Deficiency in Lymphotoxin β Receptor Protects From Atherosclerosis in apoE-Deficient Mice

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Rationale: Lymphotoxin β receptor (LTbR) regulates immune cell trafficking and communication in inflammatory diseases. However, the role of LTbR in atherosclerosis is still unclear.

Objective: The aim of this study was to elucidate the role of LTbR in atherosclerosis.

Methods and Results: After 15 weeks of feeding a Western-type diet, mice double-deficient in apolipoprotein E and LTbR (apoE−/−/LTbR−/−) exhibited lower aortic plaque burden than did apoE−/− littermates. Macrophage content at the aortic root and in the aorta was reduced, as determined by immunohistochemistry and flow cytometry. In line with a decrease in plaque inflammation, chemokine (C–C motif) ligand 5 (Ccl5) and other chemokines were transcriptionally downregulated in aortic tissue from apoE−/−/LTbR−/− mice. Moreover, bone marrow chimeras demonstrated that LTbR deficiency in hematopoietic cells mediated the atheroprotection. Furthermore, during atheroprogession, apoE−/− mice exhibited increased concentrations of cytokines, for example, Ccl5, whereas apoE−/−/LTbR−/− mice did not. Despite this decreased plaque macrophage content, flow cytometric analysis showed that the numbers of circulating lymphocyte antigen 6C (Ly6C)monocytes were markedly elevated in apoE−/−/LTbR−/− mice. The influx of these cells into atherosclerotic lesions was significantly reduced, whereas apoptosis and macrophage proliferation in atherosclerotic lesions were unaffected. Gene array analysis pointed to chemokine (C–C motif) receptor 5 as the most regulated pathway in isolated CD115+ cells in apoE−/−/LTbR−/− mice. Furthermore, stimulating monocytes from apoE−/− mice with agonistic anti-LTbR antibody or the natural ligand lymphotoxin-αβ2, increased Ccl5 mRNA expression.

Conclusions: These findings suggest that LTbR plays a role in macrophage-driven inflammation in atherosclerotic lesions, probably by augmenting the Ccl5-mediated recruitment of monocytes. (Circ Res. 2015;116:e57-e68. DOI: 10.1161/CIRCRESAHA.116.305723.)

Key Words: atherosclerosis ■ chemokine CCL5 ■ inflammation ■ lymphotoxin beta receptor ■ monocyte

Lymphotoxin α and β (LTα and LTβ), along with the classical cytokines tumor necrosis factor (TNF)-α and lymphotoxin-related inducible ligand, belong to the superfamily of TNF cytokines.1 Lymphotoxin is crucially involved in the communication between lymphocytes and the surrounding tissue by forming trimeric molecules (LTα3 or LTαβ2) that bind to various receptors in the lymphotoxin/TNF/lymphotoxin-related inducible ligand network. These specific cell surface receptors, including TNF receptor p55, TNF receptor p75, herpes virus entry mediator, and lymphotoxin β receptor (LTbR), have a partially overlapping profile of ligand interactions. Only the LTαβ2 heterotrimer and lymphotoxin-related inducible ligand can bind and activate LTbR. Both the ligands are expressed solely on activated lymphocytes, natural killer cells, or a special subset of follicular B cells.2,3 In contrast, LTbR is not expressed on lymphocytes but instead on stromal cells, such as endothelial cells, smooth muscle cells (SMCs), and cells of the myeloid lineage. Because of this characteristic...
distribution pattern, the communication is strictly unidirectional from lymphocytes to other immune cells and to the surrounding tissue. This communication orchestrates the correct trafficking of lymphoid cells and cellular interactions between immune cells.4

Besides the well-known effects of LTbR on lymphoid tissue organization, several studies also provide evidence of its proinflammatory role in various diseases characterized by acute inflammatory bursts, such as arthritis, experimental colitis, autoimmune pancreatitis, and experimental autoimmune encephalomyelitis.4 However, with respect to atherosclerosis the role of LTbR remains controversially discussed. Both, potential antiatherosclerotic (accelerated cholesterol efflux from macrophages via LTbR)9 and proatherosclerotic effects of this receptor, have been reported. For example, SMC proliferation10 and cytokine and chemokine release11 by endothelial cells are stimulated by LTbR activation. In addition, it has been shown that LTbR signaling may influence lipid homoeostasis by inhibiting the expression of hepatic lipase.12

Finally, LTbR signaling has also been linked to the formation of tertiary lymphoid organs (TLOs) in the aortic adventitia of mice deficient in apolipoprotein E (apoE).13 However, the pathophysiological role of TLOs in the aorta is currently not completely understood because both, proatherogenic and antiatherogenic lymphocyte populations, have been detected in TLOs.14

First evidence in humans points to a role of LTbR in atherosclerosis because levels of circulating LTbR in human plasma samples are associated with coronary calcium, aortic plaque, and aortic wall thickness.15 Therefore, the role of LTbR in atheroprogression and the mechanisms underlying this role remain to be clarified. From the translational perspective, it is important to note that LTbR signaling can be targeted by a blocking antibody that is currently being tested in a phase 2 clinical trial involving patients with Sjögren syndrome.

**Methods**

A detailed description of the methods used in this study are available in the Online Data Supplement.

**Animals**

Eight-week-old homozygous apoE−/− mice and apoE−/−/LTbR−/− mice on a C57BL/6J background were fed a high-fat, high-cholesterol Western-type diet (WD) (S8200-E010, sniff Spezialdiäten GmbH, Soest, Germany) containing 21% crude fat, 21% butterfat, and 0.15% cholesterol for either 4 or 15 weeks. All experiments were performed according to the guidelines for the use of experimental animals of the Deutsches Tierschutzgesetz and were approved by the local Research Board for animal experimentation (LANUV; State Agency for Nature, Environment and Consumer Protection).

**Flow Cytometric Analysis**

For flow cytometric analyses of blood, bone marrow, aortic tissue, splenic tissue, and peritoneal lavage fluid, 12-week-old apoE−/− and apoE−/−/LTbR−/− mice were euthanized after 4 weeks of feeding WD. Absolute cell concentrations were determined with Flow-Count Fluorospheres (Beckman Coulter Inc, Krefeld, Germany). Flow cytometric measurements were performed with a Gallios Flow Cytometer (Beckman Coulter Inc) and an LSRII flow cytometer (Becton–Dickinson, Heidelberg, Germany), and Kaluza Flow Analysis Software (Beckman Coulter Inc) and FlowJo software (Treestar, San Carlos, CA) were used for subsequent data analysis.

**Multiplex Analysis**

For determination of multiple cytokines in the plasma, a commercially available multiplex bead–based immunoassay (Bio-Plex Pro Cytokine Group 1 23-plex; Biorad, Hercules, CA) was used.

**Generation of Bone Marrow Chimeras**

The bone marrow of 8-week-old lethally irradiated (10 Gy) male mice was reconstituted with 5×10⁶ unfractionated bone marrow cells injected intravenously into the lateral tail vein. After the transfer, mice were fed WD for 15 weeks and were euthanized at the age of 23 weeks for the assessment of atherosclerosis and the determination of macrophage content at the aortic root.

**Microarray Gene Expression Analyses**

Peripheral blood CD115⁺ cells were isolated with a CD115 MicroBead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and aortas were removed from 12-week-old apoE−/− and apoE−/−/LTbR−/− mice. Total RNA was prepared with the RNeasy Microarray Tissue Kit (Qiagen, Hilden, Germany). cDNA syntheses, hybridizations, and scanning of Affymetrix Mouse Gene 2.0 ST Gene Expression microarrays were performed according to the manufacturer’s instructions (NuGEN Inc, San Carlos, CA; Affymetrix, Inc, Santa Clara, CA). Data analyses on Affymetrix CEL files were conducted with GeneSpring GX software (Vers. 12.5; Agilent Technologies, Santa Clara, CA). The significance threshold was set to P=0.01. Functional enrichment of differentially expressed genes within canonical pathways was analyzed with Ingenuity Pathway Analysis software (Qiagen). The complete data set was deposited at the National Center for Biotechnology Information’s Gene Expression Omnibus (accession number GSE63259).

**Quantitative Real-Time Polymerase Chain Reaction**

Specific primers for chemokine receptors and chemokines were obtained from Applied Biosystems (Carlsbad, CA) and Life technologies (Carlsbad, CA) or were designed as described (primer sequences are shown in Online Table 1) and used for quantitative polymerase chain reaction (see detailed description of methods in the Online Data Supplement).
Comparison of relative gene expression was performed with the ΔΔCt method, as previously described.16,17

Isolation of Primary Monocytes and In Vitro Stimulation With Agonistic Anti-LTbR Antibody or LTα1β2

Analyses of chemokine receptor expression and in vitro migration assays were performed with isolated bone marrow monocytes from 12-week-old male apoE−/− and apoE−/−/LTbR−/− mice by using the CD115 MicroBead Kit (Miltenyi Biotec) according to the manufacturer’s instructions.

