DOI: 10.1111/1755-5922.12201

ORIGINAL RESEARCH ARTICLE

WILEY Cardiovascular Therapeutics

Oral administration of recombinant *Mycobacterium smegmatis* expressing a tripeptide construct derived from endogenous and microbial antigens prevents atherosclerosis in ApoE$^{-/-}$ mice

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Summary

Introduction: Immunotherapy by inducing oral tolerance to atherogenic self-antigens is gaining importance as an alternative treatment modality for atherosclerosis. The use of live bacterial vectors to express the recombinant antigen in vivo will obviate the need for large-scale purification of recombinant protein and may also augment the efficacy of oral tolerance induction.

Aim: The objective of the study was to explore the use of recombinant *Mycobacterium smegmatis* as a live vector for oral delivery of antigens to induce immune tolerance.

Method and Results: We developed a *M. smegmatis* vector to secrete a recombinant tripeptide construct (AHC; peptides from Apolipoprotein B, Heat-shock protein 60 and *Chlamydia pneumoniae* outer membrane protein) expressed in a dendroaspin protein scaffold in pJH154 background. Immune response and oral tolerance to the cloned peptides were studied in C57/BL6 mice. The efficacy of this live vaccine to control atherosclerosis was studied in ApoE$^{-/-}$ knockout mice in C57/BL6 background. Oral administration of *M. smegmatis* secreting the cloned AHC antigen was found to induce tolerance to cloned protein and reduce the development of atherosclerosis by 24.0% compared to control. Protection against atherosclerosis was associated with increase in expression of regulatory T cell-associated markers including CTLA4 (1.8-fold), Foxp3 (2.6-fold), TGF-β (2.8-fold), IL10 (2.9-fold), and reduction in lipids, macrophage infiltration, and expression of inflammatory mediators in aorta.

Conclusions: Our results suggest that *M. smegmatis* can be developed as an oral carrier of recombinant proteins to treat inflammatory autoimmune diseases.

KEYWORDS

Apolipoprotein, Atherosclerosis, Autoimmune diseases, Heat-shock proteins, Immune tolerance, Live vaccine, *Mycobacterium smegmatis*, Regulatory T cells

1 | INTRODUCTION

Atherosclerosis is a chronic inflammatory disease characterized by autoimmune reactivity to endogenous antigens such as heat-shock proteins, modified lipoproteins, and exogenous microbial antigens.[1] Despite advances in medicine and interventional therapies, cardiovascular disease remains the important cause of global death and disability, emphasizing the need for novel therapies targeting the disease pathology.[2]

RK and VD contributed equally to this work.

Antigen-based therapy targeting relevant T cells without affecting the general immune system provides an effective treatment for autoimmune diseases.[3] Immunotherapy for atherosclerosis is directed toward inducing tolerance to atherogenic antigens by increasing the number of antigen-specific regulatory T cells (Tregs), which can suppress the proatherogenic immune response.[3,4] Translation of the therapeutic effects of restoring tolerance to self-antigens for autoimmune and allergic diseases from animal models to clinical trials has been highly inconsistent.[5,6] These failures are associated with the dose, purity, the amount of (auto) antigen needed, and the mode of presentation of the antigen to the mucosal immune system.[7]

Strategies using live bacterial cells as vaccine carriers are emerging as an exciting alternative for vaccine delivery. Live vaccines obviate the need for large-scale purification of recombinant protein, and can act as natural adjuvants.[8,9] Several bacterial species including salmonella, streptococci, lactococci,[10] vibrio[11,12] *Mycobacterium bovis* (BCG),[13,14] and *Mycobacterium smegmatis*[15] have been used to deliver heterologous proteins. *Mycobacterium smegmatis* is a nonpathogenic fast-growing commensal organism in humans.[16] The bacterium is rapidly cleared by the host as it is unable to arrest phagolysosome maturation and evade intracellular killing.[17] *Mycobacterium smegmatis* can be rapidly grown in vitro, and is amenable for genetic modification. The bacteria can express large amount of heterologous proteins, can activate dendritic cells, and generate long-lasting memory T cells, which render this organism an efficient vaccine carrier.[18–21] Live oral vaccines based on lactococci have been used to restore tolerance in autoimmune diabetes, experimental autoimmune encephalomyelitis, and allergic diseases.[22,23] *Mycobacterium smegmatis* has not been explored so far for its use as a live vaccine carrier for autoimmune diseases.

Several studies have demonstrated effective early reduction of atherosclerosis in mouse models by inducing tolerance to peptides derived from Apolipoprotein B (ApoB) 100, HSPs 60/65.[24–29] Immune tolerance is a default response in the gut as it is exposed to innumerable antigens through food. Antigen-presenting cells (APC) in the gut selectively induce antigen-specific Treg, which migrate and suppress damaging immune responses. We have earlier shown that oral tolerance to a combination of ApoB and HSP60 peptides[30] and to multiantigenic recombinant molecule expressing a tripeptide derived from ApoB, HSP60, and outer membrane protein from *Chlamydia pneumoniae* (AHC) in a patented dendroaspin (DSP) scaffold could prevent atherosclerosis development in animal model of atherosclerosis.[30,31] In this study, we expressed a multiantigenic construct containing three atherogenic peptides in an *M. smegmatis* secretory vector (pJH154) and explored the use of this recombinant organism in protection against atherosclerosis in ApoE$^{-/-}$ mice in C57/BL6 background.

2 | METHODS

2.1 | Generation of recombinant *M. smegmatis* expressing multiantigenic construct

The recombinant construct AHC was generated as described earlier and was used as a template for constructing the *M. smegmatis* vector.[32] The recombinant construct AHC was generated by inserting peptides from ApoB100, hHSP60, and the combined peptide from major outer membrane protein and omp5 of the *C. pneumoniae* (*Cpn*) in the DSP scaffold as described earlier. The DNA fragments containing DSP backbone and the modified DSP expressing the three peptides (AHC) were amplified using PCR and subcloned into modified pET-11d plasmid which were used as templates for cloning into pJH154 mycobacterial plasmid (Kind gift from Dr Miczak, Department of Medical Microbiology and Immunobiology, University of Szeged, Szeged, Hungary). Details of cloning are given in supplementary methods section.

