Tumor Necrosis Factor-α Does Not Mediate Diabetes-Induced Vascular Inflammation in Mice

Jenny Nilsson-Öhman, Gunilla Nordin Fredrikson, Lisa M. Nilsson-Berglund, Carin Gustavsson, Eva Bengtsson, Maj-Lis Smith, Carl-David Agardh, Elisabet Agardh, Stefan Jovinge, Maria F. Gomez, Jan Nilsson

Objective—Vascular inflammation is a key feature of both micro- and macrovascular complications in diabetes. Several lines of evidence have implicated the cytokine tumor necrosis factor (TNFα) as an important mediator of inflammation in diabetes. In the present study we evaluated the role of TNFα in streptozotocin (STZ)-induced diabetes on vascular inflammation in C57BL/6 wild-type and apoE−/− mice.

Methods and Results—Diabetes increased the expression of vascular cell adhesion molecule (VCAM)-1 in cerebral arteries >150 μm in diameter as well as the macrophage accumulation in aortic root atherosclerotic plaques in apoE−/− mice. A more pronounced vascular inflammatory response was observed in diabetic TNFα-deficient apoE−/− mice. These mice were also characterized by increased accumulation of IgG and IgM autoantibodies in atherosclerotic lesions. Diabetes also increased VCAM-1 expression and plaque formation in apoE-competent TNFα−/− mice, whereas no such effects were observed in C57BL/6 wild-type mice.

Conclusions—The present findings suggest that TNFα does not mediate diabetic-induced vascular inflammation in mice and reveal an unexpected protective role for TNFα. These effects are partly attributable to a direct antiinflammatory role of TNFα, but may also reflect a defective development of the immune system in these mice. (Arterioscler Thromb Vasc Biol. 2009;29:1465-1470.)

Key Words: diabetes ■ inflammation ■ atherosclerosis ■ tumor necrosis factor α

Complications affecting the macro- and microvasculature are the major causes of mortality and morbidity among diabetic subjects. The biological mechanisms involved in the development of diabetic vascular complication remain to be fully understood, but considerable attention has been paid to the triad of endothelial dysfunction, oxidative stress, and inflammation. One molecule that has been implicated in all of these conditions is the cytokine TNFα. TNFα plays a key role in initiating and modulating inflammatory responses including the regulation of other cytokines, adhesion molecules, and matrix metalloproteinases. It is expressed by vascular tissue in response to lipoprotein oxidation and by cultured macrophages exposed to oxidized low-density lipoprotein (LDL). TNFα is expressed in atherosclerotic plaques and has been found to be associated with atherosclerosis and cardiovascular events in epidemiological studies. Experimental studies have demonstrated that TNFα-deficient hypercholesterolemic mice, as well as mice treated with TNFα inhibitors, develop less atherosclerosis. TNFα has also been shown to be involved in the development of insulin resistance. Plasma levels of TNFα are increased in patients with both type 1 and type 2 diabetes and are significantly correlated to elevated fasting glucose levels, glycohemoglobin (HbA₁c), and markers of oxidative stress. Taken together these studies provide strong indirect support for a functional role of TNFα in the development of vascular complications in diabetes. To investigate this possibility we studied the development of early atherosclerotic changes in large arteries, as well as endothelial activation in resistance cerebral arteries, in C57BL/6 wild-type, apoE−/−, TNFα−/−, apoE−/−/TNFα−/−, and infliximab (TNFα-antibody)-treated apoE−/− mice made hyperglycemic with streptozotocin (STZ).

Methods

Animals
All animal experiments had been approved by the local Animal Care and Use Committee. C57BL/6 wild-type, apoE−/− were purchased from Taconic and TNFα−/− (B6.129-TnfmoGld) from the Jackson Laboratory (Bar Harbor, Maine). TNFα−/− and apoE−/− were intercrossed, and F7 TNFα−/− and F10 apoE−/−/TNFα−/− progeny were used in the experiments. All animals were fed normal chow diet and tap water without restrictions. The number of mice included in the studies were 28 C57BL/6 wild-type (16 STZ and 12 vehicle), 29 apoE−/− (17 STZ and 12 vehicle), 24 TNFα−/− (14 STZ and 10 vehicle), and 30 apoE−/−/TNFα−/− mice (15 STZ and 15 vehicle). A separate group of 21 apoE−/− mice (12 STZ and 9 vehicle) were treated with the TNFα antibody infliximab (Schering-Plough), in-
Inc). After 8 weeks, the animals were anesthetized with 300 μL of a mixture containing distilled water, Hypnorm, and Dormicum (2:1:1, v:v:v) and euthanized by exsanguination through cardiac puncture. Methods for tissue handling, immunofluorescence, immunohistochemistry, analysis of aortic atherosclerosis as well as serum cholesterol, triglyceride, and cytokine analyses are described in the supplemental materials (available online at http://atvb.ahajournals.org).

