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Abstract

Aims. Oxidation of LDL in the extracellular matrix of the arterial wall results in formation of malondialdehyde (MDA) that modifies surrounding matrix proteins. This is associated with activation of an immune response against modified extracellular matrix proteins present in atherosclerotic plaques. Clinical studies have revealed an inverse association between antibodies to MDA-modified fibronectin and risk for development of cardiovascular events. To determine the functional role of these immune responses in atherosclerosis we performed studies in which apoE-deficient mice were immunized with MDA-modified fibronectin.

Methods and Results. Immunization of apoE-deficient mice with MDA-modified fibronectin resulted in a 70% decrease in plaque area and a less inflammatory phenotype of remaining plaques. Immunization shifted a weak naturally occurring Th1 antibody response against MDA-fibronectin into a Th2 antibody response. Cytokine expression and flow cytometry analyses of spleen cells from immunized mice showed an activation of regulatory T cells. Immunization with MDA-fibronectin was also found to reduce plasma fibronectin levels.

Conclusions. Immunization with MDA-fibronectin significantly reduces the development of atherosclerosis in apoE-deficient mice suggesting that the immune response observed in humans may have a protective effect. MDA-fibronectin represents a possible novel target for immunomodulatory therapy in atherosclerosis.

Keywords: atherosclerosis, immunization, apoE-/- mice, MDA-fibronectin

Introduction

Retention of LDL by the extracellular matrix in the arterial wall and subsequent LDL oxidation are key steps in atherosclerotic disease. The retention of LDL involves interaction of apo B-100 protein in LDL with extracellular matrix proteoglycans. Mice expressing LDL-binding-defective proteoglycan develop less atherosclerosis [1, 2]. In addition, other extracellular matrix proteins such as collagen, laminin and fibronectin have been shown to bind lipoproteins [3, 4]. LDL oxidation is associated with decomposition of fatty acids resulting in formation of reactive aldehydes, such as malondialdehyde (MDA) and 4-hydroxynonenal, binding covalently to amino groups in the apo B-100 protein. These oxidized LDL neoantigens are targeted by the immune system and epidemiological studies have demonstrated associations between autoantibodies against oxidized LDL antigens and cardiovascular disease [5-7].

A possibility that has been largely unexplored is that reactive aldehydes released during LDL oxidation results in MDA-modifications also of the surrounding extracellular matrix proteins. These modified matrix proteins could potentially target immune responses against the extracellular matrix of the plaque. Fibronectin is an extracellular matrix protein, which is up-regulated in atherosclerotic lesions [8] and, interestingly, deposited at atherosclerosis-prone sites before others signs of atherosclerosis in mice [9]. Using an *in vitro* model, we have recently found that LDL oxidation results in MDA-modifications of fibronectin and that MDA-modified fibronectin is present in atherosclerotic lesions [10]. In addition, antibodies against MDA-modified fibronectin were associated with cardiovascular disease in a prospective case-control study. However, in contrast with our expectations baseline autoantibody levels were found to be

significantly lower in subjects that later suffered a fatal or non-fatal myocardial infarction than in controls suggesting that these autoimmune responses have a protective effect. To determine the functional role of these antibodies in atherosclerosis we immunized apo E-deficient mice with MDA-modified fibronectin.

Materials and methods

Materials

Rat fibronectin was purchased from Biomedical Technologies Inc, and mouse fibronectin from Innovative Research. Recombinant protein fragments spanning fibronectin's type III domains 1-5, 4-7, 7-10, 10-12, 12-14 and 13-15 were expressed and purified as previously described [11]. 1,1,3,3-tetraethoxy propane (Sigma) was used for chemical MDA modification.

Animals

Six-week old male C57BL/6 apoE$^{-/-}$ mice (Taconic, Denmark) were immunized with 85 µg rat MDA-modified or native fibronectin (97% identity to mouse fibronectin) or PBS using Alum (Pierce) as adjuvant, followed by two booster injections after 3 and 5 weeks. The mice were fed a high-fat diet, 21% cocoa fat, 0.15% cholesterol (AnalyCen Nordic), from 10 weeks of age and sacrificed at 12 or 26 weeks of age. One control group followed the diet program without getting injections. Tissue was preserved as described previously [12]. All animal experiments were approved by the Malmö-Lund Animal Care and Use Committee and the investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication No. 85-23, revised 1996). Plasma cholesterol was quantified colorimetrically using Infinity Cholesterol (ThermoTrace).

Immunohistochemistry

Preparation, staining and quantifications in the descending aorta, or in subvalvular plaques were performed as previously described [12]. Briefly, lipids were stained in *en face* preparations of the descending aorta with Oil Red O. Subvalvular plaques were stained with MOMA-2 antibodies

(BMA Biomedicals, Switzerland) recognizing monocytes/macrophages, with affinity purified biotinylated anti-mouse IgG (Vector Laboratories), with neutrophil antibody NIMP-R14 (abcam, 2557), with polyclonal rabbit anti-murine fibronectin antibodies (abcam, ab23750) or with IgG against MDA-fibronectin produced in rabbit [10], using rabbit immunoglobulins (DAKO) as negative control and counterstained with haematoxylin. The specificity of rabbit anti-MDA fibronectin for MDA-mouse fibronectin, but not native mouse fibronectin was verified by ELISA. Polyclonal anti-murine fibronectin antibodies recognized both native and MDA-modified fibronectin.

Splenocyte proliferation and cytokine production

Mice immunized with MDA-fibronectin with Alum, native fibronectin with Alum, PBS with Alum or left non-treated as described above were sacrificed at 12 weeks of age and spleens were harvested. Splenocytes were collected by mechanical force through a 70 μm cell strainer (BD Falcon) and erythrocytes were removed using red blood cell lysing buffer (Sigma). The cells were cultured in culture media (RPMI 1640 media containing 10% heat-inactivated fetal calf serum, 1 mmol L^{-1} sodium pyruvate, 10 mmol L^{-1} Hepes, 50 U penicillin, 50 μg mL^{-1} streptomycin, 0.05 mmol L^{-1} mercaptoethanol and 2 mmol L^{-1} L-glutamine, GIBCO) in 96-well round bottom suspension cell plates (Sarstedt). Splenocytes were cultured with Concanavalin A (Con A, Sigma), 30 μg mL^{-1} rat MDA-fibronectin, 30 μg mL^{-1} rat native fibronectin or with medium alone. CD28 monoclonal antibody (17 μg mL^{-1}) (BD Pharmingen) was added to wells with unstimulated splenocytes and cells stimulated with native or MDA-fibronectin in the cytokine assays. Splenocytes were incubated during 72 h after which cytokine assay plates were terminated and proliferation plates were pulsed with 1μCi [methyl-^{3}H] thymidine (Amershamn)

during further 20 h. Cells were then harvested (Packard) onto glass fiber filter (Wallac) and thymidine incorporation was measured using a liquid scintillation counter (Wallac). The cytokine release from cultured splenocytes were analyzed using a TGF-β ELISA kit (R&D Systems) or a multiplex kit measuring IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12, TNFα, IFNγ and KC (Meso Scale Discovery, USA). IL-1β values in measured samples were below the detection limit.

