Research Article

Comparison of Oral Tolerance to ApoB and HSP60 Peptides in Preventing Atherosclerosis Lesion Formation in *ApoB48*⁻/*Ldlr*⁻ Mice

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Antigen-specific immune modulation is emerging as an attractive therapeutic option to prevent atherosclerosis. We compared the efficacy of oral administration of peptides derived from apolipoprotein B (ApoB; 661–680) and heat shock protein 60 (HSP60; 153–163), in the prevention of atherosclerotic lesion formation hyperlipidemic low density lipoprotein receptordeficient (*LDLr*^{-/-}), apolipoprotein B-100 only (*apoB*^{100/100}) mice model. Oral administration of peptides induced tolerance as seen by an increase in regulatory T cells in the peripheral immune system. Tolerance to ApoB peptide reduced plaque development by 28.7% ($P < 0.001$) while HSP60 was effective in reducing lesion development by 26.8% in *ApoB48/LDLr*^{-/-} mice. While tolerance to HSP60 resulted in increase in anti-inflammatory cytokines (IL10 and TGF- β), ApoB tolerance was effective in reducing the lipid deposition in the lesion. Our results suggest that the two peptides have distinct mechanisms of controlling the development of atherosclerosis in mice.

1. Introduction

Coronary artery disease remains the major cause of death and disability throughout the world despite the introduction of novel therapeutics [1]. Experimental observations in the past decade have proved that both innate and adaptive immune responses play an important role in the modulation of atherosclerosis. The complex role of the immune response in atherosclerosis is highlighted by the fact that they can contribute to both atheroprotective and proatherogenic effects [2–4]. The immune system generates regulatory T cells (Tregs), which actively suppress immune activation and maintain immune homeostasis [5, 6]. An imbalance between pathogenic T cells producing proatherogenic mediators and

Treg cells with immunosuppressive properties is well established during the development of disease [7–9]. Thus antigen-specific immune modulation is emerging as an attractive therapeutic option to prevent inflammatory autoimmune diseases such as atherosclerosis [10–12].

The observation that immunization with modified low-density lipoproteins can reduce the atherosclerotic lesion in experimental models has opened the possibility that an atheroprotective vaccine can be developed [13–16]. Several antigens like the oxidized phospholipid molecules, modified apolipoprotein B-100 (ApoB) peptide, cholesteryl ester transfer protein, heat shock proteins, and vascular endothelial growth factor receptor have been used as a vaccine for the modification of immune response in atherosclerosis [4].

Immunotherapy is directed toward inducing tolerance to self-antigens mediated by protective antibodies or increasing the antigen-specific Treg cells, which can suppress the proatherogenic T-effector cells [11–16].

The mucosal (intranasal or oral) route of administration is an attractive method of inducing antigen-specific peripheral tolerance to treat autoimmune diseases [17]. Oral administration suppresses the initiation of autoimmune diseases in animal models of experimental autoimmune encephalomyelitis, uveitis, colitis, and atherosclerosis [18]. The efficiency of oral tolerance is dependent on the type of antigen and its dose. While treatment with a high antigen dose results in deletion or anergy of peripheral T cells, low-dose treatment induces antigen-specific Treg cells [19, 20]. Recent reports have demonstrated the atheroprotective role of natural Treg (nTreg) cells expressing CD25 and the transcription factor fox head box p3 (Foxp3) and suppress proliferation of effector cells by a contact-dependent mechanism [17]. Several studies have established effective early reduction of atherosclerosis in hyperlipidemic mouse models by inducing tolerance to modified lipids and peptides derived from apolipoprotein B (ApoB) 100, HSPs 60/65, and β 2-glycoprotein [20–22].

We believe that each of these self antigens has a distinct role to play in the development of the disease and using multiple peptides from these atherogenic proteins would be more effective than using either of them alone. In this study we compared the immune tolerance mediated by two peptides derived from ApoB 100 and HSP60 molecule and its effect on atheroprotection in a double-gene knockout (ApoB^{tm25gy}/LDLr^{tm1Her}) mouse model, which expresses only ApoB100 and is deficient in low-density lipoprotein receptor. The specific strain of mice was used for this study as majority of cholesterol is transported in ApoB100-containing lipoproteins (i.e., LDL fraction), and also it is reported to more closely mimic human atherosclerosis than other models [17].

2. Methods

2.1. Animals. ApoB^{tm25gy}/Ldlr^{tm1Her}/J knockout mice on a C57BL/6 background (ApoB48⁻/Ldlr⁻) were donated by the Thrombosis Research Institute, London, UK. All animal experiments were approved by the institutional animal ethics committee and in compliance with Government of India guidelines and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011). Mice were kept under standard laboratory conditions and fed a normal chow diet (Nutrilab, India) or a high-fat diet (Harlan, TD 96121, Indianapolis, IN, USA). Food and water were administered *ad libitum*. 6–8 animals were used per group.