2.2 | Bacterial strains and culture conditions

Plasmid containing *Escherichia coli* cultures were grown at 37°C in Luria broth, and *M. smegmatis* mc^2 155 was grown in Middlebrook 7H9 medium supplemented with an albumin–dextrose–catalase (ADC; Hi Media Laboratories, Mumbai, India) and 0.05% Tween 80 at 37°C. Plasmids were electroporated into competent mycobacterial cells as previously described.[6] Transformants were selected on Middlebrook 7H10 medium plates containing Oleic acid ADC supplement (OADC; Hi Media Laboratories) and 20 μg/mL of Kanamycin. Modified Sautons minimal media was used to check the expression of secreted protein (5.0 g L-asparagine, 1.5 g citric acid, 5.9 g KH_2PO_4, 0.6 g $MgCO_3$, 0.5 g K_2SO_4, 40 mmol/L Glucose, and 0.02% Tween 20 pH 8.0). Stock cultures of log-phase cells were maintained in glycerol (25% final concentration of glycerol) at −80°C.

2.3 | Colony PCR and Western blot

Single colonies of Kanamycin-resistant *M. smegmatis* were suspended in 200 μL of water, vortexed vigorously, and heated at 95°C for 5 minutes to lyse the cells for colony PCR using the primers U1 (5′ GGGTTA ACC ATGTTT GAC AGC TTA TCA TCGATA 3′) and U2 (5′ TTA GGGCCC ATGGGC AGC AG 3′). PCR-positive recombinant *M. smegmatis* containing AHC and DSP genes were grown in Sautons minimal media for 3 days. Cell-free supernatants were concentrated and loaded on 10% SDS–PAGE gel, and the separated proteins were transferred onto polyvinylidene fluoride (PVDF) membrane (Amersham Hibond-P Hibond-P, GE Healthcare Life sciences, Little Chalfont, UK) at 23 V for 45 minutes. The bound proteins were subsequently probed with primary antibody (mouse serum with known reactivity to AHC protein) at 4°C and visualized with HRP-conjugated anti-mouse IgG (1:2000) antibody (GE Healthcare).

2.4 | Animals

Experiments related to use of animals were carried out in 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, and Government of India. All the protocols were approved by Institutional Animal Ethics Committee (Registration

Number: 1261/c/09/CPCSEA) and Institutional Bio Safety Committee (BT/BS/17/467/2012PID) of the Thrombosis Research Institute. C57BL/6 wild-type mice were used for immunization experiments, and ApoE$^{-/-}$ knockout mice in C57BL/6 background were used for the assessment of atherosclerosis.

2.5 | Immunization of mice with recombinant *M. smegmatis*

Group of four C57BL/6 mice (two males and two females) were immunized intraperitoneally with 10^8 CFU of recombinant *M. smegmatis* secreting AHC protein and control *M. smegmatis* mc^2 155 in 100 μL of PBS for the evaluation of immune response to recombinant antigens. Two booster doses were given at an interval of 2 weeks each, and blood was collected after every 15 days to check antibody response. Spleen and the lymph nodes were collected 3 days after the last dose to study cell-mediated response.

2.6 | Analysis of the antibody response

Antibodies in sera from immunized mice were tested by antigen-specific ELISA. Briefly, 96-well microwell plates (Nalge Nunc International, Penfield, NY, USA) were coated with purified AHC protein (10 μg/mL) and incubated at 4°C overnight. Blocking was carried out with 5% lipid-free milk at room temperature for 1 hour, followed by the addition of diluted sera from mice. The bound immunoglobulin was assessed using horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody and tetramethylbenzidine substrate. Maleimide-activated 96-well plates (Pierce, Thermo Fisher Scientific Inc., Hampshire, UK) were used for coating the three peptides individually, and peptide-specific antibodes were detected in the plasma of immunized mice according to the manufacturer's instructions. The specificity of the antibodies from immunized mice was also confirmed by Western blot as described in the earlier section using immunized sera with appropriate controls.

2.7 | Analysis of T-cell response

Antigen-specific T-cell proliferation was evaluated by stimulating splenocytes (5×10^5/well) from the immunized mice with the purified AHC protein or concavalin A (10 μg/mL) for 72 hours, using Roche cell proliferation BrdU assay kit. Proliferating cells were labeled with 10 μL BrdU reagent for the last 18 hours of culture, fixed, and detected by anti-BrdU-POD antibody followed by substrate reaction using tetramethyl benzidine, and absorbance was recorded by ELISA reader.

2.8 | Survival of *M. smegmatis* in mice after oral dosing

C57BL/6J wild-type mice were dosed orally with 100 μL of the suspension (1×10^8 CFU/animal). The animals were sacrificed after 1, 24, 48, 72, 96, and 144 hours of dosing. Spleen, liver, kidney, lymph node, stomach, and intestine were collected, homogenized using sterile PBS, and colonies were enumerated on Middlebrook agar plates (supplemented with OADC and Kanamycin). The plates were checked for growth of *M. smegmatis* after 3 days.

2.9 | Effect of dosage on *M. smegmatis* tissue burden

The effect of dosage was studied by giving 1, 3, 5, and 7 doses of recombinant *M. smegmatis* to ApoE$^{-/-}$ animals by oral route (1×10^8 CFU/animal). Stomach and intestine were collected and homogenized using sterile PBS. Colonies were enumerated as described in the earlier section.

2.10 | Flow cytometry

Flow cytometry analyses were performed by FACS Canto II using FACS DIVA software (Becton Dickinson, Fraklin Lakes, NJ, USA) and FLOWJO software (Tree star, Ashland, OR, USA) as described earlier using anti-mouse antibodies to CD4, CD25, and fork-head box p3 (Foxp3).[33]

2.11 | Treg functional assay

Treg function was studied by antigen-specific suppression assay as described earlier.[33] Briefly, effector T cells were generated by immunizing mice with purified AHC protein. CD4-positive cells from spleen were purified using T cell isolation kit (Miltenyi Biotech, Teterow, Germany) and labeled with 10 μmol/L 5, 6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Sigma Chemicals, St. Louis, MO, USA) and used as effector cells. Spleen cells from mice orally dosed with recombinant *M. smegmatis* secreting AHC protein and regulatory T cells were isolated using the CD4$^+$CD25$^+$ regulatory T cell Isolation Kit (Miltenyi Biotech) and labeled with 6 μmol/L PKH26 (Sigma Chemicals) to discriminate the effector and regulatory CD4$^+$ population. Effector T cells (1×10^5) and regulatory cells were taken in equal ratios and activated with 10 μg/mL of antigen (purified AHC protein) in X vivo 20 serum-free medium (Lonza, Basel, Switerland). After 5 days of incubation, cells were stained with CD4-APC (eBiosciences, San Diego, CA, USA).[34] Proliferation of CD4$^+$ effector cells was measured by CFSE dilution using FACS CANTO II (Becton Dickinson) and analyzed using FlowJO software (Tree star). The proliferation index of T cells was calculated from the FlowJO software.

