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Immune responses against aldehyde-modified laminin accelerate atherosclerosis in *Apoe*^{-/-} mice

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Short title: Aldehyde-modified laminin immunity accelerates atherosclerosis in *Apoe*^{-/-} mice

Key words; Atherosclerosis, Connective tissue, Autoimmunity, Aldehyde, Acute MI

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Abstract

Background: LDL oxidation in the vascular wall is associated with aldehyde modification of surrounding extracellular matrix proteins that may target autoimmune responses against vascular tissues. Here we investigated the possible influence of immunity against a malondialdehyde (MDA)-modified form of the basement membrane protein laminin on atherosclerosis.

Methods and results: IgM and IgG autoantibodies were present in human plasma and a prospective clinical study demonstrated that individuals who later suffered from acute cardiovascular events had lower levels of MDA-laminin antibodies compared to those in the control group. Immunohistochemical analysis of atherosclerotic plaques from *Apoe*^{-/-} mice demonstrated co-localization between laminin and MDA epitopes, however MDA-laminin IgG was absent in mouse plasma. To determine the effect of MDA-laminin immunity, *Apoe*^{-/-} mice were immunized with MDA-laminin. Analysis of circulating leukocytes at 12 weeks demonstrated increased T-cell activation, expansion of Th17 cells and a lower fraction of regulatory T cells (Tregs) in mice immunized with MDA-laminin. At 25 weeks, aortic atherosclerosis was increased by more than 60% in mice immunized with MDA-laminin, together with increased levels of MDA-laminin IgG1 and MDA-laminin-specific T-cells expressing IL-2, IL-4 and IL-6 in the spleen.

Conclusion: The clinical observations suggest that immune responses against MDA-laminin may be involved in the development of cardiovascular disease in humans. Furthermore, observations in mice provide evidence for the presence of aldehyde-modified laminin in atherosclerotic lesions and demonstrate that induction of an immune

response against these structures is associated with activation of Th17 cells, reduced fraction of Tregs and a more aggressive development of atherosclerosis.

Introduction

Accumulation and subsequent oxidation of LDL in the connective tissue of the arterial wall are considered key events in the development of atherosclerosis. Oxidation of LDL results in a decomposition of fatty acids and release of highly reactive aldehydes, such as malondialdehyde (MDA) and 4-hydroxynonenal ¹. These aldehydes react with lysine and histidine residues in the LDL-associated protein apolipoprotein B-100 resulting in formation of antigens targeted by the immune system ². Both oxidized LDL-specific antibodies and T cells ³ have been shown to be common in humans ^{4,5}. Since LDL binds to extracellular matrix proteins in the arterial wall, like fibronectin, collagen and laminin ⁶, it is likely that reactive aldehydes formed during the oxidation of LDL also may modify these proteins. Immune responses against aldehyde-modified extracellular matrix proteins in the atherosclerotic plaque could potentially have an important influence on the disease process.

We have recently confirmed that LDL oxidation *in vitro* results in MDA modifications of fibronectin and that MDA-modified fibronectin is present in atherosclerotic plaques ⁷. A prospective clinical study showed that antibodies specific for MDA-modified fibronectin were associated with cardiovascular disease in humans ⁷. Subsequent studies demonstrated that immunization of *Apoe*^{-/-} mice with MDA-fibronectin resulted in an

inhibition of atherosclerosis suggesting a protective role of immune responses against the plaque extracellular matrix (Dunér et al., unpublished data). However, the interpretation of these findings was confounded by the fact that immunization also induced immunity against native fibronectin which resulted in a substantial reduction of circulating fibronectin levels.

In the present paper we have investigated the effect of immunity against MDA-modified laminin. Laminin is a major protein of basement membranes and has been shown to have a capacity to bind LDL^{6,8}. Laminin plays an important role in the vascular wall and is required for maintaining normal endothelial and smooth muscle cell function and survival. An immune attack against modified laminin epitopes could therefore have important implications for atherosclerosis.

Materials and methods

Human study population

The study subjects, born between 1926 and 1945, were recruited from the “Malmö Diet and Cancer (MDC)” study cohort as previously described⁴. Participants who had a history of myocardial infarction or stroke prior to enrolment were not eligible for the present study.

The study population consisted of 217 subjects, 75 cases that developed acute coronary heart events, i.e. fatal or non-fatal myocardial infarction or deaths due to coronary heart disease during follow-up and 142 controls matched for age, sex, smoking habits, presence of hypertension, month of participation in the screening examination and duration of follow-

up. The ethical committee of Lund University, Sweden approved the study. Risk factors for cardiovascular disease and carotid intima-media thickness were assessed as previously described ⁹.

Animals

Six-week-old male C57BL/6 *Apoe*^{-/-} mice (Taconic, Denmark) were immunized with 75 µg native or MDA-modified laminin or PBS using Alum (Pierce) as adjuvant, followed by three booster injections at weeks 9, 11 and 24. The mice were fed a high-fat diet, 21% cocoa fat, 0.15% cholesterol, from 10 weeks of age and sacrificed at 25 weeks of age. Blood samples were taken from 12 and 25-week-old mice. Tissue was preserved as described previously ¹⁰. All animal experiments were approved by the Animal Care and Use Committee. Plasma cholesterol was quantified colorimetrically using Infinity Cholesterol (ThermoTrace). Cytokine levels were analyzed in plasma from 12 and 25-week-old with mouse Th1/Th2 9-plex assay (Meso Scale Discovery) according to manufactures protocol.