ELISA

Proteins were either MDA-modified on the microtiter plate or in solution using 0.05 mol L^{-1} MDA in PBS pH 7.4. MDA modifications were assayed using thiobarbituric acid reactive substances assay (TBARS) [7]. ELISA for measuring antibody response and determining immunoglobulin isotype in immunized mice was performed using microtiter plates coated with 10 μg mL^{-1} native or MDA-modified mouse fibronectin, and plasma pooled from five or eight mice in each group or from individual mice was added. Bound antibodies were detected by biotinylated anti-mouse IgG or IgM produced in goat (Jackson ImmunoResearch), followed by alkaline phosphatase-conjugated streptavidin, or with alkaline phosphatase-conjugated anti mouse-IgG1 produced in rat (BD Pharmingen), or alkaline phosphatase-conjugated IgG2a produced in rat (BD Pharmingen). Observations that were 1.5 times the inter-quartile range beyond the appropriate quartile were regarded as outliers and excluded form the analysis. Fibronectin in plasma from mice was analyzed with AssayMax mouse fibronectin ELISA kit (DayMoon Industries Inc.).

Statistical analysis

Statistical analyses were performed with SPSS version 12.0.1. Values are presented as mean±s.d. or as individual values. We used ANOVA or Kruskal-Wallis followed by *post hoc* test to compare multiple groups. For skewed variables the non-parametric Mann-Whitney-test was used for comparisons of data.

Additional methods are presented in online Data Supplement Methods.

Results

Immunization of apoE$^{-/-}$ mice with MDA-fibronectin protects against atherosclerosis

To study the functional role of antibodies against MDA-fibronectin in atherosclerosis we immunized 6 week old apo E$^{-/-}$ mice with 85 μg rat MDA-modified fibronectin using Alum as adjuvant. Mice immunized with native fibronectin, adjuvant alone and non-immunized were included as controls. Booster immunizations were given 3 and 5 weeks later. The mice were sacrificed at 26 weeks of age and the severity of atherosclerosis was assessed by *en face* Oil Red O staining of the aorta and measurement of plaque area of subvalvular lesions. Immunization with MDA-fibronectin was found to reduce atherosclerosis in the aorta by 60% (1.0%±0.6% lesion area of total vessel area) as compared with animals injected with adjuvant alone (2.6%±2.1%) and by 69% as compared with mice given no treatment (3.3%±1.2 %) *(Figure 1A)*. The plaque area of subvalvular lesions was decreased by 27% (2.6±0.6x10^5 μm^2) in MDA-fibronectin immunized mice as compared with the adjuvant control (3.6±1.1x10^5 μm^2) *(Figure 1B)* and mean plaque area demonstrating positive immunoreactivity for macrophages was reduced from 8.2±2.8x10^4 μm^2 in the adjuvant control mice to 6.0±1.4x10^4 μm^2 (mean±s.d. P<0.05) in immunized mice *(Figure 1D).* In contrast, there was a trend towards increased T-cell accumulation (P=0.06) in the lesions of MDA-fibronectin immunized mice *(Figure 1C)*. Mice immunized with native fibronectin also had reduced atherosclerosis in aorta (0.9%±0.5%) and decreased subvalvular lesions (2.0±0.7x10^4 μm^2) compared to adjuvant treated or non-immunized mice *(Figure 1A, B)*. Moreover, macrophage immunoreactivity was reduced in plaques of native fibronectin immunized mice (4.7±1.0x10^4 μm^2, P<0.05) *(Figure 1D)* and there was also a trend towards increased T-cell accumulation *(Figure 1C)*. There was no significant difference in neutrophil content between the groups (online Data Supplement Figure S1).

Histological examinations did not reveal any vascular alterations outside atherosclerotic lesions in response to immunization with native or MDA-fibronectin.

The observation that immunization with native fibronectin inhibited atherosclerosis was unexpected. However, since it has been shown that aluminum potently induces oxidative stress and lipid peroxidation [13-17], we explored the possibility that immunization with fibronectin together with Alum as adjuvant would result in MDA-modifications of the injected native fibronectin. Therefore mice were immunized as above and tissue from the injection site was homogenized and analyzed by fluorescence measurements of aldehyde groups *(Figure 2)*. The analysis revealed presence of MDA-modified protein at the injection site of mice immunized with native fibronectin. Accordingly, it is likely that immunization with native fibronectin mimics that of immunization with MDA-fibronectin and it is even possible that this immune response more resembles that induced by MDA-modification of fibronectin in vivo.

Immunization of apoE$^{-/-}$ mice results in a Th2-type antibody response

Immunization of apoE$^{-/-}$ mice with either MDA-modified or native fibronectin resulted in high levels of IgG binding MDA-fibronectin *(Figure 3A)*. ELISA studies demonstrated that IgG antibodies binding to MDA-fibronectin could be competed by addition of MDA-fibronectin (data not shown). The antibodies were almost exclusively of the IgG1 isotype reflecting activation of a Th2-type immune response *(Figure 3B)*. Non-treated mice or mice treated with adjuvant alone had only very low levels of IgG against MDA-fibronectin and these autoantibodies were primarily of the IgG2a isotype indicative of a weak, naturally occurring Th1 response against MDA-fibronectin *(Figure 3B)*. To study whether the antibodies were recognizing site-specific

epitopes in the fibronectin protein, we tested the antibody reactivity to various MDA-modified fragments of fibronectin, which comprise the fibronectin type III domain containing part of the protein *(Figure 3C)*. The results demonstrated that the antibody response was not restricted to a single site but occurred at multiple sites along the entire sequence of the protein.

To analyze if circulating antibodies bind to MDA-fibronectin antigen in the vessel wall, we stained subvalvular lesions from MDA-fibronectin immunized mice with anti-IgG and anti-MDA-fibronectin. Although antibodies recognizing IgG showed a more widespread distribution than anti-MDA-fibronectin, the result clearly shows colocalization of MDA-fibronectin and IgG, indicating that antibodies from the plasma bind MDA-fibronectin (online Data Supplement Figure S2).

Immunized mice were also found to develop antibodies recognizing native fibronectin as assessed by ELISA. To determine if these antibodies cross-reacted with native fibronectin also in vivo we analyzed the effect of immunization on plasma fibronectin levels. Indeed, circulating fibronectin levels in 26-week old mice were found to be significantly reduced in both mice immunized with MDA-modified (640±90 μg mL^{-1}) and native fibronectin (630±160 μg mL^{-1}) as compared with adjuvant (840±180 μg mL^{-1}) or non-immunized mice (920±120 μg mL^{-1}) *(Figure 4)*. The decrease in circulating fibronectin suggests the possibility that the protective effect of immunization could be due to cross-reactivity of antibodies with the plasma protein resulting in reduced accumulation of fibronectin in plaque tissue. However, immunohistochemical analysis did not demonstrate any significant difference in native or MDA-modified fibronectin in plaques (online Data Supplement Figure S3). We also analyzed subvalvular sections from 12 week old

mice, but found only occasional and small lesions. However, in such lesions, MDA-fibronectin immuno-reactivity was frequently present (data not shown).