2.2. Antigens. The antigens used were apolipoprotein B-keyhole limpet hemocyanin (ApoB-KLH) peptide (ApoB-100 amino acids 661–680 conjugated to KLH, used at 1 mg/mL, dissolved in phosphate-buffered saline [PBS]) and heat shock protein 60-KLH (HSP60-KLH) peptide (HSP60

amino acids 153–163 conjugated to KLH, used at 1 mg/mL, dissolved in PBS) (Severn Biotech, Worcester, UK). The peptides were administered orally, (20 μ g per animal, per dose). PBS dosing was carried out as control.

2.3. Lipid Analysis. Blood from individual mice was collected by retroorbital venous plexus under 3% Isoflurane inhalant anesthesia in oxygen as per the American Veterinary Medical Association guidelines (June 2007). Plasma was obtained by centrifugation and was stored at -80°C until the assay was performed. Concentrations of plasma total cholesterol and triglycerides were determined by Siemens Dimension Flex reagent cartridge (Siemens Healthcare Diagnostics Ltd, UK) using the Cobas Fara II Clinical Chemistry auto analyzer (F. Hoffman La Roche Ltd., Basel, Switzerland), following the manufacturer's instructions.

2.4. Antibody Response Measurement. Blood samples were collected in heparinized capillaries by retro-orbital bleeding 2 and 10 weeks after the first oral dose for assessment of antibody production. Free ApoB-100 and human HSP-60 peptides containing an N-terminal cysteine were synthesized by Severn Biotech (Worcester, UK) and were coated on Maleimide-activated 96-well plates (Pierce, Thermo Fisher Scientific Inc., USA). Peptide-specific immunoglobulin IgG or IgA was measured in the serum of immunized mice using horseradish peroxidase-conjugated α -mouse IgG and horseradish peroxidase-conjugated α -mouse IgA (Sigma chemicals, St. Louis, MO, USA) as secondary antibodies.

2.5. Assessment of Atherosclerotic Lesions. Quantification of atherosclerotic lesions was carried out as per the protocol approved by the Animal Models of Diabetic Complications Consortium (<http://www.diacomp.org/>). Mice were euthanized humanely using an overdose of isoflurane inhalant anesthetic (15%). Mouse hearts were perfused with 10 mL of PBS. Hearts were collected in two mediums: optimal cutting temperature (OCT) medium (Tissue Tek, Leica, Germany) and 10% buffered formalin. Aortic root sections (10 μ m) were cut from the hearts embedded in an OCT medium in frozen conditions using a cryotome (Leica CM 1900 UV Cryotome) and used for either immunofluorescence or immunohistochemical studies. Similarly, aortic roots were sectioned from hearts in neutral buffered phenol (NBF) after embedding in paraffin blocks. For lesion analysis in each mouse, five sections 80 μ m apart were stained with Elastica van Geison (EVG). Several sections were measured and the mean is calculated and expressed as micrometer square, using Image-Pro Plus software (Media Cybernetics, Bethesda, MD, USA).

2.6. Immunohistochemical Analysis of Atherosclerotic Lesions. Immunofluorescence on frozen sections was carried out using an indirect immunofluorescence technique. Mice heart was collected in OCT (JUNG tissue freezing media). Sections (10 micron) were cut using a Leica CM 1900 UV cryotome. The frozen sections on poly-l-lysine-coated slides

(poly-prep-slides sigma) were permeabilized using 0.2% of triton X 100 for 30 min, fixed with ice-cold acetone, and blocked with 5% goat serum or 5% rabbit serum depending on the secondary antibody used. Further, the sections were incubated with primary antibodies (rat anti-mouse CD68 (AbD Serotech, Oxford, UK) anti-mouse CD4 (Abcam, Cambridge, UK), antimouse α -actin (Abcam, Cambridge, UK) for 2 h followed by incubation with an appropriate secondary antibody (Alexa-633 tagged Invitrogen), diluted 10 times that of the primary antibody for 1 h). Tissue sections were treated with Bouin's solution to intensify the final color. Nuclei were stained with Weigert's iron hematoxylin, and cytoplasm and muscle were then stained with Beibrich Scarlet-Acid Fuchsin after treatment with phosphotungstic/phosphomolybdic acid. The presence of collagen was demonstrated by staining with aniline blue.

Sections were mounted with Vector Shield. Images were captured using a Leica DMI 4000 B confocal microscope and the analysis was done using Image-Pro software, and percentage areas of fluorescence of specific antigens of interest in the plaque were calculated.

