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Oral dosing with multi-antigenic construct induces atheroprotective immune tolerance to individual peptides in mice

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ABSTRACT

Inflammatory immune response to self-antigens plays an important role in the development of atherosclerosis. Restoring immune tolerance to self-proteins reduces the pro-inflammatory response. We previously showed that oral tolerance to a combination of two peptides is atheroprotective. In the present study we expressed epitopes from apolipoprotein B 100 (ApoB), human heat shock protein (HSP60) and *Chlamydia pneumonia* outer membrane protein (Cpn) in a single protein scaffold and used this multi-antigenic construct to induce tolerance to individual peptides by oral route in $ApoB^{tm2Sgy}/Ldlr^{tm1Her/J}$ mice. Antigen specific tolerance to individual peptides was observed in treated animals as seen by an increase in regulatory T cells. Tolerance to the peptides resulted in a 46.5% ($p = 0.002$) reduction in the development of atherosclerosis compared with control. Atheroprotection was associated with a significant ($p < 0.05$) decrease in plaque inflammation and an increase in the expression of immune regulatory markers in the aorta. $CD11c^+$ cells coexpressing CD11b and CD103 increased in lymphoid organs and were found to activate regulatory T cells and reduce effector T-cell response. Adoptive transfer of $CD11c^+$ cells was atheroprotective. Our results suggest that atheroprotection by oral tolerance to a multi-antigenic construct is mediated by antigen specific regulatory T cells and $CD11c^+$ cells with immune regulatory properties.

1. Introduction

Atherosclerosis is a chronic inflammatory disease that develops in response to lipid accumulation, immune response and inflammation [1]. Recent evidence suggests that inflammation is mediated by an autoimmune response to self-antigens such as oxidized low-density lipoprotein (ox-LDL) and heat shock proteins (HSPs) in the vascular wall [2,3]. Immunotherapy for atherosclerosis is directed towards inducing tolerance to self-antigens by increasing the number of antigen-specific regulatory T cells (Tregs), which can suppress the pro-atherogenic immune response [4,5].

Oral administration of antigen leads to systemic unresponsiveness and represents a powerful tool for treating autoimmune and inflammatory diseases. Repeated administration of a low dose of antigen induces regulatory T cells, secreting transforming growth factor β (TGF-β) or interleukin (IL)-10, and $CD4^+CD25^+Foxp3^+$ Tregs [6]. Dendritic cells (DCs) play a major role in the development of antigen specific immune regulation to orally administered antigens [7]. Antigen-induced, tolerogenic $CD11c^+$, $CD11b^+$ DCs are shown to increase in Peyer's patches and confer protection against experimental arthritis by inducing regulatory T cells in mice [8]. Recent studies have shown that mucosal DCs expressing CD103 were able to induce $Foxp3^+$ Tregs in the presence of TGF-β [9].

Several studies have demonstrated an effective early reduction of atherosclerosis in mouse models by inducing tolerance to peptides derived from apolipoprotein B (ApoB) 100, HSPs 60/65, and β2-glycoprotein [10–15]. We have earlier shown that the combination of ApoB and HSP60 peptides confer better protection compared with either of the individual peptides [15] and oral tolerance to this combination could prevent development and progression of atherosclerosis in mice [16].

Abbreviations: Ox-LDL, Oxidized low-density lipoprotein; HSP, Heat shock proteins; Tregs, Regulatory T cells; TGF-β, Transforming growth factor β; IL, Interleukin; ApoB, Apolipoprotein B; Cpn, *Chlamydia pneumonia*; DSP, Dendroaspin; GST, Glutathione *S*-transferase; IFN, Interferon; MMP, Matrix metalloproteinase; MRP8/14, Myeloid related protein complex; TNF, Tumor necrosis factor; OMP, Outer membrane protein.

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We believe that each of these self-antigens has a distinct role in the pathogenesis of atherosclerosis and using multiple antigens would be more effective for immune therapy than either of the individual antigens [17]. We also consider that infection can contribute to atherosclerosis by different mechanisms including molecular mimicry between antigens from pathogens and self-proteins [18]. We chose three peptides derived from ApoB100, human HSP60 (hHSP60) and outer membrane protein (OMP) of *Chlamydia pneumonia* (Cpn) to construct our multi-antigenic molecule [19]. Immunization with ApoB peptides was reported to reduce atherosclerosis in transgenic mice expressing human ApoB100 [20]. Antigenic epitopes from hHSP60 share considerable homology with human cytomegalovirus derived immediate early protein UL122 and *Porphyromonas gingivalis* HSP60, resulting in immunological cross-reactivity, which may play a role in atherogenesis [21]. An epitope of the major outer membrane protein (MOMP) of *Cpn* (AA 67–74: GDYVFDRI) and the putative outer membrane protein 5 (Omp5) of *Cpn* (AA 284–292: QAVANGGAI) share high homology, with two sequence locations of ApoB protein (http://web.expasy.org/sim/). In addition MOMP and Omp5 peptide sequences were found to reduce atherosclerosis in *C. pneumonia* infected mice [22] and intranasal administration of MOMP was reported to induce anti-atherogenic T-cell responses and protect against atherosclerosis in ApoE$^{-/-}$ mice [23].