Statistical analysis

Statistical analyses were performed with GraphPad version 5. Values are presented as mean ± S.D, if not otherwise indicated. ANOVA followed by “Dunn's Multiple Comparison Test” *post hoc* test were used to compare multiple groups. For skewed variables the non-parametric Mann-Whitney-test was used for comparisons of data. In the clinical studies, age and sex adjusted partial correlation coefficients were computed to

assess association between antibody levels, carotid IMT and cardiovascular risk factors. Statistical significance was considered at the level * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$.

Results

To investigate if immune responses against MDA-laminin occur we first screened pooled human plasma for presence of autoantibodies against native and MDA-laminin. Both IgM and IgG autoantibodies recognizing MDA-laminin could be detected, as well as lower amounts of IgG antibodies recognizing native laminin (Fig 1A). The specificity of the assay was demonstrated by competition studies. The binding of IgG autoantibodies to MDA-laminin coated plates was reduced by more than 60% after pre-incubation of plasma on MDA-laminin compared to pre-incubation on MDA-modified collagen IV, BSA, fibronectin or elastin, which only reduced binding by 20-25% (Fig 1B). Binding of IgM autoantibodies to MDA-laminin was reduced by more than 60% after pre-incubation on MDA-laminin compared to <10% by MDA-modified BSA (data not shown). Binding of IgM and IgG antibodies to MDA-laminin were diminished by increasing dilutions of plasma (Fig 1C and D). To investigate if immune responses against MDA-laminin are associated with acute atherosclerotic events, we conducted a prospective clinical study. Individuals participating in the prospective Malmö Diet Cancer Study were recruited to the study. We measured MDA-laminin IgM and IgG antibody levels in baseline plasma samples from 75 subjects that subsequently suffered from an acute myocardial infarction or death due to coronary heart disease (mean time from baseline sample to the cardiac event was 2.8 years) matched with 142 healthy controls. The group that later suffered

from an acute cardiovascular event was showed to have significantly lower IgM and IgG antibody levels against MDA-laminin, compared to the control group (Fig 1E and F).

There were no correlations between MDA-laminin IgM or IgG antibody levels and age or carotid intima-media thickness (data not shown).

In contrast to humans, plasma from 25-week-old *Apoe*^{-/-} mice contained only low levels of native and MDA-laminin IgM and no MDA-laminin IgG (Fig. 2A).

Immunohistochemical staining of aortic root lesions from these *Apoe*^{-/-} mice showed presence of laminin subendothelially, as well as more diffusely throughout the rest of the plaque (Fig 2B and C). Immunostaining for laminin demonstrated co-localization with MDA, and to a minor extent with IgM. Only negligible staining for IgG could be detected in the plaques. Taken together these observations suggest that humans, but not *Apoe*^{-/-} mice, have a T-cell response against MDA-laminin. However, the target for such an immune response appears to be present in *Apoe*^{-/-} mice.

To determine the effect of activating an immune response against MDA-laminin in mice we immunized 6-week-old *Apoe*^{-/-} mice with 75 µg MDA-modified mouse laminin using Alum as adjuvant. Booster injections were given 3 and 5 weeks after the first immunization. A final booster immunization was given at 24 weeks to facilitate mechanistic studies of antigen-specific immune responses against MDA-laminin *in vitro*. High-fat diet was not given until 1 week after the first booster immunization to avoid unnecessary interference with the immune response. Two control groups were used in the present study. One group was given Alum alone to control for the anti-atherogenic effect

of this adjuvant observed in some studies ¹¹. A second control group was immunized with native laminin to control for the importance of the MDA modification. However, it should be kept in mind that results obtained with such a control needs to be interpreted with some caution since aldehyde modification of proteins may take place at the site of injection ¹².

The effect of immunization on systemic inflammation and immune activation was first assessed in blood 1 week after the second booster injection. As compared with the Alum group, immunization with both native and MDA-laminin were associated with increased CD3⁺ T-cell expression of IL-17, whereas expression of the regulatory markers IL-10 and FoxP3 were decreased (Fig 3). There was also an increased expression of the activation marker CD28 on CD4⁺ T cells. Immunization with native and MDA-laminin did not affect the plasma cytokine levels at 12 weeks (Table 1, online). Collectively, these observations suggest that immunization with both native and MDA-laminin activated a Th17 response, together with a reduction in the fraction of regulatory T cells, but did not induce systemic inflammation.

At 25 weeks the aortic area covered by atherosclerosis was increased by over 60% in mice immunized with MDA-laminin as compared with the Alum control ($1.17\% \pm 0.65\%$ versus $0.73 \pm 0.40\%$, $P < 0.05$; Fig 4A). The cross-sectional plaque area of aortic root lesions was also significantly increased in mice immunized with MDA-laminin compared with Alum control ($360.3 \pm 99.3 \mu\text{m}^2$ versus $276.2 \pm 64.8 \mu\text{m}^2$, $P < 0.05$, Fig 4B). Similar trends were observed in mice immunized with native laminin, but the difference against

the Alum control group was not statistically significant. Immunization with MDA-laminin did not influence general plaque inflammation as assessed by the percent of the plaque area demonstrating staining for the macrophage marker MOMA-2 (Fig 4C).