2.12 | Assessment of atherosclerosis

Groups of B6129P2-ApoE^{tm1Unc}/J (ApoE$^{-/-}$) animals (5–6 weeks of age) were orally dosed five times on alternate days with 10^8 CFU/animal/dose of recombinant *M. smegmatis* secreting AHC protein. *Mycobacterium smegmatis* secreting DSP protein, bacteria transformed with empty plasmid, and *M. smegmatis* mc^2 155 were used as controls. For comparison, purified AHC protein (1ug/dose/animal) was administered by oral route in separate set of ApoE$^{-/-}$ animals.

Atherosclerosis was induced using diet high in fat and cholesterol (TD 96121; Harlan, Indianapolis, IN, USA: 21% fat and 1.25% cholesterol), for 10 weeks. 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, and the hearts were collected in either optimal cutting temperature medium (Tissue Tek, Leica, Wetzlar, Germany) or neutral-buffered formalin. For lesion analysis, six sections 80 μm apart were stained with Elastica van Gieson in each mouse. Immunofluorescence on frozen sections was carried out using an indirect immunofluorescence technique as described earlier.[30,33] Images were captured using a Leica DMI 4000 B Confocal microscope and the analysis was performed using Image-Pro software, and percentage areas of fluorescence of specific antigens of interest in the plaque were calculated.

2.13 | Real-time reverse transcription–polymerase chain reaction analysis

Total RNA was extracted from the ascending part of the aorta using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Real-time reverse transcription–polymerase chain reaction (RT-PCR) was performed with two-step EXPRESS SYBR superscript RT-PCR kit (Invitrogen)

