Chapter 6

TNFα and atherosclerosis

Tumor Necrosis Factor-α promotes atherosclerotic lesion progression in APOE*3-Leiden transgenic mice

Lianne S.M. Boesten[1,4]; A. Susanne M. Zadelaar[2,4]; Anita van Nieuwkoop[1,4]; Marion J.J. Gijbels[5,6]; Menno P.J. de Winther[5]; Louis M. Havekes[2,4]; Bart J.M. van Vlijmen[3]

[1]Department of General Internal Medicine, [2]Department of Cardiology, [3]Department of Hematology, Leiden University Medical Center, [4]TNO-PG/Gaubius Laboratory, Leiden, The Netherlands, [5]Department of Molecular Genetics, Cardiovascular Research Institute Maastricht, [6]Department of Pathology, Maastricht University, Maastricht, The Netherlands

ABSTRACT

Tumor Necrosis Factor-α (TNFα) is a pleiotropic cytokine exerting both inflammatory and cell death modulatory activity, and is thought to play a role in the pathogenesis of atherosclerosis. Studies in mice indicated that TNFα affects atherosclerosis minimally or not under conditions that allow fatty streak formation. Here, we examined the possible role of TNFα in advanced and complex atherosclerotic lesions.

To induce atherosclerosis, TNFα-deficient (*Tnf-/-*) APOE*3-Leiden and control APOE*3-Leiden only mice were fed a cholesterol-rich diet. Comparable levels of plasma cholesterol and triglycerides and the systemic inflammatory parameters, serum amyloid A and soluble intercellular adhesion molecule-1 were found in APOE*3-Leiden*Tnf-/-* and control mice. Although absence of TNFα did not affect the quantitative area of atherosclerosis, APOE*3-Leiden*Tnf-/-* mice had a higher relative number of early lesions (46.1% vs. 21.4%) and a lower relative number of advanced lesions (53.9% vs. 78.6%, P=0.04). In addition, the advanced lesions in APOE*3-Leiden*Tnf-/-* mice showed less necrosis (9.9±12.1% vs. 23.4±19.3% of total lesion area, P=0.04) and an increase in apoptosis (1.5±1.5% vs. 0.4±0.6% of total nuclei, P=0.03).

Our data indicate that TNFα stimulates the formation of lesions towards an advanced phenotype, with more lesion necrosis and a lower incidence of apoptosis.

Inflammatory processes are involved in all stages of atherosclerotic lesion development.[1,2] Cytokine-mediated pro-inflammatory responses are thought to positively contribute to the atherogenic process. Tumor Necrosis Factor-α (TNFα) is a central mediator of inflammatory reactions.[3] TNFα is a member of the TNF ligand family and binding to its receptors TNFR1 (p55) and TNFR2 (p75) leads to activation of downstream targets.[4] Whereas binding of TNFα to TNFR1 activates responses associated with induction of adhesion molecule expression,[5] apoptosis,[6] and resistance to bacterial infection,[7,8] binding to TNFR2 activates induction of T cell proliferation,[9] induction of TNFα-mediated skin tissue necrosis,[10] and modulation of TNFα-mediated pulmonary inflammation.[11]

Although TNFα and its receptors are thought to be considerably important in a number of biological activities relevant to atherosclerosis, its function in atherogenesis remains unclear. The TNFα gene has a number of polymorphisms, some of which affect transcription and secretion.[12] These polymorphisms have been associated with a number of infectious, inflammatory and immune diseases including atherosclerosis and coronary artery disease (CHD).[12] However, human association studies are controversial varying from no,[12-14] a weak[15] or a strong[16] association between TNFα polymorphisms and CHD. Moreover, studies on the role of TNFα in atherosclerosis using several transgenic or knock out mouse models also yielded controversial results. TNFα ligand deficiency on a wild type C57BL/6 background did not affect early lesion development.[17] However, on the same background, TNF receptor 1 deficiency did affect atherosclerosis formation, resulting in enhanced (early) lesion formation. This unexpected result was attributed to increased macrophage scavenger receptor activity and consequent increased uptake of atherogenic lipoproteins.[18] Very recently, as a part of mouse studies on a non-cleavable transmembrane form of TNFα, TNFα-deficiency on a C57BL/6 background was demonstrated to reduce atherosclerosis.[19] This effect however coincided with a significant decrease in plasma VLDL and increase in plasma HDL levels. Hence, a direct anti-atherosclerotic effect of TNFα deficiency could not be concluded from this experiment. Overall, the above recombinant mouse studies suggest that TNFα may have subtle pro-atherogenic properties, possibly related to an unexpected TNFα-mediated effect on atherogenic lipoproteins, at least in a setting of a C57BL/6 background.

