



# Freunds adjuvant alone is antiatherogenic in apoE-deficient mice and specific immunization against TNF $\alpha$ confers no additional benefit

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## Abstract

TNF $\alpha$  participates in the pathogenesis of atherosclerosis. The effect of immunization against TNF $\alpha$  on development of advanced vascular lesions in atherosclerosis-susceptible apoE-deficient mice was investigated. At 5–7 weeks of age, animals received immunization with either Freunds adjuvant and a recombinant antigenic TNF $\alpha$  molecule (TNF106), Freunds adjuvant alone, or no immunization. All mice received a Western-type high-fat diet for 12 weeks. Aortic sinus lesion area was determined by microscopic morphometry, the total aortic arch cholesterol content was determined by gas chromatography, and antibodies against TNF $\alpha$ , malondialdehyde-modified low density lipoprotein, or heat shock protein 60, were assessed by ELISAs. Immunization with TNF106 induced high-titered circulating antibodies against TNF $\alpha$  ( $n = 23$ ), and these antibodies were not detected in mice immunized with Freunds adjuvant alone ( $n = 22$ ), or in non-immunized control animals ( $n = 25$ ). After 12 weeks, the atherosclerotic lesion size was significantly reduced in immunized animals, whether they had been immunized with TNF106 or Freunds adjuvant alone, and the total lesional cholesterol content was decreased in mice immunized with TNF106. There were no correlations between circulating antibody titers and plaque size, total aortic arch cholesterol content, or plasma lipid levels, respectively. Administration of Freunds adjuvant alone can thus reduce formation of mature atherosclerotic lesions in apoE-deficient mice and this response is not modified by specific immunization against TNF $\alpha$ . © 2001 Elsevier Science Ireland Ltd. All rights reserved.

**Keywords:** Atherosclerosis; Tumor necrosis factor- $\alpha$ ; Freunds adjuvant; Immunization; Apolipoprotein E knockout; Immunology; Inflammation

## 1. Introduction

Tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) is a central mediator of inflammatory reactions and host defence that is produced by many cell types as a component of the injury response repertoire [1,2]. Atherosclerosis is now

perceived as a chronic inflammatory disease involving both innate and adaptive immune mechanisms and accumulating evidence has indicated that TNF $\alpha$  plays a key role in the signaling cascades leading to plaque formation [3–6]. TNF $\alpha$  is expressed in atherosclerotic plaques and is capable of inducing a range of predominantly proatherogenic effects [2,6,7].

Treatment of inflammatory disease by blocking the biosynthesis or actions of TNF $\alpha$  has received considerable interest. The biological effects of TNF $\alpha$  are medi-

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ated by two cell surface receptors, p55 and p75 [2], and neutralization of TNF $\alpha$  activity by administration of soluble TNF $\alpha$  receptors or murine-human chimeric monoclonal antibodies against TNF $\alpha$  can reduce manifestations of tissue inflammation in rheumatoid arthritis and inflammatory gut disease [8,9]. Systemic administration of soluble TNF $\alpha$  receptors can attenuate vessel wall inflammation in experimental cardiac allograft vasculopathy [10], a condition bearing some resemblances to atherosclerosis [11], and treatment with a TNF $\alpha$  receptor homologue was recently found to inhibit the formation of early vascular lesions (i.e. fatty streaks) in apolipoprotein E (apoE)-deficient mice, the most extensively studied animal model of atherosclerosis [12]. However, these therapeutic modalities have major shortcomings because engineered proteins are expensive and the antigenicity of murine monoclonal antibodies may preclude long-term therapy.

A method for breaking the B cell tolerance toward a self protein by immunization with a recombinant construct of the self protein containing a foreign immunodominant T helper (Th) cell epitope was recently developed [13,14]. This modified self protein elicited a strong polyclonal autoantibody response cross reactive with the nonmodified self protein, and induction of anti-TNF $\alpha$  antibodies by this novel approach was found to neutralize pathobiological effects of TNF $\alpha$  in mouse models of cachexia and autoimmune arthritis [15]. A number of similarities have been observed in the immunoinflammatory response in atherosclerosis and autoimmune arthritis [16] and the objective of the present study was therefore to use a similar scheme to examine the effects of immunization against TNF $\alpha$  on development of advanced human-like atherosclerotic plaques in apoE-deficient mice.