For measurement of LTbR-induced gene expression, mouse monocytes were isolated from the bone marrow of 6- to 8-week-old male apoE−/− mice with a CD115 MicroBead Kit (Miltenyi Biotec) according to the manufacturer’s instructions.

For migration assays, 3×10^5 cells were seeded in ultralow attachment plates (Greiner, Bio-One-GmbH, Kremsmünster, Austria) and incubated with either 10 μg/mL agonistic anti-LTbR antibody (clone 5G11; Hycult Biotech, Plymouth Meeting, PA), 1 μg/mL LTα1β2 (R&D Systems, Minneapolis, MN),18 or the respective controls for 24 hours. Cells were labeled for 30 minutes with 10 μmol/L calcine at room temperature (Merck Millipore, Billerica, MA), washed twice with phosphate-buffered saline, and seeded in 5 μm inserts (Transwell Permeable Supports, Corning Incorporated, Corning, NY) in a 24-well plate in RPMI medium containing 5% fetal calf serum. Cells were allowed to migrate for 4 hours toward 10% fetal calf serum in the bottom well, after which migrated cells were measured with a Synergy Mx microplate reader (BioTek, Winooski, VT).

Recruitment of Labeled Monocytes to Atherosclerotic Lesions

For selective in vivo labeling of circulating lymphocyte antigen 6C (Ly6C) and Ly6C<sup>+</sup> monocytes, fluorescent microspheres (Fluoresbrite Polychromatic Red Microspheres; Polysciences, Warrington, PA) were used according to a protocol described by Tacke et al.19 The number of invading monocytes was calculated by dividing the number of lesional microsphere-positive macrophages by the number of circulating labeled Ly6C<sup>+</sup> or Ly6C<sup>+</sup> monocytes, respectively.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism 6 software (GraphPad Software, La Jolla, CA). Data are presented as mean±SEM of the mean (SEM). Values identified as outliers by Grubbs test (<0.05) were excluded from the analysis. Statistical significance was set at the level of P<0.05. In ANOVA followed by the Bonferroni post hoc test unless stated otherwise. Statistical significance was set at the level of P<0.05.

Results

LTbR Deficiency Reduced Atherosclerosis and Plaque Inflammation

Starting at 8 weeks of age, male apoE−/−/LTbR−/− mice and their apoE−/− littermate controls were fed a WD for 4 weeks (until 12 weeks of age) or 15 weeks (until 23 weeks of age; Figure 1A). No statistically significant changes in body mass index or plasma lipid levels were detected (Online Figure I, panels A–E).

LTbR was strongly expressed in the aortas of C57BL/6J mice and apoE−/− mice fed normal chow diet and in the aortas of apoE−/− mice fed a WD for 4 weeks (data not shown). Importantly, LTbR mRNA expression was also detectable in human atherectomy specimens (data not shown).

In 23-week-old male mice, the extent of atherosclerosis was quantified in en face preparations of the aorta. The atherosclerotic plaque area was significantly lower for apoE−/− mice deficient in LTbR than for their littermate controls (Figure 1B; area fraction: apoE−/−, 8.9±0.6%; apoE−/−/LTbR−/−, 6.6±0.7%; n=6–8) as determined by lipid staining with Oil Red O. A similar trend toward a lower atherosclerotic burden was also observed in female mice (data not shown). The plaque size at the aortic root was decreased in apoE−/−/LTbR−/− as well (Figure 1C). Importantly, at 23 weeks of age, male apoE−/−/LTbR−/− mice exhibited significantly weaker mac2 staining than did their apoE−/− littermates (positively stained area: apoE−/−, 20.1±2.8%; apoE−/−/LTbR−/−, 11.1±2.4%; n=5–7), a finding indicating lower numbers of macrophages in the atherosclerotic lesions at the aortic root in apoE−/−/LTbR−/− mice (Figure 1D).

Aortic root plaque composition was analyzed further in detail. Lipid retention was not different between the groups (Online Figure II A). Furthermore, the amount of α smooth muscle actin (αSMA)–positive SMC was reduced (Online Figure II B). The cell density (nuclei/plaque area) was assessed microscopically to search for quantitative changes in the accumulation of extracellular matrix. Cell density was, however, unchanged in apoE−/−/LTbR−/− mice (data not shown). To search for qualitative changes in the matrix composition, collagen and proteoglycans were analyzed. Plaque collagen content, and

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**Figure 1. Reduced atherosclerotic lesion size and macrophage accumulation in 23-week-old apolipoprotein E (apoE)−/−/Lymphotoxin β receptor (LTbR)−/− mice.** A, Male, 8-week-old apoE−/− and apoE−/−/LTbR−/− mice were fed a Western-type diet (WD) for 4 or 15 weeks, respectively. The experimental design is shown. B, En face preparations of Oil Red O-stained aortas of 23-week-old apoE−/−/LTbR−/− mice and their apoE−/− littermates and determination of the aortic plaque score. Representative images of Oil Red O-stained aortas are shown; n=6 to 8. C, Determination of atherosclerotic lesion size at the aortic root and D, quantification of macrophage content in atherosclerotic lesions at the aortic root at 23 weeks of age as mac2-positive area fraction. Pictures are taken at ×50-fold; n=5 to 7. Data are presented as mean±SEM; *P<0.05. In C, Mann–Whitney test was used.
collagen organization as evidenced by birefringence analysis of Sirius red staining (not shown) did not differ significantly between the genotypes (Online Figure II C). Because SMC are known to secrete large amounts of proteoglycans into the lesion, the amount of glycosaminoglycans as stained by alcian blue and of proatherogenic proteoglycans, versican, biglycan, and perlecan, was determined. Although no significant changes were detected (Online Figure II, panels D–G), alcian blue, versican, and biglycan staining revealed a trend toward reduced proteoglycan accumulation in apoE−/−/LTbR−/− mice. Therefore, the data suggest that in addition to reduced macrophages, reduced SMC accumulation occurs in the plaques of apoE−/−/LTbR−/− mice, which is paralleled by a trend to less proteoglycan accumulation and no effect on total collagen.

The mechanisms underlying the atheroprotective effects of LTbR deficiency were examined at an early time point in the development of atherosclerotic lesions. At 12 weeks of age, the genotypes are just beginning to exhibit differences in athroprogession. Therefore, in an attempt to discover underlying pathways, 12-week-old mice were chosen for mechanistic analysis. Twelve-week-old male apoE−/−/LTbR−/− mice exhibited a trend toward reduced atherosclerotic lesion size compared with their apoE−/− littermate controls (Figure 2A; area fraction: apoE−/−, 2.9±0.4%; apoE−/−/LTbR−/−, 2.2±1.8%; n=7). However, lesions at the aortic root were significantly smaller in apoE−/−/LTbR−/− mice (Figure 2B) and the percentage of invaded macrophages was markedly decreased, as determined by immunohistochemical analysis (Figure 2C; apoE−/−, 4.7±1.2%; apoE−/−/LTbR−/−, 1.2±0.4%; n=5–7).

Flow cytometric analysis of the aorta confirmed that apoE−/−/LTbR−/− mice exhibited lower numbers of invading macrophages (Figure 2D–2F), whereas the relative distribution of M1 and M2 macrophages remained unchanged between the 2 genotypes (data not shown). These findings suggest that the underlying reason for the decreased development of atherosclerosis in apoE−/−/LTbR−/− mice is a decrease in the influx or retention of macrophages, with no effect on macrophage polarization. A representative gating scheme for aortic macrophages and isotype control staining is shown in Online Figure III, panels A and B.

Chemokines and chemokine receptors differentially regulate the recruitment of inflammatory cells into the vessel wall, depending on the stage of atherosclerosis. Therefore, quantitative polymerase chain reaction was used to determine whether LTbR deficiency influences the expression profile of chemokines, chemokine receptors, or both in the aortic tissue of 12-week-old mice. Especially, the factors responsible for monocyte invasion during the initial and early phases of lesion development were investigated. Analysis of aortic mRNA expression showed that the levels of nearly all studied chemokines were significantly lower in apoE−/−/LTbR−/− mice than in their apoE−/− littermates (Figure 2G).