Variable results were also obtained upon (pharmacological) modulation of TNFα expression. Specific immunization against TNFα was not effective in preventing the formation of advanced lesions in apoE-deficient mice.[20] Capturing TNFα using a TNFα receptor homologue had only a modest and gender-dependent inhibitory effect on the formation of early lesions in the apoE-deficient setting.[21] On the contrary thalidomide, a TNFα production inhibitor, was capable of inhibiting the early atherogenesis in apoE-deficient mice.[22]

The majority of these mouse studies focuses on the role of TNFα in early lesion development in relatively atherosclerosis-resistant C57BL/6 mice[17-19] and young apoE-deficient mice[20,22] that display only minimal advanced lesion formation. Inflammation and cell death are important processes in the development and transition towards advanced and complex atherosclerotic lesions and may be modulated by TNFα. Therefore, we examined the role TNFα under conditions of advanced lesion formation. To this end, we crossbred TNFα deficient (*Tnf-/-*) mice with APOE*3-

Leiden transgenic mice. APOE*3-Leiden is a mutant form of apoE, characterized by a 7-amino acid tandem repeat of residues 120-126 and yields a mature protein of 306 amino acid residues. Family studies have demonstrated that this mutation is associated with a dominantly inherited form of familial dysbetalipoproteinemia (FD).[23-25] APOE*3-Leiden transgenic mice have a lipoprotein profile that is very similar to the profile of patients with FD in which the elevated plasma cholesterol and triglyceride levels are mainly confined to the VLDL/LDL-sized lipoprotein fractions. Therefore, APOE*3-Leiden transgenic mice are an established model for hyperlipidemia and atherosclerosis.[26] We investigated the effect of TNFα on advanced lesion formation and on lesion composition. Our data indicate that TNFα, independent of the atherogenic lipoprotein profile, stimulates lesional necrosis, decreases the incidence of apoptosis and the progression of lesions towards a more advanced phenotype.

METHODS

Mice and diet

The experimental animals were obtained by crossbreeding *Tnf-/-* mice[27] with APOE*3-Leiden transgenic mice.[28] Offspring was analyzed for their TNFα status using polymerase chain reaction (PCR) analysis and presence of the human APOE*3-Leiden transgene by ELISA.[29]

For experiments, 8 weeks old female APOE*3-Leiden*Tnf-/-* and littermate control APOE*3-Leiden*Tnf*+/+ mice were used. Mice (n=31) were fed a semi-synthetic cholesterol-rich diet composed essentially according to Nishina et al.[30] supplemented with cocoa butter (15%, by weight) and cholesterol (1%, by weight), without dietary cholate. The diet was purchased from Hope Farms, Woerden, The Netherlands. Mice were bred and housed under specific pathogen-free conditions and given food and water ad libitum. All animal work was approved by institutional regulatory authority and carried out in compliance with guidelines issued by the Dutch government. The investigation conforms 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).

Blood sampling and analysis

Blood samples were collected in EDTA-coated vials (Sarstedt, Nümbrecht, Germany) by bleeding from the tail vein. Plasma cholesterol and triglyceride levels were measured enzymatically using commercially available kits (Roche Diagnostics GmbH, Mannheim, Germany).

Total blood leukocyte ($CD45^+$), T-cell ($CD3^+$), B-cell ($CD19^+$) and monocyte/granulocyte ($CD11b^+$) numbers were determined by FACS analysis (FACSCalibur, BD Biosciences, California, USA) of whole blood using a PerCP-CY5.5-conjugated rat anti-mouse CD45 monoclonal antibody, a fluorescein isothiocyanate (FITC)-conjugated hamster anti-mouse CD3 monoclonal antibody, a R-Phycoerythrin (R-PE) conjugated rat anti-mouse CD19 monoclonal antibody, and an allophycocyanin (APC)-conjugated rat anti-mouse CD11b monoclonal antibody, respectively following standard protocol (TruCOUNT, BD Biosciences, California, USA).

Serum Amyloid A (SAA, acute phase protein) ELISA (BioSource International, Inc, Camarillo, CA) and Mouse soluble Intercellular Adhesion Molecule-1 (sICAM-1, marker for endothelial cell activation) ELISA (Pierce Biotechnology, Inc, Rockford, IL) were performed on plasma samples according to standard protocols.[31]

Atherosclerosis analysis

After 20 weeks of a cholesterol-rich diet, mice were sacrificed, heart and aorta were isolated and embedded in tissue tek (Sakura Finetek Europe BV, Zoeterwoude, The Netherlands) and stored at -80˚C. From the entire aortic root area of the heart, four 7 µm cross-sections with an interval of 42 µm were used for quantification of atherosclerotic lesion area.[32] Sections were routinely stained with toluidin blue for morphometric analysis, characterization and categorization of the lesions, and with Sirius red for quantification of collagen area. Lesion area was determined using Leica Qwin image software (EIS, Asbury, NJ). All analyses were performed blindly without prior knowledge of the genotype.

Atherosclerotic lesions were categorized on severity. Two types of categories were discerned: (1) early lesions were fatty streaks containing only foam cells (type I-II lesions) and (2) advanced lesions showed foam cells in the media and presence of fibrosis, cholesterol clefts, mineralization and/or necrosis covered by a collagenous cap (type III-V lesions).[29] The number observed in each category is expressed as a percentage of the total number of lesions observed within one group of mice (APOE*3-Leiden*Tnf-/-* or APOE*3-Leiden*Tnf*+/+ control group).

