

**Impact Of Multiple Antigenic Epitopes From ApoB100, HHSP60 And *Chlamydophila*
Pneumoniae (*Cpn*) On Atherosclerotic Lesion Development In $\text{Apob}^{\text{tm}2\text{Sgy}}\text{Ldlr}^{\text{tm}1\text{Her}}\text{J Mice}$**

Xinjie Lu^{1*}, Min Xia¹, Valeria Endresz², Ildiko Faludi², Eva Gonczol³, Lakshmi Mundkur⁴, Daxin Chen^{1,4}, and Vijay Kakkar^{1,5*}

¹The Mary and Garry Weston Molecular Immunology Laboratory, Thrombosis Research Institute, London, United Kingdom; ²Department of Medical Microbiology and Immunobiology, University of Szeged, Szeged, Hungary; ³Virology, National Center for Epidemiology, Budapest, Hungary; ⁴MRC Centre for Transplantation, King's College London. ⁵Thrombosis Research Institute, Bangalore, India;

*Corresponding authors: Xinjie Lu and Vijay Kakkar, Thrombosis Research Institute, London, SW3 6LR Email: xlu@tri-london.ac.uk, vijay@tri-london.ac.uk
Tel: 0044(0)2073518312; Fax: 0044(0)2073518324

Abstract

Aims: To assess whether immunizing $\text{ApoB}^{\text{tm2Sgy}}\text{Ldlr}^{\text{tm1Her}}\text{J}$ mice simultaneously with different atherosclerosis-related epitopes engineered in a single recombinant protein is effective in reducing atherosclerotic lesions.

Methods and results: Antigenic epitopes were incorporated into a dendroaspin scaffold: AHC (ApoB100 peptide + hHSP60 peptide [hHSP60₁₅₃₋₁₆₃] + putative epitope derived from *Chlamydomonas pneumoniae* (*Cpn*)) and AHHC (AHC + hHSP60₃₀₃₋₃₁₂) were compared with construct A (ApoB peptide), construct H (hHSP60₁₅₃₋₁₆₃), and construct AH (ApoB100 peptide + hHSP60₁₅₃₋₁₆₃). Immunization with 2 multiple-antigenic epitope constructs elicited high levels of antibodies against each epitope (apart from hHSP60₁₅₃₋₁₆₃, which induced a low antibody response) in ApoB100 only, LDLr^{-/-} mice. Histological analyses demonstrated that the mice immunized with AHHC and AHC showed significantly greater reductions in atherosclerotic lesions compared with controls (64.2% and 63.1 %; $P < 0.001$, respectively) and compared with constructs A (24.5 %; $P < 0.05$), H (27.2 %; $P = 0.013$) and AH (42.9 %; $P < 0.001$). Moreover, combination of 2 short *Cpn* peptides along with ApoB and hHSP60 peptides had an additive effect on reducing the lesion without *Cpn* infection. Reduction in plaque size correlated with cellular infiltration, and cytokine/chemokine secretion in serum or by stimulated spleen cells as well as specific cellular immune responses when compared with controls.

Conclusions: Immunization of mice with a single construct containing multiple epitopes derived from ApoB100, hHSP60 and *Cpn* was more effective in reducing early atherosclerotic lesions through the induction of a specific Treg cell response than was the construct containing either mono- or bi-

epitopes. This approach offers attractive opportunities for the design of protein-based, multivalent vaccines against atherosclerosis.


Key words: immunization, ApoB-100, hHSP60, *C. pneumoniae* and atherosclerosis.

1. Introduction

Atherosclerosis is increasingly recognized as a complex chronic inflammatory disease of the arterial walls [1-3], as evidenced by the presence of inflammatory cells, activated immune cells, and cytokines in lesions, all of which indicate involvement of the immune system [4].

Atherosclerotic plaques are known to contain macrophage-derived foam cells in which macrophages interact with T-cells to produce a wide array of cytokines that can exert both pro- and anti-inflammatory effects [5].

Macrophages interact with T-cells to produce a wide array of cytokines that can exert both pro- and anti-inflammatory effects [6]. Antibodies against aldehyde-modified ApoB100, a major constituent of low density lipoprotein, reduce atherosclerosis in mice expressing human ApoB100, suggesting both antigenic and immunogenic roles of ApoB100 [7]. Antibodies against epitopes of the human heat shock protein 60 (hHSP60) molecule (hHSP60₁₅₃₋₁₆₃: AELKKQSKPVT and hHSP60₃₀₃₋₃₁₂: PGFGDNRKNQ) are present in atherosclerotic patients and share considerable homology with human cytomegalovirus (HCMV)-derived protein (immediate early protein UL122) and *Porphyromonas gingivalis* microbial HSP60. Sequence homology between microbial HSP60 and hHSP60 has been suggested to result in immunological cross-reactivity, which may play a role in atherogenesis [8,9]. Titers of *Cpn* antibodies are not always positively associated with the *Cpn* organism in atheroma; however, these antibodies might exert cross-reactivity to non-*Cpn* antigens (10). In addition, chlamydial antigens are involved in cell-mediated immune responses; antigenic epitopes derived from the *Cpn* proteins, including the major outer membrane protein (MOMP) (residues 67-74: GDYVFDRI), and from the putative outer membrane protein 5 (residues: 283-291: QAVANGGAI) of *Cpn* are thought to elicit cytotoxic T lymphocyte response following peptide immunization [1].

Intranasal administration of recombinant MOMP was found to promote anti-atherogenic T-cell responses and halt the development and progression of atherosclerotic plaques in ApoE^{-/-} mice [1]  However, much less is known about the effect of these antigenic epitopes on atherosclerotic lesions without *Cpn* infection.

The use of recombinant antigens where antigen delivery is dependent upon epitopes of the protein scaffold is likely to prove effective due to the natural variability in loop length and conformation, and varying α -helical and β -sheet content. Recombinant antigens of this type have indeed induced protective immune responses in animal models [13]. We have used a protein scaffold-dendroaspin, a short-chain neurotoxin homologue from the venom of *Elapidae* snakes, with no detectable neurotoxicity. Its core structure is similar to that of short-chain neurotoxins, but with a novel arrangement of loops [14] that makes it a unique protein scaffold for engineering a recombinant antigen.

We have recently demonstrated that simultaneous immunization of Apob^{tm2Sgy}Ldlr^{tm1Her} J mice with KLH-conjugated ApoB peptide (residues 688-707, numbered including signal peptide) and hHSP60 peptide (residues 153-163) significantly reduced atherosclerotic lesion development [1]. In the present study we sought to incorporate the epitopes derived from ApoB100, hHSP60 and *Cpn* into a dendroaspin scaffold to generate multiple antigenic molecules and to investigate whether these constructs enhance lesion reduction without infecting the mice with *Cpn*.

2. Methods


2.1 Expression and purification of recombinant glutathione S-transferase constructs

Glutathione S-transferase (GST)-dendroaspin (*Figure S1A*) and GST-recombinant construct A (ApoB100-peptide, amino acid (AA) 688-707, numbered including signal peptide), construct H (hHSP60₁₅₃₋₁₆₃); construct AH (ApoB100-peptide + hHSP60₁₅₃₋₁₆₃); construct AHC (ApoB100-peptide + hHSP60₁₅₃₋₁₆₃ + a combination of MOMP AA 66-73 and outer membrane 5, AA 283-291 of *Cpn* [designated as *Cpn* peptide]) and construct AHHC (ApoB100-peptide + hHSP60₁₅₃₋₁₆₃ + hHSP60₃₀₃₋₃₁₂ + *Cpn* peptide) (*Figure S1B*) were generated. These recombinant molecules were expressed in *Escherichia coli* (BL-21 strain), purified by affinity and ion exchange chromatography and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (*Figure S1C*).

2.2 Animal experiments

The experiments were approved by the Animal Welfare Committee of the University of Szeged and conform with the Directive 2010/63/EU of the European Parliament.

ApoB^{tm2Sgy}Ldlr^{tm1Her}J mice were used in our study in a total of 8 groups (5 sample groups, 3 control groups) consisting of 5-6 week-old males (similar body weight, 19.2 ± 2 g; Table S1) in each group, two separate experiments were performed with a total of **8 mice**.

The immunizing antigens used were construct A, H, AH, AHC and AHHC. Alum was used as an adjuvant. The repetitive immunization multiple sites strategy (RIMMS) was adopted [1] and mice were sacrificed at the end of week 12 (a high fat diet was started at the end of week 2 and continued for 10 weeks). The 3 control groups followed the diet programme after injections with GST-dendroaspin plus Alum  and PBS, respectively.

2.3 Tissue preparation and antibody response measurements

Twelve weeks after the first immunization, tissues were harvested and mounted in OCT and in paraffin, for immunohistochemical analyses and lesion measurement, respectively. Atherosclerosis in aortic roots was examined by Image-Pro Plus TM software, version 4.0 (Media Cybernetics, Inc., Bethesda, MD, USA). The peptide-specific antibody levels in the plasma samples were measured by ELISA following the manufacturer's instructions.

2.4 Immunohistochemical and morphometric analyses, quantitative measurements of atherosclerosis and immunohistochemical analysis of forkhead box protein 3 (Foxp3) expression in CD4^{SP} T cells

OCT-embedded samples were used for immunohistochemical analyses. Sections of paraffin-embedded tissues were stained with hematoxylin and eosin (HE) and elastin/van Gieson (Sigma) for histological examination and were evaluated using an Olympus U-ULH Optical microscope (Olympus Optical Co. Ltd, Tokyo, Japan).

2.5 Flow cytometric analysis of Foxp3 expression in CD4^{SP} T cells in lymph nodes

Cells from lymph nodes were processed for staining (30 minutes at 4°C) using allophycocyanin-anti-mouse Foxp3 antibody (BioLegend, Cambridge, UK). Cells were analyzed with a Beckman-Coulter FC-500 Analyzer (Bachman Coulter, High Wycombe, UK).

2.6 Measurement of cytokines

IL-10 and TNF- α levels in the lesions were quantified by immunohistochemical analyses (rat anti-mouse TNF- α and IL-10 purchased from BioLegend, CA, USA). Levels of ConA-induced IL-10, TGF- β , TNF- α and IFN- γ in splenocyte cultures were measured. Plasma levels of the cytokines interleukin (IL)-10, transforming growth factor (TGF)- β , tumor necrosis factor (TNF)- α and interferon (IFN)- γ were also measured by ELISA following the manufacturer's instructions (R&D systems, Abingdon, UK).

Antigen-specific regulatory function assays

Antigen-specific regulatory function assay experiments were performed as previously described by Herbin et al [15] and Klingenberg et al [16] with some modifications. CD4⁺CD25⁺ Treg cells were isolated from the spleen CD4⁺ T-cells of Apob^{tm2Sgy}Ldlr^{tm1Her} J mice immunized subcutaneously with construct AHC. T-effector cells (CD4⁺CD25⁻ cells) were isolated from the spleen of mice immunized with construct AHHC. Co-cultured CD4⁺CD25⁻ cells with CD4⁺CD25⁺ cells in the presence of 2×10⁴ APC (3000 rad irradiated splenocytes) per well were stimulated with 1 μ M of either construct A or H or with PBS control measured by proliferation assay.