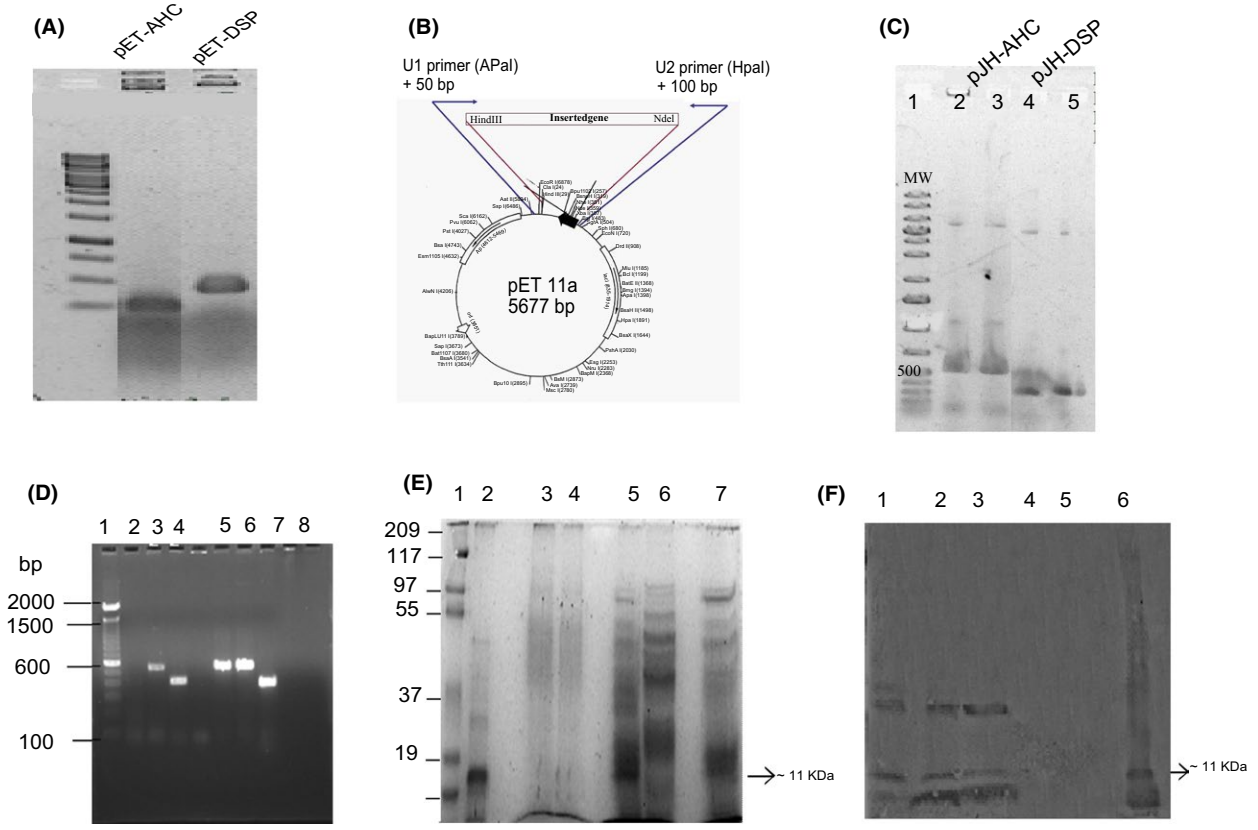

FIGURE 1 Expression of AHC protein in *Mycobacterium smegmatis*. Cloning and expression of AHC and DSP protein in *M. smegmatis*. (**A**) The recombinant construct AHC was generated by inserting peptides from ApoB100, hHSP60, and the outer membrane proteins of *Chlamydia pneumoniae* (*Cpn*) in the DSP scaffold as described earlier. The fragments containing DSP (backbone and the modified DSP expressing the three peptides (AHC) were amplified using PCR and subcloned into modified pET-11d plasmid which were used as templates for amplification of AHC and DSP genes; (**B**) plasmid map of pET-11a vector and position of the U1 and U2 primers used for amplification of genes for cloning; (**C**) the amplified fragments were digested with ApaI/HpaI and ligated into pJH154 mycobacterial plasmid to obtain pJH-AHC and pJH-DSP; (**D**) colony PCR to confirm the presence of insert; Lane 1, Marker; Lane 2, *M. smegmatis* mc^2 155 as a negative control, Lane 3, pJH-AHC-transformed *M. smegmatis*; Lane 4, pJH-DSP-transformed *M. smegmatis*; Lane 5 and 6, pJH-AHC-transformed *Escherichia coli* as a positive control; Lane 7, pJH-DSP as a positive control; Lane 8, *M. smegmatis* transformed with empty pJH154 plasmid as a negative control. (**E**, **F**) Expression and immunoblot analysis of the concentrated culture supernatant of pJH-AHC-transformed *M. smegmatis* expressing AHC protein. (**E**) SDS–PAGE analysis of *M. smegmatis* culture supernatant. Lane 1, protein molecule weight markers; lane 2, purified AHC protein; lane 3, control *M. smegmatis* mc^2 155 culture supernatant; lane 4, *M. smegmatis* transformed with empty pJH154 plasmid; Lane 5 and 6, the supernatant of pJH-AHC-transformed *M. smegmatis* expressing target protein; and Lane 7, supernatant of pJH-DSP-transformed *M. smegmatis* supernatant. The expressed AHC protein with molecular weight about 11 kDa was pointed by an arrow. (**F**) Western blot analysis of the separated proteins as in SDS–PAGE. Lane 1, supernatant of pJH-DSP-transformed *M. smegmatis* supernatant; Lane 2 and 3 supernatant of pJH-AHC-transformed *M. smegmatis* expressing target protein; Lane 4, control *M. smegmatis* mc^2 155 culture supernatant; Lane 5, *M. smegmatis* transformed with empty pJH154 plasmid; Lane 6, purified AHC protein. Data are a representative of three independent experiments. DSP, dendroaspin; AHC, dendroaspin expressing three peptides from ApoB, HSP60, and *Cpn* outer membrane protein

using the ABI PRISM 7500 sequence detection system (7500 real-time PCR system; Applied Biosystems, Foster city, CA, USA) according to the manufacturer's protocol using the standard cycling program. The sequence of primers is given in the supplementary section.

2.14 | Statistical analysis

Data are expressed as mean±standard error of mean. Differences between control and experimental groups were evaluated by Mann-Whitney *U* test and were considered statistically significant at $P<.05$. Statistical analyses were performed using Graph Pad prism software version 5.01 (GraphPad Prism Software, Inc., La Jolla, CA, USA).

3 | RESULTS

3.1 | Expression of AHC protein in *M. smegmatis*

Dendroaspin protein and the modified DSP scaffold containing the three atherogenic peptides (AHC) were cloned in pET-15A. The pET constructs were used as templates for amplification of the genes using the U1 and U2 primers. The amplicons were digested with ApaI/HpaI and ligated into pJH154 mycobacterial plasmid which secretes the cloned proteins (Figure 1A–C). PCR was used to confirm the presence of cloned insert in the transformed *M. smegmatis* colonies as shown in Figure 1D. Presence of recombinant construct was observed in *M. smegmatis* transformed with pJH-AHC and pJH-DSP as an amplified DNA of ~516 and ~384 bp, respectively (lane 3 and 4), but not in wild-type *M. smegmatis* and that transformed with empty plasmid (lane 2 and 8). *Escherichia coli* DH5α transformed with pJH-AHC and pJH-DSP were used as positive controls (lanes 5–7). The expression of the cloned proteins AHC and DSP was detected by SDS–PAGE and Western blot as shown in Figure 1E, F. Expression of protein with a molecular mass of ~11 KDa was detected in recombinant *M. smegmatis* transformed with AHC and DSP genes (lanes 5 and 6), which matched with the purified AHC protein (lane 2). *Mycobacterium smegmatis* transformed with empty plasmid and untransformed *M. smegmatis* mc^2 155 did not show any protein reacting with anti-AHC antibody (lanes 3 and 4).