Sections were stained for macrophages using rat FA11 antibody to mouse macrophages (a kind gift from S. Gordon, Oxford University, UK), for smooth muscle cells using a monoclonal α-smooth muscle cell actin antibody (Sigma-Aldrich, St. Louis, USA), for T-cells using KT3 rat antibody (a kind gift from G. Kraal, VUMC, The Netherlands), and areas were quantified as described previously.[33,34] For the detailed phenotypic sub-analysis of the advanced lesions (macrophage area, SMC area, collagen area and the number of T-cells) only those mice were included that displayed advanced atherosclerotic lesions (APOE*3-Leiden*Tnf*+/+ n=13, APOE*3-Leiden-*Tnf-/-* n=14)

Quantification of lesion necrosis and apoptosis

During pathological examination of the lesions, lesional necrosis was defined by the presence of pyknosis, karyorrhexis, or complete absence of nuclei, as described before.[33] Necrosis area was measured using the method for total lesion area measurements, as described above. Apoptosis was quantified using the TUNEL technique.[35] Only those TUNEL-positive nuclei were included that displayed morphological features of apoptosis including cell shrinkage, aggregation of chromatin into dense masses, and nuclear fragmentation. For the detailed phenotypic sub-analysis of the advanced lesions, on lesion necrosis and apoptosis, only those mice were included that displayed advanced atherosclerotic lesions (APOE*3-Leiden*Tnf*+/+ n=13, APOE*3-Leiden*Tnf-/-* n=14).

Statistical analysis

Data were analyzed using GraphPad Prism (GraphPad Software Inc., San Diego, California, USA). Data were tested for normality using the Kolmogorov-Smirnov test.

All data following normality were tested using Welch's corrected *t*-test. Data are expressed as mean±SD, unless stated otherwise. *P*-value < 0.05 was regarded as significant. Frequency data for lesion categorization were compared by means of the Fisher's exact test.

RESULTS

General

Female APOE*3-Leiden*Tnf-/-* (n=18) mice and APOE*3-Leiden*Tnf+/+* (n=13) littermate controls were fed a cholesterol-rich diet. The mice appeared healthy and displayed no signs of abnormalities. After 20 weeks feeding the cholesterol-rich diet, APOE*3-Leiden*Tnf-/-* mice were significantly lower in body weight as compared to APOE*3-Leiden*Tnf+/+* control mice (Table 1).

Following cholesterol-rich diet feeding, both APOE*3-Leiden*Tnf-/-* and control mice had comparable levels of plasma cholesterol and plasma triglyceride levels (Table 1). As determined by FPLC analysis on pooled plasma, lipoprotein distribution did not differ between APOE*3-Leiden*Tnf-/-* and APOE*3-Leiden*Tnf+/+* control mice (Figure 1).

Analysis of $CD45^+$ blood leukocytes showed that absence of TNFα resulted in an approximate doubling of circulating $CD3^+$ (P=0.01) and $CD19^+$ (P=0.06) cells (T- and B-cells, respectively), leaving the $CD11b^+$ cell population (monocytes/granulocytes) unaffected (Table 1).

Plasma levels of serum amyloid A (SAA) and soluble intercellular adhesion molecule-1 (sICAM-1) were comparable in APOE*3-Leiden*Tnf-/-* and APOE*3-Leiden*Tnf+/+* control mice indicating no difference in inflammation (SAA) or endothelial cell activation (sICAM) between the two groups (Table 1).

Table 1. Characteristics of female APOE*3-Leiden*Tnf+/+* and APOE*3-Leiden*Tnf-/-* mice after 20 weeks feeding a cholesterol-rich diet

Parameters	E3L*Tnf+/+*	E3L*Tnf-/-*
Weight (g)	25.7±2.6	22.6±1.5*
Plasma cholesterol (mmol/l)	21.7 ± 3.8	19.4 ± 4.2
Plasma triglycerides (mmol/l)	2.0 ± 0.7	2.3 ± 1.5
$CD3^+$ (10^6 cells/ml)	1.8 ± 0.6	3.2 ± 1.0*
$CD19^+$ (10^6 cells/ml)	3.8 ± 2.2	6.1 ± 2.0
$CD11b^+$ (10^6 cells/ml)	2.6 ± 0.9	2.7 ± 1.5
SAA (μg/ml)	16.0±4.5	12.8±28.4
sICAM (μg/ml)	23.8±2.1	23.6±2.0

* P<0.05, as compared to APOE*3-Leiden*Tnf+/+* control mice

Atherosclerosis analysis

Mice fed the cholesterol-rich diet for 20 weeks were sacrificed for collection of heart and aorta. Histopathological analysis of atherosclerotic lesions of the aortic valve area in both APOE*3-Leiden*Tnf-/-* and control mice revealed that the majority

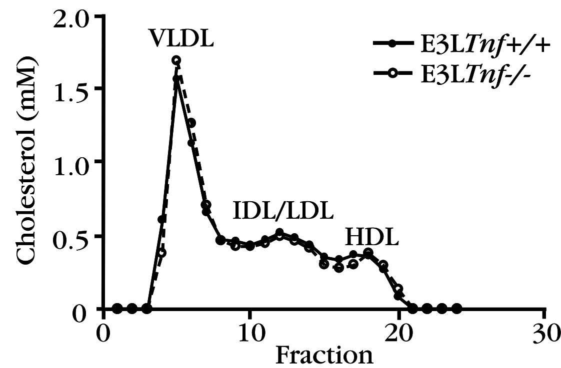

Figure 1. Plasma lipoprotein profile after size fractionation of pooled plasma samples of APOE*3-Leiden*Tnf*+/+ (closed symbols) and APOE*3-Leiden*Tnf*-/- (open symbols) after 20 weeks of feeding the cholesterol-rich diet.