Immunization of apoE$^{-/-}$ mice results in a regulatory T-cell response

To determine if the atheroprotective effect of MDA-fibronectin immunization was associated with changes in antigen-specific cellular immune responses we isolated splenocytes from 12 week old non-immunized, PBS/adjuvant, native or MDA-fibronectin immunized mice. Splenocytes from MDA-fibronectin immunized mice were characterized by an increased proliferation in response to stimulation with MDA-fibronectin or native fibronectin, the latter indicating that cellular tolerance to the native protein was broken *(Figure 5A)*. Exposure of spleen cells from MDA-fibronectin immunized mice to the polyclonal activator Concanavalin A (Con A) resulted in lower proliferation as compared to splenocytes isolated from non-immunized mice or mice given adjuvant alone *(Figure 5A)*. To characterize the presence of regulatory T cells in immunized mice, we measured FoxP3, CD4, CD25 positive cells by flow cytometry *(Figure 5B)*. Increased regulatory T cells were seen in both MDA-modified and native fibronectin immunized mice. The relative presence of these cells was not further increased by stimulation with MDA-modified protein (data not shown). To analyze if there was an increase in cytokines associated to regulatory T cells, we measured TGF-β and IL-10 secretion from cultured splenocytes of immunized mice. An increased secretion of TGF-β was observed in cultured splenocytes from both Alum (501±70 pg/500,000 cells) and MDA-fibronectin immunized mice (520±82 pg/500,000 cells) compared to non-immunized mice (286 ±53 pg/500,000 cells) *($p<0.0001$)*. The secretion of TGF-β was not further enhanced by exposing the cells to MDA-fibronectin (data not shown). Splenocytes from both native and MDA-fibronectin

immunized mice were also characterized by an increased antigen-dependent secretion of IL-10 and IFNγ *(Figure 6A,B)*. This was particularly evident following stimulation with native fibronectin. In addition, immunization with native and MDA-fibronectin induced an antigen-dependent IL4-release *(Figure 6C)* in accordance with formation a Th2-type antibody response. No significant differences were seen between the groups in TNFα, IL-5, IL-12, IL-2 or KC production. Taken together these results suggest that immunization with both native and MDA-fibronectin induces an antigen-specific T cell response, and that this occurs simultaneously with activation of a suppressive regulatory T cell response.

Immunization of apoE$^{-/-}$ mice with MDA-fibronectin results in decreased plasma cholesterol

Immunization with MDA-fibronectin or native fibronectin was associated with decreased levels of plasma cholesterol as compared to non-immunized mice at 26 weeks of age (Table 1). To further investigate the relation between plasma cholesterol and fibronectin levels we analyzed blood samples from 12 week old animals (i.e. one week after the last immunization). At this time point a significant reduction in plasma fibronectin was already present in MDA-fibronectin or native fibronectin immunized mice (811±72 or 765±221 versus 1025±93 μg mL^{-1} in non-immunized mice, $P<0.05$), while there was no significant difference in plasma cholesterol (650±173 or 619±106 versus 748±244 mg dL^{-1}). Thus, there was no direct association between cholesterol and fibronectin levels in plasma in 12-week old mice.

Discussion

We have previously shown that oxidation of LDL is associated with aldehyde-modification of surrounding extracellular matrix proteins such as fibronectin [10]. MDA-fibronectin is present in human atherosclerotic plaques and autoantibodies against MDA-fibronectin can also be detected in human plasma. In a prospective case-control study it was found that antibodies against MDA-modified fibronectin were associated with lower risk for cardiovascular events. To investigate the functional role of these antibodies in atherosclerosis we immunized apoE$^{-/-}$ mice with MDA-modified fibronectin. Increased antibody levels against MDA-fibronectin induced by immunization were found to be associated with a significant inhibition of the development of atherosclerosis supporting the possibility that immune responses against MDA-fibronectin have atheroprotective effects. The present study identified several potential mechanisms through which MDA-fibronectin immunization may protect against atherosclerosis including modulation of the Th1/Th2 balance, activation of regulatory T cells and cross-reactivity with native fibronectin resulting in lower levels of plasma fibronectin, and lower cholesterol levels. Thus, our study provides important clues which may help to explain the association between autoantibodies against MDA-fibronectin and lower cardiovascular risk observed in humans.

Immunizations with native and MDA-fibronectin were unexpectedly found to be equally effective in inhibiting the development of atherosclerosis. However, analysis of tissue extracted from the injection site from mice immunized with native fibronectin revealed presence of MDA-modified fibronectin, indicating that modifications of the protein may take place after immunization. This is in accordance with previous studies showing that aluminum induces oxidative stress and lipid peroxidation [13-17]. The presence of antibodies reacting with MDA-

modified fibronectin in native fibronectin-immunized mice further supported the notion that modification of the protein takes place locally at the injection site. Accordingly, the atheroprotective effect of immunization with native fibronectin most likely involves activation of an immune response against MDA-fibronectin.

Interestingly, immunization with MDA-fibronectin was also associated with activation of several subtypes of immunosuppressive regulatory T cells (Treg) including FoxP3-expressing natural Tregs, TGF-β producing Th3 and IL-10 producing Tr1 cells [18]. All of these types of Tregs have previously been shown to reduce the development of atherosclerosis [19-21]. In addition, stimulation of splenocytes from MDA-fibronectin immunized mice with the polyclonal activator Con A resulted in decreased proliferation as compared to the effect on cells from non-immunized or adjuvant-treated mice, supporting the notion that immunization with MDA-fibronectin induced a functional suppression through activation of regulatory T cells. Accordingly, two effects on regulatory T cells were induced by immunization with MDA-fibronectin. The first was Alum-dependent involving activation of TGF-β producing Th3 cells and may explain the trend towards an atheroprotective effect observed in the mice treated with adjuvant alone. The second was observed only in mice immunized with native or MDA-fibronectin and involved activation of FoxP3 positive natural Tregs and IL-10 producing Tr1 cells. Both natural Treg and Tr1 cells have been demonstrated to have protective roles in atherogenesis in several mouse models. Treatment of apoE-/- mice with CD25-antibody depleting natural Treg cells resulted in a 50% increase in lesion size as compared to animals given control IgG [19]. Transfer experiments using IL-10 producing Tr1 cells to apoE$^{-/-}$ mice have been shown to reduce atherosclerosis [22]. In addition, LDLr$^{-/-}$ mice with leukocyte IL-10 deficiency developed markedly increased

atherosclerosis [23]. Thus, adjuvant alone induces a regulatory T-cell response which is further enforced by immunization with native or MDA-fibronectin activating antigen-specific natural Treg and Tr1 cells. Immunization with both native and MDA-fibronectin also induces a pro-inflammatory IFNγ response, but this is counteracted by an anti-inflammatory IL-10 response. Both the anti-inflammatory IL-10 and the pro-inflammatory IFNγ responses were antigen-specific and activated by both native and MDA-modified fibronectin. It seems likely that MDA-fibronectin is formed upon immunization in native fibronectin-immunized mice, explaining the immune responses against MDA-fibronectin in these mice. However, the immune reactions against the native protein in both groups, indicate that tolerance against the native protein is broken.

It is conceivable that the reverse association between MDA-fibronectin autoantibodies and cardiovascular risk in humans can be explained by a similar mechanism as in the MDA-fibronectin immunized mice. According to this theory, the autoimmune responses against MDA-fibronectin in humans would be accompanied by counteracting activation of antigen-specific Tregs which in turn would exert plaque-stabilizing, anti-inflammatory effects in MDA-fibronectin containing plaques.

Another possibility is that immunization with MDA-fibronectin results in generation of antibodies which cross-react with native fibronectin in atherosclerotic lesions and mediates the removal of the protein. The reduced plasma level of fibronectin observed in immunized mice could potentially also contribute to a reduced presence of fibronectin in plaque tissue. Fibronectin is involved in the regulation of cell adhesion, migration and proliferation [24] and is

up-regulated in atherosclerotic lesions [8]. Although the functional role of fibronectin in atherosclerosis remains to be fully understood, recent studies demonstrating that hypercholesterolemic mice lacking the alternatively spliced fibronectin EIIIA domain have a retarded progression of disease [25] support a pro-atherogenic effect. These data was confirmed by Babaev et al who, however, also showed that mice constitutively expressing EIIIA had reduced atherosclerosis, suggesting that regulation of the splicing of the EDA exon is involved in the disease [26]. However, the finding that plaques from immunized mice did not contain less fibronectin immunoreactivity argues against the possibility that the atheroprotective effects observed in the present study is explained by immune responses against plaque fibronectin.