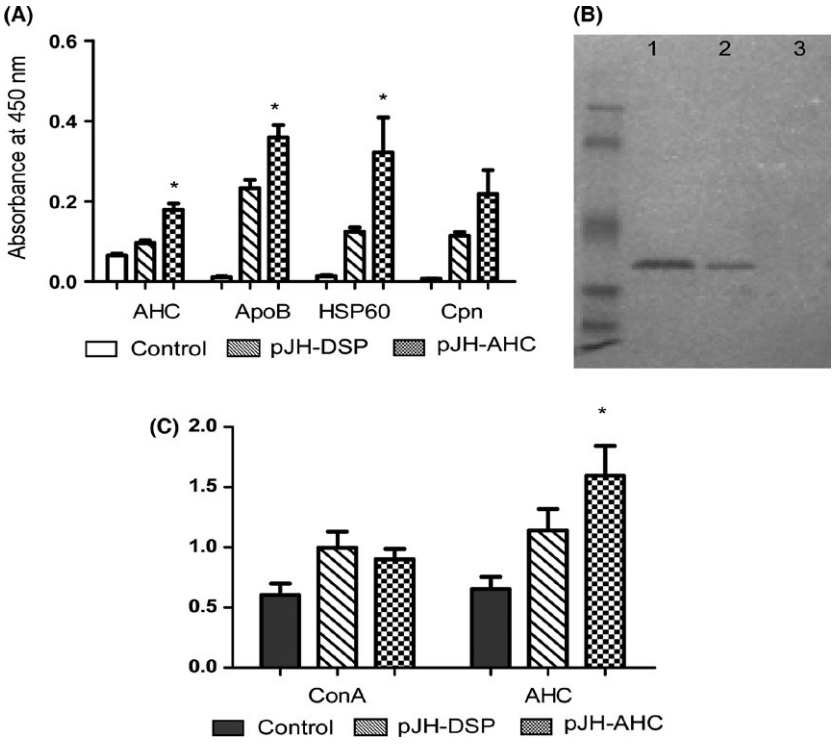

FIGURE 2 Immune response to AHC protein secreted from recombinant *Mycobacterium smegmatis*. Groups of C57BL/6 mice were given 10^8 CFU of *M. smegmatis* expressing AHC protein, DSP protein or empty plasmid (control) followed by 2 booster doses at 14 days interval by intraperitoneal route. (**A**) AHC protein and ApoB, HSP60, and *Cpn* peptide-specific IgG antibodies in serum were detected by ELISA after second booster dose of immunization. The mean absorbance at 1:100 dilution of sera is represented in the graph and significant differences in ODs produced by sera of pJH-AHC-immunized mice compared to that of pJH-DSP-immunized mice are shown. (**B**) Immunoblot: purified AHC protein was separated in 10% SDS–PAGE and transferred to PVDF membrane. Immunoblot was developed with serum from immunized mice (lane 1, pJH-AHC; lane 2, pJH-DSP) and lane 3, control. (**C**) Lymphocyte proliferation assay: splenocytes were collected 3 days after the last immunization and at the end of the study; 1×10^5 cells were stimulated with either AHC protein (10 μg/mL) or concanavalin (con) A (10 μg/mL) in vitro for 72 hours. Cell proliferation was measured by BrdU assay as quantified by measurement of fluorescence at 560 nm/590 nm. Splenocytes from each animal were plated in triplicates (N = 4 animals/group). Values represent mean±SEM. **P*≤.05, ***P*≤.01, ****P*≤.001

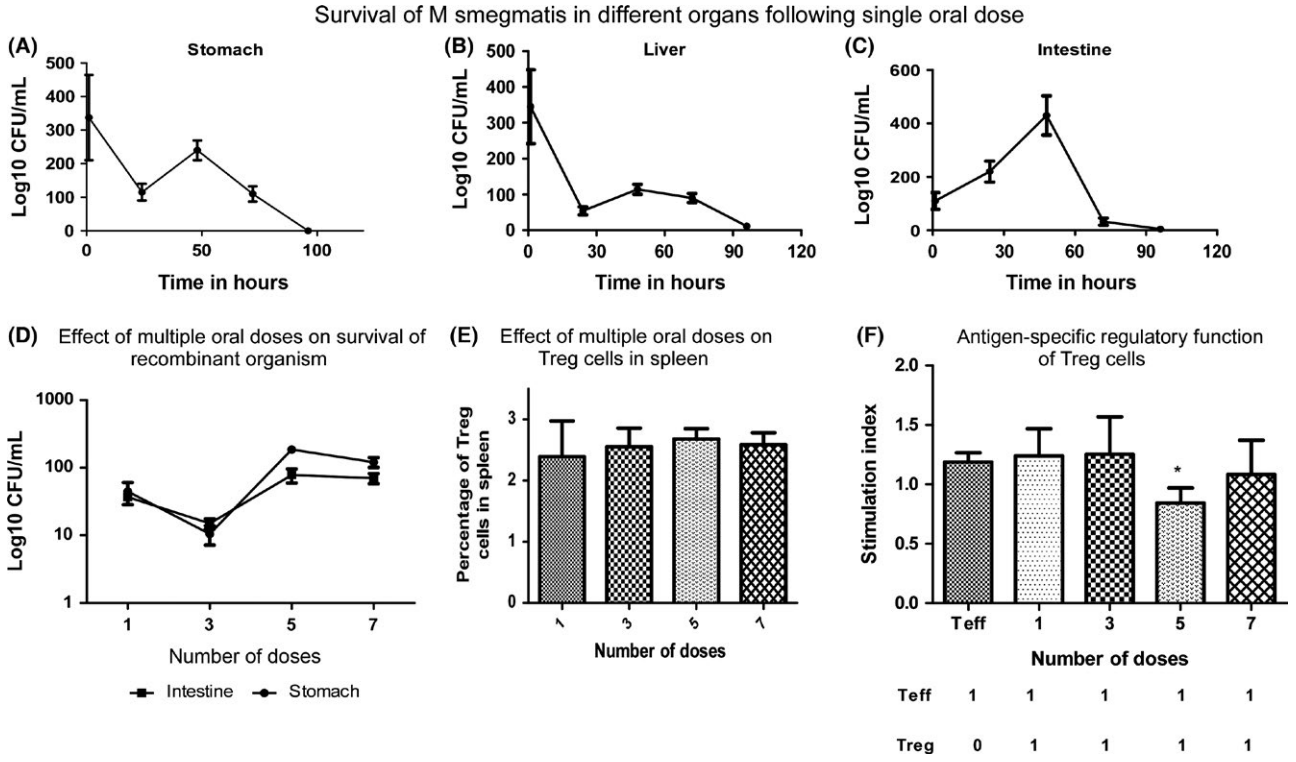

FIGURE 3 Survival of *Mycobacterium smegmatis* tissues and effect of *M. smegmatis* dosage in mice after oral dosing. Survival of *M. smegmatis* in mice: A single oral dose of *M. smegmatis* (10^8 CFU/dose/animal) was given to mice, and the survival of the bacteria was monitored in the organs Bacterial load in (**A**) stomach; (**B**) liver; (**C**) intestine obtained by plating serial dilutions of homogenized organs from mice. Effect of dosage: groups of mice were given increasing (1, 3, 5, and 7 doses) of *M. smegmatis* (10^8 CFU/dose/animal) on alternate days. (**D**) survival of bacteria in stomach and intestine, (**E**) percentage of Treg ($CD4^+$, $CD25^+$, $Foxp3^+$) cells in spleen as measured by flow cytometry, (**F**) functional activity of Tregs, that is, proliferation index produced by effector T cells from intraperitoneally immunized mice in the presence of Treg cells isolated form orally treated mice 24 hours after the last oral dose of recombinant *M. smegmatis* secreting AHC protein

3.2 | Immune response to AHC protein secreted from recombinant *M. smegmatis*

Good immunogens are generally good tolerogens. To understand the immunogenicity of secreted recombinant proteins from recombinant *M. smegmatis*, we studied the antibody and cell-mediated immune response to AHC protein following intraperitoneal immunization with recombinant bacteria. Mice were found to respond to the cloned antigen by generating a strong IgG response to AHC protein and the individual peptides, suggesting that the secreted proteins are capable of generating an immune response (Figure 2A). Western blot with the immunized sera showed reactivity to purified AHC protein further confirming the antigen-specific immune response to the cloned protein (Figure 2B). Splenocytes isolated from the immunized animals showed antigen-specific proliferation in response to stimulation with AHC protein (PI=1.46±0.11), but no proliferation was induced by the control antigen (0.53±0.06, P=.015), suggesting that the cloned protein was capable of eliciting a cell-mediated immune response (Figure 2C). Collectively, these results suggest that the recombinant protein secreted from *M. smegmatis* is immunogenic and induces an antigen-specific immune response in mice.

