p55 Tumour necrosis factor receptor in bone marrow-derived cells promotes atherosclerosis development in low-density lipoprotein receptor knock-out mice

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Aims Tumour necrosis factor (TNF) is a pivotal pro-inflammatory cytokine with a clear pathogenic role in many chronic inflammatory diseases, and p55 TNF receptor (TNFR) mediates the majority of TNF responses. The aim of the current study was to investigate the role of p55 TNFR expression in bone marrow-derived cells, in atherosclerotic lesion development.

Methods and results Irradiated low-density lipoprotein receptor knock-out mice were reconstituted with either p55 TNFR knock-out or control haematopoietic stem cells to generate chimeras deficient or wild-type for p55 TNFR specifically in bone marrow-derived cells, including macrophages. Upon high fat feeding, p55 TNFR knock-out transplanted mice developed smaller atherosclerotic lesions. These lesions were characterized by the presence of smaller foam cells and a reduced macrophage foam cell area. They did not differ in other compositional characteristics as determined by quantification of inflammatory T-cell and neutrophil influx, apoptotic and necrotic cell death, and collagen content. In vitro studies confirmed a significant defect in modified lipoprotein endocytosis by p55 TNFR knock-out macrophages due to reduced scavenger receptor class A expression. Interestingly, plasma cytokine/chemokine profile analysis indicated that monocyte chemoattractant protein-1 (MCP-1) levels, a major chemokine involved in atherogenesis, were consistently and significantly lower in p55 TNFR knock-out transplanted mice compared with controls, before and after high fat feeding.

Conclusion p55 TNFR expression in bone marrow-derived cells contributes to the development of atherosclerosis by enhancing lesional foam cell formation and by promoting the expression of pro-atherosclerotic chemokines such as MCP-1.

KEYWORDS Atherosclerosis; Macrophages; Inflammation; Leukocytes; Cytokines

1. Introduction

Inflammatory processes play a major role in atherosclerosis development and a crucial pro-inflammatory cytokine produced mainly by activated monocytes and macrophages is tumour necrosis factor (TNF). The involvement of this cytokine in atherosclerosis is supported by studies showing clear evidence of local TNF production by macrophages and smooth muscle cells within plaque microenvironments. In addition, circulating TNF levels are strongly associated with the incidence of cardiovascular and atherosclerotic events, with increased risk of recurrent myocardial infarction and with atherosclerotic thickening of carotid intima-media.

TNF could contribute to atherogenesis due to its numerous effects on different cell types (macrophages, endothelial cells, smooth muscle cells), such as induction of adhesion molecule expression, cytokine and chemokine production, induction of cell migration, proliferation, and apoptosis. This wide range of TNF activity is mediated by signalling through two receptors present on almost all nucleated cell types named p55 and p75 TNF Receptor (TNFR), and different experimental approaches have shown that p55 TNFR mediates the majority of TNF responses.

To date, the role of TNF-p55 TNFR signalling in atherosclerosis remains elusive. Using knock-out mouse models, a pro-atherosclerotic action of the cytokine TNF was found in the majority of the reports. However, studying the
role of the TNF-p55 TNFR axis in C57BL/6 mice fed an atherogenic diet, Schreyer et al. described no effect of TNF deficiency but an anti-atherogenic effect of p55 TNFR signalling, while Blessing et al. found no effect of p55 TNFR in very advanced atherosclerosis in apoE mice. Deficiency of the p75 TNFR was shown to protect from atherosclerotic lesion development.

In addition, the majority of these studies were performed in mice deficient for TNF or p55 TNFR in all cells and therefore there is little knowledge on the cell type specific role of these molecules in atherogenesis. Using an arterial grafting model, it was shown recently that arterial wall (i.e. smooth muscle and endothelial cell) p55 TNFR expression is pro-atherogenic. To investigate the role of p55 TNFR expression in bone marrow-derived cells in the immunopathogenesis of atherosclerosis, we have employed a bone marrow transplantation approach to generate low-density lipoprotein (LDL) receptor (LDLR) knock-out mice with either a p55 TNFR wild-type or deficient haematopoietic compartment. We show that p55 TNFR expression in bone marrow-derived cells promotes atherosclerosis by enhancing modified lipoprotein endocytosis by lesional macrophages and thus leading to an increased foam cell area. In addition, we show that bone marrow-derived p55 TNFR plays a role in controlling circulating levels of the crucial pro-atherogenic chemokine MCP-1 (monocyte chemoattractant protein-1), identifying another potential mechanism by which p55 TNFR may promote atherogenesis.