Statistics
Results are expressed as means±SEM if not otherwise specified. Statistical analysis was performed using GraphPad software (Prism 4.0) or SPSS version 12.0.1. Analyses of distributions were performed before decisions were made to use parametric or nonparametric tests. Statistical significance was determined using Student t test; Kruskal-Wallis followed by Dunn multiple comparison test or Mann-Whitney for nonparametric data; or 1-way ANOVA followed by Bonferroni or Tukey-Kramer post hoc tests for comparisons between ≤5 and ≥6 groups, respectively. (*P<0.05, **P<0.01, and ***P<0.001). Pearson test was used for correlation analyses.

Induction of Diabetes
At 22 weeks of age C57BL/6 wild-type, apoE−/−, TNFα−/−, and apoE−/−/TNFα−/− mice were injected ip with STZ (60 mg in citrate buffer per kg body weight, pH 4.5) or vehicle once a day for 5 days. The animals were weighed once a week, and animals with a loss of body weight >10% were excluded from the study (2 C57BL/6 wild-type, 1 TNFα−/−, and 4 apoE−/−/TNFα−/− mice). Blood glucose levels were measured using a One-Touch glucometer (LifeScan Inc). After 8 weeks, the animals were anesthetized with 300 μL of a mixture containing distilled water, Hypnorm, and Dormicin (2:1:1, administered ip) and euthanized by exsanguination through cardiac puncture. Methods for tissue handling, immunofluorescence, immunohistochemistry, analysis of aortic atherosclerosis as well as serum cholesterol, triglyceride, and cytokine analyses are described in the supplemental materials (available online at http://atvb.ahajournals.org).

Results
Effects on Glucose, Body Weight, Plasma Lipids, and Cytokines
C57BL/6 wild-type, apoE−/−, TNFα−/−, apoE−/−/TNFα−/−, and infliximab-treated apoE−/− mice were chow-fed until 22 weeks of age, injected with STZ or vehicle once a day for 5 days and then kept on chow diet for another 8 weeks. STZ increased blood glucose levels by at least 2-fold in all groups (Figure 1A). At the time mice were euthanized, diabetic TNFα−/− and apoE−/−/TNFα−/− mice had lower body weight than nontreated littermates (Figure 1B). In accordance with previous observations19,20 STZ-induced hyperglycemia was found to increase plasma cholesterol levels in apoE−/− mice (Figure 1C). Triglyceride levels remained low in infliximab-treated apoE−/−/TNFα−/− mice. Accordingly, diabetic apoE−/− mice had combined hyperglycemia and hyperlipidemia, whereas wild-type and TNFα−/− diabetic mice were characterized by hyperglycemia and no significant changes in plasma lipids. Plasma cytokine levels
in C57BL/6 wild type and apoE−/− are presented in supplemental Tables I and II. Increased plasma levels of the keratinocyte-derived chemokines (KC, also known as CXCL1) was found in diabetic TNFα−/−, apoE−/−/TNFα−/−, but not in diabetic infliximab-treated apoE−/− mice.

Effects of Hyperglycemia on Atherosclerosis
Atherosclerosis was assessed by en face Oil Red O staining of the descending aorta as well as by determining cross-sectional plaque area at the aortic root. Only very minor Oil Red O staining was observed in control and diabetic C57BL/6 wild type, and no mice in these groups had atherosclerotic plaques exceeding 0.5% of the total surface of the descending aorta (Figure 2A). The presence of Oil Red O–stained plaques among TNFα−/− mice was more variable, with 2 mice in the TNFα−/−/control group and 4 mice in the diabetic TNFα−/− group having more than 0.5% plaque staining. There was no significant difference in aortic atherosclerosis between TNFα−/− control and diabetic mice, but hyperglycemic TNFα−/− mice had more aortic atherosclerosis than hyperglycemic C57BL/6 wild-type mice (0.55 ± 0.21% versus 0.02 ± 0.04%, P < 0.005). No plaques were detected in the aortic root of control or diabetic C57BL/6 wild-type and TNFα−/− mice (data not shown).