2.7. Cell Proliferation and Cytokine Assays. Cell culture experiments were performed in Roswell Park Memorial Institute (RPMI) 1640 medium (Bio Whittaker, Walkersville, MD, USA) supplemented with 10% Fetal bovine serum, 2 mM glutamine, 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), sodium pyruvate, and antibiotics. X vivo 20 (Lonza, Basel, Switzerland) was used for the assays whenever the supernatants were collected for cytokine analysis. Splenocytes and lymph node cells passed through a sterile cell strainer, washed twice with Hanks balanced salt solution and plated in culture dishes at a concentration of 1×10^5 cells/mL in RPMI medium, and stimulated with 10 μ g/mL of concavalin A (ConA; Merck, New Jersey, USA) or ApoB and HSP60 peptides at 10 μ g/mL in triplicates for 72 hours. To measure cell proliferation, Alamar blue (AbD Serotec, Raleigh, NC, USA) was added to the wells at a dilution of 1:10. Reduction of Alamar blue was quantified by measuring fluorescence after 24 hours using filters set at 560 nm/590 nm (excitation/emission) [18]. Culture supernatants were collected at 72 h, acidified by the addition of HCl, and neutralized with NaOH. Transforming growth factor (TGF)- β concentrations was measured by enzyme-linked immunosorbent assay (ELISA) kits, as per the manufacturer's instructions (eBiosciences, CA, USA). The concentrations of interferon (IFN)- γ and interleukin (IL) 10 were also measured in the supernatant by ELISA as per the manufacturer's instructions (eBiosciences, CA, USA).

2.8. Flow Cytometry Analysis. For fluorescent activated cell scanner (FACS) analyses, splenocytes were isolated from control and peptide-treated mice at the end of the study. To study oral tolerance, splenocytes were isolated 1 week after the final dose. Cells were stained in 2% serum containing phosphate buffered saline. Flow cytometry analyses were performed by FACS Canto II using FACS DIVA software (Becton

Dickinson, NJ, USA) and FLOWJO software (Tree star Ltd., OR, USA). The antibodies used were as follows: fluorescein isothiocyanate (FITC)-conjugated CD4 (clone RM4-5; eBiosciences, San Diego, CA, USA), allophycocyanin- (APC-) anti CD25 (clone PC61.5; eBiosciences), phycoerythrin- (PE-) antifork head box p3 (Foxp3) (clone NRRF-30, eBiosciences), allophycocyanin- (APC-) anti-TGF- β 1 (R&D systems, Minneapolis, MN, USA) PE-anti-CD152 (clone UC10-4F10-11), and isotype-matched control antibodies. Intracellular staining of Foxp3 was performed using the Foxp3-staining buffer set (eBiosciences) according to the manufacturer's instructions. Surface staining was performed according to standard procedures at a density of 1×10^5 cells/100 μ L, and volumes scaled up accordingly.

2.9. Functional Immunoassays. To generate effector T cells, groups of six mice were immunized with either ApoB-KLH peptide or HSP60-KLH peptide (50 μ g/animal) via the subcutaneous route with complete Freund's adjuvant. The animals were given two booster doses of the same antigen (25 μ g/animal) in incomplete Freund's adjuvant 3 weeks apart. Six days after the last immunization, the splenocytes were collected and used as effector cells. Oral tolerance was induced in a second set of mice as described earlier. The spleen cells were collected from tolerized mice and regulatory T cells were isolated using a CD4⁺CD25⁺ regulatory T-Cell Isolation Kit (Miltenyi Biotech, Teterow, Germany). The CD4⁺CD25⁺ regulatory cells were labeled with 6 μ M PKH26 (Sigma chemicals, St. Louis, MO, USA) to discriminate the effector and regulatory CD4 population. The effector cells were labeled with 10 μ M CFSE (Sigma chemicals). The effector cells (1×10^5) and regulatory cells were taken in different ratios and activated with 10 μ g/mL of antigen (ApoB100 peptide and HSP60 peptide). After 5 days of incubation, cells were stained with CD4-APC (eBiosciences, CA, USA) [3].

The lymphocytes were gated using forward and sidescatter plots. PKH26 (Sigma, USA)-stained CD4 cells were excluded from the analysis. Proliferation of CD4 effector cells was measured by 5, 6-carboxyfluorescein diacetatesuccinimidyl ester (CFSE) dilution using FACS CANTO II (Becton Dickinson, NJ, USA) and analyzed using FLOWJO software. The proliferation index of T cells was calculated as described previously [4].