## 2. Methods

### 2.1. ApoE-deficient mice

Female apo E-deficient mice [17], backcrossed for nine generations into the C57BL/6J background (Bomholtgaard Breeding and Research Center, Denmark), were housed in a temperature-controlled facility with 12 h light–dark cycle and free access to food and water. After being weaned at 3 weeks of age, the mice received standard rodent chow for a 3–4 week adaptation period, and were subsequently placed on a Western-type high-fat diet containing 21% (wt/wt) fat and 0.15% cholesterol (Altromin C1057-157d, Møllegaarden, Denmark), without addition of cholic acid. The investigation conformed with the Guide for the care and use of laboratory animals published by the US National Institutes of Health (NIH publication No 85-23, revised 1996).

### 2.2. Immunization protocol

ApoE-deficient mice were divided into three treatment groups: Animals immunized with the recombinant TNF $\alpha$  antigen in Freund's adjuvant (TNF $\alpha$  group;  $n = 23$ ), mice receiving Freund's adjuvant alone (FA group;  $n = 22$ ), and a control group ( $n = 25$ ) that was not subjected to immunization. The modified TNF $\alpha$  antigen was prepared as previously described [15]. In brief, an oligonucleotide encoding for a well-characterized Th epitope from hen egg-white lysozyme (HEL[81–95]: SALLSSDITASVNCA) [18] was introduced into the murine TNF $\alpha$  DNA sequence by standard PCR mutagenesis procedures. The identity of the construct was verified by DNA sequencing and it was subsequently expressed by recloning into the IPTG-inducible expression vector pTrc99A (Pharmacia Biotech) as specified by the manufacturer's instructions. The resulting recombinant TNF $\alpha$  protein, TNF106, was expressed in *Escherichia coli* and purified as described elsewhere [15]. Immunization with this molecule has previously been shown to elicit high-titered antibodies that crossreact with native TNF $\alpha$  in Balb/c and C3H/Hen strains of mice and are capable of neutralizing binding of murine TNF $\alpha$  to the p55 receptor [15]. The immunization protocol began 1 week before the mice were shifted to Western-type high-fat diet, i.e. when they were 5–7 weeks of age. Animals in the TNF $\alpha$  group were immunized by subcutaneous injection with 100  $\mu$ g of TNF106 solubilized in PBS and emulsified with an equal volume (100  $\mu$ l) of complete FA (Difco, Detroit, MI). At 2, 4, and 8 weeks thereafter, booster injections were administered with 100  $\mu$ g of TNF106 emulsified in incomplete FA. Animals in the FA group were immunized with sterile water in an equal volume of complete FA, and boosted at 2, 4, and 8 weeks with sterile water in incomplete FA. After receiving a high-fat diet for 12 weeks, mice were anesthetized and exsanguinated by cardiac puncture. Plasma was separated by centrifugation at 2500 rpm for 5 min and stored at  $-20^{\circ}\text{C}$  until use. After exsanguination, animals were perfusion-fixed via the left ventricle at 100 mmHg in 4% formaldehyde and then immersed in the fixative overnight.

### 2.3. Quantification of atherosclerosis

#### 2.3.1. Lesion size

The heart and aortic root were removed, cut transversally and embedded in paraffin. The aortic root was cross-sectioned sequentially in 4  $\mu$ m-thick sections as described previously [19], and five sections from five different levels 80  $\mu$ m apart were stained with Orcein (Sigma). The aortic sinus lesion area was determined by microscopic morphometry using computer-assisted image analysis (SigmaScan Pro, Jandel Scientific, San Rafael, CA), and the median and maximum plaque

cross-sectional areas were selected as endpoints of the present study.

### 2.3.2. Total cholesterol content

After tissue fixation, the adventitial tissue was carefully removed, and the aortic arch, i.e. the aortic region extending from the aortic root to 4 mm distal to the left subclavian artery, was opened and laid out flat on a black background. The image was recorded and the percent intimal area covered by grossly discernible lesions was quantified by computerized image analysis (Image-Pro, Media Cybernetics, Silver Springs, MD). Tissue lipids were extracted with chloroform-methanol, and the content of unesterified and esterified cholesterol was determined by gas chromatography as described previously [20,21]. Aortic arch total cholesterol content was normalized to the lesion size as determined by image analysis.