In accordance with previous studies, apoE−/−/TNFα−/− mice had fewer Oil Red O–stained plaques in the aorta than apoE−/− mice (0.97 ± 0.60% versus 2.11 ± 2.09%), but this difference was not statistically significant in the present study because of large variation in staining in the apoE−/− group (Figure 2B). Diabetes had no significant effect on atherosclerosis in the descending aorta in apoE−/− mice, apoE−/−/TNFα−/−, or infliximab-treated apoE−/− mice. Furthermore, no significant difference in aortic root plaque area between apoE−/−, apoE−/−/TNFα−/−, and infliximab-treated apoE−/− mice was observed, and no effect of diabetes on aortic root plaque size was found in any of the groups (Figure 2C).

Increased inflammation is believed to be a key mechanism in diabetic-induced vasculopathy. The inflammatory response of the aorta was assessed by immunohistochemical staining of macrophages in aortic root lesions. Diabetes resulted in increased macrophage accumulation in aortic root plaques of apoE−/− mice (10.5 ± 3.1% versus 13.3 ± 2.4%, P < 0.01). Increased plaque inflammation in response to hyperglycemia was observed also in apoE−/−/TNFα−/− and infliximab-treated apoE−/− mice (Figure 3A and 3B). Interestingly, the accumulation of macrophages was higher in diabetic apoE−/−/TNFα−/− mice and infliximab-treated apoE−/− mice than in diabetic apoE−/− mice (19.7 ± 4.2% and 28.3 ± 6.4% versus 13.3 ± 2.4%, P < 0.001 and P < 0.001, respectively). Moreover, more macrophages were found to accumulate in lesions of control apoE−/−/TNFα−/− and control infliximab-treated apoE−/− mice than in control apoE−/− mice (14.9 ± 5.2% and 19.4 ± 5.0% versus 10.5 ± 3.1% P < 0.02 and P < 0.001, respectively). There were significant associations between the macrophage accumulation in subvalvular plaques and the plasma levels of glucose and cholesterol in apoE−/− mice (r = 0.41, P < 0.05 and 0.84, P < 0.001 respectively; both control and STZ mice were included in the analyses). The association between plasma glucose and plaque macrophage accumulation was even stronger in apoE−/− mice lacking TNFα (r = 0.57, P = 0.001), although the association was weaker for plaque macrophages and plasma cholesterol (r = 0.52, P < 0.005). Hyperglycemia did not significantly affect VCAM-1 expression in subvalvular plaques (supplemental Figure I).

Atherosclerosis is associated with accumulation of autoantibodies against oxidized LDL and other modified self antigens in plaque tissue. To analyze whether diabetes affected this process, aortic root lesions were stained for mouse IgG and IgM. There was no difference in IgG and IgM immunoreactivity between control and diabetic apoE−/− mice or between control and diabetic apoE−/−/TNFα−/− mice (supplemental Figures II and III). However, significantly higher IgG and IgM levels were found in lesions of control apoE−/−/TNFα−/− mice than in lesions of apoE−/− mice (supplemental Figures II and III).

Effects of Hyperglycemia on VCAM-1 Expression in Cerebral Arteries
To determine the effect of diabetes on inflammatory activation in small- to midsized arteries, we assessed the expression of VCAM-1 in cerebral arteries by confocal immunofluorescence microscopy. In accordance with previous studies, a basal expression of VCAM-1 was detected in both endothelial and smooth muscle cells in intact cerebral arteries of
control C57BL/6 wild-type mice. The expression was higher in endothelial cells than in smooth muscle cells and it was dependent on vessel diameter, with higher expression in the larger (>150 μm) cerebral arteries (Figure 4A and 4B). TNFα−/− mice had overall lower endothelial and smooth muscle cell expression of VCAM-1 than C57BL/6 wild-type mice suggesting that TNFα−/− was involved in the basal regulation of VCAM-1 expression in these vessels. However, TNFα−/− mice also deficient for apoE−/− had a higher basal expression of VCAM-1, and there was no difference as compared to C57BL/6 wild-type mice (Figure 5A and 5B). The latter observation suggests that hyperlipidemia induces VCAM-1 expression in smaller arteries by a mechanism that is independent of TNFα.