The increased atherosclerosis in mice immunized with MDA-laminin was associated with increased levels of MDA-laminin specific IgG and a moderate increase in IgG against native laminin (Fig 4). Mice immunized with native laminin had low levels of IgG against both native and MDA-laminin. Low levels of laminin and MDA-laminin IgM were present in Alum control mice and the level of these did not change in response to immunization. Analysis of IgG subtypes demonstrated that the antibodies generated by immunization with MDA-laminin were primarily of Th2 (IgG1) isotype (Fig 4E). Immunization with native or MDA-modified laminin was not associated with any changes of plasma cholesterol or weight compared to Alum control mice (Table 2, online), but mice immunized with MDA-laminin had lower levels of IL-2 in plasma (Table 3, online).

To further characterize the cellular immune response to the immunizations we isolated splenocytes from immunized mice and cultured these in the presence MDA-laminin. Splenocytes isolated from mice immunized with MDA-laminin responded with increased cell proliferation, whereas no such response was observed when splenocytes from the other two groups were exposed to MDA-laminin (Fig 5). All groups showed the same response to the polyclonal mitogen Concanavalin A (Con A) demonstrating the antigen specificity of the proliferative response to MDA-laminin. Splenocytes isolated from mice

immunized with native laminin did not respond with increasing proliferation when stimulated with native laminin (data not shown). Splenocytes from MDA-laminin immunized mice responded with an increased secretion of IL-2, IL-4 and IL-6 when exposed to MDA-laminin while there were no difference in the expression of interferon- γ and IL-10.

To determine the functional effect of MDA modification on cellular interactions with laminin we conducted *in vitro* experiments on leukocyte translocation and endothelial cell function. Leukocytes were allowed to migrate through a transwell chamber, coated with native or MDA-modified laminin and translocated cells were counted. There were no differences in migration after MDA modification, as detected by this migration model. Endothelial cells were used to study the effect of MDA modification of laminin on adherence, proliferation and apoptosis status. No significant change in endothelial cell function after MDA-modification of laminin could be detected (data not shown).

Discussion

The present findings demonstrate that immune responses against aldehyde-modified laminin are associated with cardiovascular disease. In a prospective clinical study we demonstrate that humans who later suffered from acute cardiovascular events had lower levels of antibodies against MDA-laminin than those in the control group. These findings are in line with previous studies, demonstrating an association between high levels of autoantibodies directed against Apo B peptides or aldehyde-modified fibronectin and a

lower risk to develop acute cardiovascular events ^{7, 13, 14}. Although clinical association studies need to be interpreted with due caution when it comes to mechanisms these results appears to support a protective role of autoantibodies against modified self-antigens such as MDA-laminin.

In contrast to the findings in humans, our results show that mice normally lack MDA-laminin IgG even in the presence of hypercholesterolemia. However, they also show that induction of immune responses against aldehyde-modified laminin in these mice results in a more aggressive development of atherosclerosis. Although there is evidence for the presence of aldehyde-modified laminin in atherosclerotic lesions of *Apoe*^{-/-} mice, it is not likely that autoimmune responses against these structures normally contribute to disease progression, because control *Apoe*^{-/-} mice lacked both MDA-laminin specific IgG and MDA-laminin reactive T cells.

It has previously been shown that modifications of the vascular extracellular matrix occur with age and that they are more common in diabetes and atherosclerosis ¹⁵. These modifications include covalent binding of aldehydes generated by oxidation of lipids and advanced glycosylation end-products (AGE) produced in response to hyperglycemia. Autoantibodies against MDA- and AGE-modified LDL are common in man ^{5, 16}, but their associations with cardiovascular disease remain controversial. Immunization of experimental animals with MDA-LDL or MDA-apolipoprotein B peptides has been shown to reduce atherosclerosis demonstrating that autoimmunity against aldehyde-modified LDL at least under certain conditions may be atheroprotective ^{17, 18}. One

possible explanation for this could be that immune responses against MDA-LDL epitopes may help to clear oxidized LDL from the circulation. Evidence in support of this notion has come from studies using recombinant oxidized LDL-specific IgG ¹⁹. It is likely that immune responses against modified extracellular matrix proteins in the vascular wall could be more detrimental because such antigens would be more difficult to clear and their removal would depend on inflammation-dependent matrix degradation. An immune attack against laminin may have particularly important effects since laminin-containing basement membranes have an important role in regulating endothelial cell and smooth muscle cell function and survival. The present study provides the first evidence that an immune attack against modified extracellular matrix proteins may accelerate atherosclerosis in mice.