An additional factor that may contribute to the inhibition of atherosclerosis observed here is the decrease in plasma cholesterol levels in immunized mice. Lower plasma cholesterol levels has also been observed in fibronectin EIIIA deficient mice [25] further implicating fibronectin in lipoprotein metabolism. Several clinical studies have also reported associations between plasma fibronectin and cholesterol levels [27, 28]. The mechanisms responsible for these associations remain to be fully understood but may involve interactions of fibronectin with lipoprotein receptors [29]. Taken together, these observations suggest that the athero-protective effect of immunization with MDA-fibronectin in mice is in part due to cholesterol-lowering in response to decreased levels of plasma fibronectin. Such a mechanism is unlikely to act in humans, since no relation between autoantibodies and plasma cholesterol levels were observed in association studies between MDA-fibronectin autoantibodies and cardiovascular risk [10].

Interestingly, immunization with MDA-laminin in apoE-/- mice has been found to result in increased atherosclerosis [30]. This was accompanied by an increase in proinflammatory Th17 effector cells and a decrease in Foxp3-positive regulatory T-cells. The reason why immunization with MDA-laminin results in a proinflammatory immune response increasing atherosclerosis, whereas immunization with MDA-fibronectin induces an anti-atherogenic regulatory T-cell immune response remains to be fully understood. Fibronectin is present in almost every tissue and is likely to be oxidized also in other locations than the atherosclerotic lesions. It is possible that an immune response recognizing MDA-fibronectin would have more adverse effects, than immune responses against MDA-laminin, which has a more restricted distribution. Therefore, it may be more critical for regulatory T cells to control autoimmune reactions against MDA-fibronectin.

It should be kept in mind that in a human setting this type of immunomodulatory therapy most likely would be administered when atherosclerotic lesions already are formed. In this study immunizations of mice were started at 6 weeks of age before high fat diet. Although cholesterol levels and lesion formation is enhanced upon high fat diet, it is likely that LDL-oxidation and MDA-modification are present already at the start for immunization. ApoE-/- mice have increased lipid levels compared to wt mice, and develop atherosclerosis even without high fat diet [31, 32]. In addition, lipid retention in the artery is present by 3 weeks of age and monocyte attachment to endothelium is observed at 5 weeks of age on a normal chow [33]. Interestingly, fibronectin has been shown to present in the vessel wall at a very early stage, before fatty streak development [9].

In conclusion, we demonstrate that induction of an immune response against MDA-fibronectin has an atheroprotective effect supporting the clinical observation of an inverse association between MDA-fibronectin autoantibodies and cardiovascular risk. The mechanisms involved in this protection remain to be fully elucidated, but appear to involve effects on the balance of Th1/Th2-type immunity, activation of regulatory T cells and a decrease in fibronectin and plasma cholesterol levels. Immune responses against aldehyde-modified fibronectin represent a novel mechanism that may be involved in modulating the atherosclerotic disease processes and suggest a new possibility of immunization strategy to reduce atherosclerosis in humans. However, it should be recognized that modulating immune responses against MDA-fibronectin in established human disease is likely to be more complex and needs further investigation.

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Supplementary material

Supplementary material is available at Cardiovascular Research online.

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Conflict of interest statement

Conflict of interest: none declared.

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Figure legends

Figure 1

Immunization of apoE$^{-/-}$ mice with MDA-fibronectin reduces atherosclerosis.

(A) *En face* preparations of the descending aorta of 26 week-old non-immunized mice (non-imm), alum immunized mice (Alum), native fibronectin (natFN) or MDA-fibronectin (MDA-FN) immunized mice were stained with oil-red O. (B, C) Subvalvular lesions from 26 week-old immunized mice were stained with haematoxylin or anti-CD3. (A-B) Areas were quantified by computer aided morphometry. (D) Subvalvular lesions from 26 week-old immunized mice were stained with antibodies recognizing monocytes/macrophages and counterstained with haematoxylin. Scale bar = 500 μm. $^{**}P<0.005$, $^{*}P<0.05$, *a* $P=0.06$, *b* $P=0.08$.

Figure 2

Presence of MDA-modified protein in mice immunized with native fibronectin.

Tissue (10 mg) from the immunization site of Alum (Alum; n=5) immunized mice or native fibronectin (natFN; n=5) immunized mice was homogenized in buffer containing protease inhibitors and analyzed for MDA-linked fluorescence (excitation and emission wavelengths 390 nm and 460 nm, respectively). Native fibronectin (natFN) (10 μg) and *in vitro* MDA-modified fibronectin (MDA-FN) (10 μg) were used as controls.

Figure 3

Immunization with MDA-fibronectin results in a Th2-type antibody response.

(A) Plasma from mice immunized with MDA-fibronectin (MDA-FN), native fibronectin (natFN), Alum (Alum) or non-immunized (non-imm) control mice (x-axis) was tested for IgG and IgM antibody titers against native (white bars) or MDA-fibronectin (black bars) in ELISA. Values represent plasma (dilution 1:50) pooled from five mice in each group and run in duplicates.

(B) The isotype of the antibody response was assayed by ELISA. Plasma from mice immunized with MDA-fibronectin (MDA-FN) (dilution 1:10000), native fibronectin (natFN) (dilution 1:10000), Alum (Alum) (dilution 1:5) or non-immunized mice (non-imm) (dilution 1:5) (x-axis) was tested for IgG1 (grey bars) and IgG2a (white bars) titers against MDA-fibronectin in ELISA. Values represent plasma from individual mice.

(C) The specificity of the IgG1 antibodies to various MDA-modified type III fragments of fibronectin was assayed by ELISA. Values represent plasma (dilution 1:2500) pooled from eight mice in each group (non-immunized mice, Alum immunized mice, native fibronectin immunized mice, MDA-fibronectin immunized mice; x-axis).

Figure 4

Immunization of apoE$^{-/-}$ mice with MDA-fibronectin reduces fibronectin levels in plasma. Fibronectin levels in plasma from 26-week old non-immunized (non-imm), Alum (Alum), native fibronectin (natFN) or MDA-fibronectin (MDA-FN) immunized mice was analyzed by ELISA. $^{**}P<0.005$, $^{*}P<0.05$; ANOVA followed by *post hoc* test.

Figure 5

Immunization of apoE$^{-/-}$ mice with MDA-fibronectin induces antigen-specific T-cell proliferation and regulatory T-cells.

(A) Splenocytes from12 week-old mice immunized with MDA-fibronectin (MDA-FN), Alum (Alum) or non-immunized control mice (non-imm) (x-axis) were stimulated with 30 μg mL^{-1} native fibronectin, MDA-fibronectin, or Con A and T-cell proliferation was assayed by [^{3}H]-thymidine uptake. Proliferation index is defined as the ratio of proliferation of stimulated cells to non-stimulated cells.

(B) To analyze presence of regulatory T-cells lymphocytes from spleens of 12 week old apoE$^{-/-}$ mice immunized with Alum (Alum), native fibronectin (natFN), MDA-fibronectin (MDA-FN) or non-immunized (non-imm) mice were analyzed with flow cytometry to determine the percentage of CD3^{+}/CD4^{+} cells expressing CD25/FoxP3.

n=11-12 (A) or n=4-6 (B) in each group; *P<0.05, **P<0.001, ***P<0.0001*;* Mann-Whitney-test.