We have used a unique protein scaffold to express the peptides derived from self-antigens thus creating a multi-antigenic recombinant protein [19]. Dendroaspin is a short-chain neurotoxin homologue from the venom of Elapidae snakes, with a novel arrangement of loops [24], with no detectable neurotoxicity. We have earlier reported that subcutaneous immunization with recombinant constructs expressing multiple antigens can control the development of atherosclerosis in mice [25]. In the present study we show that oral administration of a recombinant antigen expressing epitopes from ApoB, HSP60 and Cpn, induces immunological tolerance to individual peptides and protects against atherosclerosis development in ApoB^{tm2Sgy}/Ldlr$^{tm1Her/J}$ knockout mice, derived from of C57BL/6, which express only ApoB100 and is deficient in the LDL receptor, and is reported to closely mimic human atherosclerosis [26].

2. Methods

2.1. Animals

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Committee for the Purpose of Control and Supervision of Experiments on Animals (*CPCSEA*), Ministry of Environment, Government of India and conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011). The protocol was approved by the Institutional Animal Ethics Committee of the Thrombosis Research Institute (Registration Number: 1261/c/09/CPCSEA). ApoB^{tm2Sgy}/Ldlr$^{tm1Her/J}$ knockout mice on a C57BL/6 background (Jackson labs) were kept under standard laboratory conditions with controlled temperature, humidity as specified by CPCSEA, and were given chow diet (Nutrilab, India) or a high-fat diet containing 1.25% cholesterol and 21% milk fat (Harlan, TD 96121 Indianapolis, USA). Tolerance induction and efficacy studies were carried out twice in groups of 6 mice per group per study.

2.2. Expression and purification of recombinant construct

Recombinant multi-antigenic protein was constructed in the dendroaspin background as described earlier [19]. Three peptides derived from ApoB100 (AA 688–707), hHSP60 (AA 153–163), and a combination of MOMP (AA 66–73) and OMP 5 (AA 283–291) of Cpn were linked to the N-terminal, loop 2 and C terminal end respectively of dendroaspin protein referred to as AHC (Fig. SI A). The recombinant construct was expressed as glutathione *S*-transferase (GST) tagged protein in *Escherichia coli* and purified by affinity chromatography. The molecular weight of AHC-GST was 39 kDa and that of DSP-GST was 33.5 kDa.

2.3. Experimental design

Groups of ApoB^{tm2Sgy}/Ldlr$^{tm1Her/J}$ mice (5–6 weeks of age) were orally dosed five times on alternate days with 20 μg of the AHC-GST, dendroaspin-GST and GST per animal, per dose. Animals were given 2 mg of soybean trypsin inhibitor before oral dosing (Sigma Chemical Co. St. Louis. MO, USA). Dose response was carried out with 1 μg, 5 μg, 20 μg and 80 μg of AHC-GST to determine the optimum dose for tolerance induction (Fig. SII). Induction of tolerance was studied 6 days after the last dose. Mice were fed on a diet high in fat and cholesterol (Harlan, TD 96121 Indianapolis, USA: 21% fat and 1.25% cholesterol), for 10 weeks to develop atherosclerosis. DSP was used as a control for all experiments with AHC protein. GST was also included as a control in experiments related to atheroprotection.