As far as we have been able to find, there is only one previous report describing autoimmune responses against aldehyde-modified extracellular matrix proteins and their association with cardiovascular disease ⁷. In that study high levels of autoantibodies against MDA-fibronectin were associated with a lower risk for development of acute myocardial infarction. Subsequent studies in *ApoE*^{-/-} mice showed that immunization with MDA-fibronectin markedly reduced atherosclerosis supporting the athero-protective role of MDA-fibronectin immunity suggested by clinical studies (Dunér et al., unpublished data). It remains to be fully understood why immune responses against MDA-fibronectin in mice can be athero-protective while immunity against MDA-laminin has the opposite effect. However, it is interesting to note that the immune responses evoked by MDA-fibronectin and MDA-laminin differed in some important aspects. While both proteins

induced robust Th2-dependent antibody responses immunization with MDA-fibronectin resulted in activation of Tregs, whereas immunization with MDA-laminin instead down-regulated Tregs. This could imply that *Apoe*^{-/-} mice have a good ability to maintain tolerance for MDA-fibronectin but that this ability is weaker for MDA-laminin. Tregs are immunosuppressive cells that play an important role in maintaining self-tolerance and protection against autoimmunity. A functional role for Tregs in atherosclerosis is supported by finding that depletion of Tregs aggravates atherosclerosis, whereas Treg transfer has the opposite effect^{20, 21}. Accordingly, it is possible that immune responses against aldehyde-modified extracellular matrix proteins contribute to atherosclerosis only if they fail to be controlled by a compensatory Treg response. In the present study immunization with MDA-modified laminin also resulted in an increase in Th17 positive T cells, whereas we did not observe activation of IFN- γ producing Th1 cells. Th17 cells have pro-inflammatory effects and have been linked to the development of several chronic inflammatory diseases including atherosclerosis²²⁻²⁴. These findings suggest that the pro-atherogenic effect of MDA-laminin immunization is mediated by Th17 rather than by Th1 cells. Recent data have suggested that a shift in the Th17/Treg balance may play a key role in controlling inflammation, plaque destabilization, and the onset of acute coronary syndrome (ACS) in humans²². Th17 cell numbers, as well as Th17 related cytokines levels (IL-17, IL-6, and IL-23), were shown to be significantly higher in patients with ACS as compared to controls. Moreover, these patients had decreased numbers of regulatory T-cells indicating that a Th17/Treg imbalance exists in patients with ACS. Our results suggest that Th17 / Treg imbalance might influence also the progression of atherosclerosis. However, it should be kept in mind that the role of Th17

cells in atherosclerosis remains to be fully established and that recent studies also have provided evidence for an atheroprotective effect of Th17 cells ²⁵.

Another characteristic of MDA-laminin immunized mice was an increased expression of IL-2 in splenocytes exposed to MDA-laminin. Treatment with IL-2 has previously been shown to increase atherosclerosis whereas anti-IL-2 antibodies had an anti-atherosclerotic effect in *Apoe*^{-/-} mice ²⁶ suggesting that the increased IL-2 expression may have contributed to the atherogenic effect of MDA-laminin immunization. A strong IL-6 response was also seen when spleen cells isolated from MDA-modified laminin immunized mice were challenged with antigen in vitro. The direct role of IL-6 effects in atherosclerosis is not clear. IL-6 levels has been shown to correlate with lesion area as measured in aorta ²⁷, but *Apoe*^{-/-}*IL-6*^{-/-} mice had enhanced atherosclerotic plaque formation ²⁸. IL-6 and TGFβ are crucial for the differentiation of naïve T cells into Th17 cells at the same time as they are inhibiting the generation of Tregs ²⁹.

There is an apparent contradiction between the findings that high levels of autoantibodies in man is associated with a lower risk for development of acute cardiovascular events and the finding in hypercholesterolemic mice that immunization with MDA-laminin results in an aggravation of atherosclerosis. However, in view of the complex role of the immune system in atherosclerosis these inconsistencies are not entirely unexpected. Accumulating evidence suggest that both protective and disease-promoting immune responses against modified self-antigens exist and may function in parallel in atherosclerosis ³⁰ and that the rate progression of disease is dependent on the balance between these immune responses.

There are some limitations to the present study that needs to be considered. First, we were only able to provide indirect evidence for the presence of MDA-laminin in atherosclerotic lesions of *Apoe*^{-/-} mice based on co-localization of laminin and MDA epitopes. Attempts to generate MDA-laminin specific antibodies were not successful as these antibodies reacted also with epitopes in native laminin. However, it has previously been shown that LDL oxidation is associated with aldehyde modification of surrounding extracellular matrix proteins ⁷ and that oxidized LDL is a major constituent of atherosclerotic lesions in these mice suggesting that all prerequisites for the generation of MDA-laminin are in place. Second, control mice immunized with native laminin also showed a trend towards increased atherosclerosis. Mice immunized with native laminin also showed a modest increase in antibodies against both native and MDA-laminin, increased activation of T cells and a decrease in circulating Tregs. Previous studies have shown that antigens co-injected with Alum are susceptible to aldehyde modification at the injection site ¹². Accordingly, it is possible that the atherogenic effect of laminin immunization is explained by a MDA modification of laminin subsequent to the injection. The generation of antibodies against MDA-laminin in mice immunized with native laminin is in line with such a possibility. Another possibility is that immunization with laminin indeed results in an immune response against native laminin and that this has the same atherogenic effect as immune responses against MDA-laminin. However, this seems less likely since only a minor antibody response against native laminin was present and splenocytes from laminin immunized mice did not respond with increasing proliferation when stimulated with native laminin. Finally, cholesterol levels tended to be

somewhat higher in the immunized groups compared to the Alum control. However, even if there were no significant differences in cholesterol between any of the groups it can not be completely excluded that our results in parts were influenced by effects on cholesterol metabolism.