2.10. Plasma Cytokine Concentrations. Cytokine concentrations in the plasma such as IL-10, TGF- β , IFN- γ , and TNF- α were measured using paired antibodies specific for the corresponding cytokines by ELISA kits, as per the manufacturer's instructions (eBiosciences).

2.11. Statistical Analysis. Results are expressed as Mean $\pm$ SEM. Statistical significance was determined using Student's *t*-test or the Mann-Whitney test using Prism software version 5.01 (CA, USA). *P* value < 0.05 was considered to be statistically significant.

3. Results

3.1. Oral Administration of ApoB and HSP60 Peptides Increases the Number of Treg Cells in Lymphoid Organs. To evaluate whether oral tolerance induction to the peptides was associated with an effect on Tregs, flow Cytometry analysis was performed. (Gating strategy for the analysis is presented in Supplementary Figure 1 in supplementary Material available online at <http://dx.doi.org/10.1155/2013/212367>). HSP60 and ApoB peptide-treated ApoB^{tm2Sgy/Ldlr^{tm1Her/J}} mice were euthanized 3, 8 days and 10 weeks after last oral dose. During this period mice were given a diet rich in cholesterol to induce the development of atherosclerosis. Three days after oral dosing the number of CD4⁺CD25⁺ Foxp3⁺ T cells increased significantly ($P < 0.001$) in Peyer's patches ($37.62\% \pm 3.09$ versus $15.9\% \pm 1.54$), spleen ($28.4\% \pm 1.80$ versus $9.44\% \pm 0.41$), and in blood ($26.23\% \pm 5.79$ versus $4.50\% \pm 0.62$) of HSP60-treated animals compared to control. The increase in Treg cells in ApoB-treated animals was lower compared to HSP60-treated mice but were still significantly higher ($P < 0.05$) than the control. Increase was seen in Peyer's patches ($22.75\% \pm 0.96$ versus $15.9\% \pm 1.54$), spleen ($13.8\% \pm 0.99$ versus $9.44\% \pm 0.41$), and in blood ($14.52\% \pm 2.10$ versus $4.50\% \pm 0.62$). The number of Treg cells was not significantly different in the mesenteric lymph nodes. Eight days after the last dose, the number of Treg cells decreased in all the organs but were still significantly higher ($P < 0.05$) only in HSP60-treated animals compared to control. The increase in FoxP3 positive cells compared to control was maintained for 10 weeks after the oral dosing and was found to be significantly higher ($P < 0.05$) in the spleen ($5.18\% \pm 0.91$ and $4.48\% \pm 0.4$ versus 2.25 ± 0.29) and blood ($7.56\% \pm 0.68$ and $5.11\% \pm 0.56$ versus $2.28\% \pm 0.22$) of ApoB- and HSP60-treated animals, respectively (Figure 1 and Supplementary Figure 2). The percentage of Treg cells was consistently higher with HSP60 treatment compared to ApoB. Antibodies specific for ApoB and HSP60 peptides were not detected in the serum after oral administration of peptides, further supporting the induction of tolerance to individual peptides (Supplementary Table 1).

3.2. Oral Tolerance to Peptides Reduces the Proliferative Response of the Splenocytes. To determine the effect of oral tolerance induction on the proliferative response of splenocytes, mice were orally dosed with peptides and PBS. Splenocytes were collected 3 days and 10 weeks after the last dose and cultured with ApoB peptide, HSP60 peptide, and Concanavalin A *in vitro*. Control mice showed a proliferation index of 1.12 ± 0.028 to ApoB peptide 3 days after last dose which increased to 1.65 ± 0.078 by the end of 10 weeks, suggesting that ApoB peptide reactive T cells accumulate in the lymphoid organs as the lesion develops. In the ApoB-treated mice the proliferative index moderately increased from 0.95 ± 0.016 to 1.19 ± 0.047 on day 3 and 10 weeks after the last dose, respectively. The reduction in ApoB-specific proliferation of splenocytes was significant ($P < 0.05$) for

3 days and ($P < 0.01$) for 10 weeks after the last dose. Similar reduction in HSP60 peptide-specific proliferation was observed in the splenocytes of HSP60-treated mice compared to control (0.95 ± 0.02 versus 1.12 ± 0.028 on day 3 and 1.19 ± 0.04 versus 1.62 ± 0.07 by 10 weeks). Splenocytes collected 3 days after last dose were comparable for their proliferative response to Concanavalin A but a significant reduction was observed in those taken after 10 weeks (1.43 ± 0.07 for control, 1.21 ± 0.09 for ApoB, and 1.38 ± 0.09 for HSP60 treatment on day 3 and 1.51 ± 0.01 , 1.04 ± 0.14 and 0.98 ± 0.11 after 10 weeks) (Figure 2(a)).