2. Methods

2.1 Mice, transplantation, and diet

p55 TNFR knock-out mice (p55 TNFR−/−) on a C57BL/6 background were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and have been described elsewhere. C57BL/6 mice were obtained from in-house breeding. LDLR knock-out mice (LDLR−/−) have been described elsewhere and were crossed to C57BL/6 background for at least 10 generations. Transplantation was performed as described previously with 12 × 105 bone marrow cells from pools isolated from either wild-type or p55 TNFR−/− mice (five donor mice/group). All LDLR−/− recipients used were females. High fat diet (Hope Farms, Woerden, The Netherlands) contained 16% fat, 25% cholesterol and no cholate. All experiments were approved by the Committee for Animal Welfare of Maastricht University. The investigation conforms to 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 Chimaerism determination, genotyping, and blood analysis

Chimaerism and mouse genotyping was performed on blood deoxyribo nucleic acid (DNA) 4 weeks after transplantation (before onset of diet). For determination of wild-type (470 bp product) or p55 TNFR deleted alleles (300 bp product) the following primers were used: 5′-TGGTGAAGGGCACCTTTACGCC-3′; 5′-GGTGGACGTCCAGCACCATCGG-3′; 5′-ATTGCCAATGACAAAGGCTG-3′. Cholesterol and triglyceride levels were determined on plasma, after an overnight fast, using enzymatic kits (Sigma-Aldrich; cat. no. 401 and 337). Lipoprotein profiles were determined on pooled plasma samples using an AKTA Basic chromatography system with a Superose 6PC3.2/30 column (Amersham Biosciences). Leukocyte analysis was performed as described previously. Total leukocyte counts were determined using a standard haemocytometer. Plasma cytokine levels were determined with the Cytometric Bead Array kit (BD Biosciences, Pharmingen) according to manufacturer’s instructions.

2.3 Quantitative gene expression

RNA (ribonucleic acid) was isolated using Tri-Reagent (Sigma). cDNA synthesis was performed using the iScriptTM cDNA synthesis kit (Bio-Rad) and quantitative polymerase chain reaction (PCR) was performed using the qPCR IQ™ Custom SYBR Green Supermix (Bio-Rad) on an iCycler thermal cycler (Bio-Rad). Cyclophilin A was used as housekeeping gene. Primer sequences are available upon request.

2.4 Histology and immunohistochemistry

Atherosclerosis was quantified as previously described. Sections were stained with toluidine blue for morphometric analysis, with Sirius-red for collagen, and with antibodies against T-cells (KT3; cultured and purified in-house), granulocytes (Ly-6G; BD Biosciences, Pharmingen) or macrophages (MOMA2 and Mac1; gifts from Dr G. Kraal; VUMC, Amsterdam) or FA11 (a gift from S. Gordon; Oxford University), CD45 (PharMingen cat no. 553076). Terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling (TUNEL)-positive cells were determined as described previously. The average foam cell size in the lesions was calculated by dividing the foam cell area by the number of nuclei in the area in toluidine-stained sections from 10 randomly selected mice per group. Liver sections of 7 μm were fixed in acetone and stained with Oil Red O (Sigma) for 30 min or with different antibodies as mentioned previously. Oil Red O quantification was performed by measuring the positive area in four different fields/mouse at magnification ×200 (stained pixels were measured using Adobe Photoshop 6.0). For Mac1 quantification we counted the positive cells in four different fields/mouse at magnification ×200. Nuclei were stained with Mayer’s haematoxylin. All analyses were performed without prior knowledge of the genotype.