of the lesions were fibrous plaques with a lipid core and a cap covering necrotic material (pyknosis, karyorrhexis, or complete absence of nuclei), cholesterol clefts and extra-cellular lipids. As determined by computer-assisted morphometry, mean total lesion area did not differ significantly between APOE*3-Leiden*Tnf*+/+ and APOE*3-Leiden*Tnf*-/- mice (5.8±2.8 and 5.1±4.5x104 μm^2, respectively, *P*=0.64, Figure 2A). While a difference in total lesion area was absent, lesion categorization (early vs. advanced) revealed a difference between APOE*3-Leiden*Tnf*-/- and control mice (Figure 2B). APOE*3-Leiden*Tnf*-/- mice had a significant higher incidence of early lesions (46.1% vs. 21.4%) and a significant decreased incidence of advanced lesions (53.9% vs. 78.6%) as compared to APOE*3-Leiden*Tnf*+/+ controls (*P*=0.04).

A more detailed phenotypic sub-analysis analysis of the advanced lesions only, did not yield any effect of TNFα status on lesion macrophage area, lesion smooth muscle cell area or collagen area. In addition, the number of lesional T cells did not differ between the groups (Table 2). However, pathological analysis revealed that advanced lesions in APOE*3-Leiden*Tnf*-/- mice had smaller areas of pyknosis, karyor-

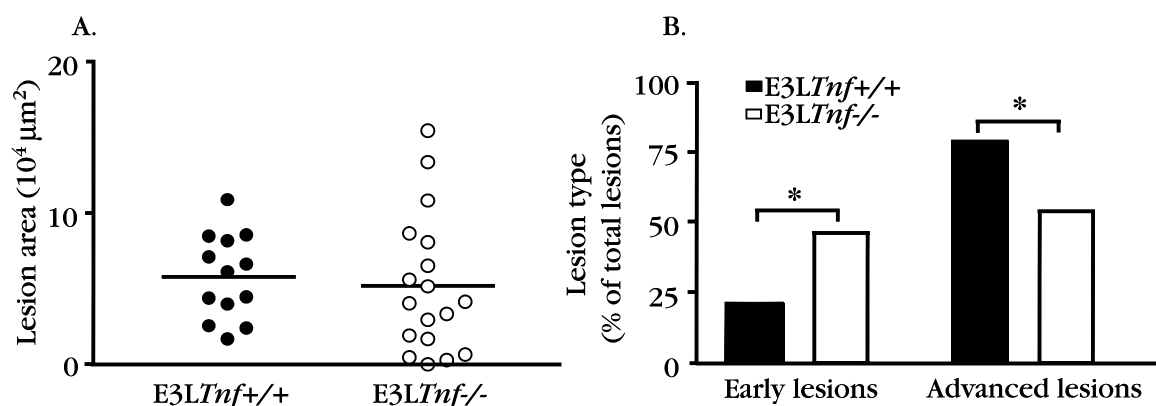

Figure 2. (A.) After 20 weeks of feeding the cholesterol-rich diet, the extent of atherosclerosis in APOE*3-Leiden*Tnf*+/+ (closed symbols, n=13) and APOE*3-Leiden*Tnf*-/- (open symbols, n=18) mice was quantified at the level of the aortic valves. Each data point represents the lesion area per mouse. Line represents mean area for each group. (B.) Lesion categorization of APOE*3-Leiden*Tnf*+/+ (black bars) and APOE*3-Leiden*Tnf*-/- (white bars) mice. Lesions were categorized according to severity and are shown as percentage of the total number of lesions present. Frequency data were compared by means of the Fisher's exact test, *P*=0.04.

Table 2. Characteristics of advanced atherosclerotic lesions in APOE*3-Leiden*Tnf*+/+ and APOE*3-Leiden *Tnf*-/- mice

Lesion composition	**E3L*Tnf*+/+**	**E3L*Tnf*-/-**
Lesion macrophage area (% of total lesion area)	39.0±14.0	38.7±18.3
Lesion SMC area (% of total lesion area)	9.0±5.6	10.9±7.1
Collagen area (% of total lesion area)	73.5±21.9	78.9±13.9
T-cells (number of cells/total lesion area)	1.8±1.1	1.3±1.4