### 2.4. Plasma lipid analysis

Plasma concentrations of total cholesterol, triglycerides, and high density lipoprotein (HDL)-cholesterol at the end of the study were measured on a Cobas Fara analyzer using commercially available kits (Roche Diagnostics, Copenhagen, DK).

### 2.5. Determination of autoantibody titers

#### 2.5.1. Antibodies against TNF $\alpha$

Polystyrene microtiter plates (MaxiSorp, Nunc, Roskilde, Denmark) were coated overnight with highly purified recombinant murine TNF $\alpha$  (0.1  $\mu$ g/well) dissolved in PBS. Remaining binding sites were blocked with 200  $\mu$ l/well of washing buffer containing 1% bovine serum albumin, 0.5 M NaCl, 2.5 mM KCl, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 7 mM Na<sub>2</sub>HPO<sub>4</sub>, and 1% Triton X-100 (pH 7.2). Three-fold dilutions of plasma samples (100  $\mu$ l/well in washing buffer starting at 1:100) were added and incubated for 1 h at room temperature. After washing, 100  $\mu$ l of horse radish peroxidase-labelled rabbit anti mouse immunoglobulin (DAKO) was added and incubated for 1 h at room temperature. The wells were washed, and binding was visualized with *o*-phenyldiamine (Sigma). The reaction was stopped with 150  $\mu$ l 2 M H<sub>2</sub>SO<sub>4</sub>/well and absorbance was measured at 492 nm. A standard curve with dilutions of a high-titered anti-murine TNF $\alpha$  antiserum was included on each plate and the anti-TNF $\alpha$  antibody titer of each plasma sample was expressed as the reciprocal of the dilution of the standard that gave the same absorbance as the sample.

#### 2.5.2. Antibodies against recombinant human heat shock protein 60 (rhHSP60)

Amplification of the human pancreatic HSP60 gene

and subsequent expression and purification of rhHSP60 was performed as described elsewhere [22]. Microtiter plates (MaxiSorp) were coated with rhHSP60 (2  $\mu$ g/ml) in isotonic PBS. Nonspecific binding was blocked by incubation with diluting buffer (0.1% Tween-20 in PBS, pH 7.2) for 1 h and wells were subsequently incubated for 2 h with plasma samples in 1:100-fold dilution in diluting buffer. After washing, peroxidase-labelled rabbit anti mouse IgG (DAKO) was added and binding was detected with *o*-phenyldiamine. The colour reaction was terminated with 1 M H<sub>2</sub>SO<sub>4</sub> and background-corrected optical density at 492 nm was measured using an automatic plate reader. All reactions were performed at room temperature in volumes of 100  $\mu$ l. Antibody titers were determined by use of a standard curve obtained with 2-fold dilutions (1:100–1:3200) of a high-titered rat immune serum.

#### 2.5.3. Antibodies against malondialdehyde (MDA)-modified LDL

Purified human LDL (Fluka, Buchs, Switzerland) was treated with protein G Sepharose (Pharmacia, Lund, Sweden) to remove traces of immunoglobulins and oxidized by treatment with MDA generated by acid hydrolysis of MDA-bis (diethylacetal) [23]. One volume of native LDL (1 mg/ml in PBS, pH 7.4) was incubated with one volume of freshly prepared MDA for 2 h at 30°C and dialyzed against PBS before use. Microtiter plates (MaxiSorp) were with MDA-modified LDL and native LDL (one-half plate each) by adding 100  $\mu$ l/well of MDA-modified LDL or native LDL in suitable dilution with 50 mM carbonate buffer, pH 9.6. Wells were incubated for 2 h at 37°C followed by overnight incubation at 4°C. After washing of wells with isotonic saline, 0.05% Tween 20, plasma samples were incubated for 1 h after 1:100 dilution in incubation buffer containing 0.04% Tween 20, 0.2% bovine serum albumin. After washing, bound IgG was detected by incubation with alkaline phosphatase-conjugated goat anti-mouse IgG (1:4000, Sigma) and enzyme activity was measured at 405 nm using *p*-nitrophenyl phosphate as substrate. The absorbance differences between wells coated with MDA-modified LDL and native LDL were converted to arbitrary antibody titers with use of a standard curve obtained with a high-titered positive human serum included on all plates.