2.5 Peritoneal macrophage isolation and modified low-density lipoprotein uptake

Macrophages were isolated 4 days after intraperitoneal administration of 1 mL 4% thioglycollate solution and plated in 24-well plates at a density of 5 × 105 cells/well. After 6 h the culture medium was removed and cells were incubated overnight with new medium that either contained no lipopolysaccharide (LPS) (non-activated cells) or 100 ng/mL LPS (O111:B4; Sigma) (6 h LPS stimulation). The following morning medium was removed and cells were incubated in Optimem-1 (Gibco-BRL) containing 25 μg/mL of DII-labelled modified LDL for either 3 or 24 h. Uptake of modified LDL and foam cell formation was visualized using fluorescence microscopy and quantified by flow cytometry. LDL isolation, modification, and labelling were performed as described previously.

2.6 Statistical analysis

Statistical analyses were performed using Graphpad Prism (Graphpad Software) or SigmaPlot t-tests. For lesion area and characterization, differences were evaluated using a Welch corrected t-test. Data are expressed as means ± SEM or SD. A P-value of <0.05 is considered statistically significant.

3. Results

3.1 General characterization of transplanted mice

Bone marrow from either wild-type or p55 TNFR knock-out (p55 TNFR−/−) donor mice was transplanted to lethally irradiated LDL receptor knock-out recipients (LDLR−/−) in order to generate chimaeric LDLR−/− mice with p55 TNFR−/+.
(p55 TNFR\textsuperscript{+/+}-tp) or p55 TNFR\textsuperscript{−/−} (p55 TNFR\textsuperscript{−/−}-tp) hematopoietic stem cells. After 4 weeks of recovery, chimerism determination showed that the percentage of white blood cells of donor origin was 93.4 ± 3.55 for the p55 TNFR\textsuperscript{+/+}-tp group and 90.9 ± 2.7 for the p55 TNFR\textsuperscript{−/−}-tp group (mean ± SD), indicating a correct donor stem cell engraftment in the recipient mice. PCR analysis of leukocyte DNA confirmed the wild-type or p55 TNFR\textsuperscript{−/−} genotype of these cells in the p55 TNFR\textsuperscript{+/+}-tp or p55 TNFR\textsuperscript{−/−}-tp mice, respectively (data not shown). In addition, PCR analysis of genomic DNA isolated from atherosclerotic lesions of either p55 TNFR\textsuperscript{+/+}-tp or p55 TNFR\textsuperscript{−/−}-tp mice, at the end of the experiment, confirmed the incorporation of donor mice-derived cells in atherosclerotic lesions (see Supplementary material online, Figure S1). Mice were fed a high fat diet for 11 weeks and blood samples were collected at regular time points for the different analyses. Absolute numbers and relative percentages of the different white blood cell populations were determined after 4 and 11 weeks of high fat diet. As shown in Table 1, absolute leukocyte counts and relative percentages of monocytes, granulocytes, and T-cells were similar between the groups at all time points. The relative percentage of B-cells was significantly lower in p55 TNFR\textsuperscript{−/−}-tp mice after 4 weeks of high fat diet, which is in accordance with a previous report indicating that generation of B-cells in the bone marrow depends on p55 TNFR signalling.\textsuperscript{20} However, after 11 weeks of high fat diet, the relative percentage of B-cells became normalized. Body weights were also not significantly different between the groups (Table 1).

3.2 Liver analysis
Mice were sacrificed after 11 weeks of high fat diet and hearts and livers were isolated. To investigate the degree of liver fat accumulation, liver sections of p55 TNFR\textsuperscript{−/−}-tp and control mice were stained with Oil Red O for neutral lipids and the positive area was quantified (Figure 1A). In addition, we wanted to evaluate recruitment of inflammatory cells in the liver in response to the diet. Therefore, we compared four antibodies recognizing inflammatory cells (see Supplementary material online, Figure S2). Mac1 turned out to recognize best the recruited inflammatory cells without staining of resident Kupffer cells. Thus, liver sections from p55 TNFR\textsuperscript{−/−}-tp or control mice were stained with Mac1 antibody and the inflammatory response was quantified (Figure 1B). Both fat accumulation and number of Mac1-positive inflammatory infiltrates were similar between the groups. To characterize more in detail the liver inflammatory response, quantitative gene expression analysis was performed. Most classic pro-inflammatory effects of TNF, including the release of other inflammatory molecules, are mediated through p55 TNFR.\textsuperscript{21} Expression levels of TNF (P = 0.01) were significantly decreased in p55 TNFR\textsuperscript{−/−}-tp livers while for IL-6 (P = 0.07) and IL-10 (P = 0.06) differences did not reach significance. IL-12 and MCP-1 expression were comparable between both groups (Figure 1C).