Detailed flow cytometric analysis demonstrated not only lower numbers of macrophages but also important changes in the composition of aortic immune cells: apoE−/−/LTbR−/− mice exhibited a trend toward fewer CD45+ cells and higher lymphocyte counts than did the apoE−/− controls (Online Figure IV, panels A–C). This trend was primarily driven by a significant

Figure 2. Reduced macrophage accumulation and chemokine/chemokine receptor expression in 12-week-old apoE−/−/LTbR−/− mice. A, En face preparations of Oil Red O-stained aortas of apoE−/−/LTbR−/− mice and their apoE−/− littermates and determination of the aortic plaque score. Representative images of Oil Red O-stained aortas are shown; n=7. B, Determination of atherosclerotic lesion size at the aortic root; n=8. C, Macrophage content in atherosclerotic lesions at the aortic root and quantification of macro2-positive area fraction. Pictures are taken at ×50-fold; n=5 to 7. D and E, Flow cytometric analysis of aortic macrophage content, determined as CD11b+/F4/80+ cells per aorta; n=13. E, Representative plots gated on CD45+ cells are shown. F, Ratio of invaded lesional macrophages/number of circulating monocytes per microliter blood; n=13 to 18. G, mRNA expression of multiple chemokines and chemokine receptors in aortas from apoE−/− and apoE−/−/LTbR−/− mice as determined by quantitative polymerase chain reaction; n=4 to 6. Data are presented as mean±SEM; *P<0.05 vs apoE−/−. In D, Mann–Whitney test was used. In G, 1-way ANOVA followed by Holm–Sidaks multiple comparison test was used.
increase in the number of B lymphocytes in apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> mice (Online Figure IV, panels D–F; a representative gating scheme for aortic lymphocytes and isotype control staining is shown in Online Figure VA and B).

Because of this observation of altered immune cell subsets in the aorta, also other immune cell compartments were analyzed. In line with the described homing defect caused by changes in the architecture of the spleen and the absence of lymph nodes in apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> mice, we detected fewer leucocytes and significantly lower numbers of splenic lymphocytes (Online Figure VI, panels A–C) because of decreased B- and T-cell numbers (Online Figure VI, panels D–F). In parallel, the relative percentage of B cells compared with T cells was increased (Online Figure VI G). We observed no changes in the numbers of macrophages (Online Figure VI H) and with respect to M1/M2 polarization in the spleen between both the genotypes (data not shown).

The spleen serves as a known reservoir for monocytes. It has been described that during atheroprogression extramedullary monopoiesis in the spleen increases leading to an efflux of monocytes to the circulation and their recruitment to the inflammatory sites. Flow cytometric analysis of Ly6C<sup>high</sup> and Ly6C<sup>low</sup> monocytes in the spleen of apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> mice revealed a significant decrease in both the subsets compared with apoE<sup>−/−</sup> controls (Online Figure VI, panel I and VI, panel J). Representative plots and isotype control staining are shown in Online Figure VI, panel K.

The peritoneal cavity is an important immune cell compartment, in which many immune cell subsets can be found. Peritoneal lavage fluid was analyzed by flow cytometry for the distribution of immune cell subsets. Increased leukocytes (Online Figure VII, panels A and B) and, as was the case for the aortic wall, more lymphocytes (Online Figure VII C) and in particular more B cells were detectable in apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> mice than in apoE<sup>−/−</sup> mice (Online Figure VII, panels D–G). However, no changes in the basal content of macrophages were observed (Online Figure VII, panels H and I).

In contrast to the results in atherosclerotic plaques, a trend toward an even stronger invasion of immune cells was observed in apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> mice with thioglycollate-induced peritonitis as a model of acute inflammation (Online Figure VIII A). However, after normalization of the results to circulating monocyte counts, no differences in thioglycollate-triggered macrophage invasion could be observed (Online Figure VIII B). On the basis of these findings, it might be assumed that a reduction in the numbers of macrophages in atherosclerotic plaque does not represent a general recruitment defect of macrophages in apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> mice but is rather because of factors and mechanisms specific for the progression of atherosclerosis representing chronic low-grade inflammation.

**LTbR Deficiency Leads to Decreased Systemic Inflammation During Atheroprogression**

To evaluate whether the inflammatory response is generally reduced in apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> mice, analysis of important circulating cytokines was performed with a multiplex bead-based assay (Figure 3; Online Figure IX). Cytokine concentrations at early stages of atherosclerosis (12 weeks of age) were generally unchanged between genotypes. Importantly, during atheroprogression, a strong increase of circulating proinflammatory chemokine (C–C motif) ligand 5 (Ccl5), interleukin (IL)-2, IL-6, and interferon (IFN)-γ was detected at 23 weeks compared with 12 weeks in apoE<sup>−/−</sup> mice (Figure 3A–3D). In contrast, in apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> mice this increase of Ccl5, IL-2, IL-6, and IFNγ was missing at 23 weeks (Figure 3A–3D). Furthermore, the concentration of anti-inflammatory IL-10 was significantly higher in apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> mice than in apoE<sup>−/−</sup> mice at 23 weeks (Figure 3E).

**Hematopoietic LTbR Expression Promotes Atherosclerosis**

Next, it was determined whether LTbR expression in cells of the hematopoietic lineage or in stromal cells of the arterial compartment promotes atherosclerosis and plaque inflammation in apoE<sup>−/−</sup> mice. For these studies, bone marrow chimeras were generated. Successful reconstitution of recipient bone marrow with donor bone marrow was verified by PCR for the deleted or wild-type LTbR allele on genomic DNA isolated from whole blood from the chimeras (Online Figure X). In addition, LTbR expression on circulating monocytes was analyzed by flow cytometric analysis of bone marrow chimeras; this analysis demonstrated a reconstitution rate of 90% to 100% (data not shown). Homologous transfers of apoE<sup>−/−</sup> bone marrow in apoE<sup>−/−</sup> mice and of apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> bone marrow in apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> mice served as controls and demonstrated a reduction in atherosclerosis caused by global deletion of LTbR, as shown above for nonirradiated apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> mice (Figure 4A). Importantly, the atherosclerotic plaque burden was decreased to a similar extent in apoE<sup>−/−</sup>mice receiving...
apoE−/−/LTbR−/− bone marrow, whereas no effect on atherosclerosis was observed in chimeras with apoE−/− bone marrow on an apoE−/−/LTbR−/− background (Figure 4A). In line with these findings were the results of lesional macrophage content, which showed that plaques from apoE−/− mice reconstituted with bone marrow from apoE−/−/LTbR−/− mice contained significantly smaller numbers of macrophages than did plaques from apoE−/−/LTbR−/− animals receiving apoE−/− bone marrow (Figure 4B).

**LTbR Deficiency Promotes Monocytosis in the Peripheral Blood of apoE−/−/LTbR−/− Mice**

The results of the above presented experiments using bone marrow chimeras strongly suggested that the hematopoietic expression of LTbR plays a causal role in atherogenesis and atheroprotection. Therefore, we examined the immune cell populations in peripheral blood. At 12 weeks of age, apoE−/−/LTbR−/− mice exhibited stronger leukocytosis than their apoE−/− littermate controls (Figure 5A and 5B). Analysis of the relative contribution of various cell subsets to the total leukocyte fraction demonstrated that leukocytosis, in particular, was based on a strong increase in lymphocyte counts (Figure 5C). As observed for the aorta, in peripheral blood B cells were the lymphocyte subset that was strongly increased, and the ratio of B/T cells shifted toward B lymphocytes (Figure 5D–5F). However, in apoE−/−/LTbR−/− mice also the absolute numbers of circulating neutrophils and monocytes were elevated compared with their apoE−/− littermate controls (Figure 5G and 5H) at 12 weeks.

Ly6Cslow monocytes (also referred to as nonclassical monocytes) and Ly6Cchib monocytes (or classical monocytes), are known to be differently involved in the early steps of plaque development. Therefore, we performed flow cytometric analysis of circulating monocyte subpopulations to determine whether the more inflammatory Ly6Cchib subset might be reduced in apoE−/−/LTbR−/− mice. However, absolute numbers of Ly6Cchib monocytes were significantly higher in apoE−/−/LTbR−/− mice, whereas absolute number of Ly6Cchib cells did not differ between apoE−/−/LTbR−/− mice and their apoE−/− littermates (Figure 5I–5K). This increase in the numbers of Ly6Cchib monocytes resulted in a shift in the ratio of Ly6Cchib/Ly6Cslow monocytes in apoE−/−/LTbR−/− mice (Figure 5J). A representative gating scheme for flow cytometric analysis of circulating immune cells is provided in Online Figure XI.