Diabetes enhanced VCAM-1 expression in cerebral arteries of apoE−/−/TNFα−/− mice and to a lesser extent also in mice deficient for either TNFα or apoE, but not in infliximab-treated apoE−/− mice (Figure 6). Induction of diabetes was found to reduce VCAM-1 expression in cerebral arteries of C57BL/6 wild-type mice (Figure 5 and supplemental Figure IV). This reduction was observed in small (<100 μm) and in the largest (>150 μm) cerebral arteries (supplemental Figure IV), whereas the enhanced expression seen in apoE-deficient mice was more prominent in the largest (>150 μm) vessels (Figure 6A). A significant association was found between blood glucose levels and endothelial VCAM-1 expression in apoE−/−/TNFα−/− and TNFα−/− mice (r=0.53, P<0.05 and 0.58, P<0.05, respectively), but no such associations were observed in TNFα-competent C57BL/6 wild-type or apoE−/− mice (both control and STZ mice were included in the analyses). There was no correlation between endothelial expression of VCAM-1 in cerebral arteries and plasma cholesterol or triglyceride levels.

Discussion

The present study confirms previous observations that diabetes enhances macrovascular inflammation in hyperlipidemic mice22–25 and extends these findings by demonstrating aggravated inflammation also in smaller vessels such as the cerebral arteries. The main focus of our study was to determine whether the vascular inflammatory response to hyperglycemia is mediated by TNFα. Previous studies have demonstrated that TNFα is critically involved in the development of atherosclerotic lesions in hypercholesterolemic animals.14,15,26,27 TNFα has also been suggested to represent an important link between metabolic dysregulation and inflammation in diabetes,4,18,28 and plasma levels of TNFα are increased in type 1 diabetes patients suffering from proliferative retinopathy.29,30 Collectively, these studies suggest that hyperglycemia or other metabolic changes associated with diabetes may contribute to the development of diabetic vasculopathy through TNFα-induced vascular inflammation. However, in contrast to this notion the present findings clearly demonstrate that TNFα does not mediate vascular inflammation in response to hyperglycemia.

The present studies also reveal a complex role for TNFα in the regulation of vascular homeostasis and response to metabolic stress. In apoE-competent mice, TNFα appeared to have a protective effect on hyperglycemia-induced athero-
A number of studies have reported that TNFα deficiency in hypercholesterolemic mice results in reduction of atherosclerotic plaque size without decreasing the plaque content of inflammatory cells, and in line with the present observations some of these studies even observed a trend toward increased plaque inflammation in TNFα-deficient mice. The reason why a reduced plaque inflammation was less evident in previous studies remains to be fully clarified, but it is possible that the use of a proinflammatory high-fat diet (as opposed to the chow diet used in this study) masked any possible influence of TNFα deficiency in those studies.

TNFα is a key mediator of inflammatory responses able to target cells in the vascular wall. Because it activates the expression of a number of adhesion molecules, chemotacticants, and proinflammatory cytokines, the present observation of increased plaque inflammation in apoE−/−/TNFα−/− mice was unexpected. One possible explanation to the increased vascular inflammation in TNFα-deficient mice described here is that they suffer from a more general dysregulation of the immune system. However, the finding of increased plaque inflammation also in infliximab-treated apoE−/− mice argue against this possibility and provide support for a direct antiinflammatory role of TNFα in these lesions. In mouse models of autoimmune demyelination, autoimmune lupus nephritis, and type I diabetes, TNFα was found to protect animals from severe injury. Suggested mechanisms include transactivation of NF-κB leading to the induction of ant apoptotic genes improving cell survival and induction macrophage expression of alternative cytokine signaling including antiinflammatory cytokines such as IL-10 and TGFβ.

Several previous studies have reported that STZ-induced hyperglycemia increases the development of atherosclerosis in apoE−/− mice. The reasons for the lack of such an effect in our studies remain to be clarified but may involve differences in diet (ie, chow versus high fat) and duration of the study (ie, early versus late stages of atherosclerosis).