### 2.6. Statistical analysis

Data were expressed as mean  $\pm$  S.E.M. for normally distributed data, and median (interquartile range) for non-normally distributed data. The lesion area, plasma lipid values, and mean antibody titers were compared between treatment groups with the nonparametric Kruskal–Wallis test. The correlation between antibody

Table 1  
Effect of immunization of apoE-deficient mice against TNF $\alpha$  (TNF $\alpha$  group) or administration of Freund's adjuvant alone (FA group) on body weight, plasma lipids, and antibody (ab) titers against TNF $\alpha$ , malondialdehyde-modified low density lipoprotein (MDA-LDL), or recombinant human heat shock protein 60 (rhHSP 60)

	Control group (n = 25)	TNF $\alpha$ group (n = 23)	FA group (n = 22)
Body weight (g)	20.6 $\pm$ 0.3	21.2 $\pm$ 0.3	21.2 $\pm$ 0.3
P-total-cholesterol (mM)	13.3 $\pm$ 0.9	13.0 $\pm$ 0.5	12.0 $\pm$ 0.3
P-HDL-cholesterol (mM)	2.9 $\pm$ 0.2	2.9 $\pm$ 0.1	3.7 $\pm$ 0.2* $\dagger$
P-Triglycerides (mM)	0.8 $\pm$ 0.1	0.5 $\pm$ 0.1 $\ddagger$	0.5 $\pm$ 0.1 $\ddagger$
Anti-TNF $\alpha$ -ab (titer)	1.5 $\pm$ 0.1	2367.6 $\pm$ 483.7 $\ddagger$ , $\S$	0.9 $\pm$ 0.1
Anti-MDA-LDL-ab (titer)	171.3 $\pm$ 103.9	66.0 $\pm$ 19.3	116.3 $\pm$ 35.8
Anti-rhHSP60-ab (titer)	0.96 $\pm$ 0.12	4.56 $\pm$ 0.78* $\S$	2.42 $\pm$ 0.46

\*  $P < 0.005$  vs. control group;

$\dagger$   $P < 0.001$  vs. TNF $\alpha$  group;

$\ddagger$   $P < 0.001$  vs. control group;

$\S$   $P < 0.001$  vs. FA group.

titers and other parameters was examined by Spearman's rank correlation test. A value of  $P < 0.05$  was considered statistically significant.

### 3. Results

As indicated in Table 1, immunization of apoE-deficient mice with the recombinant TNF $\alpha$  protein, TNF106, resulted in development of a high-titered circulating anti-TNF $\alpha$  antibody response. At the termination of the study (i.e. 12 weeks after the first immunization), elevated circulating anti-TNF $\alpha$  antibody titers in the TNF $\alpha$  group were comparable to titers previously observed in Balb/c mice subjected to a similar immunization protocol [15], whereas animals immunized with FA alone or non-immunized controls failed to demonstrate antibody activity against TNF $\alpha$ . Mice immunized with TNF106 or FA alone generally displayed subcutaneous granulomata at the immunization sites, but otherwise appeared healthy with no overt pathology, and the body weight did not differ between the three treatment groups. Considerable titers of circulating antibodies to rhHSP60 and MDA-LDL were detected in all apoE-deficient mice, and the titers of anti-rhHSP60 were slightly but significantly increased in the TNF $\alpha$  group, whereas antibody titers against MDA-LDL were not different between the groups. Plasma total cholesterol levels were not significantly affected by immunization with TNF106 or FA, but a significant decrease in triglyceride levels was observed in immunized mice compared with control animals, and plasma HDL-cholesterol levels were increased in mice immunized with FA alone (Table 1).

Mature atherosclerotic plaques containing all components of advanced human plaques, e.g. foam cells, vascular smooth muscle cells, a collagen-rich matrix, and extracellular lipid accumulation with cholesterol crystals, were present in sections from the aortic root

(Fig. 1) and as shown in Figs. 2 and 3, the median and maximum cross-sectional areas per lesion were significantly reduced in immunized animals, whether they had been immunized with TNF106 or FA alone. There was no significant difference between plaque size in the