We then assessed the ability of Treg cells recovered from either HSP60 or ApoB-tolerized mice to suppress the proliferation of T effector CD4⁺CD25[−] cells recovered from HSP60 or ApoB-sensitized (immunization with adjuvant) mice (Figure 2(b)). The suppression of effector cell proliferation was more pronounced in the HSP60 treatment group. The Treg cells from HSP60-treated mice reduced the effector cell proliferation by 73.5% at a ratio of 1:1, while those from control mice suppressed proliferation by 7.8%. The suppression of T effector cells by regulatory T cells from ApoB treated mice was 51.9% compared to 6.4% by control. The functional activity of Treg cells isolated from HSP60-treated mice gave higher degree of suppression compared to ApoB treatment.

3.3. Effect of Tolerance on Cytokine Production. Since cytokines play an important role in atherosclerosis, we investigated whether oral tolerance to peptides would result in a change in cytokine profile in serum. Concentration of IL10 in the serum was comparable between ApoB- and HSP60-treated mice and higher than control 3 days after the last dose (69.92 ± 2.91 and 67.69 ± 2.7 pg/mL versus 21.36 ± 1.9 pg/mL). IL10 concentrations increased significantly to (267.94 ± 18.35 pg/mL) by the ends of 10 weeks with HSP60 treatment while the increase was moderate for ApoB treatment (86.64 ± 13.9 pg/mL). Serum IFN- γ concentrations were comparable between the groups initially (11.32 ± 2.5 , 11.52 ± 1.7 , and 12.32 ± 2.6 pg/mL respectively for control, ApoB and HSP60-treated mice). The concentrations increased in the control group to 19.52 ± 2.1 pg/mL and 14.94 ± 2.08 pg/mL in HSP60 treated groups but were found to decrease slightly to 10.8 ± 1.4 pg/mL in ApoB-treated group. Serum concentrations of TNF- α were found to increase in control (14.69 ± 0.45 to 24.55 ± 2.69 pg/mL) but remained stable in the peptide-treated animals (14.92 ± 0.73 and 14.36 ± 1.98 pg/mL) (Figure 3(a)). We then investigated the cytokine profile in the supernatant of splenocytes isolated from mice at the end of 10 weeks after immunization stimulated with respective peptides. Interestingly the concentrations of TGF β and IL10 were significantly higher in the supernatants of mice treated with the peptides compared to control while those of TNF α and IFN γ were lower in the treated groups compared to control. The TNF- α and IFN γ concentrations decreased by 47.7% and 22.5% in ApoB-treated group compared to control while the decrease was higher at and 55.8% and 58.2% in HSP60-treated groups.

FIGURE 1: Oral Administration of ApoB and HSP60 peptides increases the number of Treg cells in lymphoid organs. Flow cytometry analysis: (a) percentage of CD4⁺CD25⁺ FoxP3⁺ cells within the CD4 population (total Treg cells) in lymph nodes, spleen, Payer's patches, and PBMC in ApoB peptide and Hsp60 peptide treated compared to control (PBS) three days after the last dose ($N = 6$). (b) Percentage of CD4⁺CD25⁺ FoxP3⁺ cells within the CD4 population (total Treg cells) in lymph nodes, spleen, Payer's patches and PBMC in ApoB peptide and Hsp60 peptide treated compared to control (PBS) eight days after the last dose ($N = 6$). (c) Percentage of CD4⁺CD25⁺ FoxP3⁺ cells within the CD4 population (total Treg cells) in lymph nodes, spleen, Payer's patches, and PBMC in ApoB peptide and Hsp60 peptide treated compared to control (PBS) at the end of the study after 10 weeks of high fat diet feed to induce atherosclerosis ($N = 6$). * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

The concentration of IL10 and TGF- β increased by 23.9% and 24.3% in the supernatants of ApoB-treated splenocytes while it was 24.3% and 38.7% in that of HSP60-treated splenocytes (Figure 3(b)). These results further reiterate that tolerance to HSP60 results in a relatively higher anti-inflammatory profile compared to ApoB tolerance.

3.4. Oral Tolerance to ApoB and HSP60 Reduces Atherosclerosis Development and Lipid Accumulation in Lesion. Oral administration of ApoB peptide resulted in 27.2% reduction of lesion development on aortic sinus. The total lesion area reduced to $346622.8 \pm 21530.3 \text{ sq } \mu\text{M}$ from $473071.4 \pm 32819.3 \text{ sq } \mu\text{M}$, ($P = 0.006$). Similar reduction of 25.5% was seen with HSP60 peptide also (352103.2 ± 20141.0 from 473071.4 ± 32819.3 , $P = 0.009$) (Figures 4(a) and 4(c)).