In contrast to peripheral blood, in bone marrow the numbers of Ly6Cchib and Ly6Cslow monocytes did not differ between apoE−/−/LTbR−/− mice and apoE−/− mice (Online Figure XII A). In addition, monocyte progenitors (monocyte dendritic cell progenitor and circulating monocyte progenitor) in the bone marrow were not affected in apoE−/−/LTbR−/− mice (Online Figure XII B and C; a representative gating scheme is shown in XIID). These findings suggest that the pronounced Ly6Cchib monocytosis in apoE−/−/LTbR−/− mice is because of a peripheral recruitment defect of monocytes rather than to an increase in hematopoiesis. Next, we analyzed the expression of LTbR on circulating monocyte subsets. We found that LTbR was expressed on both Ly6Cchib and Ly6Cslow monocytes (data not shown).

**LTbR Promotes the Recruitment of Ly6Cslow Monocytes into Atherosclerotic Lesions**

On the basis of the findings of an increase in the numbers of circulating monocytes concurrent with a reduction in lesional macrophages, we next considered whether a reduction in monocyte recruitment, a decrease in macrophage proliferation in the atherosclerotic plaque, or an increase in the rate of apoptosis in the atherosclerotic lesion might be responsible for the atheroprotective phenotype.

No changes in macrophage proliferation were observed, as determined by the thymidine analog 5-bromo-2-deoxyuridine.
staining of lesional macrophages (Online Figure XIII A). In addition, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining of the aortic root detected no alterations in the rate of apoptosis (Online Figure XIII B).

Because peripheral monocytosis was caused especially by high numbers of circulating Ly6C<sub>low</sub> monocytes, we performed additional analyses focused specifically on the recruitment of Ly6C<sub>low</sub> and Ly6C<sub>high</sub> monocytes. The Ly6C<sub>low</sub> monocyte subset was preferentially labeled using an in vivo approach with intravenous injections of fluorescent microspheres, which was previously described by Tacke et al. Mice were treated with clodronate 18 hours before application of the fluorescent microspheres to specifically label Ly6C<sub>high</sub> monocytes. Three days after intravenous injection of fluorescent microspheres, monocyte invasion into the developing atherosclerotic lesion was analyzed. Serial sections of the aortic root were stained for mac2 and the number of macrophages labeled by the uptake of fluorescent microspheres was counted (Figure 6A).

This experiment demonstrated that the invasion of Ly6C<sub>low</sub> monocytes into the plaques of apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> mice was significantly lower than into the plaques of apoE<sup>−/−</sup> controls, as calculated by the ratio of the number of labeled lesional macrophages/circulating Ly6C<sub>low</sub> monocytes. In contrast, the recruitment of Ly6C<sub>high</sub> monocytes was not affected (Figure 6C).

**LTbR Stimulates Chemokine Release in Monocytes**

Next, we addressed the underlying pathways leading to decreased monocyte influx and atheroprotection by performing gene array analyses of isolated CD115<sup>+</sup> cells from peripheral blood and isolated aortas from apoE<sup>−/−</sup> and apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> mice (Online Figure XIV; Online Tables II–VII). Importantly, in CD115<sup>+</sup> cells, the chemokine (C–C motif) receptor 5 (Ccr5) signaling pathway in macrophages emerged as the most highly regulated pathway (Online Table II) that has been shown to be involved in the recruitment of both the monocyte subsets to the atherosclerotic plaque. However, Ccr<sub>5</sub>mRNA expression on isolated monocytes was not significantly different between apoE<sup>−/−</sup> and apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> mice (Figure 7A), a finding suggesting that expression of the receptor itself is not crucial. In addition, the chemokine receptors Ccr1, Ccr2, Ccr3, and Cx3cr1 were also unaffected by LTbR deficiency (Figure 7A). These findings indicate that regulation of chemokine receptors...
is not responsible for the decrease in monocyte recruitment to atherosclerotic lesions in apoE−/−/LTbR−/− mice.

The results of the gene array analysis of isolated aortas confirmed a pronounced downregulation of multiple genes, as also observed for various chemokines and chemokine receptors in the aortas of apoE−/−/LTbR−/− mice (Figure 2G). In line with the observed changes in lymphocyte subsets in various compartments, and once more underlining the importance of the LTbR pathway for lymphoid tissue and function, the findings showed that the pathways involved in B-cell development, CD28 T-helper cell signaling, and T-cell receptor signaling were predominantly regulated in apoE−/−/LTbR−/− aortas (Online Table III).

Ccl3/macrophage inflammatory protein-1α, Ccl4/macrophage inflammatory protein-1β, Ccl8/MCP-2, and Ccl5 are known Ccr5 ligands. It has been proposed that Ccl5 plays a crucial role in promoting monocyte entry. Accordingly, as mentioned above, the missing increase in circulating Ccl5 in apoE−/−/LTbR−/− mice and a reduction in Ccl5 mRNA expression occurred in apoE−/−/LTbR−/− aortas (Figures 2G and 3). To determine whether LTbR directly increases Ccl5 expression, bone marrow-derived monocytes were isolated from apoE−/− mice and stimulated with agonistic anti-LTbR antibody or the natural ligand LTα1β2. Indeed, Ccl5 and TNF-α were upregulated after 4 hours of stimulation (Figure 7B). In contrast, no significant changes in mRNA expression of the other Ccr5 ligands were detected (data not shown). These findings demonstrate direct induction of inflammatory cytokines in monocytes by LTbR signaling. In line with this finding are the direct promigratory effects of agonistic LTbR antibody (Figure 7C) and LTα1β2 (data not shown) on the migration of isolated monocytes, which strongly supports the concept of LTbR-stimulated monocyte recruitment.

Neointimal Hyperplasia is Unaffected in apoE−/−/LTbR−/− Mice

To determine whether the inhibitory effect of LTbR deficiency on atherosclerotic plaque formation was specific for atherosclerosis or could also affect other vascular pathologies, we studied apoE−/−/LTbR−/− mice in a model of neointimal hyperplasia. In this model, which represents primarily the migration and proliferation of SMCs and matrix synthesis, identical results were obtained for both the genotypes (Online Figure XV).

Discussion

Although previous studies have described LTbR-dependent modulation of various cell functions related to the development of atherosclerotic lesions, the role of LTbR in atherosclerosis is still unclear. Therefore, this study focused on the role of LTbR during atheroprogession. Aortic plaque burden was significantly lower in apoE−/−/LTbR−/− mice on WD than in apoE−/− controls, and the number of lesional macrophages was lower in early and late atherosclerosis. On the basis of these findings, we can assume a protective, anti-inflammatory role for LTbR deletion during atherosclerosis. Online Figure XVI summarizes a working hypothesis of the role of LTbR in atheroprogession.

It has been previously reported that LTbR promotes autoimmune responses by regulating immune cell trafficking via chemokine expression,23 coordinating immune cell interactions, and exerting a direct effect on cytokine secretion.24 LTbR has been suggested to play a proinflammatory role in a variety of inflammatory disorders, such as rheumatoid arthritis,25 autoimmune pancreatitis, and autoimmune hepatitis.26 In contrast, other studies have found that LTbR signaling by macrophages during acute colitis induced by dextran sodium sulfate exerts protective, anti-inflammatory functions by activating the tripartite motif containing 30α signaling pathway.27 Therefore, the immunomodulatory functions of LTbR seem to be context-specific.

One interesting aspect of the phenotype of apoE−/−/LTbR−/− mice was that the increase in circulating cytokines and chemokines that occurred in apoE−/− mice between 12 and 23 weeks was blunted in apoE−/−/LTbR−/− mice. This effect, together with a reduction in the number of lesional macrophages, suggested inhibition of the systemic proinflammatory state and of local macrophage-driven inflammation. This inhibition is the proposed effector mechanism leading to the reduction of atheroprogession in apoE−/−/LTbR−/− mice. Twelve-week-old apoE−/−/LTbR−/− mice were chosen to analyze the pathways and mechanisms that might be responsible for reduced atherosclerosis.