2.4. Cell isolation and purification

Mice were euthanized humanely using an overdose of isoflurane inhalant anesthetic. Cells were harvested from the spleen, Peyer's patches, mesenteric lymph nodes and lamina propria six days or 10 weeks after the last oral dose. For the isolation of lamina propria cells, Peyer's patches were excised, the intestine opened longitudinally and incubated thrice for 15 min in Hank's balanced salts with 10% FCS and 2 mM ethylenediaminetetraacetic acid (EDTA) at 37 °C to remove epithelial cells. After each incubation step, tubes were shaken for 10 s and media containing epithelial cells and debris were discarded. The remaining tissue was incubated for 45 min in Roswell Park Memorial Institute medium (RPMI1640), with 10% fetal bovine serum, 0.24 mg/ml collagenase A and 40 U/ml DNAse I (Roche Applied Sciences, Penzberg, Germany), the tubes shaken for 10 s and cell suspensions collected. This step was repeated one more time and the pooled cell suspensions were used for flow cytometry [27]. The Peyer's patches were removed and treated for 90 min at 37 °C with media containing dithiothreitol and EDTA to remove epithelial cells, and washed with Hank's balanced salt solution, followed by digestion with 0.24 mg/ml collagenase A and 40 U/ml DNAse I and incubated in the presence of 5 mM EDTA for 5 min at 37 °C. Mononuclear cells were analyzed by flow cytometry. Single cell suspensions of spleen and lymph nodes were prepared by passing the cells through a sterile cell strainer; erythrocytes were lysed in ammonium chloride buffer (Sigma Chemical Co., St.Louis. MO, USA) and washed twice with Hank's balanced salt solution. To isolate CD11c positive cells, mononuclear cells from gut lymphoid organs (mesenteric lymph node, Peyer's patches and lamina propria) were pooled and incubated with anti-mouse CD11c-coated magnetic beads (Miltenyi Biotec, Teterow, Germany) and then subjected to selection through MACS separation columns. Cells selected on the basis of CD11c expression routinely showed greater than 95% viability. Regulatory T cells were isolated using a CD4$^+$CD25$^+$ regulatory T Cell Isolation Kit (Miltenyi Biotec, Teterow, Germany).

2.5. Functional immunoassays

To generate peptide specific effector T-cells, groups of six mice were injected with a mixture of equal concentration of ApoB, HSP60 and Cpn-KLH peptides (100 μg per animal) emulsified in complete Freund's adjuvant by the subcutaneous route The animals were given two booster doses (50 μg each per animal) in incomplete Freund's adjuvant 3 weeks apart. Six days after the last immunization, the splenocytes were collected, CD4 positive cells purified using T Cell Isolation Kit (Miltenyi Biotech, Teterow, Germany) and used as effector cells. The T effector cells were labeled with 10 μM 5, 6-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Sigma Chemical Co., St.Louis. MO, USA).

Oral tolerance was induced in a second set of mice as described in the previous section. The spleen cells were collected from tolerized mice and regulatory T cells were isolated using the CD4$^+$CD25$^+$ regulatory T Cell Isolation Kit (Miltenyi Biotech, Teterow, Germany). The CD4$^+$CD25$^+$ regulatory cells were labeled with 6 μM PKH26 (Sigma Chemical Co., St.Louis. MO, USA) to discriminate the effector and regulatory CD4 population. Effector T cells (1×10^5) and regulatory cells were taken in different ratios, and activated with 10 μg/ml of antigen (ApoB100 peptide, HSP60 peptide or Cpn peptide) in X vivo 20 serum free medium (Lonza, Basel, Switzerland). After 5 days of incubation, cells were stained with CD4-APC (eBiosciences, California, USA) [28]. Lymphocytes were gated using forward and side-scatter plots. PKH26 stained CD4 cells were excluded from the analysis. Proliferation of CD4 effector cells was measured by CFSE dilution using FACS CANTO II (Becton Dickinson, New Jersey, USA) and analyzed using FlowJO software. The proliferation index of T cells was calculated from the FlowJO software. The proliferation index of control wells without an antigen was subtracted from the test wells for analysis. The culture supernatants from the wells were collected for cytokine analysis. The functional assay with CD11c positive cells were carried out using the same protocol, by replacing Tregs with purified CD11c positive cells.

2.6. Cytokine assays

The concentrations of TGF-β and IL-10, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α in the culture supernatants or plasma were measured by enzyme-linked immunosorbent assay (ELISA) as per the manufacturer's instructions (eBiosciences, San Diego, CA, USA).

2.7. Flow cytometry

Flow cytometry analyses were performed by FACS Canto II using FACS DIVA software (Becton Dickinson, New Jersey, USA) and FlowJo software (Tree Star Ltd, Oregon, USA). The antibodies used were as follows: fluorescein isothiocyanate (FITC)-conjugated CD4 (clone RM4-5), allophycocyanin (APC)-anti-CD25 (clone 3C7), phycoerythrin (PE)-anti-fork head box p3 (Foxp3) (clone NRRF-30), APC-anti-CD11c (clone N418), PE-anti-CD11b (clone M1/70) USA, FITC-CD103 (clone 2E7), PE-IFN-γ (clone XMG1.2) all from eBiosciences, San Diego, CA, USA, PE-anti-CD152 (clone UC10-4 F10-11) and APC-Cy™7 IL-17A (clone TC11-18H10, BD Pharmingen, San Jose, USA) and isotype-matched control antibodies. Intracellular staining of Foxp3 was performed using the Foxp3-staining buffer