In conclusion, the present observations demonstrate that high MDA-laminin IgM and IgG antibody levels are associated with a lower risk to develop acute cardiovascular events in humans. Moreover, the study in *ApoE*^{-/-} mice provide evidence for the presence of aldehyde-modified laminin in atherosclerotic lesions in mice and demonstrate that activation of an immune response against such structures is associated with induction of Th17 cells, decreased fraction of Tregs and a more aggressive development of atherosclerosis. Taken together, these observations provide support for a role of immune responses against modified extracellular matrix proteins, such as MDA-laminin, in atherosclerosis and suggest that this role could be complex involving both protective and atherogenic immunity.

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Conflict of interest statement

No conflict of interest was declared.

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Figure legends

Figure 1: Presence of immune responses against MDA-laminin in plasma and their association with risk of future cardiovascular events in man.

(A) Human plasma pooled from 50 healthy individuals contains IgM and IgG autoantibodies recognizing native and MDA-laminin (the assay was repeated 10 times). (B) The specificity of IgG autoantibodies to MDA-laminin was tested with overnight incubation with different MDA-modified ECM proteins. (C and D) IgM (C) and IgG (D) titers to native and MDA-modified laminin in increasing dilutions of plasma pooled from healthy blood donors (n=50). (E and F) Plasma from 75 cases of individuals, who developed acute myocardial infarction during a 5-year follow-up and 142 matched controls were analyzed for the presence of IgM (E) and IgG (F) autoantibodies specific to MDA-laminin. Data represents mean \pm SEM.

Figure 2: Presence of immune responses against MDA-laminin in mice.

(A) Plasma from 25-week-old mice contains low level of IgM and no IgG autoantibodies recognizing native or MDA-laminin. (B and C) Immunohistochemical staining against laminin, MDA, IgG and IgM in aortic root lesions of *Apoe*^{-/-} mice.

Figure 3: Immunization with MDA-modified laminin induces pro-inflammatory T cells and results in less regulatory T cells.

(A) Flow cytometry analyses of blood lymphocytes in 12-week-old mice show significantly increased levels pro-inflammatory Th17 cells. The increase in inflammatory T cells was accompanied by a significant decrease in the regulatory marker (B) IL-10 and (C) FoxP3 on T cells. (D) There was an increased expression of the CD28 activation marker on CD4⁺ cells.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Figure 4: Immunization of *Apoe*^{-/-} mice with MDA-modified laminin accelerates atherosclerosis and results in a Th2-type antibody response.

(A) En face preparations of the descending aorta of 25-week-old immunized mice were stained with Oil Red O and the percent stained area of total area determined by image analysis. (B) Total aortic root lesions area and (C) monocytes/macrophages stained area in percent of total area plaque area from cross-sections were determined by image analysis. (D) Plasma from 25-week-old mice immunized with Alum, native laminin and MDA-modified laminin were tested for IgG and IgM antibody titers against native or MDA-modified laminin by ELISA. (E) The IgG isotype of the antibody response was assayed by ELISA. Plasma from individual mice was tested for IgG1 and IgG2a titers against native and MDA-modified laminin by ELISA. * $P < 0.05$

Figure 5: Immunization of *Apoe*^{-/-} mice with MDA-modified laminin induces antigen-specific T-cell proliferation and cytokine production.

Proliferation index from mice immunized with Alum, native laminin or MDA-modified laminin, simulated with (A) 30 $\mu\text{g/mL}$ MDA-modified laminin or (B) Con A. (C) IL-2 cytokine release from splenocytes stimulated with MDA-modified laminin. (D-G) IL-6, IL-4, IL-10 and IFN- γ release from splenocytes stimulated with MDA-modified laminin, Con A or PBS. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