2.8 Statistical analyses

Data are reported as mean±standard error of the mean (\pm SEM), unless otherwise indicated.

Figures were plotted using graph-pad Prism 5.01 and Sigma plot 9.0. For atherosclerotic lesion size, data were compared and intergroup differences were conducted using one-way ANOVA for multiple comparisons and post hoc bonferroni test. Others data were analyzed using Student's *t*-test (2-tailed analyses). Non-parametric distributions were analyzed using Mann-Whitney *U*

test for pairwise comparisons and the Kruskal-Wallis test for multiple comparisons. Differences between groups were considered significant at P values below 0.05.

An extended description of the methods is available as an online supplement.

3 Results

3.1 Peptide-specific immunoglobulin G in the sera of immunized mice

Antibody levels were measured by the ELISA test in mice immunized with either dendroaspin (GST tagged) or constructs (GST-tagged) at weeks 2 and 12. Construct A–induced ApoB peptide-specific antibody was observed when ApoB peptide was used as an ELISA antigen (*Figure 1A*); construct AH–induced ApoB-specific antibodies were also detected (*Figure 1C*). Constructs H and AH did not induce detectable levels of the hHSP60₁₅₃₋₁₆₃-specific IgG (*Figures 1B and 1C*); neither did constructs AHC and AHHC which contain hHSP60₁₅₃₋₁₆₃ peptide 1 (*Figures 1D and 1E*). IgG against hHSP60₃₀₃₋₃₁₂ was detectable in AHHC-immunized animals.


High antibody levels against *Cpn* peptide were detected in mice immunized with constructs AHC or AHHC at weeks 2 and 12 (*Figures 1D and 1E*). The GST-dendroaspin control-immunized mice showed very low levels of antibody responses against any peptide antigen (*Figure 1*).

3.2 Reduction of atherosclerotic lesion size in the aortic sinus

Following immunization with constructs A, H, AH, AHC, and AHHC and after a 10-week high-fat diet, the aortic sinuses of the mice were evaluated for the extent of atherosclerosis. The calculated plaque sizes from the immunized animals were compared with those of the controls. Representative sections from experimental groups are shown in *Figure 2A*. Lesion size was similar in three control

mice immunized with adjuvant (Alum) only ($31.5 \pm 2\%$), GST–dendroaspin + Alum ($32.0 \pm 1.9\%$) or PBS ($29.1 \pm 2\%$) (*Figure S3*). As three control groups of mice showed a similar lesion size, GST–dendroaspin was taken as a representative control hereafter. Valvular plaque areas and the ratio of plaque areas in μm^2 versus total areas are shown in *Figures 2B* and *2C*, respectively. Lesion size was significantly smaller in mice immunized with either construct A ($P=0.018$) or H ($P=0.013$) compared with controls ($32.0 \pm 2.0\%$), and significantly reduced lesion areas were observed in mice immunized with construct AH ($18.3 \pm 1.9\%$), compared with mice immunized with construct A ($P=0.018$), but not in the case of construct H ($P=0.076$) (*Figure 2C*). However, there was no significant difference in lesion size between mice immunized with construct A ($24.2 \pm 0.86\%$) or H ($23.3 \pm 1.85\%$; $P=0.704$). The smallest lesion area was observed in mice immunized with either construct AHHC ($11.46 \pm 0.79\%$) or construct AHC ($11.81 \pm 0.70\%$); this difference was significant ($P<0.001$) when compared with controls as well as with the other constructs (*Figure 2C*). Reduction in lesion size was expressed as a percentage of decrease in the lesion size in the aortic sinus compared with lesions in the control mice: 24.5% following immunization with construct A and 27.2% with construct H (*Figure 2D*). Mice immunized with construct AH showed significant reductions in lesion size (42.9%), and immunization with constructs AHC and AHHC resulted in greater reductions than with construct AH (63.1% and 64.2%, respectively versus 42.9%) (*Figure 2D*).

3.3 Amount of inflammatory cells and CD4⁺ cells expressing Foxp3 in local (in the lesions of aortas) and remote organs: splenocytes and lymphocytes

The percentage of anti-CD68  stained area in the lesions showed significant decreases of 69.7%, 68.8%, 66.3%, 47.0%, and 41.0% in mice immunized with constructs AHHC ($P<0.001$),

AHC($P<0.001$), AH ($P=0.003$), A (all $P=0.005$) and H ($P=0.021$), respectively (*Figures 3A and 3D*); the anti-CD68⁺-stained area in lesions in control mice (19.5%) was set as at 100% (0% reduction).

Measurement of the anti-CD11c⁺-stained lesion area showed a significant reduction in mice immunized with all of recombinant antigens with reductions of 72.6%, 67.8%, 62.5%, 40.3%, and 50.2% for immunization with constructs AHHC, AHC, AH, A, and H, respectively (*Figures 3B and 3C*), compared with the CD11c⁺ content of the lesions in the control group. Double immunostaining for CD68 and CD11c clearly revealed that most of macrophages were CD68⁺CD11c⁺, indicating the myeloid origin of this cell type in lesions (*Figures 3A-3D*) [17].

The proportion of CD4⁺ cells expressing Foxp3 was approximately 9-, 7-, and 4-fold higher (19.1%, $P<0.001$; 13.5%, $P<0.001$; 8.2%, $P<0.001$) in mice immunized with constructs AHHC, AHC and AH, respectively, compared with controls (2.0%) (*Figures 3D and 3E*), whereas the proportion of CD4⁺ cells expressing Foxp3 was approximately 2.5-fold higher (5.0% versus 2.0%, $P=0.002$; 5.2% versus 2.0%, $P=0.012$, respectively) in construct A- and H-immunized mice (*Figures 3D-F*).

With the exception of AH, which did not show a significant increase of Foxp3 expression when compared with construct A ($P=0.240$) and H ($P=0.114$)(*Figures 3H-I*), significantly higher levels of Foxp3 expression were observed in spleen CD4⁺ cells from mice immunized with the multi-epitope constructs AHHC ($20.4 \pm 0.9\%$), AHC ($20.3 \pm 1.3\%$), and AH ($18.8 \pm 1.9\%$), when compared with those of single-epitope constructs A ($15.4 \pm 0.9\%$) and H ($15.3 \pm 0.7\%$). In addition, expression of Foxp3 in spleen CD4⁺ cells from mice immunized with the recombinant antigen constructs was significantly higher ($P<0.001$) than that in controls immunized with GST-dendroaspin (*Figures 3H-I*).

Similarly, mice immunized with the recombinant antigens showed significantly increased Foxp3 expression in lymph node CD4⁺ cells immunized with GST-dendroaspin (*Figure 3J*, $P<0.001$). Significantly increased foxp3 expression levels in mice immunized with tri- or quadri-epitope antigens AHC and AHHC were also observed when compared with those immunized with the mono-epitope antigen A or H (*Figure 3J*, $P<0.05$).

The results of statistical analysis showed that the increased levels of Foxp3 contents either in the lesion or in spleen CD4⁺ cells as well as lymph node CD4⁺ cells correlated with a decreased percentage of lesion-occupied area in aorta sinus (*Figures S4F-H*, correlation $\alpha=0.05$, $P<0.05-0.001$).

3.4 Expression of anti-inflammatory cytokines and pro-inflammatory cytokines in lesion sites and levels of cytokines in plasma and in the supernatants of stimulated splenocytes

IL-10 expression in the aortic lesions of mice immunized with constructs A, H, AH, AHC and AHHC detected by immunohistochemistry is shown in *Figure 4A*. The proportion of CD4⁺ cells expressing IL-10 was significantly higher in mice immunized with construct AH (~14-fold; 11.9% versus 0.8%; $P<0.001$), A (~8-fold; 7.2% versus 0.8%; $P<0.001$), and H (~8-fold; 6.7% versus 0.8%; $P<0.001$), compared with that in controls. The proportion of CD4⁺ cells expressing IL-10 was >24-fold higher ($P<0.001$) in mice immunized with either construct AHC or AHHC, showing 19.2% and 21.0%, respectively, compared with controls at 0.8% (*Figure 4B*).



Immunohistochemical analyses of TNF- α showed significantly smaller TNF- α occupied areas in lesions of mice immunized with constructs compared with controls ($9.0 \pm 1.1\%$ for A; $9.9 \pm 1.0\%$ for H, $6.0 \pm 0.5\%$ for AH, $6.2 \pm 0.5\%$ for AHC and $6.1 \pm 0.5\%$ for AHHC, and $17.3 \pm 1.4\%$ for controls)

(Figures 4C and 4D). These data represent a percent reduction of 48.1%, 42.8%, 65.4%, 64.2% and 64.8%, respectively, compared with controls (total lesion area defined as 100%, and 0% reduction). Higher degree of reduction was produced by constructs AH, AHC and AHHC versus construct A or H.

Plasma levels of atheroprotective cytokines IL-10 ($P<0.001$ or $P=0.001$) and TGF- β ($P<0.001$) were significantly increased in mice immunized with these constructed antigens compared with controls (Figures 4E and 4F). Immunization with the single epitope constructs A or H had significantly less effect than with the bi- or multi-epitope containing constructs (AH, AHC and AHHC) on promoting the secretion of IL-10 and TGF- β ($P<0.05$ -0.001). Plasma levels of the atherogenic cytokine TNF- α were significantly reduced by immunization with constructs AH, AHC and AHHC (Figures 4G). A similar trend was obtained for these constructs in respect of the plasma levels of IFN- γ (Figures 4H). Although no statistically significant difference was found between mice immunized with construct AH and the 2 multi-epitope constructs (AHC and AHHC) in plasma levels of these 2 atherogenic cytokines, a significant difference was found in mice immunized with construct AHHC versus A or H in plasma levels of IFN- γ (Figures 4G and 4H, $P<0.05$).



Supernatants of splenocytes from mice immunized with these constructs individually showed significantly increased secretion of IL-10 (Figure 4I) and TGF- β (Figure 4J), stimulated with 10 or 100 $\mu\text{g/mL}$ of ConA ($P<0.05$ -0.001). The incremental secretion of IL-10 in construct AHHC-immunized mice was significantly higher than in the case of the other constructs tested. In contrast, significantly decreased secretion of TNF- α (Figure 4K) and IFN- γ (Figure 4L) was found in supernatants of splenocytes in mice immunized with these protein antigens. Again, a significantly

greater decrease in the secretion of TNF- α was observed in mice immunized with constructs AH, AHC, and AHHC versus those in construct A-immunized mice (*Figure 4K*; $P<0.05-0.01$) while a significantly greater decrease in secretion of IFN- γ was detected in constructs AH-, AHC- and AHHC-immunized mice versus construct A-or construct H-immunized mice (*Figure 4L*, $P<0.05-0.01$). Both construct AHHC- and construct AHC-immunized mice demonstrated significantly less IFN- γ secretion than AH-immunized mice ($P<0.05$).