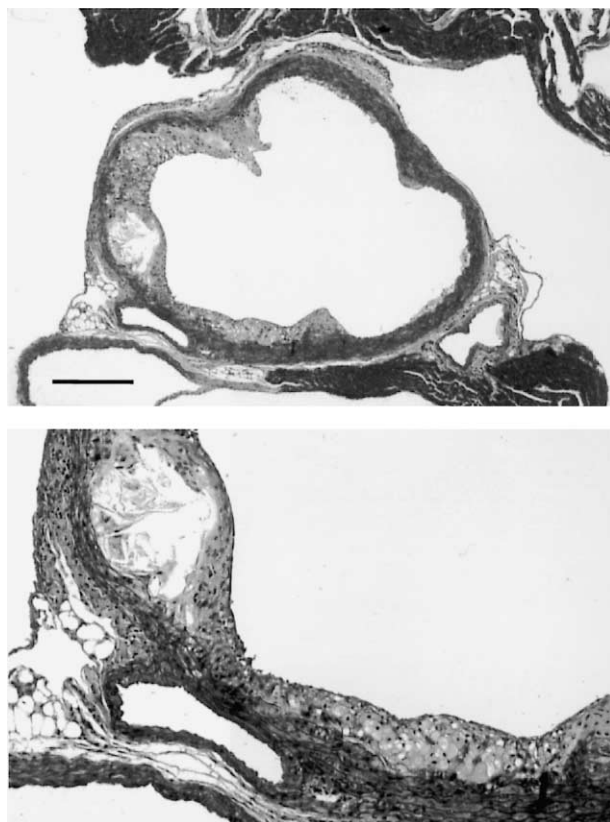


Fig. 1. Cross-section of aortic root from mouse immunized with TNF $\alpha$  illustrating a mature atherosclerotic plaque (top) containing many foam cells and a hypocellular atheromatous core with cholesterol crystals covered by a thin fibrous cap (bottom). Although plaques were larger in non-immunized mice, their morphology was similar in all three treatment groups. Trichrome staining (elastin black, collagen blue, and smooth muscle cells red), bar = 300  $\mu$ m.

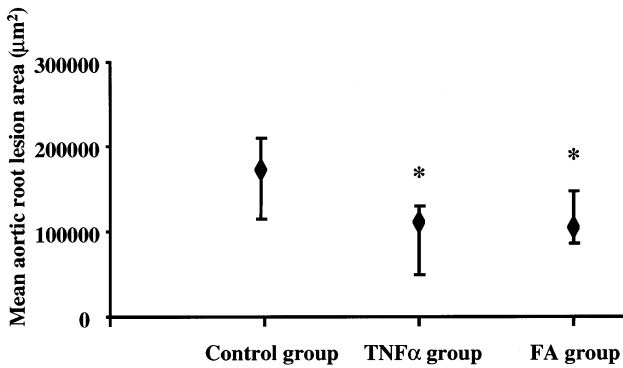


Fig. 2. Mean aortic root lesion area in apoE-deficient mice that were not subjected to immunization (Control group;  $n = 25$ ), after immunization against TNF $\alpha$  (TNF $\alpha$  group;  $n = 23$ ), or after administration of Freund's adjuvant alone (FA group;  $n = 22$ ). Values are expressed as medians and interquartile ranges. \*  $P < 0.05$  vs. control group.

TNF $\alpha$  group as compared with the FA group. No differences in histological features were apparent among the treatment groups, and plaque rupture or thrombosis were not observed. In agreement with the data on aortic sinus plaque size, a significant decrease was found in the total cholesterol content of aortic arch lesions in the TNF $\alpha$  group as compared with control mice and a similar albeit nonsignificant trend was observed in the FA group ( $0.47 \pm 0.05 \mu\text{g}/\text{mm}^2$  vs.  $0.51 \pm 0.05 \mu\text{g}/\text{mm}^2$ ,  $P = 0.15$ ; Fig. 4). Irrespective of the treatment group, correlation analysis failed to disclose any significant association between circulating titers of antibodies against TNF $\alpha$ , MDA-LDL, or rhHSP60, and the plaque size, total cholesterol content/ $\text{mm}^2$ , or plasma lipid levels, respectively (not shown).