Figure 6

Cytokine release from splenocytes of immunized mice.

Splenocytes from 12 week-old non-immunized (white bars), Alum (light-grey bars), native fibronectin (dark-grey) or MDA-fibronectin (black bars) immunized mice were isolated. The splenocytes were left un-stimulated, or were stimulated with either 30 μg native or MDA-fibronectin. Medium from the splenocyte cultures were assayed for cytokine release of IL-10, (A) IFNγ (B), and IL-4 (C). n=4-6 in each group; *P<0.05, **P<0.01; Kruskal-Wallis followed by Dunn's Multiple Comparison Test.

Table 1

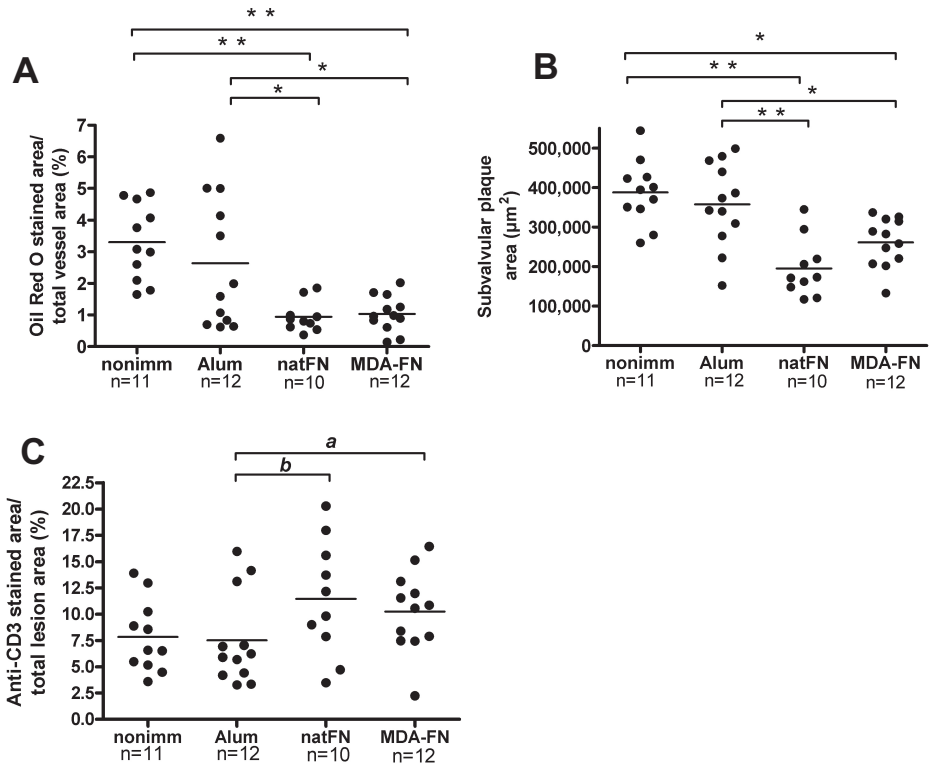
Plasma cholesterol in 26-week old mice

Mouse group	Plasma cholesterol (mg dL^{-1}) mean±s.d.
Non-immunized	715±111
Alum	637±198
Native fibronectin	425±85 * †
MDA-fibronectin	471±156 *

*P<0.005 versus non-immunized

† P<0.05 versus Alum

ANOVA followed by *post hoc* test.


A
B
C
Oil Red O stained area/ total vessel area (%)
Subvalvular plaque area (μm²)
Anti-CD3 stained area/ total lesion area (%)
nonimm n=11
Alum n=12
natFN n=10
MDA-FN n=12
a
b

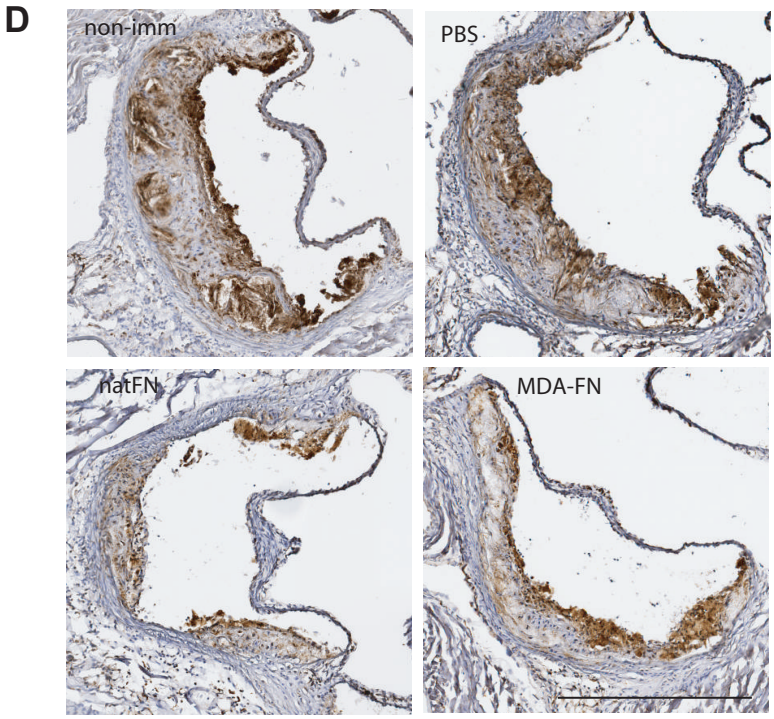

D
non-imm
PBS
natFN
MDA-FN

Figure 1

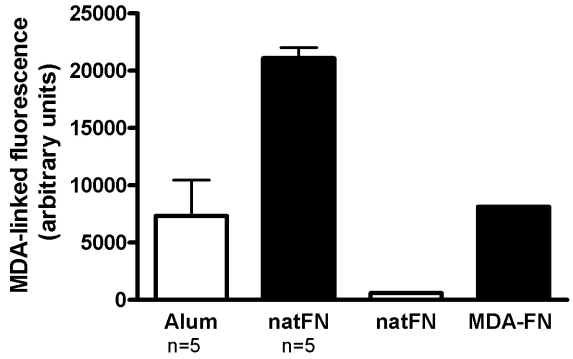

MDA-linked fluorescence
(arbitrary units)
25000
20000
15000
10000
5000
0
Alum
n=5
natFN
n=5
natFN
MDA-FN