3.3 | Survival and dosage effect of *M. smegmatis* after oral administration

The main objective of the study was to induce oral tolerance to the cloned protein, which requires availability of the antigen at a low dose for sufficiently long period of time. As *M. smegmatis* is rapidly cleared by the host, we wanted to understand the survival of the bacteria in different organs following oral administration and the optimum dosage that would generate a tolerogenic response. Bacterial load was detected in stomach and intestine as early as an hour after oral administration of 10^9 CFU/animal (Figure 3A–C). *Mycobacterium smegmatis was* found to spread to the liver at 24 and 48 hours and was eliminated from all the organs by 96 hours postdosing (Figure 3A–C). *Mycobacterium smegmatis* could not be detected in spleen, lymph nodes, and kidneys at any time point (data not shown).

Antigen dosage plays a very important role in inducing tolerance. To optimize the number of oral doses of recombinant *M. smegmatis* for effective induction of tolerance, we studied the effect of dose on the regulatory T cell activation and function. We observed that the number of colonies in stomach and intestine at 72 hours after the last dose increased with the number of oral doses and stabilized at five doses

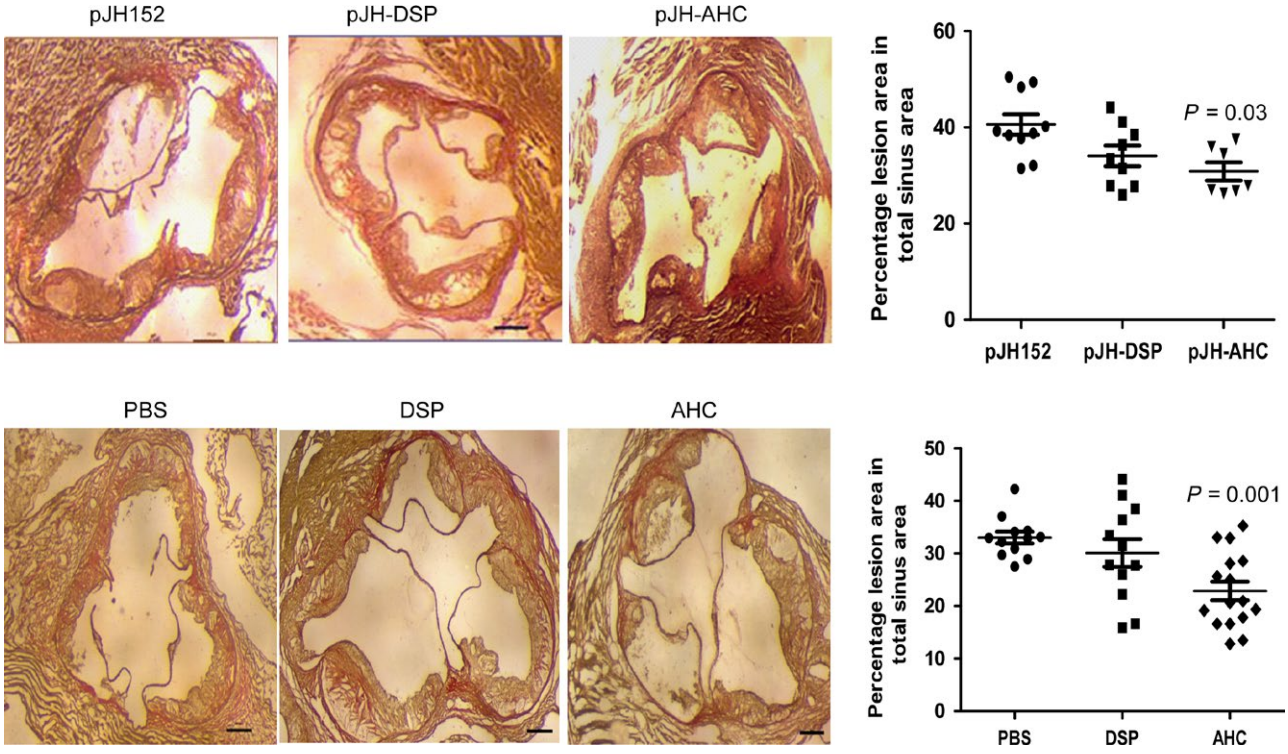

FIGURE 4 Effect of oral dosing with recombinant *Mycobacterium smegmatis* on development of atherosclerosis. Groups of ApoE$^{-/-}$ mice were orally dosed five times on alternate days with 10^8 CFU/animal/dose of recombinant *M. smegmatis* secreting AHC protein. *Mycobacterium smegmatis* secreting DSP protein, bacteria transformed with empty plasmid, and purified AHC and DSP proteins (1 μg/dose/animal) in phosphate-buffer saline (PBS). Animals were given a diet rich in cholesterol to induce atherosclerosis for 10 weeks. Aorta were collected and plaque area were enumerated by histochemical analysis. Representative photomicrographs of EVG-stained plaque area and its quantitative analysis in aortic sinus sections of 18-week-old ApoE$^{-/-}$ mice (N=6 per group). (A) Groups of ApoE$^{-/-}$ mice were given five oral doses of recombinant *M. smegmatis* secreting AHC protein (10^8 CFU/animal/dose), (B) purified AHC protein (1 μg/animal/dose). Animals were given a diet rich in cholesterol to induce atherosclerosis for 10 weeks. Scale bar represents 200 μm

on alternate days. Further increase in bacterial load was not observed after increasing the number of dose (Figure 3D). The proportion of regulatory T cells ($CD4^+CD25^+Foxp3^+$) was not found to change with the number of oral doses of the recombinant *M. smegmatis* (Figure 3E). To understand the functional activity of the Tregs in suppressing the AHC-specific T-cell proliferation, we carried out the Treg functional assay. T cells isolated from mice immunized with purified AHC protein was used as effector T cells (Teff). Antigen-specific reduction in Teff cell proliferation was observed with Tregs from five doses (32%, *P*=.045) and seven doses (11%), but no reduction was observed from single and three doses of oral bacteria (Figure 3F). Based on these results, we chose administration of five doses of live bacteria for further experiments.