3.3 Reduced aortic atherosclerosis in p55 TNFR\textsuperscript{−/−}-tp mice
High fat diet administration induced an increase in plasma cholesterol but with no significant differences in levels or lipoprotein cholesterol distribution between the groups. Plasma triglycerides levels were also similar (Figure 2A–C). Atherosclerotic lesions in the aortic root were analysed for size and other parameters. Toluidine staining indicated a 22% reduction in lesion area in p55 TNFR\textsuperscript{−/−}-tp mice compared with controls (Figure 2D; P = 0.02). Thus, p55 TNFR signalling in bone marrow-derived cells contributes to the development of atherosclerosis.

3.4 Composition of p55 TNFR\textsuperscript{−/−}-tp mice does not differ from controls
To characterize the atherosclerotic plaques more in detail, lesions were quantified for inflammatory T-cell and neutrophil content, for the presence of apoptotic nuclei and for the degree of necrosis and fibrosis. No differences in numbers of T-cells in plaques of p55 TNFR\textsuperscript{+/+}-tp or p55 TNFR\textsuperscript{−/−}-tp mice were observed (Figure 3A). In addition, staining with anti-Ly-6G monoclonal antibody, a neutrophil-specific marker, indicated similar numbers of neutrophils in the two groups (Figure 3B). Since p55 TNFR contains a death domain that can lead to cell apoptosis, the number of TUNEL-positive apoptotic nuclei and the extent of the necrotic core were also quantified, but both parameters were found to be comparable (Figure 3C and D). Finally, to quantify lesions for fibrosis, the deposition of collagen was measured and it did not differ between the groups (3E). Therefore, bone marrow-derived p55 TNFR deficiency results in smaller atherosclerotic plaques that however

<table>
<thead>
<tr>
<th>Table 1</th>
<th>General analysis of transplanted mice (body weight and absolute and relative blood leukocyte values in p55 TNFR\textsuperscript{+/+}-tp and p55 TNFR\textsuperscript{−/−}-tp mice after 4 or 11 weeks of high fat diet administration)</th>
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<tr>
<td></td>
<td>4 weeks HFD</td>
</tr>
<tr>
<td></td>
<td>p55 TNFR\textsuperscript{+/+}-tp</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>17.4 ± 1.79</td>
</tr>
<tr>
<td>Blood leukocytes (×10\textsuperscript{6}/mL)</td>
<td>16.8 ± 4.8</td>
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<tr>
<td>Monocytes (%)</td>
<td>8.4 ± 2.4</td>
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<tr>
<td>Granulocytes (%)</td>
<td>5.1 ± 1.8</td>
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<tr>
<td>T-cells (%)</td>
<td>23.7 ± 4.4</td>
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<tr>
<td>B-cells (%)</td>
<td>59.3 ± 3.3</td>
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Values represent means ± SD; \textsuperscript{*}P < 0.05 by Student’s t-test.
have a similar cellular composition and do not differ qualitatively from control plaques.