Figure 2

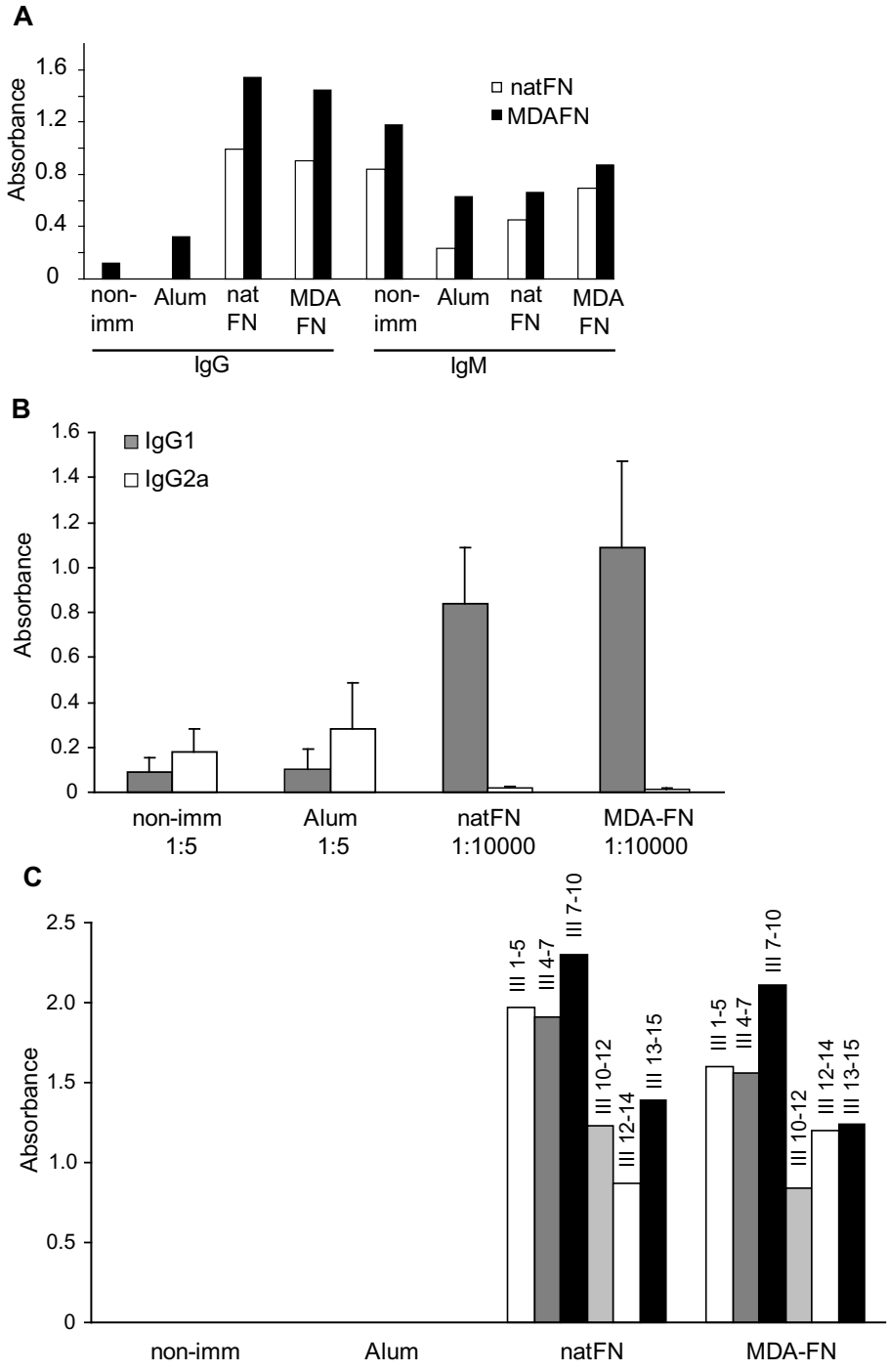

A
Absorbance
1.6
1.2
0.8
0.4
0
natFN
MDAFN
non-imm
Alum
nat FN
MDA FN
non-imm
Alum
nat FN
MDA FN
IgG
IgM
B
IgG1
IgG2a
Absorbance
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
non-imm 1:5
Alum 1:5
natFN 1:10000
MDA-FN 1:10000
C
Absorbance
2.5
2.0
1.5
1.0
0.5
0
III 1-5
III 4-7
III 7-10
III 10-12
III 12-14
III 13-15
non-imm
Alum
natFN
MDA-FN

Figure 3

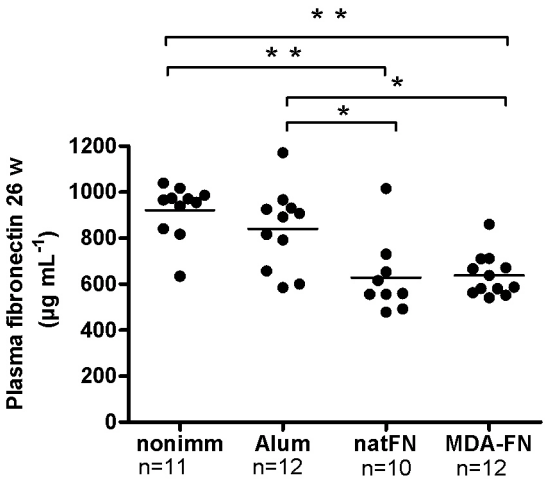

* *
* *
*
*
Plasma fibronectin 26 w
(µg mL-1)
1200
1000
800
600
400
200
0
nonimm
n=11
Alum
n=12
natFN
n=10
MDA-FN
n=12