We enumerated the lipid content in the lesion by staining with Oil red O (ORO). ApoB treatment was able to reduce the lipid content in the lesion by 32.64% (34.10 ± 3.18 versus 50.63 ± 2.77 , $P = 0.028$) but HSP60 treatment resulted in only 15.21% reduction in the lipid content of the plaque (42.93 ± 2.43 versus 50.63 ± 2.77 , $P = \text{NS}$) (Figures 4(b) and 4(d)). The circulating lipid analysis revealed a reduction in plasma triglyceride levels in mice treated with ApoB but it was not statistically significant (Figure 4(e)).

3.5. Oral Tolerance Reduces Inflammatory Cells in the Lesion. To confirm that the arrest in the plaque progression is due to reduced inflammation in the plaque, macrophage infiltration and cytokine expression were studied. Oral administration of ApoB peptide resulted in 82.4% reduction in macrophages

FIGURE 2: Oral tolerance to peptides reduces the proliferative response of the splenocytes. Splenocytes were collected 3 days and at the end of the study after 10 weeks of high fat diet feed to induce atherosclerosis. (a) Splenocytes (1×10^5 cells) were stimulated with either ApoB peptide ($10 \mu\text{g/mL}$), HSP60 peptide ($10 \mu\text{g/mL}$), or concavalin A ($10 \mu\text{g/mL}$) *in vitro* for 72 h. Cell Proliferation was measured by Alamar blue reduction as quantified by measurement of fluorescence at 560 nm/590 nm. Splenocytes from each animal were plated in triplicates. ($N = 6$ animals/group). Values represent Mean $\pm$ SEM. (b) Splenic effector cells were generated from ApoB48/LDLr^{-/-} mice immunized subcutaneously with the peptides. Treg cells were purified from spleen of ApoB- and HSP60-treated mice at the end of the study (after 10 weeks of high fat diet feed to induce atherosclerosis). Addition of purified Treg cells from oral tolerant mice is indicated at different ratios to effector cells. Proliferation of effector-cell alone is indicated by the black bar; proliferation index represents the percentage carboxyfluorescein succinimidyl ester reduction in culture stimulated with ApoB or HSP60 peptides ($10 \mu\text{g/mL}$) relative to unstimulated culture. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

in the lesion ($9.49\% \pm 1.67$ to $1.91\% \pm 0.65$, $P = 0.02$) as compared to the control. The reduction in macrophages as observed by CD68 staining was 55.8% for HSP60 treatment ($9.49\% \pm 1.67$ to $4.19\% \pm 0.91$, $P = 0.03$) (Figure 5(a)). The

percentage of CD 68 positive area in the lesion was less in ApoB peptide as compared to HSP 60 peptide (Figure 5(a)). In contrast lower percentage of CD4 cells was found in the aortic sinus of HSP60- ($2.05\% \pm 0.18$ to $1.31\% \pm 0.12$)

FIGURE 3: Effect of tolerance on cytokine production. (a) Concentration of the cytokines (TNF- α , IFN- γ , and IL-10) in the plasma was determined by ELISA as per the manufacturers instructions, three days after the last dose and at the end of the study after 10 weeks of high fat diet feed to induce atherosclerosis following oral administration of ApoB and Hsp60 peptides ($N = 6$). (b) Splenocytes isolated from orally treated animals at the end of the study were stimulated with ApoB or Hsp60 peptide ($10 \mu\text{g/mL}$) *in vitro* for 72 hrs. Cytokine concentrations (TNF- α , IFN- γ TGF- β , and IL-10) in the supernatant were estimated by ELISA. ($N = 6$) * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

FIGURE 4: Oral tolerance to ApoB and HSP60 reduces atherosclerosis development and lipid accumulation in lesion. (a) Representative photomicrographs of aortic sinus plaque area stained with EVG (hearts were sectioned at the end of the study) in control, ApoB- and Hsp60-treated mice, respectively. Scale bar represents 200 μm ($N = 6$). (b) Representative photomicrographs of aortic sinus plaque area stained with Oil red O (ORO) (hearts were sectioned at the end of the study) in control, ApoB- and Hsp60-treated mice, respectively. Scale bar represents 200 μm ($N = 6$). (c) Plaque area in the aortic sinus in control, ApoB- and Hsp60-treated mice, respectively. (d) The lipid content in the lesion was detected by staining with Oil red O (ORO). Percentage of ORO positive area in the lesion in control, ApoB- and Hsp60-treated mice, respectively. (e) Concentrations of cholesterol and triglycerides were determined by Siemens Dimension Flex reagent cartridge (Siemens Healthcare Diagnostics Ltd, UK) using the Cobas Fara II Clinical Chemistry auto analyzer (F. Hoffman La Roche Ltd., Basel, Switzerland) as shown in the table. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