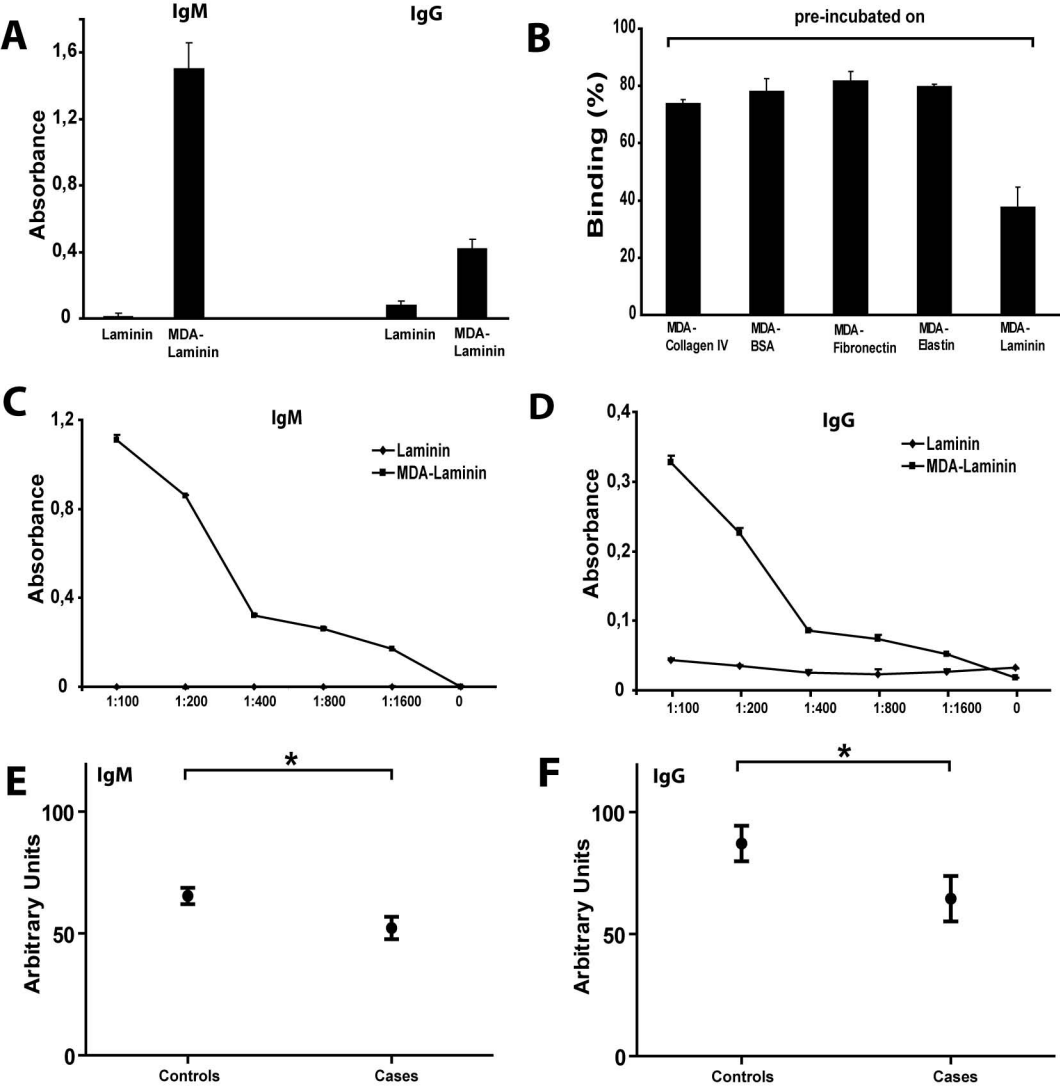


Figure 1

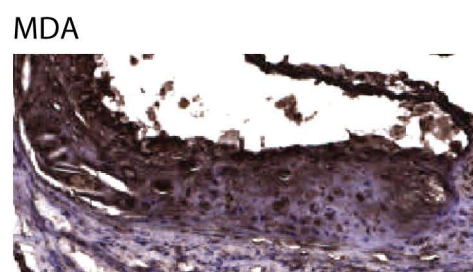
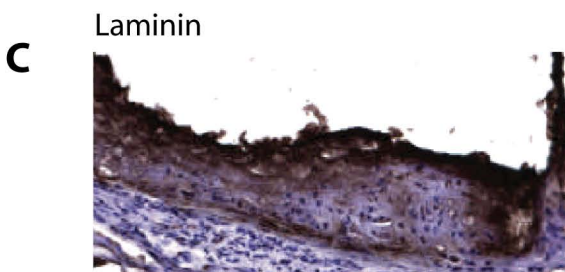
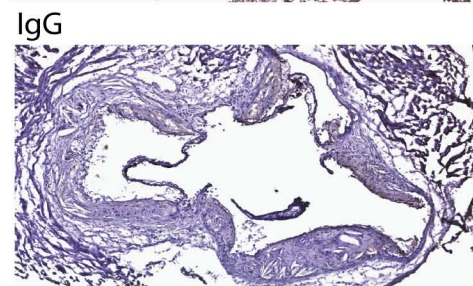
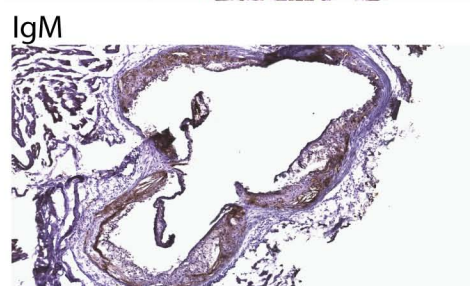
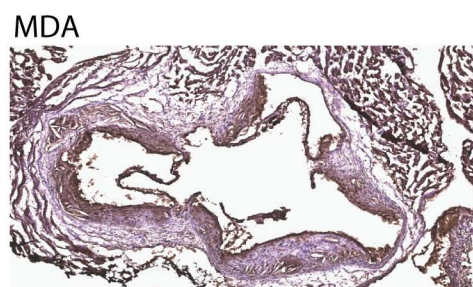
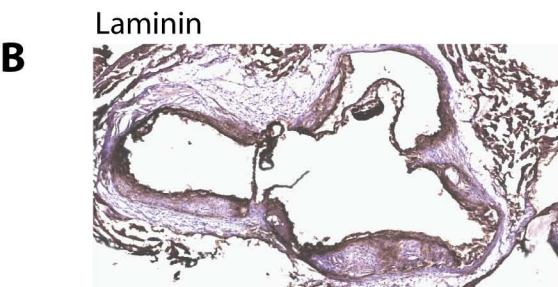
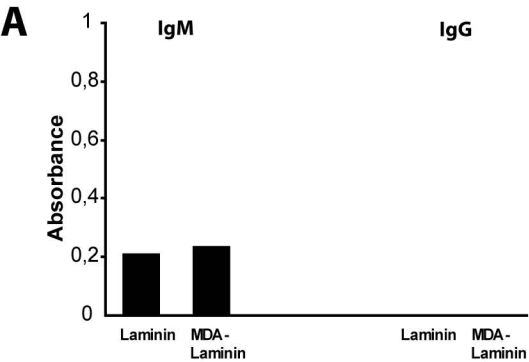