The results of statistical analysis showed that the increased levels of IL-10 and TGF- β in the plasma 
supernatants of splenocytes stimulated by CoA  at 10 $\mu\text{g/ml}$ as well as the IL-10 positive area co-localised with CD4⁺ in the lesion correlated correlated with a decreased percentage of lesion-occupied area in aorta sinus (*Figures S4A-E*, correlation $\alpha=0.05$, $P<0.05-0.001$).

3.5 Evaluation of antigen specific regulation function

CD4⁺ T-cells from mice immunized with the recombinant constructs were incubated with the corresponding immunizing antigens at 0 to 10 $\mu\text{M/L}$ and a dose-dependent significant increase in proliferative response was shown between 0.1-1 $\mu\text{M/L}$. CD4⁺ T-cell proliferative responses were observed from antigen-immunized mice incubated with the corresponding immunizing antigen and the response was specific compared with those incubated with PBS in which the responses were smaller and non-specific (*Figures S5 A-F*).

To assess whether functional Treg cells were induced by immunization, we co-cultured antigen-specific Treg cells with CD4⁺ effector T-cells (CD4⁺CD25⁻ T-cells)  effector-cell proliferation was suppressed in a dose-dependent manner in the presence of Treg cells from AHC-immunized mice  stimulated by either construct A or H (*Figure 5A*) compared Treg cell

from GST-control stimulated by either construct A or H (Figure 5B). The differences were significant when adding Tregs into the effector cells at the ratios between 1:1~8:1


($P<0.05\sim<0.001$) apart from stimulation by H at the ratio of 8:1 ($P=0.06$) compared that without addition of Tregs. No such suppression was observed from GST-immunized control mice (Figure 5B)

4. Discussion

The effect of ApoB peptide- and HSP peptide-based immunization on atherosclerotic lesion reduction has been reported by our and other laboratories [1, 18,19]. In this study, we have investigated the effect of protein-based, multiple epitope-containing antigens on atherosclerotic lesions and established an additional effect of *Cpn* epitope in construct AHC and AHHC compared with construct AH in *Apob^{tm2Sgy}Ldlr^{tm1Her}J* mice.

The selection of construct AH (i.e. ApoB and hHSP60 peptides introduced into a constrained protein scaffold) was based on an extension of our previous work [1], which showed a synergistic effect of these peptide epitopes on atherosclerotic lesion reduction. This mouse strain was used because the *Apob^{tm2Sgy}Ldlr^{tm1Her}J* mice show significantly high degree of atherosclerosis after being fed a high-fat diet [7]. Furthermore, our data also demonstrated that a *Cpn*-derived peptide attached to the C-terminus of constructs AHHC and AHC evoked a strong immune response and significantly reduced atherosclerotic lesions without *Cpn* bacterial infection. An additive effect was found with AHC containing a *Cpn*-derived peptide compared with that of AH without a *Cpn*-derived peptide. This finding suggests that *Cpn*-derived peptide may have a synergistic effect through the interaction with other epitopes such as ApoB peptide or hHSP60₁₅₃₋₁₆₃. In addition, high cross-reactivity was observed

in the case of ApoB peptide-specific antibody with *Cpn*-peptide antigen, some degree of cross-reactivity of *Cpn* antibody observed with ApoB peptide antigen (*Figures S2A and S2B*), indicating that the additive effect of the *Cpn* epitope on lesion reduction in AHC and AHHC may be due to molecular mimicry. However, this molecular mimicry has been shown to occur in the absence of any true sequence homology between *Cpn*-derived peptide and ApoB peptide using computer-based software SIM-Alignment Tool for protein or peptide sequences (<http://expasy.org/tools/sim-prot.html>), this finding concurs with the report by Kohm et al [20]. In this case, it could explain the finding that antigenic surfaces, rather than sequence homology dictate molecular mimicry [21]. The mechanism of the effect of *Cpn*-derived epitope in AHC and AHHC constructs on reducing atherosclerotic lesions therefore remains under investigation at this point.



Our results suggest that immunization with ApoB-containing constructs induces an antigen-specific antibody response that is associated with an anti-atherogenic effect, detected as a significant reduction in the size of the atheromatous lesion area, while the construct containing  HSP60₁₅₃₋₁₆₃ peptide did not induce significant levels of antibody response. A specific antibody response was not observed in earlier studies using HSP60 peptides as an anti-atherosclerotic vaccine [19], wherein the mechanism of protection was reported to be mediated by regulatory T-cells. Prevention of lesion development by HSP60 epitopes is likely to be mediated by a reduction in inflammation through a regulatory T-cell response.



Immunization with construct AHHC containing a second hHSP60 epitope (hHSP60₃₀₃₋₃₁₂) in our 10-week, high-fat diet (HFD)-based **ApoB^{tm2Sgy}Ldlr^{tm1Her}J** mouse model failed to show an added reduction in lesion area despite the antibody response and the increase in anti-atherogenic cytokines.

Our study demonstrates that an antigen-specific humoral response and a modified cellular immune response accompany reduced lesion size. However, considering the facts that increased **level** of IL-10 **level** was found in the plasma and was produced by ConA-stimulated splenocytes of the AHHC immunized mice compared with that in AHC-immunized mice, it is reasonable to speculate that hHSP60₃₀₃₋₃₁₂ may either have an additive role or its effect is a consequence of internal interactions with other introduced epitopes. A further study to discriminate between AHC from AHHC-immunized mice fed for a longer period with a HFD is **planned**.

Cellular infiltration into atherosclerotic lesions results in increased levels of macrophages, activated CD4⁺ T-cells and dendritic cells (markers of early lesion formation) [22]. We observed that low numbers of macrophages, CD4⁺ T-, and CD11c⁺-cells, and high numbers of Treg cells, in mice were due to immunization with constructs AHHC, AHC and AH. In agreement with the results of the present study, Cybulsky et al reported that cells bearing markers of DCs, including CD11c, take up residence just beneath the endothelium in the lesser curvature of mice [23]. Moreover, the results of our study show that CD68⁺CD11c⁺ cells are the main macrophage subset in lesions, but their functions, and whether their function is affected by the constructs, need to be further explored [17]. Vaccination using mature DCs pulsed with oxLDL-induced, oxLDL-specific T-cells with a lowered Th1 response increased the levels of oxLDL-specific antibodies and reduced lesion size [24]. Therefore, it will be interesting in future studies to determine whether vaccination using mature DCs pulsed with these recombinant construct-induced-specific T-cells could reduce lesion size.

The significant difference observed between the effects of immunization with bi- or multi-epitope constructs concurs with an additive effect seen in our previous study where immunization of mice with

a combination of ApoB and hHSP60 peptides  had a significantly greater effect on early lesion formation compared with the use of each peptide individually [1]. In agreement with our present study, Klingenberg et al recently reported that subcutaneous immunization with HSP65 reduces atherosclerosis in ApoE^{-/-} mice [25], but we used HSP60 peptide rather than HSP65 protein. One possible explanation could be that the HSP60 peptide incorporated in the dendroaspin scaffold may serve as a protein (HSP60)-like antigen through molecular mimicry. However, the mechanism of  HSP60/65 on lesion formation is still under investigation as other studies have reported increased fatty streak formation after immunization with HSP65 using a similar subcutaneous route of administration in mice [26,27].

Cytokines TNF- α and IFN- γ activate  other cells in the vasculature, thus orchestrating an inflammatory response to atherogenesis  [28]. Based on the cytokine profiles from either sera or spleen cells in immunized mice, the TNF- α and IFN- γ release may well be linked to the ability of the immune animals to release TNF- α and IFN- γ from pre-primed cells, leading to a decrease in the need to synthesize significant amounts of new TNF- α and IFN- γ . Our results show that vaccination with any of the constructs containing inserts promoted a major shift away from pro-inflammatory cytokines (ie TNF- α and IFN- γ), towards anti-inflammatory cytokines (particularly IL-10 and TGF- β), that is evident not only in the plaque but systemically too. Although these results strongly suggest that this generalized anti-inflammatory response is responsible for the reduction in plaque size and inflammatory cell content observed, they do not discriminate the reduction is dependent on which anti-inflammatory cytokine, as both IL-10 and TGF- β were implicated to play a role in atherosclerosis [29,30]. A strategy using IL-10 or TGF- β knock out mice will be required to delineate the relative contributions of these two cytokines.

Our present study further demonstrates that both termini of the dendroaspin protein scaffold can be extended and that antigenic epitopes can be substituted into loop III to produce functional multiple-epitope recombinant protein antigens. A higher immune response can be expected with more epitopes introduced into dendroaspin scaffold before probably reaching a saturated immune response. Our experiments do not exclude the possibility that other epitopes and other positions within the dendroaspin structure may be more beneficial in immunizing against atherosclerosis.

The discovery of Foxp3 as the key transcription factor controlling Treg cell development and function is one of the most significant advances in Treg immunobiology. The mechanism of reducing atherosclerotic lesion formation by immunization with these recombinant antigens is, at least in part, due to increasing Treg cell concentration in immunized mice, either locally (in the lesions) or in remote organs (splenocytes): we observed that concentrations of CD4⁺-expressing Treg cells were significantly higher in mice immunized with these recombinant constructs than in those of control mice immunized with GST-dendroaspin either locally (in lesion site of aorta) or in remote organs (splenocytes or lymphocytes). In addition, the atheroprotective effect paralleled an induction of Treg suppression of antigen-specific effector T-cells. This mechanism has been demonstrated in intranasal immunization [31] and subcutaneous immunization with ApoB peptide [32]. In agreement with our present findings, it appears that modulation in atherosclerosis-related autoimmunity by antigen-specific activation of Tregs represents a novel approach for the treatment of atherosclerosis.

Notably, our findings have demonstrated that the *Cpn* epitope in constructs AHHC and AHC contributed to lesion reduction in uninfected mice. Assuming the possibility that the mice are already infected with *Cpn*, then the *Cpn* epitope is still capable of playing a role in lesion reduction. In a separate study we found that the *Cpn* peptide reduces lesion formation in the same mouse strain

infected with *Cpn* (Xia et al unpublished data). Our preliminary results suggest that the *Cpn* epitope has the capacity to induce regulatory T-cells and contribute to prevention of early lesion formation by inducing an antigen-specific regulatory immune response (data not shown). Therefore, constructs AHHC and AHC containing a *Cpn* epitope may also be used to develop a vaccine against atherosclerosis in which *Cpn* infection plays an important role. *Cpn* protein, one of a variety of disease-related antigens, together with oxidized low-density lipoprotein and hHSP60 may stimulate CD4⁺ T-cells in the context of atherogenesis [33]. Antigen-specific immunotherapy involves the use of inverse vaccination for a specific autoantigen such as ApoB and HSP or their specific epitopes. Application of antigen-specific immunotherapy has an attractive advantage of permitting selective inactivation of autoreactive T-cells without interfering with normal immune function [34]. Our findings have suggested that developing a protein-based vaccine using a dendroaspin scaffold may offer attractive opportunities for the development of multivalent vaccines against atherosclerosis.