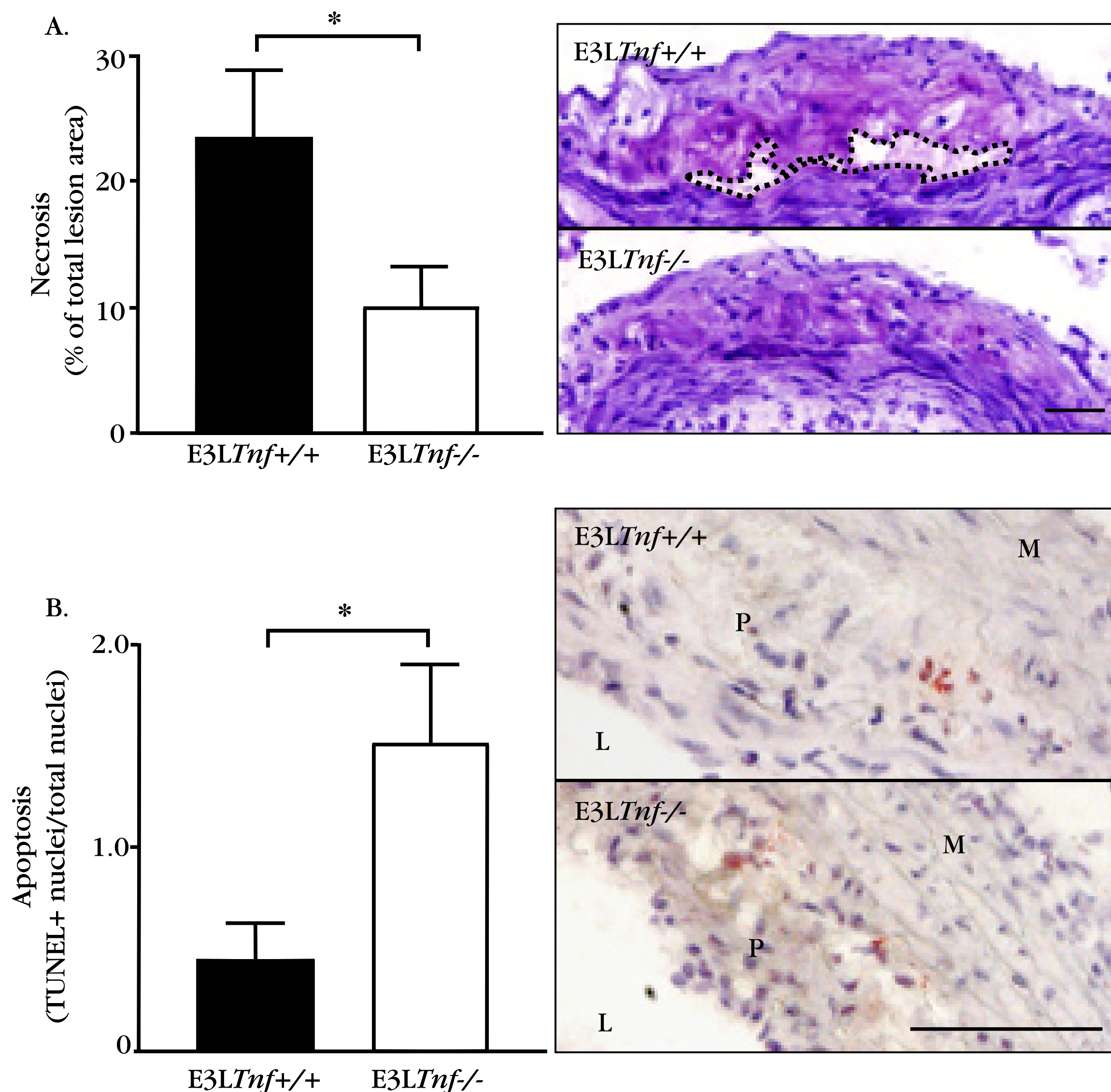

Figure 3. (A.) Necrosis (*P*=0.04) and (B.) Apoptosis (*P*=0.03) in advanced lesions of APOE*3-Leiden*Tnf*+/+ (black bar, n=13) and APOE*3-Leiden*Tnf*-/- (white bar, n=14). Error bars indicate SEM. Inserts: representative pictures of advanced lesions in APOE*3-Leiden*Tnf*+/+ and APOE*3-Leiden*Tnf*-/- mice. (A.) The black dotted line indicates the necrotic area (magn. 40x, scale bar 100 μm). (B.) The arrows indicate TUNEL-positive nuclei. L: lumen, P: plaque, M: media, (magn 100x, scale bar 100 μm).

rhexis, or complete absence of nuclei as compared to advanced lesions in control mice, suggesting that the TNFα-status affects lesional necrosis. The early atherosclerotic lesions did not contain any necrotic area in both groups. Computer-assisted morphometric analysis demonstrated that advanced lesions in APOE*3-Leiden*Tnf-/-* mice had significant smaller areas covered by necrosis as compared to advanced lesions in controls (9.9±12.1% vs. 23.4±19.3% of total lesion area, P=0.04, Figure 3A).

TUNEL-positive nuclei (apoptotic cells) were only found in advanced lesions of both APOE*3-Leiden*Tnf-/-* and control mice. The location of the TUNEL-positive nuclei (i.e. core or cap) did not differ between APOE*3-Leiden*Tnf-/-* and control mice (data not shown). In addition, the majority of the TUNEL-positive nuclei was located in the core of the advanced lesions, in both APOE*3-Leiden*Tnf-/-* and control mice. Remarkably, the incidence of TUNEL-positive nuclei in advanced lesions of APOE*3-Leiden*Tnf-/-* mice was 3.4-fold higher as observed for lesions in control mice (1.5±1.5% vs. 0.4±0.6% of total number of cells, P=0.03, Figure 3B). Hence, in APOE*3-Leiden*Tnf-/-* mice a decrease in lesional necrosis coincides with increased apoptosis.

DISCUSSION

In the present study we examined the role of TNFα in the formation of advanced lesions. Using atherosclerosis-susceptible APOE*3-Leiden mice crossbred with TNFα-deficient mice we found that TNFα modulates lesional cell death by increasing necrosis and decreasing the incidence of apoptosis. Moreover, TNFα progresses the lesions towards a more advanced phenotype.

Very recently, Branén et al. also showed that TNFα stimulates atherosclerosis development using apoE/*Tnf* knock out mice.[36] ApoE deficiency affects local cholesterol homeostasis in macrophages and inflammatory reactions within the atherosclerotic vessel.[37] In the current study the role of TNFα was assessed specifically in advanced atherosclerotic lesion development on an APOE*3-Leiden background (with endogenous apoE still present) preventing the possible local effects due to the absence of apoE. Importantly, besides the differences in study-design, both studies demonstrate that TNFα can be considered a pro-atherogenic cytokine in atherosclerosis development.

