antibodies have provided evidence for a key role of T_{reg} in mediating the atheroprotective effect of peptide-BSA immunization. These studies suggest that T_{reg} generated in response to peptide-BSA 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 atheroprotective action of these T_{reg} 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 peptide-BSA in plaques or a combination of both.

Studies using FITC-labeled peptide-BSA demonstrated that binding and uptake of peptide-BSA primarily occurs in APCs. Inhibition of IL-12 release was identified as the key mediator of the suppressive effect is not restricted to peptide-BSA-loaded APCs but also applies to peptide-BSA incubated DCs exposed to peptide-BSA and treatment with IL-10 blocking antibodies. However, we observed no increase in IL-10 release in APCs exposed to peptide-BSA and treatment with IL-10 blocking antibodies did not affect the ability of peptide-BSA loaded APCs to inhibit T effector cell proliferation. Collectively, these observations demonstrate that the effect of peptide-BSA 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_{reg} 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 peptide antigen could thus play a protective role in atherosclerosis.

An important question is whether the suppressive effect of peptide-BSA loaded APC is antigen-specific in the respect that only the proliferation of peptide-specific T effector cell is inhibited. Although pre-activated naïve T effector cell started to proliferate when co-cultured with APC in absence of peptide-BSA it cannot be excluded that these APCs presented apoB antigens derived from the serum-containing medium. However, peptide-BSA 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 peptide-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-protection 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 peptide-BSA inhibits proliferation of naïve T effector cells through suppression of IL-12 and a reduced frequency of CD86 on MHCII^{+} cells and a reduced expression of MHCII^{+} on CD11c^{+} cells. Moreover, peptide-BSA treated CD11c^{+} cells have an increased ability to induce T_{reg} from CD4^{+}CD25^{+} T cells. This T_{reg} induction can, however, not be reversed by addition of IL-12. Taken together, these observations suggest that immunization with peptide-BSA-based vaccines inhibits atherosclerosis by inducing tolerogenic APCs that in turn generate T_{reg} that suppress T effector cells and inflammation in atherosclerotic lesions.

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