Supplementary material

Supplementary material is available online.

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Conflict of interest:

None declared.

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References:

1. Lu X, Chen D, Endresz V, Xia M, Faludi I, Burian K, Szabo A, Csanadi A, Miczak A, Gonczol E, Kakkar V. Immunization with a combination of ApoB and hHSP60 epitopes significantly reduces early atherosclerotic lesion in Apobtm2SgyLdlrtm1Her/J mice. *Atherosclerosis* 2010;**212**:472-80.
2. Zernecke A, Weber C. Chemokines in the vascular inflammatory response of atherosclerosis. *Cardiovasc Res* 2010;**86**:192-201.
3. Libby P. Inflammation in atherosclerosis. *Nature* 2002;**420**:868-74.
4. Chyu KY, Nilsson J, Shah PK. Immunization for atherosclerosis. *Current Atherosclerosis Reports* 2007;**9**:104-9.
5. Grundtman C, Wick G. The autoimmune concept of atherosclerosis. *Curr Opin Lipidol* 2011;**22**:327-34.
6. Gordon S, Clarke S, Greaves D, Doyle A. Molecular immunobiology of macrophages: Recent progress. *Curr Opin Immunol* 1995;**7**:24-33.
7. Schiopu A, Bengtsson J, Söderberg I, Janciauskiene S, Lindgren S, Ares MP, Shah PK, Carlsson R, Nilsson J, Fredrikson GN. Recombinant human antibodies against aldehyde-modified apolipoprotein B-100 peptide sequences inhibit atherosclerosis. *Circulation* 2004;**110**:2047–52.
8. Bason C, Corrocher R, Lunardi C, Puccetti P, Olivieri O, Girelli D, Navone R, Beri R, Millo E, Margonato A, Martinelli N, Puccetti A. Interaction of antibodies against cytomegalovirus with heat shock protein 60 in pathogenesis of atherosclerosis. *Lancet* 2003;**362**:1971-7.
9. Choi JI, Chung SW, Kang HS, Rhim BY, Park YM, Kim US, Kim SJ. Epitope mapping of Porphyromonas gingivalis heat-shock protein and human heat-shock protein in human atherosclerosis. *J Dent Res* 2004;**83**:936-40.

10. Campbell LA, Campbell LA, O'Brien ER, Cappuccio AL, Kuo CC, Wang SP, Stewart D, Patton DL, Cummings PK, Grayston JT. Detection of *Chlamydia pneumoniae* TWAR in human coronary atherectomy tissues. *J Infect Dis* 1995;**172**:585-8.
11. Sette A, Vitiello A, Rehman B, Fowler P, Nayarsina R, Kast WM, Melief CJ, Oseroff C, Yuan L, Ruppert J, Sidney J, del Guercio MF, Southwood S, Kubo RT, Chesnut RW, Grey HM, Chisari FV. The relationship between class I binding affinity and immunogenicity of potential cytotoxic T cell epitopes. *J Immunol* 1994;**153**:5586-92.
12. Kadri R, Bermudez-Fajardo A, Puolakkainen M, Stewart G, Oviedo-Orta E. Effect of immunisation with *Chlamydia pneumoniae* recombinant major outer membrane protein on atherosclerosis development. *Heart* 2010;**96**:e27.
13. Binz HK, Pluckthun A. Engineered proteins as specific binding reagents. *Curr Opin Biotechnol* 2005;**16**:459-69. Chang JW, Mechling DE, Bächinger HP, Burrows GG. Design, engineering, and production of human recombinant T cell receptor ligands derived from human leukocyte antigen DR2. *J Biol Chem* 2001;**276**:24170-6.
14. Sutcliffe MJ, Jaseja M, Hyde EI, Lu X, Williams JA. Three-dimensional structure of the RGD-containing neurotoxin homologue dendroaspin. *Nat Struct Biol* 1994;**1**:802-7.
15. Herbin O, Ait-Oufella H, Yu W, Fredrikson GN, Aubier B, Perez N, Barateau V, Nilsson J, Tedgui A, Mallat Z. Regulatory T-cell response to apolipoprotein b100-derived peptides reduces the development and progression of atherosclerosis in mice. *Arterioscler Thromb Vasc Biol*. 2012;**32**:605-12.
16. Klingenberg R, Lebens M, Hermansson A, Fredrikson GN, Strodthoff D, Rudling M, Ketelhuth DF, Gerdes N, Holmgren J, Nilsson J, Hansson GK. Intranasal immunization with an

apolipoprotein B-100 fusion protein induces antigen-specific regulatory T cells and reduces atherosclerosis. *Arterioscler Thromb Vasc Biol.* 2010;**30**:946-52.

17. Wang F, Lee E, Lowes MA, Haider AS, Fuentes-Duculan J, Abello MV, Chamian F, Cardinale I, Krueger JG. Prominent production of IL-20 by CD68+/CD11c+ myeloid-derived cells in psoriasis: Gene regulation and cellular effects. *J Invest Dermatol* 2006;**126**:1590-9.
18. Fredrikson GN, Schiopu A, Berglund G, Alm R, Shah PK, Nilsson J. Autoantibody against the amino acid sequence 661-680 in apo B-100 is associated with decreased carotid stenosis and cardiovascular events. *Atherosclerosis* 2007;**194**:e188-92.
19. van Puijvelde GH, van Es T, van Wanrooij EJ, Habets KL, de Vos P, van der Zee R, van Eden W, van Berkel TJ, Kuiper J. Induction of oral tolerance to HSP60 or an HSP60-peptide activates T cell regulation and reduces atherosclerosis. *Arterioscler Thromb Vasc Biol* 2007;**27**:2677-83.
20. Kohm AP, Fuller KG, Miller SD. Mimicking the way to autoimmunity: an evolving theory of sequence and structural homology. *Trends Microbiol* 2003;**11**:101-5.
21. Zhao R, Loftus DJ, Appella E, Collins EJ. Structural evidence of T-cell xeno-reactivity in the absence of molecular mimicry. *J Exp Med* 1999;**189**:359-70.
22. Bobryshev YV. Dendritic cells in atherosclerosis: current status of the problem and clinical relevance. *Eur Heart J* 2005;**26**:1700-4.
23. Cybulsky MI, Jongstra-Bilen J. Resident intimal dendritic cells and the initiation of atherosclerosis. *Curr Opin Lipidol* 2010;**21**:397-403.
24. Habets KL, van Puijvelde GH, van Duivenvoorde LM, et al. Vaccination using oxidized low-density lipoprotein-pulsed dendritic cells reduces atherosclerosis in LDL receptor-deficient mice. *Cardiovasc Res* 2010;**85**:622-30.

25. Klingenberg R, Ketelhuth DF, Strodtzoff D, Gregori S, Hansson GK. Subcutaneous immunization with heat shock protein-65 reduces atherosclerosis in Apoe^{-/-} mice. *Immunobiology* 2012;**217**:540-7.
26. Afek A, George J, Gilburd B, Rauova L, Goldberg I, Kopolovic J, Harats D, Shoenfeld Y. Immunization of low-density lipoprotein receptor deficient (LDL-RD) mice with heat shock protein 65 (HSP-65) promotes early atherosclerosis. *J Autoimmun* 2000;**14**:115-21.
27. George J, Afek A, Gilburd B, Shoenfeld Y, Harats D. Cellular and humoral immune responses to heat shock protein 65 are both involved in promoting fatty-streak formation in LDL-receptor deficient mice. *J Am Coll Cardiol* 2001;**38**:900-5.
28. Zhou X, and Hansson GK. Detection of B cells and proinflammatory cytokines in atherosclerotic plaques of hypercholesterolaemic apolipoprotein E knockout mice. *Scand J Immunol* 1999;**50**:25-30.
29. Pinderski Oslund LJ, Hedrick CC, Olvera T, Hagenbaugh A, Territo M, Berliner JA, Fyfe AI. Interleukin-10 blocks atherosclerotic events in vitro and in vivo. *Arterioscler Thromb Vasc Biol* 1999;**19**:2847-53.
30. Robertson AK, Rudling M, Zhou X, Gorelik L, Flavell RA, Hansson GK. Disruption of TGF-beta signaling in T cells accelerates atherosclerosis. *J Clin Invest* 2003;**112**:1342-50.
31. Klingenberg R, Lebens M, Hermansson A, Fredrikson GN, Strodtzoff D, Rudling M, Ketelhuth DF, Gerdes N, Holmgren J, Nilsson J, Hansson GK. Intranasal immunization with an apolipoprotein B-100 fusion protein induces antigen-specific regulatory T cells and reduces atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2010;**30**:946-952.
32. Herbin O, Ait-Oufella H, Yu W, Fredrikson GN, Aubier B, Perez N, Barateau V, Nilsson J, Tedgui A, Mallat Z. Regulatory T-cell response to apolipoprotein B 100-derived peptides reduces the

development and progression of atherosclerosis in mice. *Arterioscler Thromb Vasc Biol.* 2012;**32**:605-612.

33. Chang JW, Mechling DE, Bächinger HP, Burrows GG. Design, engineering, and production of human recombinant T cell receptor ligands derived from human leukocyte antigen DR2. *J Biol Chem* 2001;**276**:24170-6.
34. Paulson KE, Zhu S, Chen M, Nurmohamed S, Jongstra-Bilen J, Cybulsky MI Resident intimal dendritic cells accumulate lipid and contribute to the initiation of atherosclerosis. *Circ Res* 2010;**106**:383-90.

Figure legends

Figure 1: Levels of constructed protein-induced IgG antibodies in the sera of **ApoB^{tm2Sgy}Ldlr^{tm1Her}J** mice at 2 and 12 weeks after the first immunization and in controls (GST-dendroaspin(den)-immunized mice). The mean optical densities (ODs) and SEM obtained from plasma samples of constructs A-, H-, AH-, AHC- and AHHC-immunized mice on ApoB peptide, hHSP60₁₅₃₋₁₆₃ and hHSP60₃₀₃₋₃₁₂, *Cpn* peptide-coated ELISA plates are shown. Dilution ratio: 1:100.

Figure 2: Detection and quantitation of the lesion areas in the aorta of **ApoB^{tm2Sgy}Ldlr^{tm1Her}J** mice fed on a high-fat diet after immunization with constructs versus controls (GST-dendroaspin and Alum).

A: Photomicrograph of lesions observed in atherosclerotic aortas as analyzed with elastin/van Gieson staining. Bar = 250μm.

B: Scatter plot showing mean of lesion area (μm²) in the aortic sinus of mice immunized with constructs compared with those in control (GST-dendroaspin + Alum) (N=8 mice). **P*<0.05; ***P*<0.01; ****P*<0.001.

C: Lesion area versus total area in the sections of the aortic sinus (%) (ratio of plaque areas in mm² versus total areas) (N=8 mice).