There are some limitations to the present study that need to be considered. It has not been entirely established that the mouse is a fully appropriate model for studies of vascular complications in diabetes. Accordingly, although the present observations exclude TNFα as the mediator of diabetes-induced vascular inflammation in mice, they do not necessarily exclude that TNFα may be actively involved in the development of diabetic vascular complications in man. Another limitation of the present study is that the use of hyperglycemic apoE−/− mice does not allow us to differentiate whether the proinflammatory effect is induced by hyperglycemia or hyperlipidemia. However, it is of interest to note that whereas inflammation in the aorta correlated with cholesterol and glucose levels, inflammation in the smaller cerebral resistance arteries was only associated with plasma glucose and not lipid levels. Moreover, the association between vascular inflammation and plasma glucose levels was stronger in mice lacking TNFα again pointing to a paradoxical protective role of this cytokine in hyperglycemia-induced vascular inflammation.

In summary, these findings demonstrate that TNFα does not mediate diabetes-induced vascular inflammation in mice. They also show that TNFα deficiency is associated with a propensity.
to increased vascular inflammation and in particular in response to metabolic stress such as hyperglycemia or hyperlipidemia.

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**Disclosures**

Expert technical assistance was provided by Bodil Israelsson, Ingrid Söderberg, and Irena Ljungkранtz.

**References**


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Supplement Material

Methods

Tissue handling
After whole body perfusion with phosphate-buffered saline (PBS) followed by Histochoice (Amresco, Solon, Ohio), the heart, aortic arch and brain were dissected out and stored in Histochoice at 4 °C until processing. The descending aorta was dissected free of external fat and connective tissue, cut longitudinally and mounted en-face lumen side-up on ovalbumin (Sigma, St. Louis, Missouri) coated slides (termed flat preparation).

Immunofluorescence
Cerebral arteries (anterior, middle, posterior, cerebellar and basilar) were dissected from the brain and cleaned of connective tissue in PBS. The arteries were immunostained as previously described. Briefly, arteries were permeabilized with 0.2% Triton-X-100 in PBS for 10 min, and blocked for 1 h with 2% bovine serum albumin (BSA) in PBS. The primary antibody BCA12 rat anti-VCAM-1 (clone M/K-2, Millipore) was diluted 1:200 in 2% BSA/PBS and applied overnight at 4 °C. The secondary antibody, Cy5-anti-
rat IgG (Jackson ImmunoResearch Laboratories), was diluted 1:500 and applied for 1 h at room temperature. For identification of nuclei, the fluorescent nucleic acid dye SYTOX Green (Molecular Probes, 1:3000) was used. After washing, the vessels were mounted (Aqua Polymount mounting medium, Polysciences) and examined at x63 magnification using a Zeiss LSM 5 Pascal laser scanning confocal microscope. VCAM-1 was detected by monitoring Cy5 fluorescence using an excitation wavelength of 633 nm and an emission wavelength of >650 nm. Specificity of immune staining was confirmed by the absence of fluorescence in arteries incubated with primary or secondary antibodies alone. At least five images were taken from each artery and subsequently analyzed for mean pixel intensity (range 0 to 255 grayscale values after background subtraction) and vessel size. Three measures of mean pixel intensity and two measures of vessel diameter were taken in each image using the Zeiss LSM5 analysis software. Orientation of the nuclei was used to distinguish smooth muscle cells from endothelial cells.

Staining of the descending aorta

En-face preparations of the descending aorta were washed in distilled water, incubated briefly in 78% methanol and stained for 40 min in 0.16% Oil-Red-
O dissolved in 78% methanol/0.2 M NaOH as previously described. The cover slides were mounted with a water-soluble mounting media L-550A (Histolab). Lipids are stained red, which makes the plaques Bordeaux colored. Stained area (bordeaux colored) and total aortic areas were quantified by microscopy and computer aided morphometry (Olympus Micro Image) under blind conditions.