Inflammation and cell death are important processes in the development and transition towards advanced and complex atherosclerotic lesions.[38-40] Our observation that TNFα promotes advanced lesion formation, and on top of that increases the extent of necrosis in these lesions, is in line with the role of TNFα function in modulating inflammatory processes and cell death.[3,4,6-8,10,11]

Studies on human endarterectomy specimens showed that unstable atherosclerotic lesions are characterized by a necrotic core consisting of dead macrophages, macrophage debris and extracellular lipid covered by a fibrocellular cap.[41] Therefore, necrosis is often considered to be a negative risk factor for plaque stability. The observation that TNFα increases the ratio of necrosis versus apoptosis suggests that TNFα modulates the lesion towards a more instable phenotype. Moreover, *in vitro* studies have shown that TNFα stimulates both macrophages and smooth muscle cells to synthesize matrix proteases[42,43] and in such a way can contribute to plaque instability by degrading the fibrotic cap. Hence, both through increased necrosis

and increased matrix protease activation TNFα may contribute to the formation of instable plaques that are prone to rupture.

The reduction in lesional necrosis upon TNFα deletion coincided with an increase in lesional apoptosis. TNFα is known to exert its effector actions, partially, through the activation of the pro-inflammatory transcription factor, NF-κB.[4] Absence of TNFα may promote apoptotic cell death via a reduction in NF-κB activation. Reduced NF-κB activation does not only lead to a beneficial reduced transcription of pro-inflammatory cytokines and chemokines, but also to a reduction in transcription of anti-apoptotic factors (i.e. Bcl-xL, Bcl-2, IAPs).[44] Apoptosis is often considered to be beneficial for atherosclerosis, since in contrast to necrosis, it may prevent the release of matrix-degrading enzymes and pro-inflammatory substances from the dying foam cells. Our data suggest that apoptosis forms part of a beneficial process since it coincides with a less advanced lesion phenotype.

Uysal et al. demonstrated that the total weight gain in obese *Tnf+/+* mice on a high fat diet was larger than that of obese *Tnf-/-* mice, which was related to a decrease in fat-pad weight.[45] Also our APOE*3-Leiden mice, fed a cholesterol-rich diet, that additionally contained 15% fat, displayed reduced body weight upon TNFα deletion (Table 1). Hence, our data support a role for TNFα in body weight development. Although body weight is not shown to be a predictive parameter for atherosclerosis development in mice,[46,47] it remains open to question whether TNFα-mediated reduction in body weight contributes to the observed TNFα-mediated reduction in advanced lesion formation.

Upon characterization of the TNFα deficient mice Marino et al. reported that these mice have no significant abnormalities in the distribution of lymphocytes, granulocytes or monocyte population in thymus, spleen or peripheral blood.[48] Remarkably, we found an increase in circulating T-cells in TNFα deficient mice, that was independent of the diet and of the APOE*3-Leiden transgene (data not shown). This suggests that TNFα might play a role in determining circulating T-cells in mice already under basal conditions. In this light it is worthwhile to mention that the higher levels of circulating T-cells did not translate to a higher number of T-cells in the advanced atherosclerotic plaque.

In conclusion, we have demonstrated that TNFα enhances progression of lesions towards a more advanced phenotype. One may speculate that drugs that inhibit TNFα expression or capture TNFα biological action may inhibit advanced lesion formation. Future studies are required to demonstrate whether, next to a reduction in early lesions,[21,22] TNFα can be used as a target to prevent the formation of advanced lesions.

ACKNOWLEDGEMENTS

This work was supported by the Netherlands Heart Foundation (NHS 2000.051), the Netherlands Organization for Scientific Research (NWO 902-26-242), M.P.J. de Winther is a NWO-fellow (906-02-075), and B.J.M. van Vlijmen is a fellow of the Royal Netherlands Academy of Arts and Sciences. The authors wish to thank Ingeborg van der Made for her technical assistance.