D: Percentage of reduction in lesion size in the aortic sinus (the reduction of control [GST-dendroaspin+Alum] group was set at zero).

Figure 3: Assessment of inflammation-associated cells in the lesions of **ApoB^{tm2Sgy}Ldlr^{tm1Her}J** mice fed on a high-fat diet after immunization with constructs A, H, AH, AHC and AHHC.

A: Photomicrographs showing immunohistochemical staining of CD68 (green) and CD11c (red) markers.

B: Scatter plot showing anti-CD68-stained area in lesion versus total lesion area (%); Data are given as the mean of 8 mice. One-way analysis of variance, $R^2=0.611$.

C: Scatter plot showing anti-CD11c-stained area in lesion versus total lesion area (%); Data are given as the mean of 8 mice. One-way analysis of variance, $R^2=0.584$.

D: CD68⁺ and CD11c⁺ co-localization; Data are given as mean \pm SEM (derived from Figures 3B and 3C).

E: Photomicrographs showing immunohistochemical staining of CD4⁺ T-cells (green) and Foxp3⁺ Treg cells (red) (magnificent x8).

F: Scatter plot showing anti-Foxp3-stained area versus anti-CD4⁺ stained area in lesion (N=8 mice). One-way analysis of variance, $R^2=0.656$.

G: Assessment of Treg cells as fold increase for percentage of Foxp3⁺ area compared with controls.

H: Photomicrographs showing immunohistochemical staining of CD4⁺ T-cells (green) and Foxp3⁺ Treg cells (red) in splenocytes (magnificent x16).

I: Scatter plot showing Foxp3⁺ cell expression levels in spleen CD4⁺ cells (N=6 mice). One-way analysis of variance, $R^2=0.745$. Data are given as mean \pm SEM.

J: Flow cytometric analysis of Foxp3⁺ expression by CD4⁺ T-cells in lymph nodes. Data represent mean \pm SEM of data from three independent samples. * P <0.05; *** P <0.001.

Figure 4: Assessment of IL-10-producing T cells, TNF- α expression in the lesions and cytokine levels in **Apob^{tm2Sgy}Ldlr^{tm1Her}J** mice fed a high-fat diet after immunization with constructs A, H, AH, AHC and AHHC compared with controls.

A: Photomicrographs showing dual-immunohistochemical staining for IL-10 (red) and CD4 (green) (magnificent x8).

B: Scatter plot showing mean of IL-10-positive area co-localized with CD4⁺ area (%) (N=8 **mice**). One-way analysis of variance, **$R^2=0.659$** .

C: Photomicrographs showing dual-immunohistochemical staining of lesions in **Apob^{tm2Sgy}Ldlr^{tm1Her}J** mice fed a high-fat diet after immunization with constructs A, H, AH, AHC and AHHC for TNF- α (green).

D: Scatter plot showing mean of anti-TNF- α stained area in the lesion versus total lesion area (N=8 **mice**). One-way analysis of variance, **$R^2=0.725$** .

E-H: Cytokine levels measured in plasma.

I-L: Cytokine levels measured in the supernatant of splenocytes stimulated with ConA.

Data given as Mean of 6 analyses \pm SEM for E-L. * P <0.05; ** P <0.01; *** P <0.001.

Figure 5. Assessment of antigen-specific regulatory function. Splenic effector cells at 2×10^5 cells/well generated from either AHC-immunized mice or GST-immunized control mice. Proliferation of effector cells alone is indicated in the leftmost bar of each group. Addition of isolated Treg cells from construct A- or H-immunized animals or KLH-immunized controls is indicated at different ratios to affect cells. Data are expressed as mean of 5 analyses \pm SEM. Differences between groups are shown; $P < 0.05$, $P < 0.01$ and $P < 0.001$.