Figure 2

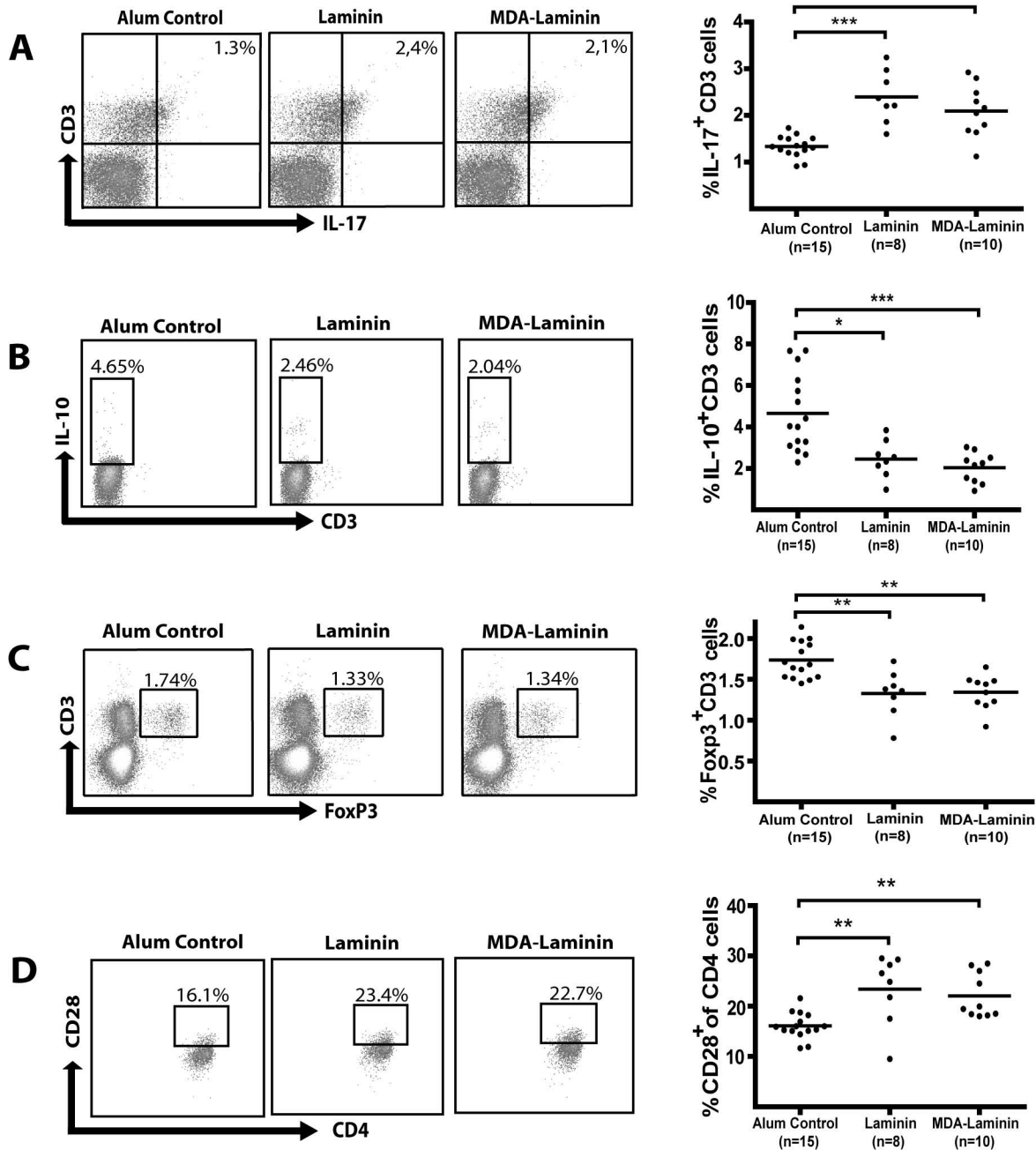


Figure 3

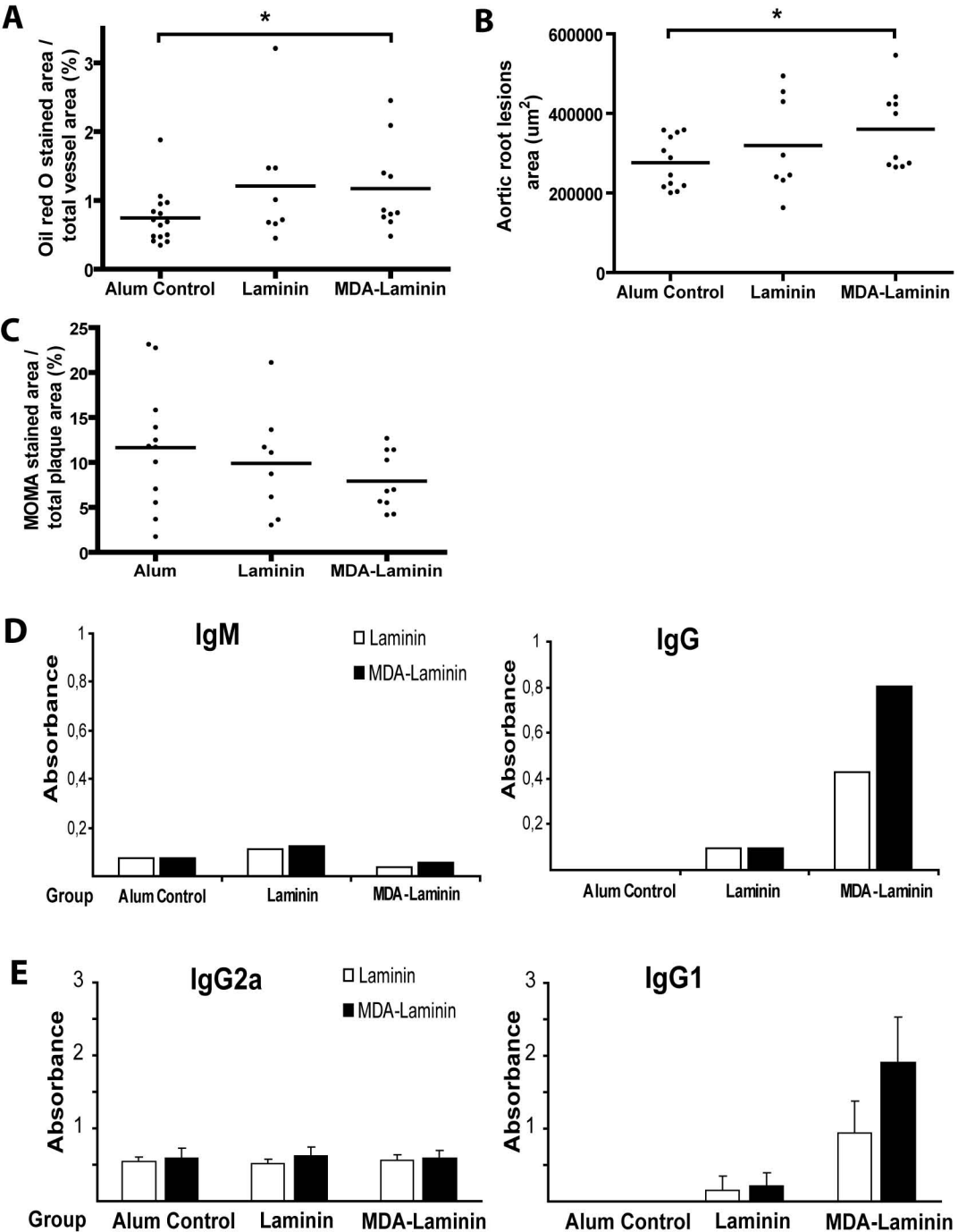