REFERENCES

1. Ross R. Atherosclerosis is an inflammatory disease. Am Heart J 1999;138:S419-S420.
2. Wick G, Schett G, Amberger A, Kleindienst R, Xu Q. Is atherosclerosis an immunologically mediated disease? Immunol Today 1995;16:27-33.
3. Camussi G, Albano E, Tetta C, Bussolino F. The molecular action of tumor necrosis factor-alpha. Eur J Biochem 1991;202:3-14.
4. Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. Science 1998;281:1305-1308.
5. Mackay F, Loetscher H, Stueber D, Gehr G, Lesslauer W. Tumor necrosis factor alpha (TNF-alpha)-induced cell adhesion to human endothelial cells is under dominant control of one TNF receptor type, TNF-R55. J Exp Med 1993;177:1277-1286.
6. Tartaglia LA, Ayres TM, Wong GH, Goeddel DV. A novel domain within the 55 kd TNF receptor signals cell death. Cell 1993;74:845-853.
7. Pfeffer K, Matsuyama T, Kundig TM, Wakeham A, Kishihara K, Shahinian A, Wiegmann K, Ohashi PS, Kronke M, Mak TW. Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to L. monocytogenes infection. Cell 1993;73:457-467.
8. Rothe J, Lesslauer W, Lotscher H, Lang Y, Koebel P, Kontgen F, Althage A, Zinkernagel R, Steinmetz M, Bluethmann H. Mice lacking the tumour necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by Listeria monocytogenes. Nature 1993;364:798-802.
9. Tartaglia LA, Goeddel DV, Reynolds C, Figari IS, Weber RF, Fendly BM, Palladino MA, Jr. Stimulation of human T-cell proliferation by specific activation of the 75-kDa tumor necrosis factor receptor. J Immunol 1993;151:4637-4641.
10. Erickson SL, de Sauvage FJ, Kikly K, Carver-Moore K, Pitts-Meek S, Gillett N, Sheehan KC, Schreiber RD, Goeddel DV, Moore MW. Decreased sensitivity to tumour-necrosis factor but normal T-cell development in TNF receptor-2-deficient mice. Nature 1994;372:560-563.
11. Peschon JJ, Torrance DS, Stocking KL, Glaccum MB, Otten C, Willis CR, Charrier K, Morrissey PJ, Ware CB, Mohler KM. TNF receptor-deficient mice reveal divergent roles for p55 and p75 in several models of inflammation. J Immunol 1998;160:943-952.
12. Allen RA, Lee EM, Roberts DH, Park BK, Pirmohamed M. Polymorphisms in the TNF-alpha and TNF-receptor genes in patients with coronary artery disease. Eur J Clin Invest 2001;31:843-851.
13. Koch W, Kastrati A, Bottiger C, Mehilli J, von Beckerath N, Schomig A. Interleukin-10 and tumor necrosis factor gene polymorphisms and risk of coronary artery disease and myocardial infarction. Atherosclerosis 2001;159:137-144.
14. Herrmann SM, Ricard S, Nicaud V, Mallet C, Arveiler D, Evans A, Ruidavets JB, Luc G, Bara L, Parra HJ, Poirier O, Cambien F. Polymorphisms of the tumour necrosis factor-alpha gene, coronary heart disease and obesity. Eur J Clin Invest 1998;28:59-66.
15. Keso T, Perola M, Laippala P, Ilveskoski E, Kunnas TA, Mikkelsson J, Penttila A, Hurme M, Karhunen PJ. Polymorphisms within the tumor necrosis factor locus and prevalence of coronary artery disease in middle-aged men. Atherosclerosis 2001;154:691-697.
16. Vendrell J, Fernandez-Real JM, Gutierrez C, Zamora A, Simon I, Bardaji A, Ricart W, Richart C. A polymorphism in the promoter of the tumor necrosis factor-alpha gene (-308) is associated with coronary heart disease in type 2 diabetic patients. Atherosclerosis 2003;167:257-264.
17. Schreyer SA, Vick CM, LeBoeuf RC. Loss of lymphotoxin-alpha, but not tumor necrosis factor-alpha reduces atherosclerosis in mice. J Biol Chem 2002;277(14):12364-8.
18. Schreyer SA, Peschon JJ, LeBoeuf RC. Accelerated atherosclerosis in mice lacking tumor necrosis factor receptor p55. J Biol Chem 1996;271:26174-26178.
19. Canault M, Peiretti F, Mueller C, Kopp F, Morange P, Rihs S, Portugal H, Juhan-Vague I, Nalbone G. Exclusive expression of transmembrane TNF-alpha in mice reduces the inflammatory response in early lipid lesions of aortic sinus. Atherosclerosis