Figure 1

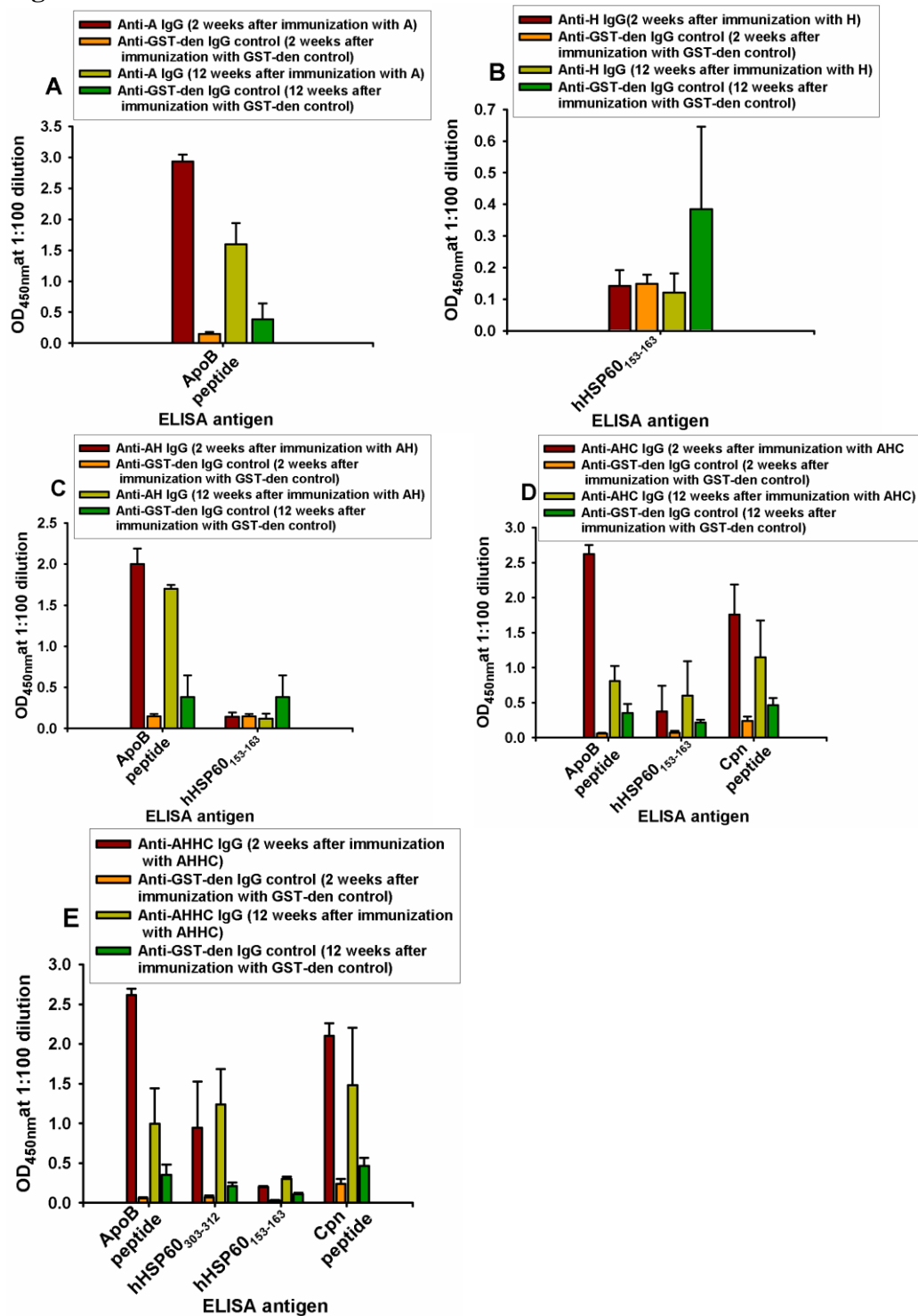


Figure 2

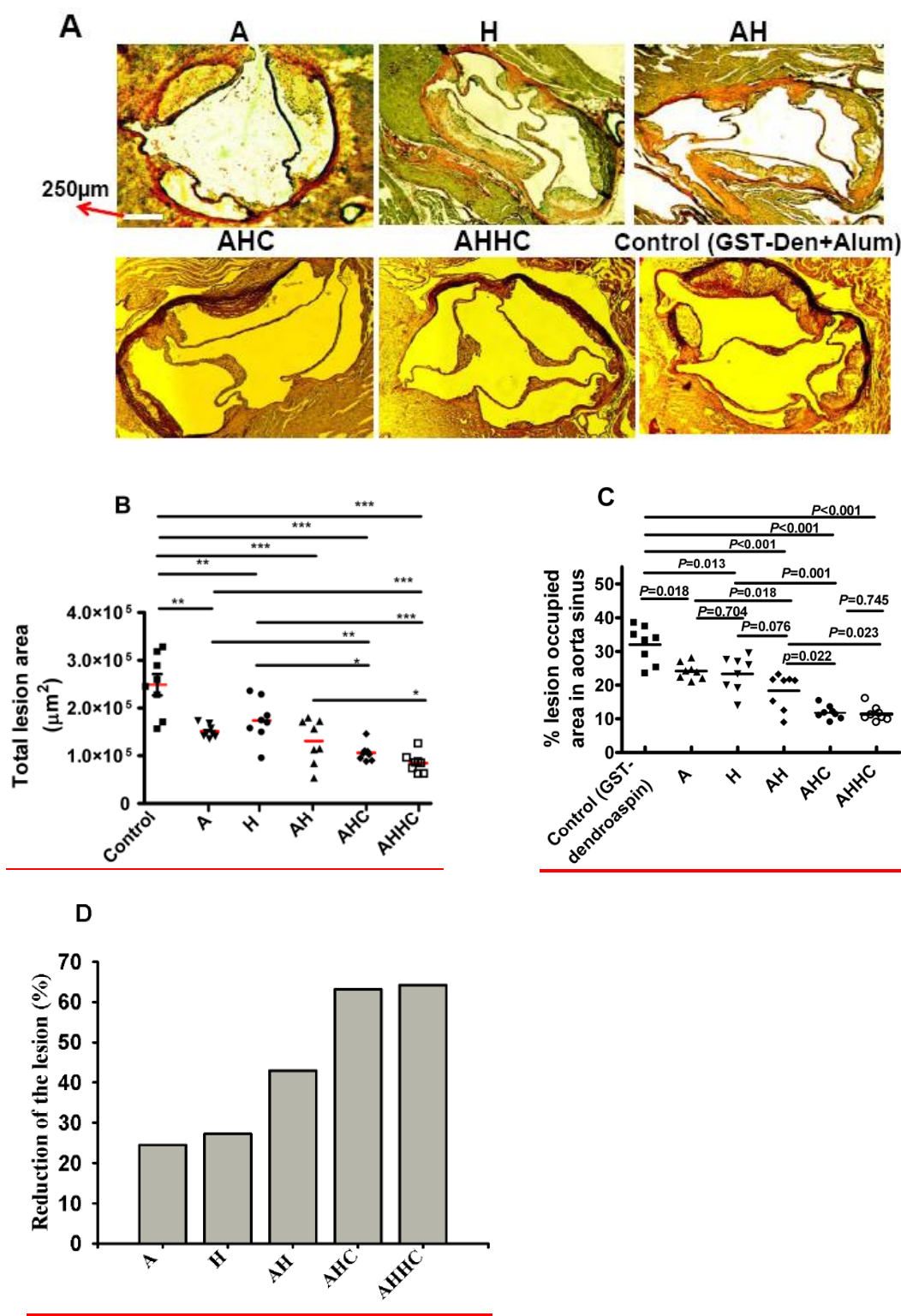
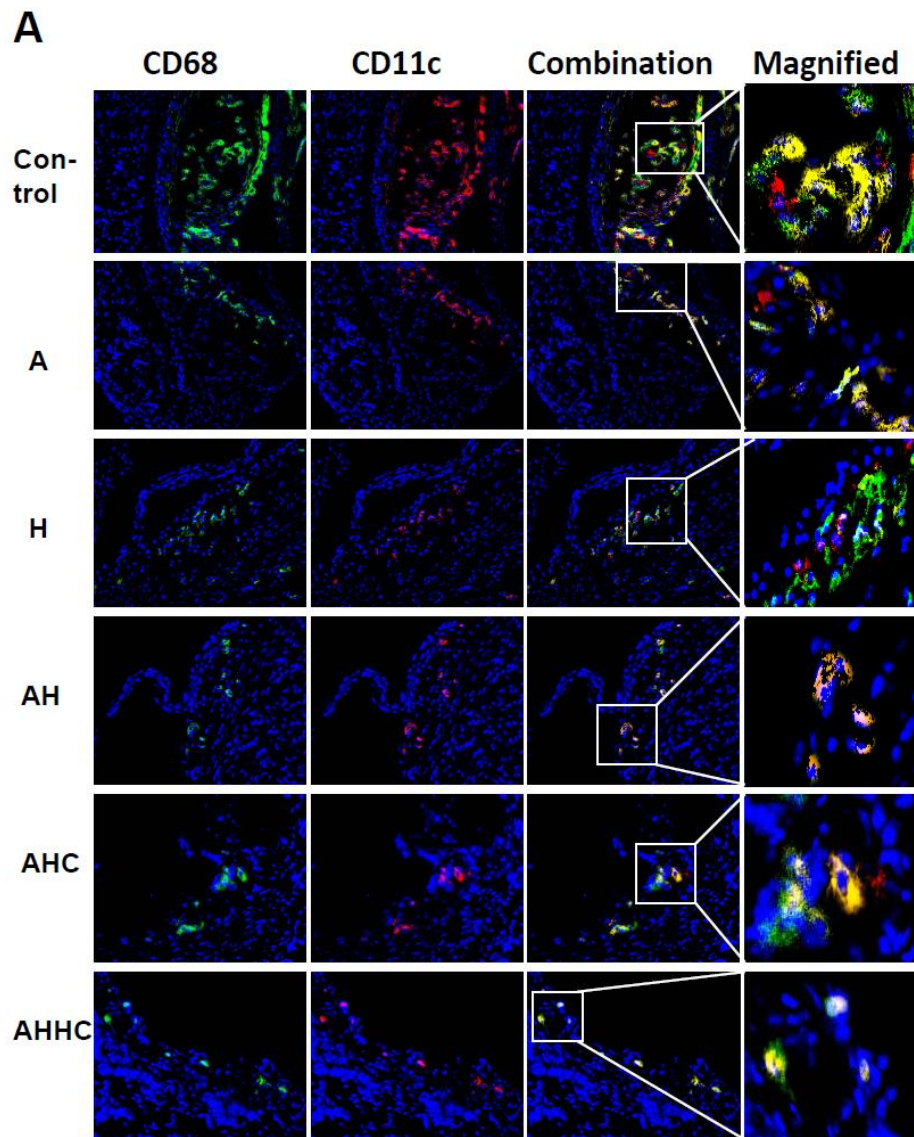
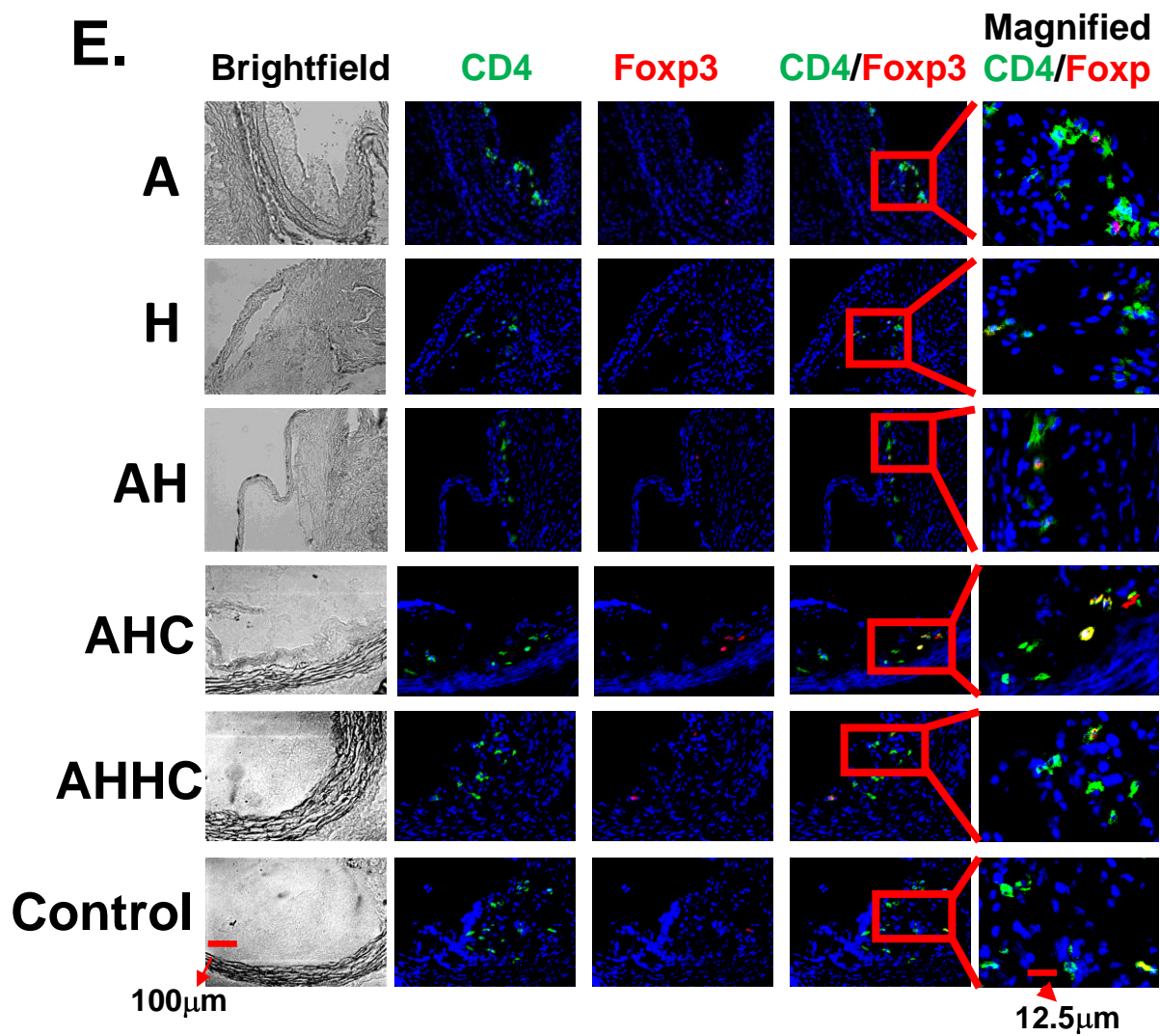