Figure 4

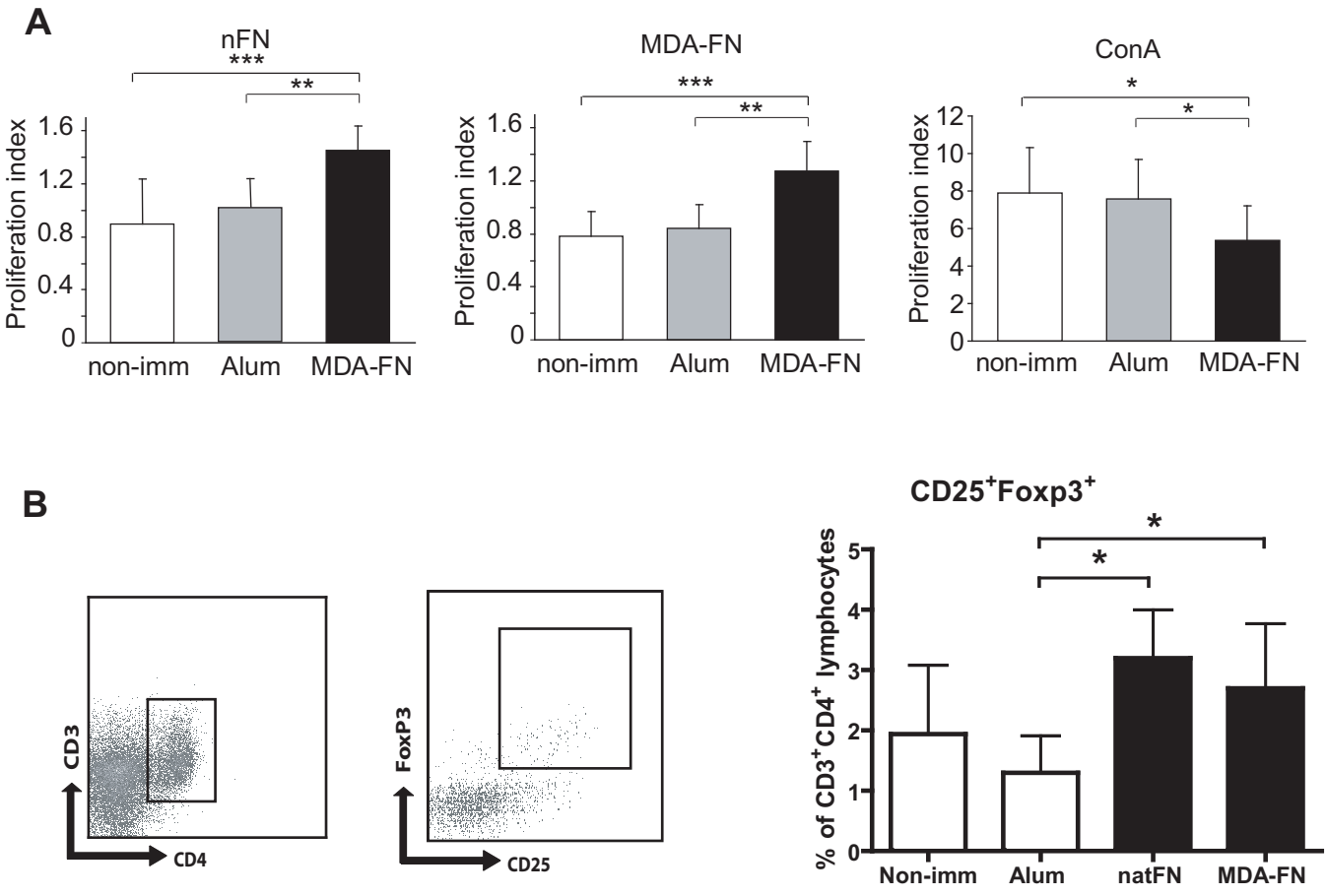
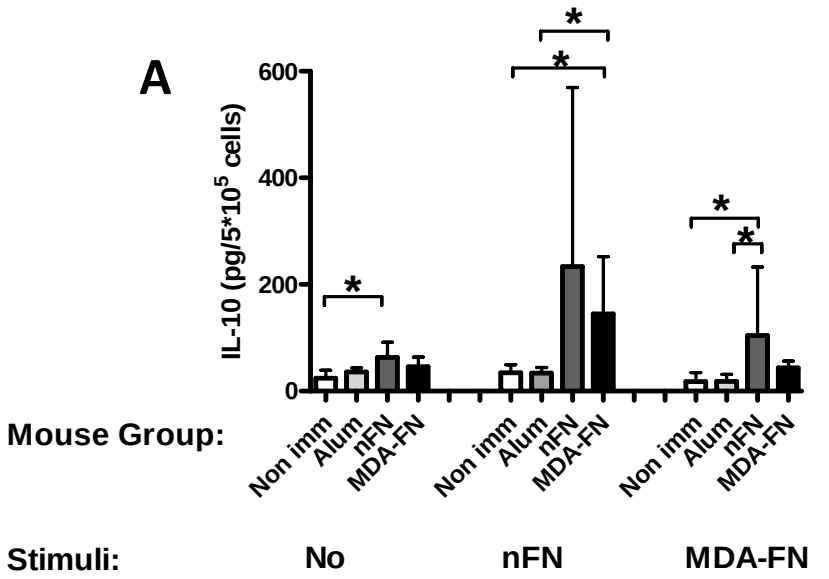
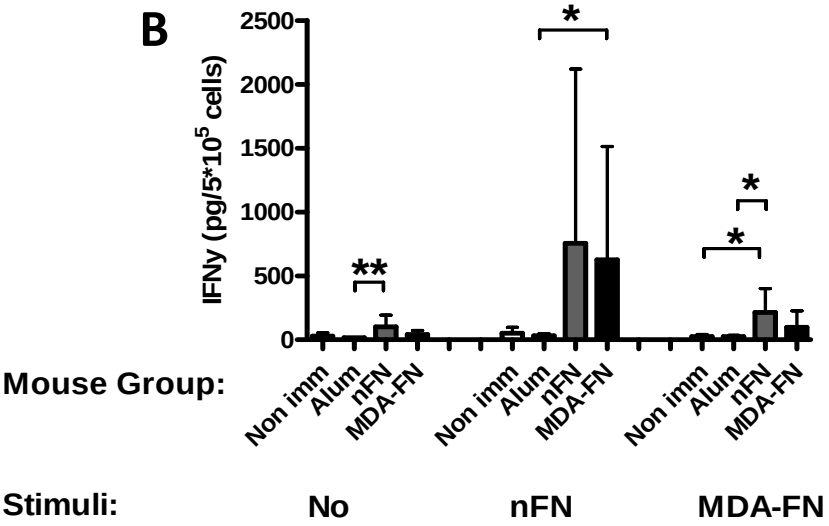

A
nFN
***
**
Proliferation index
1.6
1.2
0.8
0.4
0
non-imm
Alum
MDA-FN
MDA-FN
***
**
Proliferation index
1.6
1.2
0.8
0.4
0
non-imm
Alum
MDA-FN
ConA
*
*
Proliferation index
12
10
8
6
4
2
0
non-imm
Alum
MDA-FN
B
CD3
CD4
FoxP3
CD25
CD25+Foxp3+
*
*
% of CD3+CD4+ lymphocytes
5
4
3
2
1
0
Non-imm
Alum
natFN
MDA-FN

Figure 5


A
IL-10 (pg/5*10^5 cells)
600
400
200
0
*
*
*
*
Mouse Group:
Non imm
Alum
nFN
MDA-FN
Stimuli:
No
nFN
MDA-FN


B
IFNy (pg/5*10^5 cells)
2500
2000
1500
1000
500
0
**
*
*
Mouse Group:
Non imm
Alum
nFN
MDA-FN
Stimuli:
No
nFN
MDA-FN

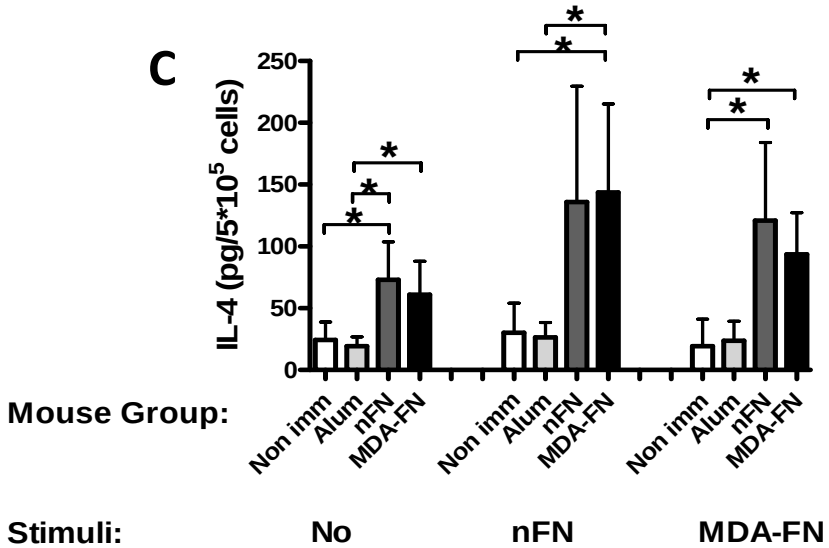

C
IL-4 (pg/5*10^5 cells)
250
200
150
100
50
0
*
*
*
*
*
*
Mouse Group:
Non imm
Alum
nFN
MDA-FN
Stimuli:
No
nFN
MDA-FN

Figure 6

Supplementary data

Methods S1

MDA-linked fluorescence analysis of precipitate from the immunization site

The white gelatinous precipitate formed at the immunization site was removed one week after the last injection. The collected precipitate was homogenized and dissolved in a protein isolation buffer (1 mol L^{-1} Tris, 0.5 mol L^{-1} EDTA, 5 mol L^{-1} NaCl, 10% Brij96, 10% NP40 and 20% protease inhibitory cocktail from Sigma). Homogenized precipitate (10 mg), native and MDA-modified fibronectin (10 µg) was diluted in PBS and the fluorescence intensities (excitation and emission wavelengths 390 nm and 460 nm, respectively) were measured with Infinite M200 (TECAN). The buffer background value was subtracted.