2004;172:211-218.
20. Hansen PR, Chew M, Zhou J, Daugherty A, Heegaard N, Jensen P, Mouritsen S, Falk E. Freunds adjuvant alone is antiatherogenic in apoE-deficient mice and specific immunization against TNFalpha confers no additional benefit. Atherosclerosis 2001;158:87-94.
21. Elhage R, Maret A, Pieraggi MT, Thiers JC, Arnal JF, Bayard F. Differential effects of interleukin-1 receptor antagonist and tumor necrosis factor binding protein on fatty-streak formation in apolipoprotein E-deficient mice. Circulation 1998;97:242-244.
22. Chew M, Zhou J, Daugherty A, Eriksson T, Ellermann-Eriksen S, Hansen PR, Falk E. Thalidomide inhibits early atherogenesis in apoE-deficient mice. APMIS Suppl 2003;113-116.
23. Havekes L, de Wit E, Leuven JG, Klasen E, Utermann G, Weber W, Beisiegel U. Apolipoprotein E3-Leiden. A new variant of human apolipoprotein E associated with familial type III hyperlipoproteinemia. Hum Genet 1986;73:157-163.
24. van den Maagdenberg AM, de Knijff P, Stalenhoef AF, Gevers Leuven JA, Havekes LM, Frants RR. Apolipoprotein E*3-Leiden allele results from a partial gene duplication in exon 4. Biochem Biophys Res Commun 1989;165:851-857.
25. Wardell MR, Weisgraber KH, Havekes LM, Rall SC, Jr. Apolipoprotein E3-Leiden contains a seven-amino acid insertion that is a tandem repeat of residues 121-127. J Biol Chem 1989;264:21205-21210.
26. Groot PH, van Vlijmen BJ, Benson GM, Hofker MH, Schiffelers R, Vidgeon-Hart M, Havekes LM. Quantitative assessment of aortic atherosclerosis in APOE*3 Leiden transgenic mice and its relationship to serum cholesterol exposure. Arterioscler Thromb Vasc Biol 1996;16:926-933.
27. Pasparakis M, Alexopoulou L, Episkopou V, Kollias G. Immune and inflammatory responses in TNF alpha-deficient mice: a critical requirement for TNF alpha in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response. J Exp Med 1996;184:1397-1411.
28. van den Maagdenberg AM, Hofker MH, Krimpenfort PJ, de B, I, van Vlijmen B, van der BH, Havekes LM, Frants RR. Transgenic mice carrying the apolipoprotein E3-Leiden gene exhibit hyperlipoproteinemia. J Biol Chem 1993;268:10540-10545.
29. van Vlijmen BJ, van den Maagdenberg AM, Gijbels MJ, van der BH, HogenEsch H, Frants RR, Hofker MH, Havekes LM. Diet-induced hyperlipoproteinemia and atherosclerosis in apolipoprotein E3-Leiden transgenic mice. J Clin Invest 1994;93:1403-1410.
30. Nishina PM, Verstuyft J, Paigen B. Synthetic low and high fat diets for the study of atherosclerosis in the mouse. J Lipid Res 1990;31:859-869.
31. Horie Y, Chervenak RP, Wolf R, Gerritsen ME, Anderson DC, Komatsu S, Granger DN. Lymphocytes mediate TNF-alpha-induced endothelial cell adhesion molecule expression: studies on SCID and RAG-1 mutant mice. J Immunol 1997;159:5053-5062.
32. Paigen B, Morrow A, Holmes PA, Mitchell D, Williams RA. Quantitative assessment of atherosclerotic lesions in mice. Atherosclerosis 1987;68:231-240.
33. Kanters E, Pasparakis M, Gijbels MJ, Vergouwe MN, Partouns-Hendriks I, Fijneman RJ, Clausen BE, Forster I, Kockx MM, Rajewsky K, Kraal G, Hofker MH, de Winther MP. Inhibition of NF-kappaB activation in macrophages increases atherosclerosis in LDL receptor-deficient mice. J Clin Invest 2003;112:1176-1185.
34. van Vlijmen BJ, Gerritsen G, Franken AL, Boesten LS, Kockx MM, Gijbels MJ, Vierboom MP, van Eck M, van De WB, van Berkel TJ, Havekes LM. Macrophage p53 deficiency leads to enhanced atherosclerosis in APOE*3-Leiden transgenic mice. Circ Res 2001;88:780-786.
35. Kockx MM, Muhring J, Knaapen MW, De Meyer GR. RNA synthesis and splicing interferes with DNA in situ end labeling techniques used to detect apoptosis. Am J Pathol 1998;152:885-888.
36. Branen L, Hovgaard L, Nitulescu M, Bengtsson E, Nilsson J, Jovinge S. Inhibition of

Tumor Necrosis Factor-{alpha} Reduces Atherosclerosis in Apolipoprotein E Knockout Mice. Arterioscler Thromb Vasc Biol 2004;24(11):2137-42.
37. Curtiss LK, Boisvert WA. Apolipoprotein E and atherosclerosis. Curr Opin Lipidol 2000;11:243-251.
38. Geng YJ, Libby P. Progression of atheroma: a struggle between death and procreation. Arterioscler Thromb Vasc Biol 2002;22:1370-1380.
39. Libby P. Inflammation in atherosclerosis. Nature 2002;420:868-874.
40. Libby P. Vascular biology of atherosclerosis: overview and state of the art. Am J Cardiol 2003;91:3A-6A.
41. Tabas I. Apoptosis and plaque destabilization in atherosclerosis: the role of macrophage apoptosis induced by cholesterol. Cell Death Differ 2004;11 Suppl 1:S12-S16.
42. Galis ZS, Muszynski M, Sukhova GK, Simon-Morrissey E, Unemori EN, Lark MW, Amento E, Libby P. Cytokine-stimulated human vascular smooth muscle cells synthesize a complement of enzymes required for extracellular matrix digestion. Circ Res 1994;75:181-189.
43. Galis ZS, Muszynski M, Sukhova GK, Simon-Morrissey E, Libby P. Enhanced expression of vascular matrix metalloproteinases induced in vitro by cytokines and in regions of human atherosclerotic lesions. Ann N Y Acad Sci 1995;748:501-507.
44. Karin M, Ben Neriah Y. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. Annu Rev Immunol 2000;18:621-663.
45. Uysal KT, Wiesbrock SM, Marino MW, Hotamisligil GS. Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. Nature 1997;389:610-614.
46. Lyngdorf LG, Gregersen S, Daugherty A, Falk E. Paradoxical reduction of atherosclerosis in apoE-deficient mice with obesity-related type 2 diabetes. Cardiovasc Res 2003;59:854-862.
47. Schreyer SA, Lystig TC, Vick CM, LeBoeuf RC. Mice deficient in apolipoprotein E but not LDL receptors are resistant to accelerated atherosclerosis associated with obesity. Atherosclerosis 2003;171:49-55.
48. Marino MW, Dunn A, Grail D, Inglese M, Noguchi Y, Richards E, Jungbluth A, Wada H, Moore M, Williamson B, Basu S, Old LJ. Characterization of tumor necrosis factor-deficient mice. Proc Natl Acad Sci U S A 1997;94:8093-8098.