FIGURE 5: Oral tolerance reduces inflammatory cells in the lesion. (a) Upper panels: representative photomicrographs showing immunofluorescence staining of aortic sinus sections with CD68 (red) in control, ApoB-, Hsp60-treated mice. Scale bar represents $50\ \mu\text{m}$ ($N = 6$), lower panel: percentage of CD68-positive areas from control and treated groups. (b) Upper panels: representative photomicrographs showing immunofluorescence staining of aortic sinus sections with CD4 (red) in control, ApoB-, Hsp60-treated mice. Scale bar represents $50\ \mu\text{m}$ ($N = 6$), lower panel: percentage of CD4-positive areas from control and treated groups. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

treated animals compared to ApoB treatment ($2.05\% \pm 0.18$ to $1.91\% \pm 0.23$) (Figure 5(b)).

3.6. Effect of Oral Tolerance on Smooth Muscle Cells and Collagen in the Lesion. Next we looked at the effect of tolerance on accumulation of smooth muscle cells (SMC) and collagen in the plaque. Tolerance to HSP60 peptide resulted in 63% reduction in the smooth muscle cells in the lesion ($1.06\% \pm 0.05$ from $2.87\% \pm 0.31$, $P = 0.004$), while tolerance to ApoB showed insignificant change in the SMCs in the lesion ($2.53\% \pm 0.39$ from $2.87\% \pm 0.31$, $P = \text{NS}$) (Figure 6(a)). The collagen content in the lesion as seen by masons trichrome staining increased slightly from

$29.02\% \pm 0.78$ to $31.83\% \pm 0.99$, $P = 0.04$ in mice tolerized to HSP60 while it was not found to change with ApoB 100 tolerance ($29.02\% \pm 0.78$ to $28.90\% \pm 1.07$, $P = \text{NS}$) (Figure 6(b)).

4. Discussion

The present study focuses on the induction of oral tolerance to ApoB and Hsp60 peptides which helps in reducing the development of atherosclerosis in ApoB^{tm2Sgy/Ldlr^{tm1Her/J}} mice. Several studies have demonstrated effective early reduction of atherosclerosis in hyperlipidemic mouse models by inducing tolerance to modified lipids and peptides derived

FIGURE 6: Effect of oral tolerance on smooth muscle cells and collagen in the lesion. (a) Upper panels: representative photomicrographs showing immunofluorescence staining of aortic sinus sections with α -Actin (red) in control, ApoB-, Hsp60-treated mice. Scale bar represents $50\ \mu\text{m}$ ($N = 6$), lower panel: percentage of α -Actin-positive areas from control and treated groups. (b) Upper panels: representative photomicrographs showing staining of aortic sinus sections with masons trichrome for collagen (blue) in control, ApoB-, Hsp60-treated mice. Scale bar represents $50\ \mu\text{m}$ ($N = 6$), lower panel: percentage of collagen positive areas from control and treated groups. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

from ApoB100, HSPs 60/65, and β 2-glycoprotein, but they have never been compared in the same study [19–21, 23–25]. We observed distinct differences in the response induced by the oral administration of the two peptides. Tolerance to HSP60 peptide resulted in higher number of Treg in the peripheral lymphoid organs compared to ApoB100. While tolerance to ApoB peptide reduced the lipid content in the lesion significantly, HSP60 tolerance was not very effective in reducing the plaque lipids. We also observed a decrease in the circulating lipids in mice tolerized to ApoB, but this reduction was not statistically significant. Our results suggest that immune tolerance to these peptides derived from two important self-antigens involved in the development of atherosclerosis has distinct mechanisms of reducing the lesion development in mice.

Natural T regulatory cells (nTreg) develop in the thymus and recognize self antigens. They are characterized by the expression of CD4, CD25 and Foxp3 transcriptional factor [26]. They maintain self-tolerance and prevent autoimmunity by inhibiting pathogenic lymphocytes [6]. These Treg cells are anergic *in vitro* and inhibit the proliferation of effector T cells [27]. During an active immune response, a subset of Treg cells is generated in the periphery, called the induced Treg cells (iTreg). Naive CD4 CD25 cells from the periphery can be converted to iTreg cells in the presence of IL10 and TGF- β or low dose of antigenic peptides [6]. These cells mediate a suppressor function by the secretion of IL10 and TGF- β , respectively [9]. Natural Treg cells with high expression of CD25 on their surfaces and transcription factor Foxp3 play protective roles in atherosclerosis [22, 28]. Besides these cells,

adaptive regulatory cells, Tr1 and Th3 cells secreting IL-10 and TGF- β , respectively, have also been implicated in protection against atherogenesis [20, 24, 25].