Figure 7. Lymphotoxin β receptor (LTbR) directly stimulates Ccl5 and Tnfa mRNA expression and monocyte migration. A, Determination of Ccr5, Ccr1, Ccr2, Ccr3, and Cx3cr1 mRNA expression in isolated monocytes from apolipoprotein E (apoE)−/− and apoE−/−/LTbR−/− mice at 12 weeks of age. Data are presented as means±SEM; n=6 to 7; *P<0.05 vs apoE−/−. B, Stimulation of isolated monocytes from apoE−/− mice with 10 μg/mL agonistic anti-LTbR antibody (anti–LTbR-Ab), LTα1β2 (LTα1β2) or respective control for 4 hours and determination of Ccl5 and tumor necrosis factor (TNF)-α mRNA expression by quantitative polymerase chain reaction; n=5 to 8; *P<0.05 vs respective control. C, Isolated monocytes were stimulated with 10 μg/mL agonistic anti–LTbR-Ab or control IgG for 24 hours followed by migration of the cells for 4 hours. Data are presented as mean±SEM; n=8; *P<0.05.
As depicted in Online Figure XVI, monocytic LTbR expression is a key element in the current working hypothesis, which is based on the finding that absence of LTbR expression in hematopoietic cells confers atheroprotection on bone marrow chimeras. Furthermore, atherosclerotic plaques from apoE−/−/LTbR−/− mice exhibited reduced amounts of CD11b+/F4/80+ macrophages, as detected by flow cytometry, and of macrophage-derived chemokines, as detected by immunohistochemistry. Therefore, a decrease in the recruitment of monocytes/macrophages was assumed to underlie the inhibition of atheroprotein in apoE−/−/LTbR−/− mice. This assumption is in line with the present in vivo finding that neither apoptosis in atherosclerotic plaques nor proliferation of macrophages was affected by LTbR deficiency. Flow cytometric analysis of the blood showed monocytosis and especially a strong increase in the number of Ly6Clow monocytes in apoE−/−/LTbR−/− mice. Ly6Clow monocytes are known to patrol the endothelium and healthy tissues; however, their role in atherosclerosis remains controversial, and both proatherosclerotic and anti-atherosclerotic functions have been proposed. Ly6Clow monocytes have been suggested to enter atherosclerotic plaques to a much lesser extent than Ly6Cshb monocytes18,20 and may exert their antiatherosclerotic function by triggering the production of natural antibodies in B cells independently of T cells.30 In contrast, Ly6Cshb monocytes have been reported to take up oxidized low-density lipoprotein, thereby promoting atherosclerosis by entering the developing lesion as cholesterol-loaded cells.31 Furthermore, Combadière et al32 found that the number of Ly6Cshb monocytes is strongly correlated with the size of atherosclerotic lesions in mice. Labeling Ly6Cshb monocytes according to a procedure established by Tacke et al32 suggested that recruitment of Ly6Cshb monocytes into atherosclerotic lesions was reduced in apoE−/−/LTbR−/− mice. In contrast, the recruitment of Ly6Cshb monocytes was not affected. Ly6Cshb monocytes use leukocyte function–associated antigen (LFA)-1 and intracellular adhesion molecule-1 for adhesion to the vessel wall during atherosclerosis.33 As shown by gene array analysis of circulating CD115+ cells and the aorta expression of LFA-1 and intracellular adhesion molecule-1 was not differentially regulated in apoE−/−/LTbR−/− mice.

The amount and proportion of Ly6Clow and Ly6Cshb monocytes, as well as monocyte progenitors in the bone marrow of apoE−/−/LTbR−/− mice were not significantly different from those in apoE−/− mice a finding suggesting that hematopoiesis of myeloid cells is not affected. In addition, during atheroprotein extramedullary hematopoiesis in the spleen has been described leading to efflux of Ly6Cshb monocytes to the circulation and their recruitment to inflammatory sites.22 Analysis of Ly6Cshb and Ly6Clow monocytes in the spleen of apoE−/−/LTbR−/− mice revealed a significant decrease in both the subsets compared with apoE−/− controls (Online Figure VI, panels I–K). However, these findings do not allow to conclude whether the disturbed architecture of the spleen in apoE−/−/LTbR−/− mice is causal for monocytosis and whether it was based on either reduced homing capacity of monocytes to the spleen or reduced extramedullary monocytopoiesis and/or increased release of monocytes from the spleen into the circulation. Furthermore, it cannot be excluded that other mechanisms, such as a decrease in the recruitment of monocytes into nonvascular tissues or an increase in the monocyte life span, may underlie the development of monocytosis in apoE−/−/LTbR−/− mice. Supporting this hypothesis was beyond the scope of this study.

Whole-genome gene array analysis of circulating CD115+ cells demonstrated differential regulation of 267 genes. In CD115+ cells, a transcriptional signature indicating generally reduced inflammation was identified. Among the downregulated genes that indicated proinflammatory functions were many genes associated with the predominantly proatherosclerotic IFN-γ pathway, for example, interferon-activated gene 205 (Ifi205); 2′-5′ oligoadenylate synthetase 1A (Oas1a), -1G (Oas1g), and -2 (Oas2); and receptor transporter protein 4 (Rtp4).34 This gene regulation is in line with the blunted increase in circulating IFN-γ in apoE−/−/LTbR−/− mice. In addition, upregulation of anti-inflammatory mediators was indicated by, for example, interleukin 9 receptor (IL9r) and stathmin 1, related sequence 1 (Stmnt1-1s1). IL9r was shown to inhibit both the production of reactive oxygen species and the release of TNF-α by LPS-stimulated human monocytes.35 Via toll-like receptor 3,36 stathmin is thought to mediate IL-10 induction,37 which is increased in the plasma of apoE−/−/LTbR−/− mice. Possible sources of IL-10 are next to macrophages, also B and T cells.

Interestingly, Ccr5 signaling in macrophages was the most regulated pathway, a finding hinting that Ccr5, its ligands, or both may play a role. Ccl5 mRNA expression was decreased in the aorta of apoE−/−/LTbR−/− mice at 12 weeks, a result that may reflect the decreased influx of macrophages into the aorta. In addition, the increase of circulating Ccl5 was blunted in apoE−/−/LTbR−/− mice at 23 weeks of age. Ccl5 is known to attract leukocytes, to mediate leukocyte arrest and transendothelial leukocyte recruitment, and to activate lesional leukocytes during atherosclerosis.38 Blocking the Ccr5 receptor reduced macrophage-driven inflammation during atherosclerosis,39,40 and Ccr5 deletion protected mice against atherosclerosis.41 In addition, Ccr5 deletion has been suggested to support a stable plaque phenotype, in part, by augmenting IL-10 expression.42 In light of these findings, it seems relevant that Ccr5 is particularly important for the accumulation of Ly6Clow monocytes in the lesions.19 In support of the suggestion that the Ccr5 pathway plays an important role, in vitro stimulation of monocytes with an LTbR ligand increased the expression of Ccl5 but not of the other Ccr5 ligands, Ccl3, Ccl4, and Ccl8. This finding is in line with LTbR-mediated upregulation of Ccl5 in hepatic stellate cells, an upregulation that was shown to promote leukocyte recruitment and thereby wound healing during chronic liver injury.43 In addition, several in vitro studies have demonstrated an increase in cytokine expression after targeting of LTbR. SMCs costimulated with agonistic anti-LTbR and TNF-α also upregulated multiple chemokines, such as Ccl2 and Ccl5, as well as vascular cell adhesion molecule-1 and intracellular adhesion molecule-1.44 Although not addressed experimentally, the reduction of both local and circulating levels of Ccl5 may contribute to the observed phenotype in apoE−/−/LTbR−/− mice. Furthermore, this present study found that migration of monocytes was stimulated by LTbR
ligands. Therefore, as depicted in Online Figure XVI, LTbR-mediated expression of Ccl5 by monocytes may create a positive feedback loop to augment the arrest and transmigration of monocytes and the activation of lesional macrophages. In line with this conclusion was the finding that the expression of a variety of proinflammatory cytokines and chemokines was significantly reduced in the aorta of apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> mice, as shown by quantitative polymerase chain reaction and gene array analysis. In addition to chemokines and cytokines, the histocompatibility 2, class II antigens, and adhesion molecules involved in leukocyte recruitment, such as glycosylation-dependent cell adhesion molecule-1<sup>15</sup> and selectin<sup>49</sup> were also downregulated in apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> mice, as determined by gene array analysis. The genes that were upregulated in the aorta of apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> mice also included genes associated with reduced progression of atherosclerosis, such as fetuin B<sup>57</sup>, an inhibitor of calcification, and genes involved in the fine-tuning of inflammatory responses, such as <i>inter α trypsin inhibitor</i><sup>58</sup> and the lectins <i>Lgals6</i> and <i>Lgals4</i>.<sup>49</sup>

Taken together, these findings support the hypothesis that LTbR on monocytes stimulates monocyte recruitment into atherosclerotic lesions and also promotes plaque inflammation, in part, by activating the Ccl5/Ccr5 pathway, thereby contributing to atheroprogession. Interestingly, the recruitment of macrophages into the peritoneal cavity after thioglycollate-induced acute peritonitis was not affected, a finding suggesting that a deficiency in LTbR plays a different role in acute inflammation driven by Ccr2 and Ly6C<sup>hi</sup> monocytes<sup>50</sup> than in the chronic low-grade inflammation typical of atherosclerosis.