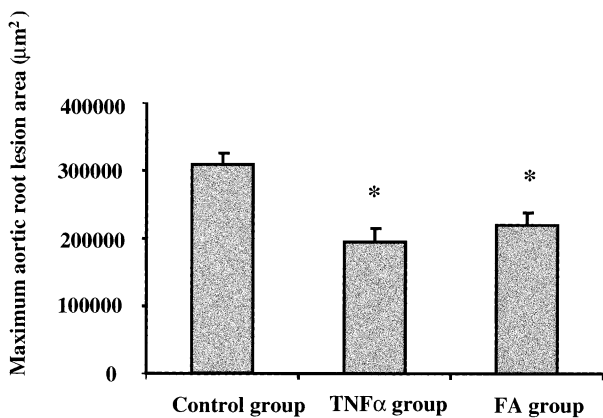


Fig. 3. Maximum aortic root lesion area in apoE-deficient mice that were not subjected to immunization (Control group;  $n = 25$ ), after immunization against TNF $\alpha$  (TNF $\alpha$  group;  $n = 23$ ), or after administration of Freund's adjuvant alone (FA group;  $n = 22$ ). Values are expressed as means  $\pm$  S.E.M. \*  $P < 0.05$  vs. control group.

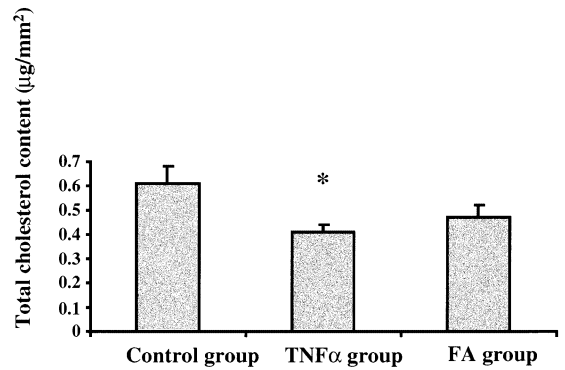


Fig. 4. Aortic arch total cholesterol content in apoE-deficient mice that were not subjected to immunization (Control group;  $n = 25$ ), after immunization against TNF $\alpha$  (TNF $\alpha$  group;  $n = 23$ ), or after administration of Freund's adjuvant alone (FA group;  $n = 22$ ). Values are expressed as means  $\pm$  S.E.M. \*  $P < 0.01$  vs. control group.

#### 4. Discussion

Immunoinflammatory mechanisms involving TNF $\alpha$  are believed to play a dominant role in atherogenesis, and the current study demonstrates that immune activation by administration of FA alone suppresses the formation of mature atherosclerotic plaques in apoE-deficient mice, while adding specific immunization against TNF $\alpha$  contributes no further to the antiatherogenic effect of FA in this model.

In order to elicit a specific immune response, exogenous antigens are proteolytically processed by antigen presenting cells (e.g. macrophages), followed by exposure of antigen peptide fragments on the macrophage surface in association with major histocompatibility complex (MHC) class II molecules, which are then recognized by Th cells that provide help to relevant B cells leading to specific antibody formation. B cells can also act as antigen presenting cells by endocytosis of antigens via surface immunoglobulin receptors, with subsequent cell surface presentation of antigen peptide fragments by MHC class II molecules producing direct T cell help [24]. Self proteins are processed and presented the same way as foreign proteins, but because of T cell tolerance, this usually does not result in antibody formation [25]. However, it was recently demonstrated that B cell non-responsiveness toward self proteins could be overcome by immunization with a recombinant construct of the self protein with a foreign immunodominant Th epitope inserted into its sequence [13,14]. With use of this immunization strategy, it was shown that TNF106 was capable of eliciting a robust polyclonal antibody response against TNF $\alpha$  which neutralized binding of TNF $\alpha$  to its receptor and suppressed the biological effects of TNF $\alpha$  in mouse models of cachexia and autoimmune arthritis [15].