3.5 Reduced macrophage area in p55 TNFR\textsuperscript{−−}−tp lesions

In order to quantify the macrophage-foam cell area in the lesions, we have performed immunohistochemical staining with different macrophage markers in order to establish which antibody stains most homogenously macrophages and foam cell area (see Supplementary material online, Figure S3). We therefore chose to stain lesions with a MOMA2 antibody. Comparable with the reduction in lesion size, immunohistochemical detection of macrophages and macrophage foam cells indicated a significant 23% reduction in positive area in p55 TNFR\textsuperscript{−−}−tp lesions (Figure 4A; \(P=0.02\)). Regression analysis showed correlation of lesion area with macrophage content in both groups, indicating that larger lesions have more MOMA2 staining (Figure 4B). Yet, no differences in the slopes were found (\(R^2=0.57, P<0.0001\) for the p55 TNFR\textsuperscript{++}−tp group and \(R^2=0.61, P<0.0001\) for the p55 TNFR\textsuperscript{−−}−tp group), indicating that equal sized lesions from p55 TNFR\textsuperscript{++}−tp and p55 TNFR\textsuperscript{−−}−tp mice have a similar macrophage content and do not differ in composition. However, although the relative macrophage area was not different between the two groups, the absolute macrophage area was smaller in the p55 TNFR\textsuperscript{−−}−tp lesions, as shown in Figure 4A.

3.6 Reduced uptake of modified lipids by p55 TNFR knock-out macrophages

To determine if the reduced macrophage foam cell area observed in p55 TNFR\textsuperscript{−−}−tp lesions could be attributed to a reduced lipoprotein uptake by p55 TNFR\textsuperscript{−−}−macrophages, we examined the size of foam cells in p55 TNFR\textsuperscript{++}−tp and p55 TNFR\textsuperscript{−−}−tp plaques. As shown in Figure 5A, lesional macrophages in p55 TNFR\textsuperscript{−−}−tp mice were 25% smaller than in control p55 TNFR\textsuperscript{++}−tp mice, indicating a reduced lipid accumulation in these cells. To study more in detail the mechanisms leading to reduced lipid accumulation in p55 TNFR\textsuperscript{−−}−cells, in vitro lipid uptake experiments were performed. Thioglycollate-elicited peritoneal macrophages from wild-type or p55 TNFR\textsuperscript{−−} cells were incubated with fluorescently labelled modified lipoproteins (LDL) for 3 or 24 h and foam cell formation was visualized by fluorescence microscopy and quantified by fluorescence-activated cell sorting (FACS). p55 TNFR\textsuperscript{−−}−cells appeared on an average smaller compared with p55 TNFR\textsuperscript{++}−cells both after 3 and 24 h of LDL loading (see Supplementary material online, Figure S4). Quantification by FACS analysis indicated a reduction in both short-term uptake (3 h) as well as
accumulation (24 h) of both oxidized and acetylated LDL by p55 TNFR−/− macrophages (Figure 5B). In addition, to examine modified LDL uptake by activated macrophages, cells were incubated overnight with LPS prior to modified-LDL loading. Activated macrophages of both genotypes were able to endocytose substantially higher amounts of modified LDL compared with non-activated cells. However, LDL uptake by p55 TNFR−/− cells remained significantly lower compared with controls (Figure 5C). To determine which receptors were responsible for the reduced modified LDL uptake, the expression of scavenger receptor class A (SR-A) and CD 36 was assessed by quantitative PCR in non-activated or activated cells after 3 h of oxLDL loading. In both non-activated and activated cells we could measure an ~30% reduction in SR-A expression while CD 36 levels were not different (Figure 5D and E).

3.7 Lower systemic inflammatory state in p55 TNFR−/−-tp mice

p55 TNFR signalling induces the production of inflammatory mediators, among that cytokines and chemokines which are important in the development and progression of atherosclerosis. We therefore sought to determine whether p55 TNFR deficiency in bone marrow-derived cells, including macrophages, could alter the homeostasis of cytokine/chemokine production towards a less inflammatory and athero-protective environment. Plasma concentrations of different mediators were measured before and after 4 and 8 weeks of high fat diet. High fat feeding increased the production of MCP-1 and IL-6 in both groups, but not of the other cytokines measured (Figure 6A–E). Interestingly, levels of MCP-1 were already ~25% lower after 4 and 8 weeks of high fat diet (Figure 6A). In addition, we measured plasma MCP-1 levels in non-transplanted p55 TNFR−/− mice on a normal chow diet and these were also ~40% lower compared with control C57BL/6 mice (mean levels ± SD: 87.6 ± 29.6 for C57BL/6 and 54.6 ± 32.2 for p55 TNFR−/− mice; P < 0.05; Figure 6F). These data indicate that p55 TNFR signalling in bone marrow-derived cells is necessary for full systemic MCP-1 production, and its specific deletion in these cells shifts the balance towards a more athero-protective environment.