Flow cytometric analysis of the blood of apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> mice also demonstrated lymphocytosis characterized by higher B cell counts. In apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> mice, not only the blood but also the spleen and peritoneal cavity contained larger numbers of B lymphocytes, a finding suggesting a general B-cell phenotype.<sup>52</sup> In particular, increased amounts of B1 cells may contribute to the observed antiatherosclerotic phenotype in apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> mice. B1a cells have been described to produce large amounts of atheroprotective natural IgM antibodies against oxidized low-density lipoprotein.<sup>51</sup> In addition, also a strong increase in circulating neutrophils was observed. Because invasion of neutrophils precedes and stimulates inflammation of atherosclerotic lesions in apoE-deficient mice,<sup>52</sup> it may be considered that also defect in neutrophil recruitment occurs in the apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> mice and may subsequently contribute to decreased lesional macrophages. In favor of this hypothesis, Ccl5 binding to Ccr5 and Ccr1 on neutrophils is thought to play a role also in neutrophil recruitment.<sup>53</sup> However, this has not been addressed experimentally in this study.

It is known that the inflammatory microenvironment in atherosclerotic plaques promotes phenotypic switching of SMC into the synthetic phenotype, characterized by loss of SMC differentiation markers and increased extracellular matrix synthesis.<sup>38,55</sup> Inhibition of phenotypic switching of SMC and reduction of extracellular matrix deposition in the plaque may contribute to inhibition of atherosclerosis. Therefore, the composition of atherosclerotic plaques was analyzed in apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> mice. Indeed, in addition to reduced macrophage content, also decreased αSMA staining was detected in plaques of apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> mice at 23 weeks. Moreover, a trend to decreased alcin blue positive glycosaminoglycans and decreased amounts of proatherogenic proteoglycans versican and biglycan,<sup>54,55</sup> was observed. Whether these changes in plaque composition are merely a consequence of decreased inflammation and atherosclerosis in apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> mice at 23 weeks or contribute to the antiatherosclerotic phenotype cannot be concluded from this data. In addition, collagen and perlecan were not changed in apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> mice. Overall, LTbR deficiency in the apoE<sup>−/−</sup> background inhibits plaque inflammation and progression of atherosclerosis resulting in plaques characterized mainly by less macrophages and SMC.

Limitations of this study are that the mechanistic data originate mainly from 12-week-old apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> mice and that the experiments with apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> mice could not determine which of the LTbR ligands, LTα1β2 or lymphotoxin-related inducible ligand, activates proatherosclerotic LTbR signaling. Both ligands may be presented by lymphocytes such as T cells, as suggested in the scheme presented in Online Figure XVI, or may be present as soluble forms. Furthermore, LTbR has been shown to play a role in the development of TLOs<sup>13,44</sup> in mice >32 weeks of age. The fact that haematopoietic LTbR expression was crucial for the antiatherosclerotic effect observed in apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> mice and that these findings are obtained in 12- and 23-week-old mice argues against the involvement of TLOs.

All considered, in hyperlipidemic apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> mice increased circulating B cells, neutrophils, and Ly6Ch<sup>−</sup> monocytes were detected and found to be associated with reduced plaque inflammation and atherosclerosis. Although not addressed experimentally in this study, B cells and especially B1 cells may contribute, for example, via natural antibodies to the atheroprotective phenotype. With respect to monocytes/macrophages, these data strongly suggest that reduced recruitment of Ly6Ch<sup>−</sup> monocytes to atherosclerotic lesions via the Ccl5/Ccr5 pathway is responsible for decreased macrophage-mediated inflammation.

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Disclosures
None.

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Novelty and Significance

What Is Known?

- Atherosclerosis is driven by chronic low-grade inflammation.
- Recruitment of leukocytes into the arterial wall initiates and promotes disease progression.
- One important function of the lymphotxin β1 receptor (LTβR), that orchestrates lymphocyte trafficking, is the resolution of acute viral and bacterial infections.

What New Information Does This Article Contribute?

- LTβR deficiency protects from atherosclerosis in hyperlipidemic mice.
- Monocytes deficient in LTβR are recruited to a lesser extent to atherosclerotic lesions.
- The chemokine (C–C motif) receptor 5 pathway is critically involved in LTβR-mediated monocyte recruitment.

The LTβR is known to be crucial for immune cell function and trafficking. Although LTβR has known important roles in many acute inflammatory diseases, the role of LTβR in atherosclerosis remains unclear. Mice on a hypercholesteremic backgound and deficient in LTβR (apoE−/−/LTβR−/−) exhibited significantly reduced atherosclerotic plaque burden and showed significantly reduced lesion macrophage content compared with apoE−/− controls. Experiments using bone marrow chimeras pointed to hematopoietic cells as major mediators of atheroprotection. Indeed, despite having increased circulating leukocyte numbers, apoE−/−/LTβR−/− mice exhibited reduced recruitment of nonclassical Ly6Clow monocytes into atherosclerotic lesions. Gene array analysis and in vitro experiments suggested that LTβR mediates recruitment of Ly6G+monocytes into atherosclerotic lesions by chemokine (C–C motif) receptor 5/chemokine (C–C motif) ligand 5 (Ccl5/Cc5). These findings demonstrate a novel LTβR-dependent recruitment mechanism for monocytes into atherosclerotic lesions. LTβR enhances inflammation in atherosclerosis and may provide a new therapeutic target.
Deficiency in Lymphotoxin β Receptor Protects From Atherosclerosis in apoE-Deficient Mice

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SUPPLEMENTAL MATERIAL

Deficiency in lymphotoxin beta receptor protects from atherosclerosis in apoE-deficient mice

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Short title: Lymphotoxin beta receptor promotes atherosclerosis

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Detailed Methods

Animals

Homozygous apolipoprotein E–deficient (apoE<sup>−/−</sup>) mice were obtained from Jackson Laboratory (Bar Habour, ME, USA). LTbR-deficient (LTbR<sup>−/−</sup>) mice were kindly provided by K. Pfeffer (Institut für Medizinische Mikrobiologie und Krankenhaushygiene, Düsseldorf, Germany) and crossbred with apoE<sup>−/−</sup> mice (C57BL/6J background) for 4 generations. Genetic background strain characterization was performed with a 1449 Single-Nucleotide Polymorphism (SNP) Panel provided by Taconic (Hudson, NY, USA), which showed that the character of the animals was more than 99% C57BL/6J.

At the age of eight weeks, male apoE<sup>−/−</sup> mice and their double-deficient littermates (apoE<sup>−/−</sup>/LTbR<sup>−/−</sup>) were fed a high-fat, high-cholesterol diet (Western-type diet, WD) containing 21% saturated fat and 0.15% cholesterol (ssniff Spezialdiäten GmbH, Soest, Germany) and were allowed water ad libitum. Mice were analyzed at the age of either 12 weeks (4 weeks WD) or 23 weeks (15 weeks WD), time points that represented early atherosclerosis and late advanced atherosclerosis (Figure 1A). Mice were kept on a normal 12-hour light-and-dark cycle. All experiments were performed according to the guidelines for the use of experimental animals as given by the “Deutsches Tierschutzgesetz” and were approved by the local Research Board for animal experimentation (LANUV; State Agency for Nature, Environment and Consumer Protection).

Blood sample collection

Blood was collected by heart puncture and was anti-coagulated with 100 mM EDTA in isotonic sodium chloride solution. Plasma was prepared via centrifugation at 800 × g for 15 min at 4°C. The supernatant was carefully removed and centrifuged at 15,700 × g for 5 min. Plasma samples were then stored at -20°C for determination of cytokine levels and plasma cholesterol concentration.

Determination of plasma cholesterol concentrations

Levels of low-density lipoprotein (LDL), very low density lipoprotein (VLDL) and high-density lipoprotein (HDL) cholesterol were determined with the HDL and LDL/VLDL Cholesterol Quantification Kit (Biovision, Milpitas, CA, USA) according to the manufacturer’s instructions.