Figure 4

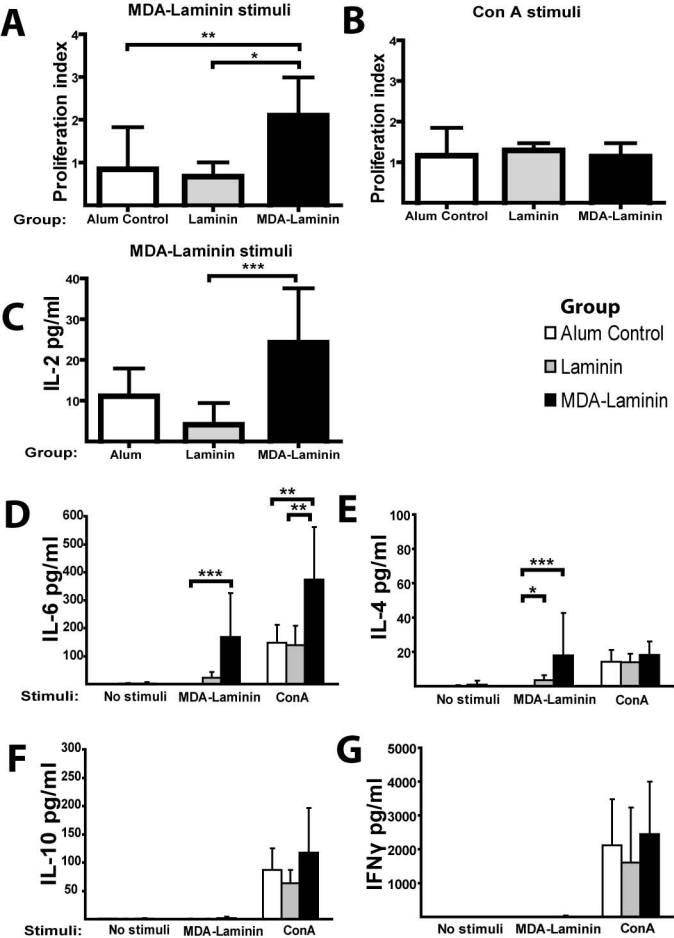


Figure 5