3.4 | Effect of oral treatment with recombinant *M. smegmatis* on development of atherosclerosis

In animals treated with recombinant *M. smegmatis* secreting AHC, 30.85% of aortic sinus was covered with lesion compared to 40.62% in controls treated with *M. smegmatis* harboring pJH 152 resulting in 24% (*P*=.003) reduction in the disease development. Interestingly, oral treatment with *M. smegmatis* secreting DSP also resulted in reduction in atherosclerosis by 16% compared to control, but the difference was not significant (Figure 4A). In comparison, oral tolerance induced by purified AHC protein in ApoE$^{-/-}$ mice was observed to be 31% (*P*=.001) and 6% for purified DSP protein compared to PBS control (Figure 4B).

3.5 | Oral treatment with recombinant *M. smegmatis* reduces inflammatory mediators in aorta

To understand the mechanism of protection mediated by oral treatment with *M. smegmatis* secreting AHC protein, we studied the expression of inflammatory and regulatory molecules in the aorta by immunohistochemistry and real-time PCR. Deposition of lipids was reduced by 29.5% (*P*=.004) in the aorta of animals treated with *M. smegmatis* secreting AHC protein compared to plasmid control (Figure 5A). AHC-treated animals showed a decrease in macrophage infiltration and MMP9 expression while the collagen content was found to be higher (Figure 5B–D). The aortic expression of inflammatory cytokines including IFN-γ (1.7-fold) and IL17 (2.3-fold) were reduced while that of regulatory T cell activity including CTLA4(1.8-fold), Foxp3 (2.6-fold), TGF-β (2.8-fold), and IL10 (2.9-fold) were higher in AHC-treated animals (Figure 5E).