Tissue processing and fixation

For immunohistochemical staining, hearts were fixed in 4% neutral buffered paraformaldehyde (PFA) overnight. Subsequently, they were either transferred in phosphate-buffered (PBS) and embedded in paraffin for sectioning (5 µm) or transferred into 20% sucrose in PBS solution and frozen in tissue-freezing medium (Tissue-Tek<sup>®</sup> O.C.T. Compound, Sakura<sup>®</sup> Finetek, Alphen aan den Rijn, The Netherlands) in liquid isopentane for preparation of cryosections (14 µm) of the aortic root.

Lipid accumulation in the aorta and the aortic root

The aorta was excised and fixed in 4% neutral-buffered PFA. Lipid accumulation was determined by Oil Red O staining (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) of the aorta and the aortic root sections. For assessment of atherosclerotic lesions and the aortic plaque score the aorta was analysed from the aortic arch to the renal artery. Quantification was performed using ImageJ 1.37v software (National Institutes of Health, Bethesda, MD, USA).
Determination atherosclerotic lesion size at the aortic root

Cryosections of the aortic root were used for determination of lesion size. Three slices per animal at intervals of 28 µm were analysed. Atherosclerotic lesion area as well as the area of the whole aortic root were analysed using AxioVision software (Carl Zeiss, Jena, Germany). Data are expressed as ratio of atherosclerotic lesion area to whole aortic root area.

Histochemical and immunohistochemical analysis

Cryosections of the aortic root were stained with Sirius Red. Qualitative analysis of collagen deposition was performed with polarized light microscopy and birefringence analysis. Macrophage accumulation was assessed with an antibody against macrophage 2 (1:400, Cedarlane, Burlington, Canada) and a horseradish peroxidase (HRP)-conjugated secondary antibody (Goat-anti-rat IgG2a HRP, NB7126, Novus biologicals).

For immunohistochemistry of proteoglycans sections were pretreated with chondroitinase to expose epitopes of the core proteins. Slides were incubated with chondroitinase ABC (Sigma-Aldrich, St. Louis, MO) for 1 h at 37°C. Biglycan was detected with polyclonal antiserum against murine biglycan (rabbit, LF 159) kindly provided by Larry Fisher (National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, USA). Versican was stained with a polyclonal antibody (rabbit, ab19345, Abcam, Cambridge, UK) and perlecan with a monoclonal antibody (rat, Seikagaku, Tokyo, Japan). Horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-rabbit, Santa Cruz, CA, USA; goat-anti-rat IgG2a, NB7126, Novus biological) and 3,3'-diaminobenzidine (DAB) (Zytomed, Berlin, Germany) were used for detection.

Acetone-fixed frozen sections were stained with alcian blue 8GX (Sigma-Aldrich, St. Louis, MO) at pH 2.7 as described before. Pictures were taken at 100× magnification and quantification of positive stained area was performed using analysis software (ImageJ 1.37v software, NIH).

For investigating the fraction of proliferating macrophages within the atherosclerotic lesions of 12-week-old apoE⁻/⁻ and apoE⁻/⁻LTbR⁻/⁻ mice, 5-bromo-2-deoxyuridine (BrdU; 50 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) was injected intraperitoneally (i.p.) 24 hours and one hour before organ harvesting. Proliferating macrophages were detected by immunohistochemical co-staining against macrophage 2 (1:200; Cedarlane) and BrdU (abcam, Cambridge, UK) using goat anti-rat Alexa Fluor® 647 and Rhodamine Red™-X-conjugated goat anti-rat IgG (Jackson ImmunoResearch, West Grove, PA, USA) as secondary antibodies. Fiji software (Fiji-win64) was used to count total nuclei per mac2-positive area and the fraction of proliferating macrophages was calculated as the ratio between BrdU-positive nuclei and total nuclei in the mac2-positive area.

In situ cell death detection

Apoptotic cells within the atherosclerotic lesions of 23-week-old apoE⁻/⁻ and apoE⁻/⁻LTbR⁻/⁻ mice were stained after proteinase K treatment (5 µg/ml, 5 min room temperature) by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) with the in situ cell death detection kit TMR-red (Roche Diagnostics, Basel, Switzerland) according to the manufacturer’s instructions. The enzyme solution was diluted 1:3 with TUNEL dilution buffer (Roche Diagnostics) for minimizing the non-specific background. Pictures were taken at 200× magnification and analyzed with Axio Vision Software. Only TUNEL-positive cells that colocalized with 4',6-diamidino-2-phenylindole (DAPI)-stained nuclei were considered positive. Mean values of two consecutive sections were calculated, and the number of nuclei positive for both TUNEL and DAPI was determined per area of the atherosclerotic lesion.
Flow cytometric analysis

For flow cytometric analyses after 4 weeks of WD, the organs of 12-week-old apoE⁻/⁻ and apoE⁻/⁻LTbR⁻/⁻ mice were harvested, and single-cell suspensions were obtained as follows.

After collection of a blood sample by heart puncture, erythrocytes were lysed with hypotonic ammonium chloride solution.

Cells collected from the peritoneal cavity were centrifuged at 500 × g for 10 minutes at 4°C and were resuspended in PEB (PBS containing 2 mM EDTA and 0.5% bovine serum albumin (BSA)).

Spleen tissue was homogenized with the gentle MACS Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) and meshed through a 70-µm cell strainer (Biologix Plastics Co., Ltd., Jiangsu, China). Erythrocyte lysis was performed as described above.

Flow cytometric analysis of the aorta was performed as recently described. The aorta was dissected and cut into small pieces after periaortic tissue had been removed. After digestion of tissue for 60 minutes at 37 °C in a collagenase solution (Hank’s Balanced Salt Solution containing 600 U/ml collagenase II (Worthington Biochemical Corporation, Lakewood, NJ, USA) and 60 U/ml DNase I (Roche Applied Science, Mannheim, Germany), aortic tissue was meshed through a 70-µm cell strainer (Biologix Plastics Co., Ltd., Jiangsu, China) and centrifuged at 300 × g for 10 minutes at 4°C.

Splenic and aortic cells were resuspended in RPMI medium (Life Technologies™, Thermo Fischer Scientific, Waltham, MA, USA) and incubated for 30 minutes at 37°C. After another centrifugation step at 300 × g for 10 minutes at 4°C, cell pellets were resuspended in PEB and stained with LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Invitrogen™ Life Technologies Corporation) for exclusion of dead cells.

Absolute cell concentrations were determined with Flow-Count™ Fluorospheres (Beckman Coulter Inc., Krefeld, Germany). Unless stated otherwise, all antibodies were purchased from Biolegend (San Diego, CA, USA). For detection of macrophages the following antibodies were used: anti-F4/80-AlexaFluor®488 (clone BM8), CD45-PE (30-F11), CD86-PECy7 (GL-1), CD206-AlexaFluor®647 (C068C2) and CD11b-PacificBlue™ (M1/70). Lymphocytes were labeled with CD45-PE (30-F11), CD3-APC/Cy7 (17A2), and CD19-PacificBlue™ (6D5). For the detection of splenic monocytes, cells were labelled using anti-Ly6C-AlexaFluor®488 (HK1.4), CD45-PE (30-F11), CD115-APC (AFS98; eBioscience, San Diego, CA, USA) and CD11b-PacificBlue™ (M1/70). In all analyses isotype control staining and fluorescence minus control staining was performed. For analyses of lymphocytes and macrophages in aortic tissue and spleen isotype control staining is shown in Supplemental Figure III, V and VI. Cells were gated on living singlets according to LIVE/DEAD® staining and forward (FSC)/side laser light scatter (SSC) gating. Flow cytometric measurements were performed on a Gallios™ Flow Cytometer (Beckman Coulter Inc., Krefeld, Germany) and Kaluza® Flow Analysis Software (Beckman Coulter Inc., Krefeld, Germany) was used for subsequent data analysis. The amount of immune cells is expressed as cells/µl peritoneal lavage, cell numbers/mg spleen, cells/µl blood, or number of cells/aorta, respectively.

Flow cytometric analysis of neutrophils, monocyte subsets in whole blood and bone marrow, and monocyte progenitors in bone marrow from 12-week-old apoE⁻/⁻ and apoE⁻/⁻LTbR⁻/⁻ mice was performed after 4 weeks of WD. Absolute bone marrow nucleated cell numbers were determined with a Z2 cell counter (Beckman Coulter Inc., Krefeld, Germany). Mixes including antibodies (purchased from BD Bioscience or eBioscience) against the following surface molecules were used: Ly6G (clone 1A8), MHCII (AF6-120.1), CD3 (145-2C11), Nk1.1 (PK136), CD19 (1D3), c-kit (2B8), CD135 (A2F10.1), B220 (RA3-6B2), Mac-1 (M1/70),
CD115 (AFS98), Ly6C (AL-21), and CCR2 (475301, R&D Systems). Flow cytometric data were acquired on an LSRII flow cytometer (Becton-Dickinson, Heidelberg, Germany), and subsequent data analysis was performed with FlowJo software (Treestar, San Carlos, CA, USA). Dead cells, debris, and doublets were excluded by FSC and SSC and propidium iodide gating.