4. Discussion

In this study we addressed the role of p55 TNFR expression specifically in bone marrow-derived cells in the development of atherosclerosis. We show that LDLR knock-out mice carrying p55 TNFR knock-out bone marrow develop smaller atherosclerotic plaques but have a similar composition in control plaques, with comparable inflammatory T-cell and neutrophil influx, degree of apoptotic or necrotic cell death and extent of fibrosis.

Macrophage staining indicated that p55 TNFR−/−-tp plaques had a reduced macrophage area and presence of smaller macrophage foam cells. In vitro foam cell assays confirmed the in vivo observations of a reduced capacity of p55 TNFR−/− macrophages to endocytose modified lipoproteins and foam cell formation. Previous studies have shown that TNF can induce the expression of SR-A in cultured vascular smooth muscle cells. However, the role of TNF-p55 TNFR signalling in scavenger receptor expression and lipoprotein uptake by macrophages has given contrasting results. In murine J774A.1 macrophages, short-term p55 TNFR
stimulation increased scavenger receptor expression through mitogen-activated protein kinase-dependent pathways. Contrarily, prolonged stimulation down-regulated scavenger receptor expression and led to reduced foam cell formation. In mouse peritoneal macrophages, p55 TNFR-deficient cells were able to accumulate higher amounts of acetylated LDL, while Ohta et al. showed that TNF-deficient macrophages had reduced endocytosis of oxidized LDL due to reduced expression of SR-A in these cells. Our results are in agreement with this latter finding.

Figure 3  Phenotypic characterization of p55 TNFR \(^{+/+}\)-tp and p55 TNFR \(^{-/-}\)-tp plaques. p55 TNFR \(^{+/+}\)-tp (n = 15) and p55 TNFR \(^{-/-}\)-tp (n = 16) plaques were analysed for (A) T-cell content; (B) neutrophil content; and (C) apoptosis (TUNEL-positive cells). Shown is the number of positive cells per lesion area. (D) Necrotic area and (E) collagen (Sirius-red-positive area) are indicated as percentage of the total area. Representative pictures of immunohistochemical stainings are shown. Original magnification \( \times 100 \). Arrows indicate positive cells. Error bars indicate SEM.
identifying an important role for TNF-p55 TNFR signalling in promoting SR-A expression and modified lipoprotein accumulation and foam cell formation, in both non-activated and activated macrophages. A possible explanation for the discrepancies in the different studies might reside in the duration of p55 TNFR stimulation or in the type of modified LDL used in the different experiments. In our experiments, p55 TNFR knock-out cells endocytosed significantly less oxidized lipoproteins compared with wild-type cells, while this difference was less pronounced for the acetylated lipoproteins.

In addition, in this study we show that p55 TNFR deficiency in bone marrow-derived cells leads to reduced systemic levels of the major pro-atherosclerotic chemokine MCP-1. In line with our findings, previous reports have also identified a pivotal role for TNF and p55 TNFR signalling in controlling MCP-1 expression. Chemokines promote the migration of leukocytes in inflammatory processes and studies in mice with a targeted disruption of MCP-1 or its receptor have established the crucial role of this chemokine in atherogenesis. MCP-1 is mainly produced by macrophages and endothelial cells and TNF is reported to induce its expression. In atherosclerosis, two different studies have shown that TNF and p55 TNFR signalling are necessary for normal MCP-1 expression levels in vascular wall cells (i.e. smooth muscle cells and endothelial cells), and inhibition of this signalling results in reduced monocyte recruitment to the artery wall and smaller plaques. In our p55 TNFR\( ^{-/-} \)-tp mice we measured ~40% lower plasma levels of MCP-1 before a high fat diet was started, and protein levels remained ~25% lower during high fat feeding. In addition, plasma MCP-1 levels in non-transplanted p55 TNFR\( ^{-/-} \) mice on a chow diet were also 40% lower compared with controls. These data indicate that p55 TNFR expression in bone marrow-derived cells has a unique role in immune regulation since it controls physiological levels of systemic MCP-1. Whether, p55 TNFR has a similar role in regulating other chemokines remains to be elucidated.