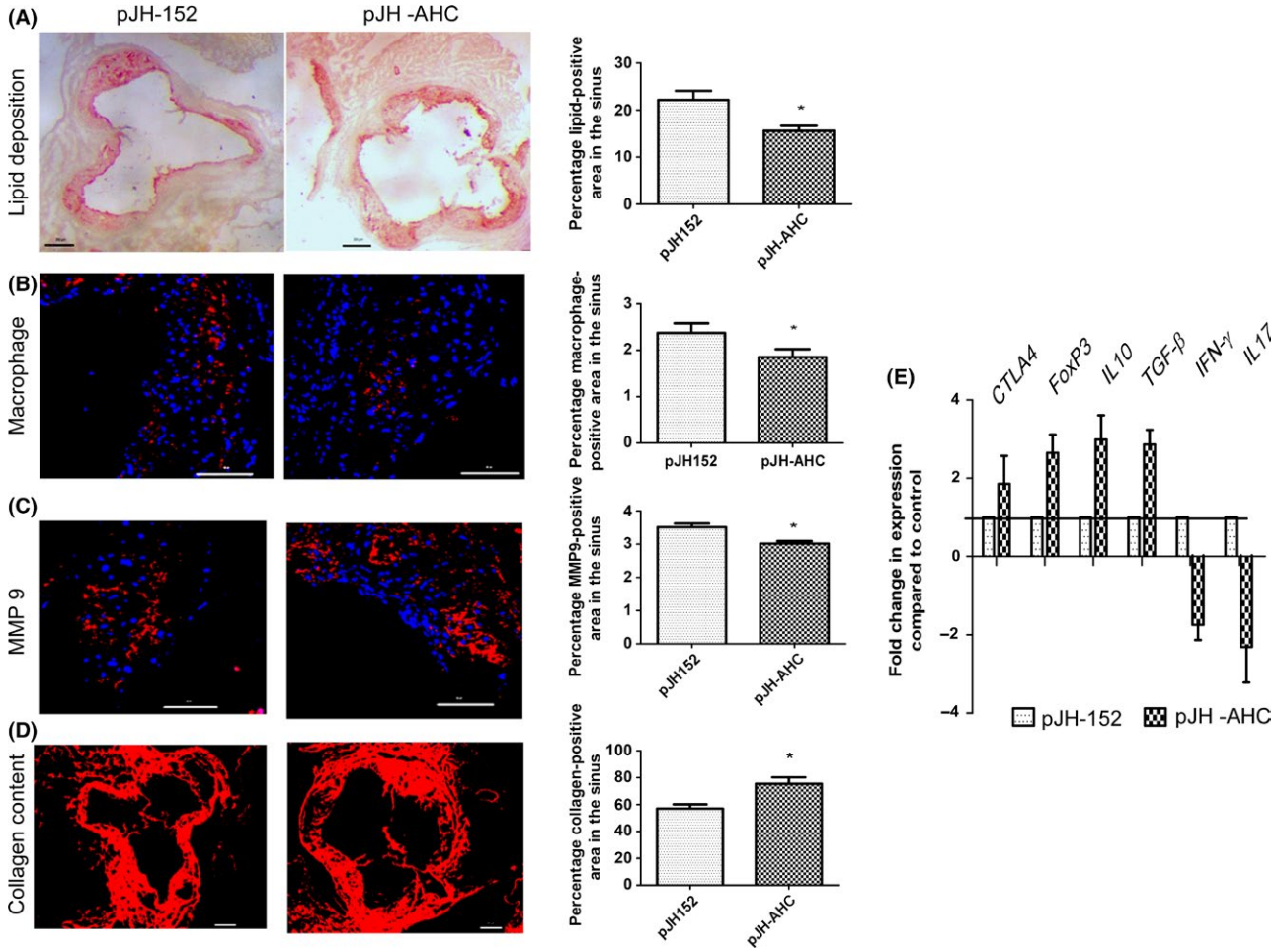

FIGURE 5 Oral treatment with recombinant *Mycobacterium smegmatis* reduces the level of inflammatory mediators in aorta. Groups of ApoE$^{-/-}$ mice were given five oral doses of recombinant *M. smegmatis* secreting AHC protein 10^8 CFU/animal/dose as treatment and *M. smegmatis* harboring pJH 152 plasmid as control. Animals were given a diet rich in cholesterol to induce atherosclerosis for 10 weeks. (**A**) Representative photomicrographs of aortic sinus plaque area stained with Oil red O (ORO) in control (pJH154), and pJH-AHC-treated mice, respectively, and its quantification. Scale bar represents 200 μm (N=6). (**B**) Representative photomicrographs and quantitative evaluation of aortic sinus plaque area stained with anti-CD68 antibody for macrophages in control and treated animals. Scale bar represents 50 μm (N=6). (**C**) Representative photomicrographs and quantitative evaluation of aortic sinus plaque area stained with anti-MMP9 antibodies in control and treated animals. Scale bar represents 50 μm (N=6). (**D**) representative photomicrographs and quantitative evaluation of aortic sinus plaque area stained with picrosirius for collagen content in control and treated animals. Scale bar represents 50 μm (N=6). (**E**) Relative mRNA concentration of Foxp3 (2.6-fold), CTLA4 (1.8-fold), TGF-β (2.8-fold), IL10 (2.9-fold), IFN-γ (−1.7-fold), and IL17 (−2.3-fold) in the ascending aorta quantified by RT-PCR and normalized to GAPDH. Fold changes in mRNA expression in treated mice are calculated relative to control (n=4 mice per group). *$P \leq .05$

4 | DISCUSSION

The present study provides evidence that recombinant *M. smegmatis* secreting a multiantigenic protein is immunogenic, induces a regulatory immune response on oral administration, and reduces the development of atherosclerosis in mice model, which was comparable to that of purified AHC protein. The strategy to use live recombinant vectors secreting heterologous proteins is emerging as a promising alternative to the use of purified antigens as vaccines.[8] Several species of Lactobacilli have been investigated as antigen delivery vehicles for inducing immune response to infectious diseases as well as inducing tolerance to human proinsulin.[22,35,36] In this study, for the first time, we have used *M. smegmatis* to induce tolerance to self-peptides and to control atherosclerosis development.

Mycobacterium smegmatis is a rapidly growing saprophyte which has been engineered to express antigens from viruses and bacteria and shown to protect against infection by efficiently cross-presenting the recombinant antigens.[20,37–41] This strain was also found to be nontoxic in immunodeficient animal models lacking NK or T cells, thus making this vehicle a safe and suitable vaccine vector.[42]

Repeated administration of a low dose of antigen is known to induce regulatory immune response involving transforming growth factor beta (TGF-β) and IL-10-producing CD4$^+$CD25$^+$ T regulatory cells.[6,43,44] Due to the ease of administration and low toxicity, oral tolerance is gaining importance as a therapeutic method to control unwanted immune

response.[45] Although oral tolerance has demonstrated protection against several autoimmune and allergic diseases, clinical translation of results from animal models has failed mainly due to problems with dose, purity, and delivery of antigen to mucosal immune system.[7,46] Transgenic plants, synthetic nanoparticles, and live bacterial carriers are being explored for effective delivery of oral antigen to the gut.[47–49]

Immune tolerance is an adaptive immune response and requires an immunogenic antigen. Good immunogens are also good tolerogens.[7] Recombinant AHC protein secreted by *M. smegmatis* was found to be immunogenic and induced specific antibody as well as cell-mediated immune response. These results suggest that the cloned proteins are recognized by the immune system of the mice and therefore are likely to be recognized by the immune system of the gut to induce a suppressive response.

Atherosclerosis is now recognized as a chronic inflammatory disease with immune response playing a major role in its development and progression. Self-reactive Th1 cells are known to migrate to arterial intima and amplify the inflammation during the progression of the disease. Oral delivery of recombinant *M. smegmatis* secreting AHC protein was able to reduce the development of atherosclerosis by 24.1% compared to vehicle control in ApoE$^{-/-}$ mice. Protection was associated with 29.5% lower lipid deposition in the aorta as seen by Oil red O staining of the sinus. Treated mice showed an increase in the expression of regulatory T cell markers (Foxp3 and CTLA4), anti-inflammatory cytokines (IL10 and TGF-β), and reduced expression of inflammatory markers in aorta (IFN-γ and IL17).

Orally administered antigens can directly cross the epithelium and enter the circulation, reach the liver via portal vein, or can be taken up by the intestinal microfold (M) cells, dendritic cells, and enterocytes. Intestinal macrophages and dendritic cells play a key role in antigen uptake, presentation, and inducing antigen-specific Treg, which migrate and suppress damaging immune responses. Thus, it is very essential that the orally delivered antigen is processed and presented to the APCs in the gut for tolerance induction. *Mycobacterium smegmatis* is readily eliminated by phagosomal proteases, and therefore, unlike pathogenic mycobacteria, these bacteria are processed upon infection and their antigens are presented very rapidly by APC[50] which could result in effective induction of tolerance. Our results suggest that *M. smegmatis* can be developed as an oral carrier for inducing regulatory immune response to self-proteins.

In conclusion, we have shown that oral administration of a recombinant *M. smegmatis* secreting a protein scaffold carrying three atherogenic peptides can control the development of atherosclerosis by inducing a regulatory immune response and reducing aortic inflammation. These preliminary results warrant further validation and improvement through optimization of dose and expression of recombinant construct.

ETHICS

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), with an approval number: TRI/IAEC/017/09/2012.

ACKNOWLEDGMENTS

We gratefully acknowledge the support of the trustees of Thrombosis Research Institute, London and Bangalore, and the Tata Social Welfare Trust, India (TSWT/IG/SNB/JP/Sdm). TP and LT acknowledge Bharti foundation, India for the support extended towards their PhD fellowship.

FUNDING

The study was supported by Department of Biotechnology, Ministry of Science and Technology, Government of India (BT/01/CDE/08/07), and Garry Weston foundation UK.

AUTHOR CONTRIBUTIONS

VE, XL, and LM were involved in concept, design, and interpretation of results. IF VD, RK, SP, TP, and LT carried out various experiments and statistical analysis. VD drafted the article. LM and VE were involved in critical revision of article. Funding was secured by VVK who also approved the article.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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