Three days after the oral HSP60-peptide (AA 153–163) treatment, the number of CD4⁺CD25⁺ Foxp3⁺ Tregs increased significantly in Payer's patches, spleen, and blood. After a week, their number reduced in all the organs but remained significantly higher compared to control. Treg cells were found to be maintained at higher level in the lymphoid organs during development of atherosclerosis (observed after 10 weeks of high fat diet feeding). We observed an increase in Treg cells following ApoB (AA 661–680) treatment also but their numbers were distinctly lower than HSP60-treated mice. The decrease in the number of Treg cells after a week of dosing could be attributed to the migration of the activated Treg cells to the site of inflammation (atherosclerotic lesions) where they may recognize self-antigens. Similar results were reported for a different peptide epitope from HSP60 (253–268) by van Puijvelde et al. [23].

Increase in Treg cells in lymphoid organs of the peptide-treated animals does not ensure their antigen specificity. However, we observed that these Treg cells recovered from lymph nodes and spleens of peptide-treated ApoB^{tm2Sgy/Ldlr^{tm1Her}} mice significantly suppressed antigen-specific T effector cell proliferation *in vitro*, the suppressive function being higher for HSP60 tolerance compared to ApoB100. Plasma concentration of IL10 increased significantly in HSP60-tolerized mice suggesting the role of induced Treg cells in atheroprotection. The increase in IL10 concentrations was moderate in ApoB tolerized mice, while the concentrations of inflammatory cytokines were reduced in the plasma. In addition, oral treatment with HSP60 and ApoB peptides also reduced the proliferative response of splenocytes to the respective peptides. Reduction in splenocytes proliferative response to HSP65 protein following oral administration was also reported by Maron et al. and Harats et al. [19, 29]. Furthermore, splenocytes of HSP60 and ApoB treated mice also produced increased levels of TGF- β and IL-10 after *in vitro* restimulation with the respective peptides. All these data suggest that oral administration of HSP60- and ApoB-peptides induced Tregs, which can dampen the inflammatory immune response to HSP60 and ApoB100 proteins in atherosclerosis prone mice and thus reduce the development of atherosclerosis.

We observed significant reduction in lipid deposition in the lesion in mice orally treated with ApoB peptide. This finding was further corroborated by the decrease in number of CD68 positive macrophages in the lesion. These results suggest that tolerance to ApoB peptide has an effect on the macrophage migration into the lesion and consequently the foam cell formation and lipid deposition. Reduction in lipid content in the lesion was not significant with HSP60 peptide treatment. Although the number of CD68 positive macrophages in the lesion was lower than control, it was moderate compared to ApoB peptide treatment. Oral tolerance to HS60 peptide resulted in reduction in CD4 positive T

cells, smooth muscle cells, and an increase in collagen content in the lesion, which could be correlated to the effective Treg activity seen in these mice. Treg cells producing TGF- β have been shown to inhibit smooth muscle proliferation and promote extracellular matrix formation, thus stabilizing the plaque [30, 31].

Atherosclerosis is a multifactorial, chronic inflammatory disease. Inflammation mediated by a pathogenic immune response to endogenous antigens such as HSP, modified lipids, as well as exogenous antigens from pathogens, have been implicated in the initiation of immune response during atherogenesis [3]. Each molecule has a distinct role to play in the initiation and progression of the disease. It is likely that these molecules also induce a different mechanism of atheroprotection. We believe that to address a multifactorial disease, a combination of multiple epitopes would have a better efficacy compared to single peptides. Based on our results we think that tolerance to two different proteins induces atheroprotection by diverse mechanisms. While HSP60 tolerance resulted in increase in Treg cells and anti inflammatory cytokine secretion ApoB tolerance was effective in reducing the lipid deposition in the lesion. Both were equally effective in controlling the disease development. We have earlier reported that repeated subcutaneous immunization with a combination of peptide epitopes from ApoB100 and HSP60 had a synergistic effect in reducing the development of atherosclerosis in mice [32]. Similar increase in protection was observed by administration of the combination of these two peptides by mucosal route also (unpublished observation). It is likely that the combination of peptides would control different aspects of disease development and result in a synergistic increase in protection compared to individual peptides.

5. Conclusions

In conclusion, based on our present and earlier results we believe that using multiple antigenic epitopes from different molecules involved in atherogenesis would be a novel and attractive strategy for immunoprotection against atherosclerosis. Additional studies will be required to completely delineate the mechanisms of protection mediated by tolerance to these individual peptides and the mechanisms that control their maintenance *in vivo*.

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