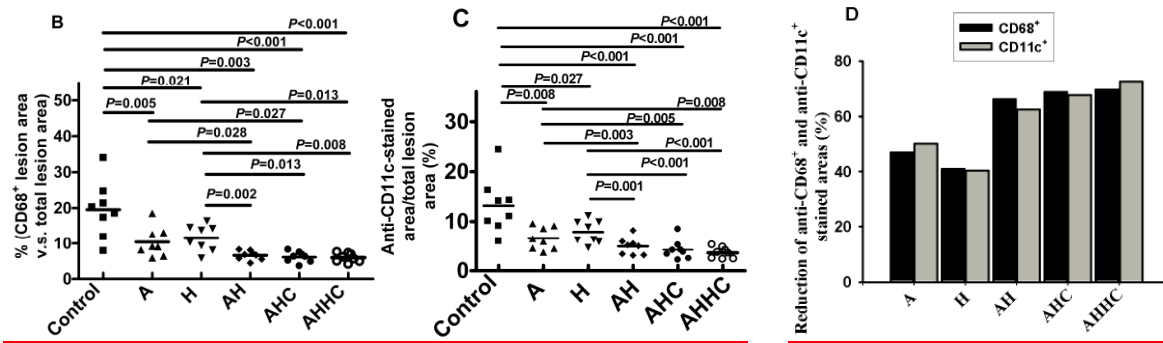
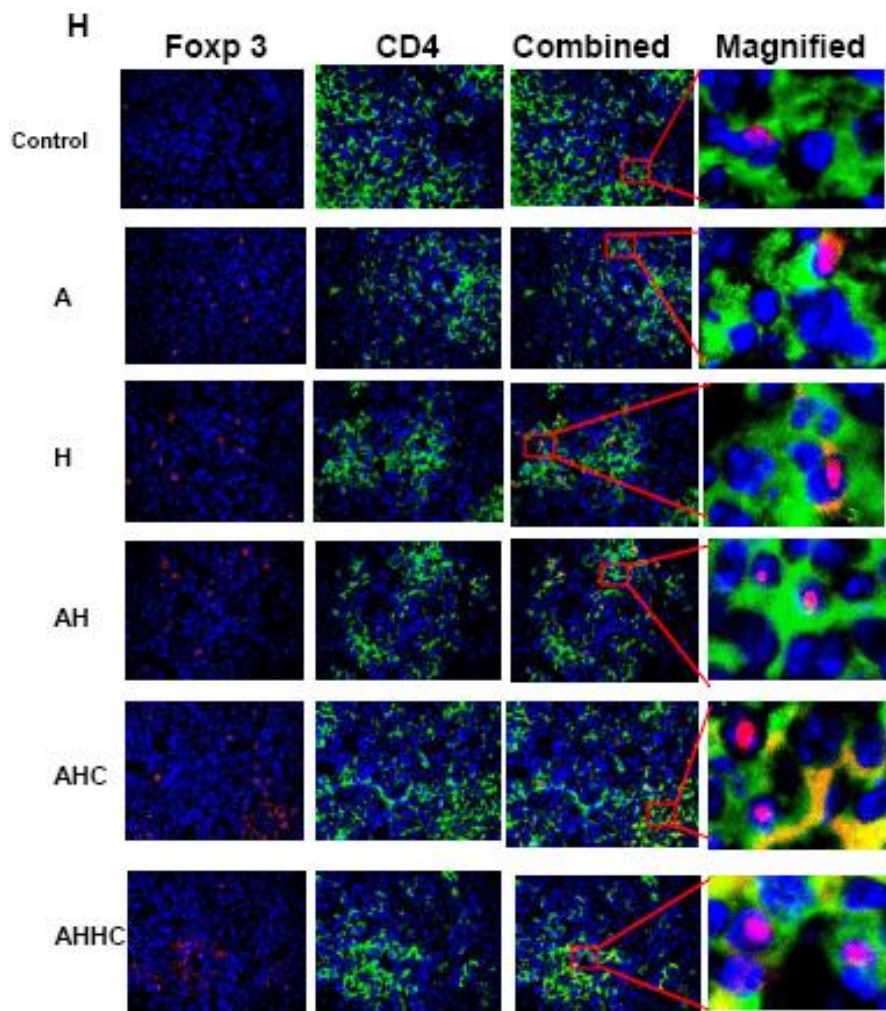
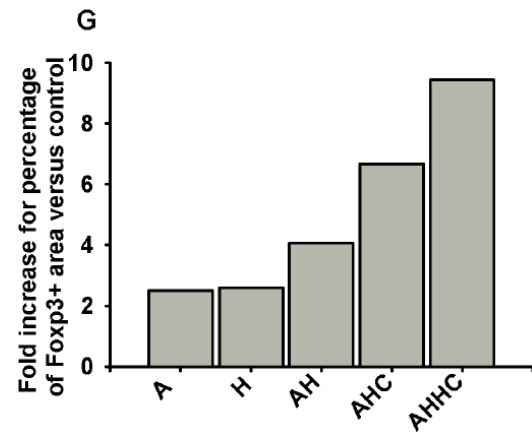
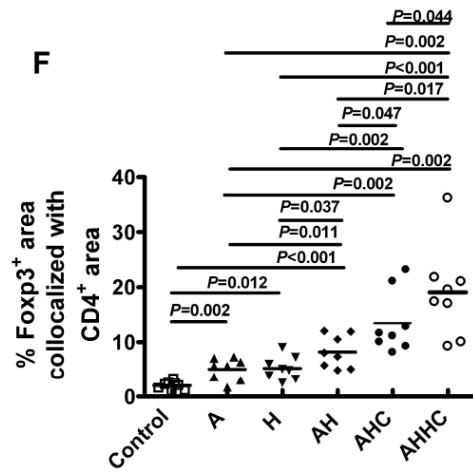
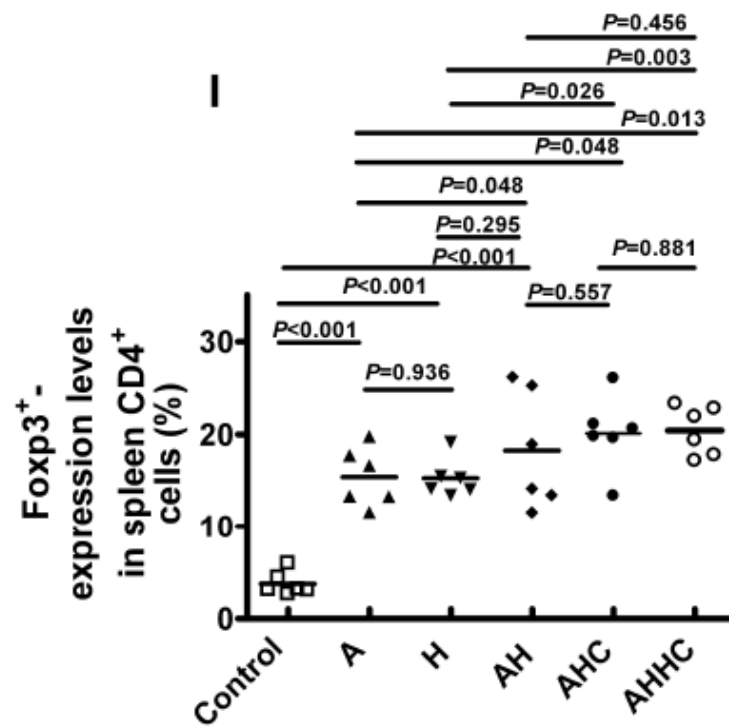