Flow Cytometry

Splenocytes were cultured during 72 h followed by 5 h of stimulation with 10 µg mL^{-1} anti-CD3e coated in the wells and 2 µg mL^{-1} anti-CD28 in suspension (BD Pharmingen). Splenocytes were then stained with fluorochrome-conjugated antibodies against CD3e-PE/Cy5 (clone 145-2C11), CD25-APC (clone PC61) (BioLegend) and CD4-Alexa700 (clone RM4-5) and FoxP3-Pacific Blue (clone FJK-16S) (EBioscience) and analyzed with a CyAn flow cytometer (Beckman Coulter).

Figure legends

Fig. S1

Immunization of apoE-/- mice with MDA-fibronectin does not alter neutrophil content in lesions.

Subvalvular lesions from 26 week old immunized mice (non-immunized, Alum control, native fibronectin or MDA-fibronectin immunized mice) were stained for neutrophils and areas were quantified by computer aided morphometry.

Fig. S2

Colocalization of IgG and MDA-fibronectin in subvalvular lesions of MDA-fibronectin immunized mice.

Fig. S3

Native and MDA-fibronectin stained areas in subvalvular plaques.

Native (A) and MDA-fibronectin (B) stained areas in subvalvular plaques of immunized mice were measured by computer aided morphometry.

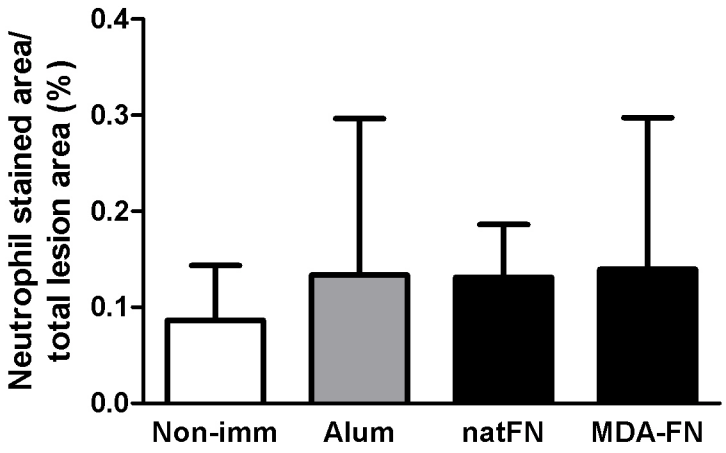
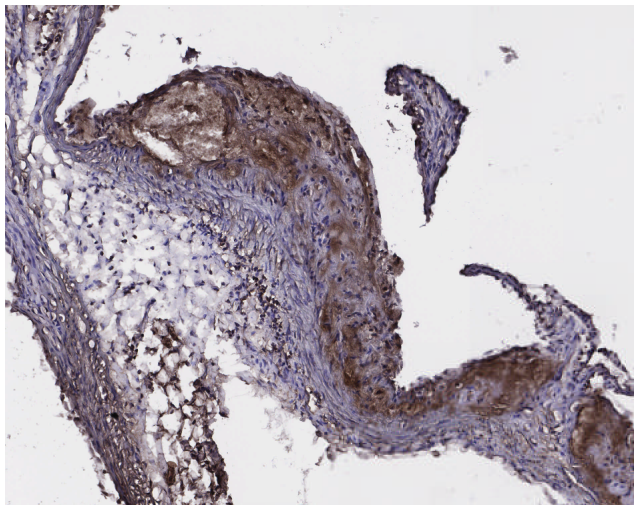

Neutrophil stained area/
total lesion area (%)
0.4
0.3
0.2
0.1
0.0
Non-imm
Alum
natFN
MDA-FN

Figure S1

α-IgG



α-MDA-fibronectin

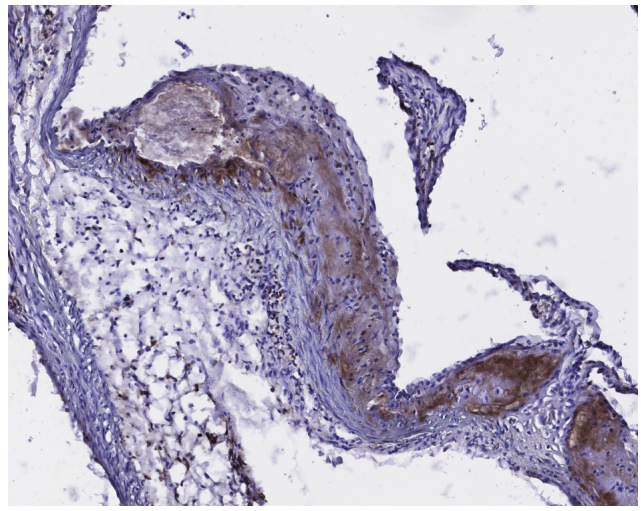


Figure S2

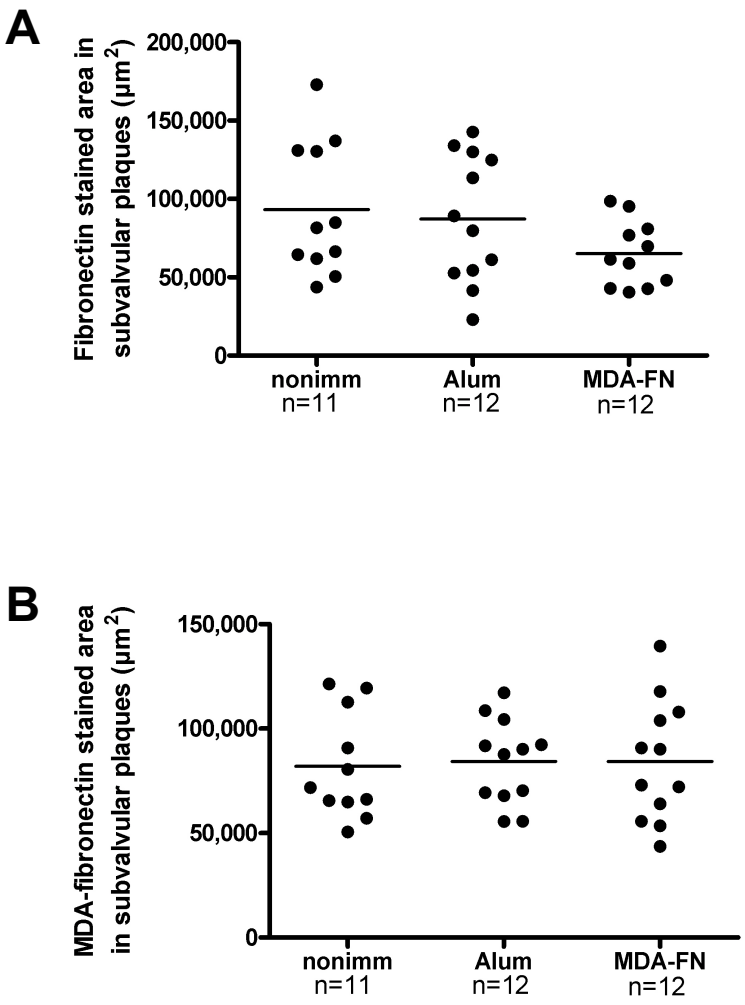

A
Fibronectin stained area in subvalvular plaques (μm2)
200,000
150,000
100,000
50,000
0
nonimm
n=11
Alum
n=12
MDA-FN
n=12
B
MDA-fibronectin stained area in subvalvular plaques (μm2)
150,000
100,000
50,000
0
nonimm
n=11
Alum
n=12
MDA-FN
n=12

Figure S3