*Analysis of plaque macrophage, VCAM-1 and autoantibody content*

The heart and proximal part of the aortic arch was embedded in OCT (Tissue-Tek). Frozen sections of 10 µm were collected from the subvalvular region. For detection of monocyte/macrophage, VCAM-1 and IgG or IgM, slides were fixed in ice-cold acetone for 5 min and blocked with 10% mouse serum in PBS for 30 min. To detect monocytes/macrophages the slides were incubated with rat anti-mouse MOMA-2 antibodies (BMA Biomedicals, diluted in 10% rat serum in PBS) and for VCAM-1 with rabbit anti-mouse VCAM-1 (Santa Cruz Biotech) at +4°C over night, followed by incubation in room temperature for 50 min with a secondary biotinylated rabbit anti-rat IgG (Vector Laboratories) and biotinylated goat anti rabbit IgG (DAKO), respectively. For detection of IgM and IgG autoantibodies, slides were incubated with biotinylated anti-mouse IgM or IgG antibodies (Vector
Laboratories) for 50 min in room temperature. The reaction products were visualized with Vectastain ABC elite kit (Vector Laboratories) using DAB as substrate (Vector Laboratories). Slides were counter-stained with hematoxylin and omission of primary or secondary antibodies were used as controls. Stained area was quantified by microscopy and computer aided morphometry (Olympus Micro Image) under blind conditions.

Plasma cholesterol, triglyceride and cytokines

Total plasma cholesterol and plasma triglycerides were quantified with colorimetric assays, Infinity™ Cholesterol and Triglyceride (INT and Sigma, respectively). Plasma cytokine concentrations were measured using an inflammation 7-plex kit (Meso Scale Discovery, USA) according to the instructions of the manufacturer. The lower detection limit for all cytokines in this assay is ~0.5 pg/ml.

References

Figure legends

Supplemental figure I. *STZ-induced hyperglycemia does not significantly affect VCAM-1 expression in aortic root atherosclerotic plaques.* Subvalvular lesions from control (vehicle) and diabetic (STZ) ApoE<sup>−/−</sup> and ApoE<sup>−/−</sup>-TNF<sub>α</sub><sup>−/−</sup> mice stained with rabbit anti-mouse VCAM-1 and percentage of positively stained areas were quantified.

Supplemental figure II. *TNFa-deficiency results in increased IgG in atherosclerotic lesions.* (A) Subvalvular lesions from control (vehicle) and diabetic (STZ) ApoE<sup>−/−</sup> and ApoE<sup>−/−</sup>-TNF<sub>α</sub><sup>−/−</sup> mice were stained with an antibody against IgG and stained area, expressed of the percent of the total plaque area, quantified by computerized image analysis. (B) Representative stains of data presented in A. Scale bar = 500 μm.

Supplemental figure III. *TNFa-deficiency results in increased IgM in atherosclerotic lesions.* (A) Subvalvular lesions from control (vehicle) and diabetic (STZ) ApoE<sup>−/−</sup> and ApoE<sup>−/−</sup>-TNF<sub>α</sub><sup>−/−</sup> mice were stained with an antibody against IgM and stained area, expressed of the percent of the total
plaque area, quantified by computerized image analysis. (B) Representative stains of data presented in A. Scale bar = 500 µm.

Supplemental figure IV. *Effect of hyperglycemia on VCAM-1 expression in cerebral arteries from control C57BL/6 wild type (wt)*. Summarized data from confocal immunofluorescence experiments showing VCAM-1 expression in arteries of different size. **P<0.005 and ***P<0.001 diabetes compared to corresponding vehicle of the same size.
Supplemental figure I.
Supplemental figure II

A

\[ \text{IgG stained area (\%)} \]

- \( \bullet \) vehicle
- \( \bigcirc \) STZ

B

ApoE\(^{-/-}\) vehicle
ApoE\(^{-/-}\) STZ
ApoE\(^{-/-}\) TNF\(\alpha\)\(^{-/-}\) vehicle
ApoE\(^{-/-}\) TNF\(\alpha\)\(^{-/-}\) STZ
Supplemental figure III
VCAM-1 expression (mean fluorescence intensity)

- **vehicle**
- **STZ**

Supplemental figure IV
Supplemental table I. Plasma cytokine levels in C57BL/6 wild type and TNFα⁻/⁻ mice at 30 weeks

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Values are presented as mean±standard deviation. *P<0.05 versus corresponding vehicle group using Mann-Whitney-test. WT; wild type, n.d.; not detectable.
Supplemental table II. Plasma cytokine levels in apo E<sup>-/-</sup>, apo E<sup>-/-</sup>/TNFα<sup>-/-</sup> and infliximab-treated apo E<sup>-/-</sup> mice at 30 weeks

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<td>n.d.</td>
<td>n.d.</td>
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</table>

Values are presented as mean±standard deviation.*<sup>P<0.05</sup> and **<sup>P<0.001</sup> versus corresponding vehicle group using Mann-Whitney-test. WT; wild type, n.d.; not detectable.