Figure 3









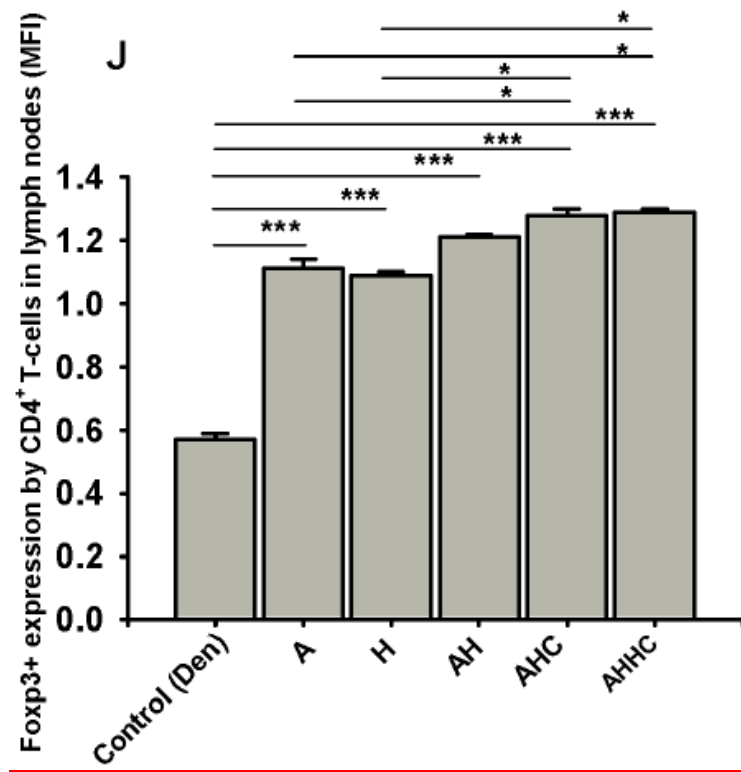
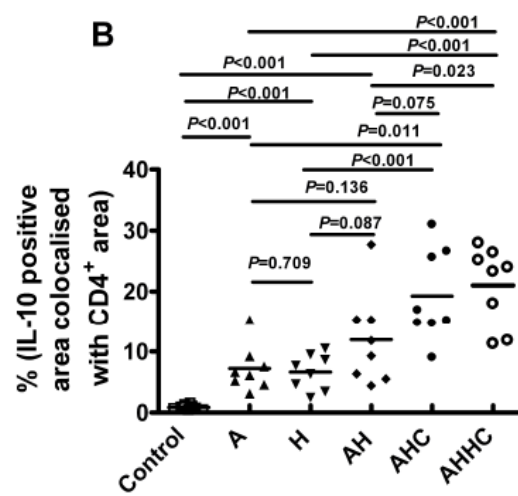
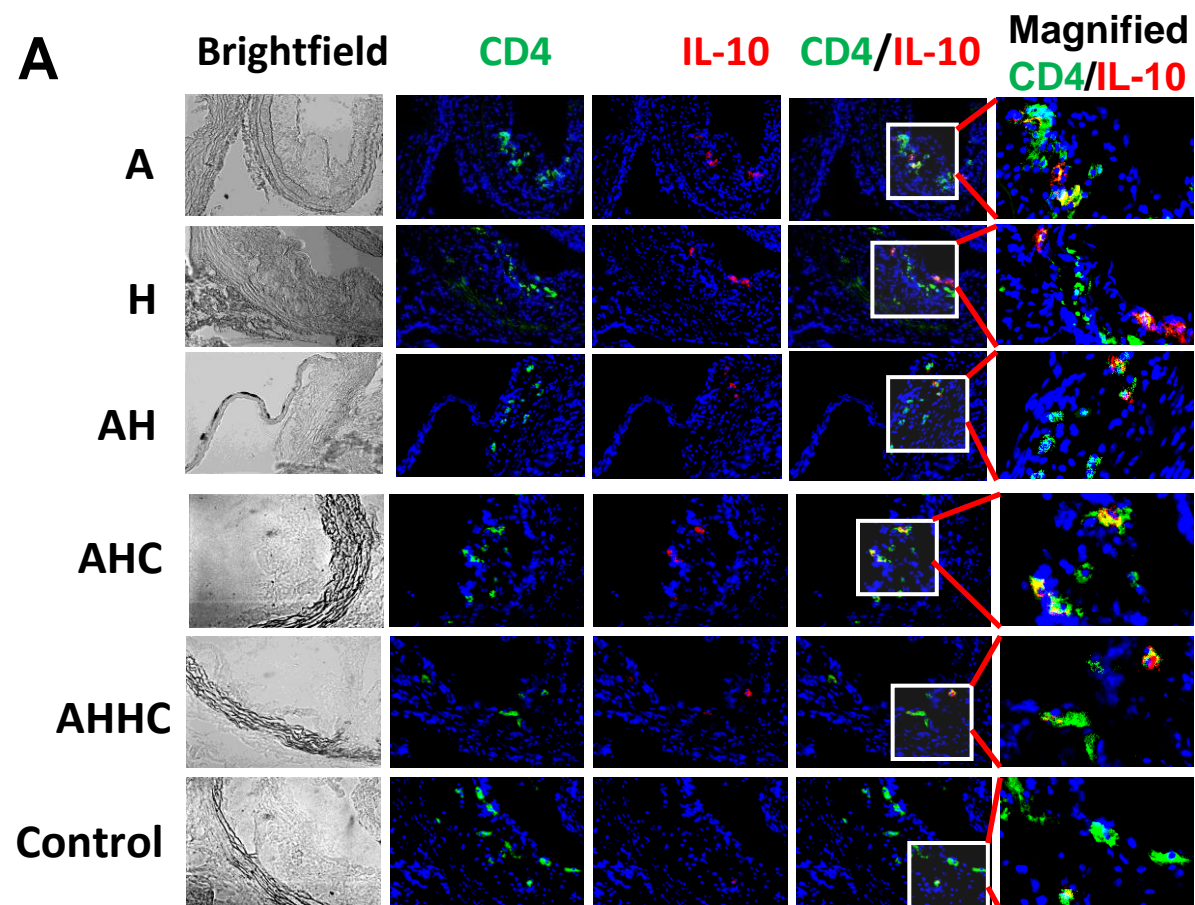
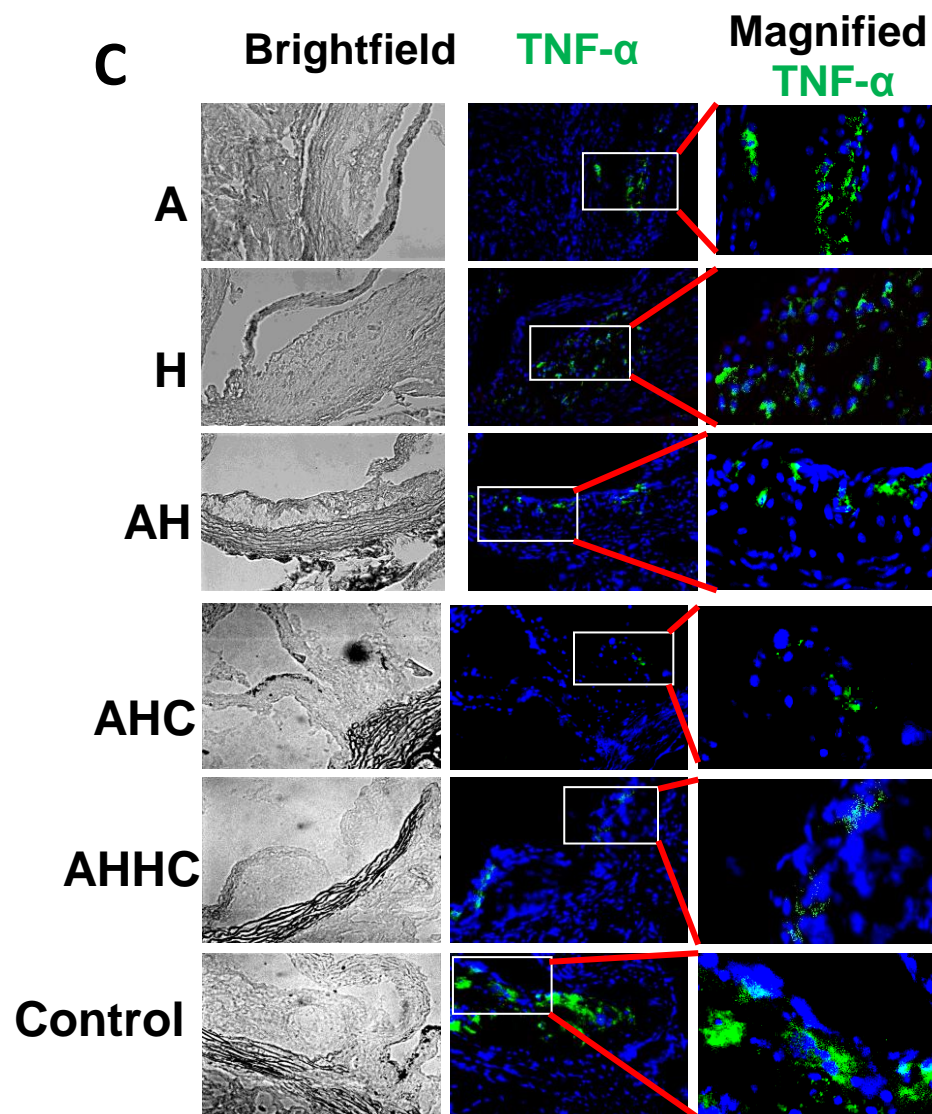
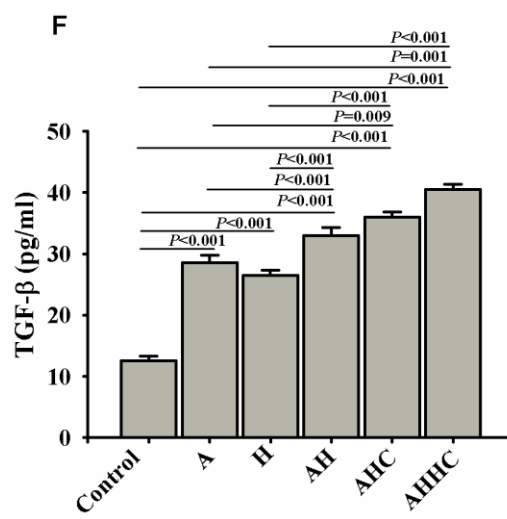
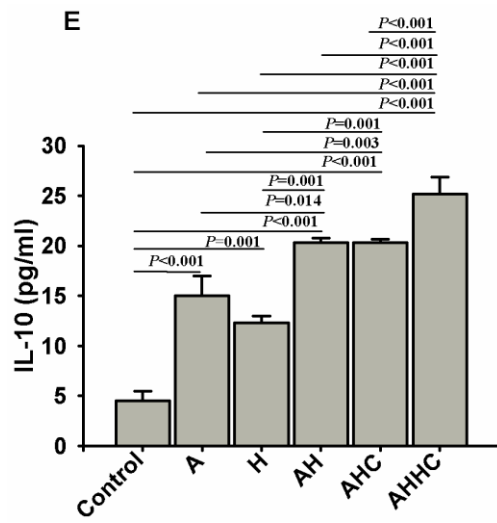
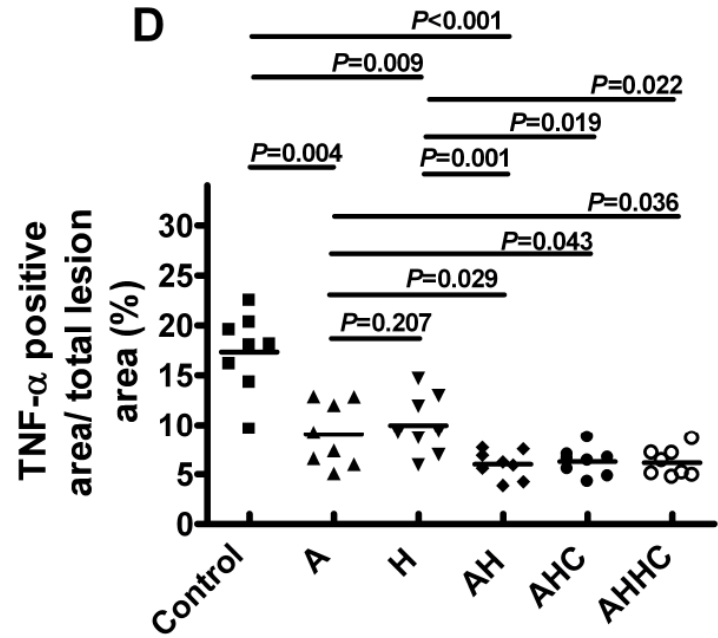
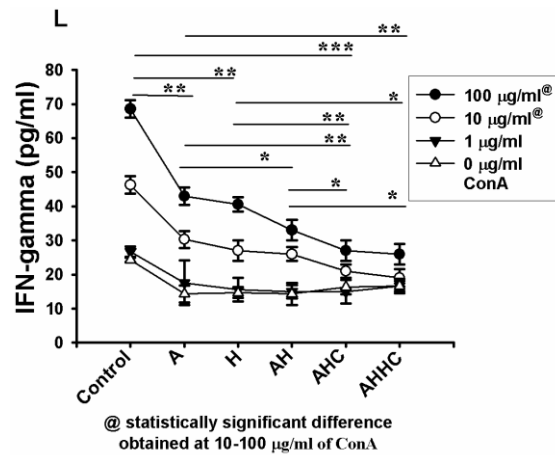
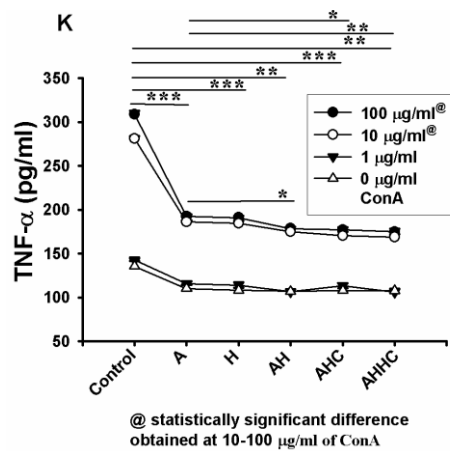
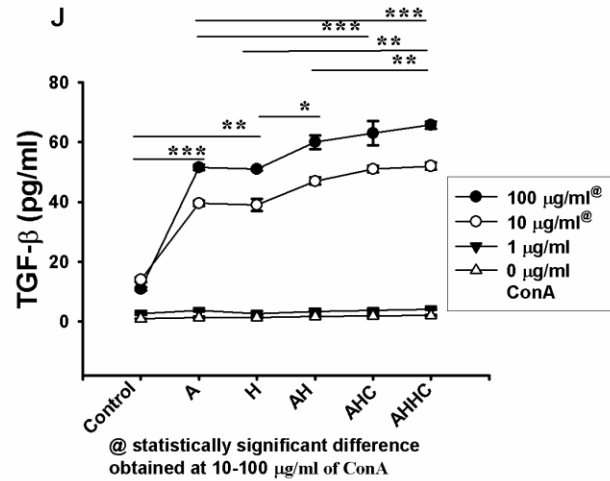
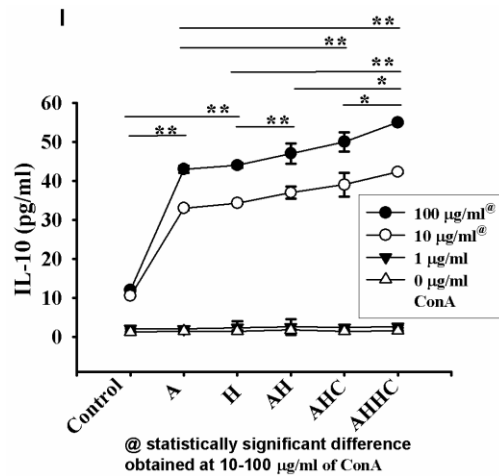
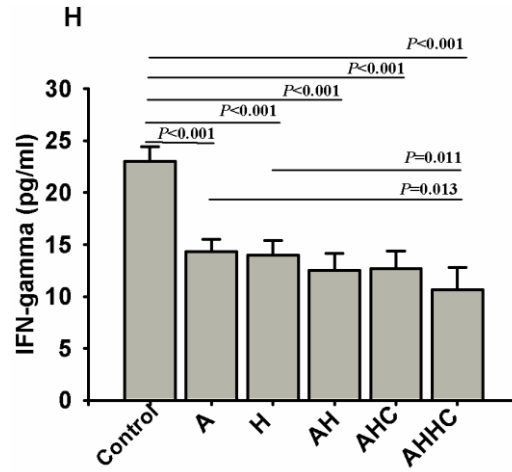
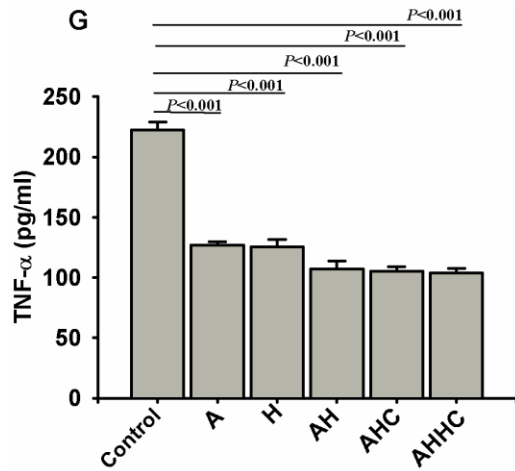


Figure 4









* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 5