TNF $\alpha$  is synthesized by all cells found in atherosclerotic lesions, i.e. endothelial cells, macrophages, vascu-

lar smooth muscle cells, T lymphocytes, and mast cells, and may exert proatherogenic effects via its ability to promote, for example, monocyte recruitment, vascular smooth muscle cell proliferation, angiogenesis, continued production of cytokines and growth factors (e.g. macrophage colony-stimulating factor), vascular smooth muscle cell release of metalloproteinases, and apoptosis [2,6,7,26–30]. Importantly, TNF $\alpha$  promotes endothelial expression of intercellular adhesion molecule-1 (ICAM-1) [2], and in apoE-deficient mice, ICAM-1 is upregulated in lesion-prone arterial sites [31] and treatment with antibodies against ICAM-1 diminishes recruitment of macrophages to vascular lesions [32]. These actions have made TNF $\alpha$  a natural target for anticytokine therapy in atherosclerosis, and administration of a TNF $\alpha$  receptor homologue was recently shown to attenuate early atherosclerosis (i.e. fatty streak formation) in female, but not male, gonadectomized apoE-deficient mice fed an unphysiological, cholate-containing diet [12]. In the current study, however, it was found that induction of a strong autoantibody response against TNF $\alpha$  reduced the formation of advanced plaques in female apoE-deficient mice, but a similar effect was observed in animals immunized with FA alone (i.e. in the absence of detectable autoantibodies against TNF $\alpha$ ) indicating that the antiatherogenic effect was elicited by the adjuvant alone.

FA has previously been shown to induce [33] or inhibit [34] atherosclerosis in rabbit models and complete FA appears to promote nonspecific immune activation by a Th1 pattern of cytokine production that is primarily dependent on interferon- $\gamma$  [35,36], with the minimal structural requirement for FA activity being a muramyl dipeptide present in the mycobacterial wall [37]. In addition, FA can induce specific immune responses against mycobacterial antigens (e.g. HSPs and bacterial lipids resembling those present in oxidatively modified LDL) whereas other adjuvants may differentially modulate the immune response and functional diversity of Th cells (e.g. aluminium salts induce a Th2 type response) [35,36]. The Th cell repertoire was not examined in the current study, and the mechanisms responsible for the observed reduction of atherosclerosis by FA and the effects of immunization with TNF106 in adjuvant vehicles of non-biological origin remain to be determined.

TNF $\alpha$  has multiple endocrine effects and chronic overproduction of this cytokine induces a whole-body catabolic state (cachexia) [38]. Moreover, TNF $\alpha$  inhibits lipoprotein lipase, a key enzyme in the hydrolysis of triglycerides [39], and decreases the activity of macrophage scavenger receptors that mediate internalization of oxidized-LDL and subsequent foam cell formation [40]. Suppression of these activities by induction of neutralizing antibodies against TNF $\alpha$

could potentially promote atherogenesis, and, indeed, mice deficient in p55 TNF $\alpha$  receptors display increased macrophage scavenger activity and increased fatty streak formation when fed a high-fat diet [41]. On the other hand, TNF $\alpha$  is a candidate mediator of insulin resistance [42] and inhibition hereof may suppress atherogenesis. In the current study, body weights of apoE-deficient mice did not change significantly and only minor changes in plasma lipids were observed. There appears to be no substantial correlation between plasma lipoprotein levels and atherosclerotic lesion size in apoE-deficient mice [43] and these alterations are unlikely to have played an important role in the postimmunization reduction of atherosclerosis.

HSPs and oxidized LDL are present in human and experimental atherosclerotic plaques, where they are recognized by infiltrating T-cells and contribute to an autoantibody response that has been proposed as a marker of disease activity [3,5]. These antibodies are capable of eliciting proatherogenic cellular effects [3,44–46] and in the present study, a small increase in anti-HSP antibody titers was observed in mice immunized against TNF $\alpha$  but not in animals receiving FA alone whereas antibody titers against MDA-LDL were not significantly influenced by the immunization procedures. The observation that antibody titers against MDA-LDL and rhHSP60 were not significantly diminished in despite of the immunization-induced reduction of atherosclerosis would appear to indicate that these antibodies may not be valid markers of vascular disease activity in apoE-deficient mice. Interestingly, protection against atherosclerosis was recently observed in LDL-receptor deficient mice after immunization with MDA-LDL in FA, but a similar effect was found after immunization with native LDL, indicating in agreement with the present findings that the antiatherogenic effect of immunization was primarily dependent on the activation of the immune responses [47].

In conclusion, induction of a strong autoantibody response against TNF $\alpha$  in apoE-deficient mice by immunization with a recombinant TNF $\alpha$  antigen construct emulsified in FA adds no further to the reduction of mature plaque formation observed after administration of FA alone. Further studies are required to clarify the mechanisms underlying the antiatherogenic effects of FA in this model.

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