Lower circulating levels of MCP-1 and possibly other pro-atherosclerotic chemokines, might shift the balance towards a less atherosclerosis prone environment in the p55 TNFR\( ^{-/-} \)-tp mice and may lead to reduced monocyte recruitment to the artery wall. Additionally, our study suggests that the monocytes that are recruited into the developing lesions are hindered in their ability to scavenge modified lipoproteins in the absence of p55 TNFR.

Lesion analysis showed that despite the difference in macrophage foam cell area, the cellular composition of the lesions appeared similar between wild-type and p55 TNFR\( ^{-/-} \)-tp mice indicating that other inflammatory cells, such as lymphocytes or neutrophils are efficiently recruited to the developing plaques. Moreover, cell death-related features such as the number of TUNEL-positive nuclei or the extent of the necrotic core were also similar in the two groups. Macrophage and foam cell death plays an important but still not well-defined role in atherosclerosis, and due to the presence of a death domain p55 TNFR is a major mediator of apoptosis. Using TNF knock-out APOE*3-Leiden mice, Boesten et al. found an increased incidence of TUNEL-positive nuclei in the absence of TNF. In combination with our findings, these results suggest that in atherosclerotic...
lesions, TNF signalling through the p75 TNFR alone is sufficient for the regulation of pro-survival signals, possibly through activation of the transcription factor NF-κB and synthesis of anti-apoptotic proteins.

Our results are in line with the majority of previous reports that identify a pro-atherosclerotic role of TNF; in particular, a bone marrow transplantation approach has pointed out the importance of bone marrow-derived TNF in atherogenesis. Together with our study these data suggest that TNF produced by bone marrow-derived cells induces a feedback signalling through p55 TNFR on the same cells to mediate at least part of the pro-atherogenic activities of this...
cytokine, as the systemic release of chemokines such as MCP-1 and the increased uptake of modified lipoproteins. However, since the effect on atherosclerosis is relatively mild, it might be that other factors, such as p55 TNFR expression in additional non-bone marrow-derived cells as recently shown for arterial wall p55 TNFR,16 or signalling through the p75 TNFR15 might contribute to the full atherogenic action of TNF.

Figure 6 Analysis of systemic inflammatory markers: (A–E) plasma levels of inflammatory mediators in p55 TNFR<sup>+/+</sup>-tp and p55 TNFR<sup>−/−</sup>-tp mice (n = 10/group), before and after 4 and 8 weeks of high fat feeding; (F) plasma levels of inflammatory mediators in non-transplanted mice on a normal chow diet (n = 10/group). Error bars indicate SEM. *P < 0.05 by Student’s t-test.

Our results are not in line with Schreyer et al.13 who determined a protective role of p55 TNFR but no involvement of the TNF ligand in atherosclerosis.1 However, they used as an atherosclerosis model C57BL/6 mice fed an atherogenic diet containing cholate, which may explain discrepancies between the studies. In addition, Blessing et al.14 have shown that in 64-week-old apoE mice, p55 TNFR does
not seem to mediate plaque progression or destabilization in innominate arteries. We have focused on early atherosclerosis in the aortic valve area in younger mice, which may explain the differences observed.

Due to its central role in inflammation and the pathogenesis of different inflammatory disorders such as rheumatoid arthritis (RA) or inflammatory bowel disease, TNF blockade has been successfully introduced into the clinic. Atherosclerosis and RA share similar inflammatory mechanisms and RA patients show increased cardiovascular mortality and morbidity. Consequently, anti-TNF therapy has been proposed for the treatment of cardiovascular diseases. However, in parallel with its pro-inflammatory action, TNF also exerts immune-suppressive functions as well and, in patients with multiple sclerosis, TNF inhibition results in autoimmune complications and disease exacerbation.

In addition, TNF blockade shows lack of improvement of symptoms and increased risk of death in patients with heart failure. Since p55 TNFR seems to mediate the majority of TNFs' pro-atherogenic actions, we suggest that selective p55 TNFR over TNF blockade could be considered a more valuable approach in atherosclerosis therapy.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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Conflict of interest: none declared.

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