**Multiplex analysis**

A commercially available multiplex bead-based immunoassay (Bio-Plex Pro™ Cytokine Group 1 23-plex; Biorad, Hercules, CA, USA) was used to determine plasma levels of interleukin (IL)-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-17α, eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), interferon γ (IFN-γ), platelet-derived growth factor-inducible protein KC (KC), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein 1α (MIP-1α), macrophage inflammatory protein 1β (MIP-1β), regulated on activation, normal T-cell expressed and secreted (RANTES; CCL5), and TNF-α. Analysis was performed with a Bioplex 200 suspension array system (Biorad, Hercules, CA, USA) according to the manufacturer’s instructions. Protein concentrations were calculated from the appropriate optimized standard curves with Bio-Plex Manager Software version 6.0 (Biorad, Hercules, CA, USA). Cytokines below the detection limit were excluded from the analysis.

**Microarray gene expression analyses**

Gene array analyses were performed in 12-week-old apoE−/− and apoE−/−/LTbR−/− mice after 4 weeks of WD. Aortas were rapidly removed from the aortic arch to the bifurcation and were snap-frozen in liquid nitrogen. Blood was collected by heart puncture followed by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare, UK), and the mononuclear cell fraction was subsequently used for isolation of CD115+ cells according to the manufacturer’s instructions (CD115 MicroBead Kit, Miltenyi Biotec, Bergisch Gladbach, Germany). Total RNA was prepared with the RNeasy Microarray Tissue Kit (Qiagen, Hilden, Germany) from 4 (aorta) and 3 (CD115+) biological replicates. For obtaining sufficient amounts of RNA for microarray cDNA synthesis, two of three apoE−/− CD115+ replicated samples had to be prepared from two littermate animals each, and enriched CD115+ cells were pooled before isolation of total RNA.

RNA preparations were checked for RNA integrity by Agilent 2100 Bioanalyzer quality control. All samples in this study showed high-quality RNA Integrity Numbers (RIN; aorta: mean, 7.2; monocytes: mean, 7.8). RNA was further analysed by photometric Nanodrop measurement and quantified by a fluorimetric Qubit RNA assay (Life Technologies, Darmstadt, Germany).

Synthesis of cDNA and subsequent biotin labeling was performed according to the manufacturer’s instructions (Ovation Pico WTA System V2/Encore Biotin Module, NuGEN Inc., San Carlos, CA, USA). Briefly, 1 ng of total RNA was converted to cDNA, followed by isothermal amplification, fragmentation, and biotin labeling of cDNA. Labeled cDNA was hybridized to Affymetrix Mouse Gene 2.0 ST Gene Expression Microarrays for 18 h at 45°C, stained by streptavidin/phycoerythrin conjugate, and scanned as described in the manufacturer’s instructions (GeneChip Whole Transcript (WT) Sense Labeling Assay User Manual, Affymetrix, Inc., Santa Clara, CA, USA).

Data analysis of Affymetrix CEL files was conducted with GeneSpring GX software (Vers. 12.5; Agilent Technologies). Probes within each probe set were summarized by GeneSprings’ ExonRMA16 algorithm after quantile normalization of probe-level signal intensities across all samples to reduce inter-array variability. Preprocessing of input data was concluded by baseline transformation to the median of all samples.
After replicated samples had been grouped according to their respective experimental condition, a given probe set had to be expressed above background (i.e., fluorescence signal of a probe set was detected within the 20th and 100th percentiles of the raw signal distribution of a given array) in all three replicates in either one of two, or both, conditions before additional analyses could be carried out in pairwise comparisons.

Differential gene expression was statistically determined by moderated t-tests. The significance threshold was set at p=0.01. Hierarchical cluster analysis of differentially expressed genes was performed with Euclidian similarity measures and Ward’s linkage.

Functional enrichment of differentially expressed genes within canonical pathways was analysed with Ingenuity Pathway Analysis (IPA) software (Qiagen, Hilden, Germany). The population of genes to be considered for p-value calculations was set to the IPA-provided Affymetrix Mouse Gene 2.0 ST Arrays reference set.

The complete data set was deposited at the National Center for Biotechnology Information’s Gene Expression Omnibus database (accession number GSE63259).

**Generation of bone-marrow chimeras**

Bone marrow cells were obtained by flushing the femurs and tibias of 8-week-old mice. For reconstituting the bone marrow of 8-week-old lethally irradiated (10 Gy) male mice, 5×10^6 unfractionated bone marrow cells were injected intravenously (i.v.) into the lateral tail vein. The transfer was performed from apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> mice to apoE<sup>−/−</sup> mice and vice versa. Because of the known effects of the bone marrow transfer itself and the irradiation on atherogenesis, control transfers from apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> to apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> and vice versa were performed. After the transfer, mice were fed WD and were sacrificed after 15 weeks for the assessment of atherosclerosis.

**RNA extraction**

Whole aortas of apoE<sup>−/−</sup> and apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> mice were rapidly removed from the aortic arch to the aortoiliac bifurcation and were immediately snap-frozen in liquid nitrogen. Total RNA from homogenized aortic tissue was isolated using peqGOLD TriFast (PEQLAB Biotechnologie GmbH, Erlangen, Germany) according to the manufacturer’s instructions.

RNA concentration and quality were determined via photometric measurement of the ratio of absorbance at 260 and 280 nm with a NanoDrop™ Photospectrometer (Thermo Scientific). After this, 1 µg of total RNA was transcribed into cDNA with the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany).

**Isolation of primary monocytes and in vitro stimulation with agonistic anti-LTbR antibody or LTα1β2**

For gene expression cells (3x10^5) were seeded in 24 well plates in DMEM supplemented with 5% FCS, 100 U/ml penicillin and 100 U/ml streptomycin and stimulated with either 10 µg/ml anti-LTbR antibody, clone 5G11 (Hycult Biotech, Plymouth Meeting, PA, USA) or 1 µg/ml LTα1β2 (R&D Systems, Minneapolis, MN, USA) or the appropriate negative control (rat IgG; Sigma-Aldrich, St. Louis, MO, USA; or PBS/BSA) for 4 hours in humidified CO<sub>2</sub> at 37° C.

RNA was isolated, and analysis of gene expression was performed as described above.

**Analysis of human atherectomy specimen**

After informed consent had been obtained from patients undergoing carotid endarterectomy, carotid atherectomy specimens were immediately frozen, and total RNA was extracted as described previously (ethics vote 3944).
Quantitative real-time polymerase chain reaction

Specific primers for chemokine receptors and chemokines were obtained from Applied Biosystems (Applied Biosystems, Darmstadt, Germany). In addition, primer pairs were designed (Primer3Plus software and Primer-BLAST). The respective sequences are given in Supplemental Table I.

Quantitative polymerase chain reaction (qPCR) was performed with the Applied Biosystems 7300 or StepOne Plus™ Real-Time PCR System using Platinum® SYBR® Green qPCR SuperMix-UDG (Life Technologies GmbH, Carlsbad, CA, USA) and with the Applied Biosystems ABI Prism 7000 using Power SYBR® Green PCR Master Mix. PCR reactions and subsequent comparison of relative gene expression were performed with the ∆∆Cq method, as previously described.

Thioglycollate-induced peritoneal macrophages

After 4 weeks on WD, 12-week-old apoE−/-LTbR−/- mice and their apoE−/- littermates were given i.p. injections of 3% sterile thioglycollate. Cells were isolated on day 5 by injecting 3 ml PBS into the peritoneal cavity, aspirating the peritoneal lavage fluid, and counting the aspirated cells. Purity of peritoneal cells was analyzed by flow cytometry with CD11b and F4/80 as macrophage markers, as described above. The total numbers of invading macrophages and the number of invading macrophages related to the circulating monocyte counts were calculated.

Recruitment of labelled monocytes to atherosclerotic lesions

For in vivo labelling of circulating Ly6Clow monocytes, fluorescent microspheres (Fluoresbrite Polychromatic [PC] Red Microspheres; Polysciences, Warrington, PA, USA) were used according to a protocol by Tacke et al. Briefly, microspheres were injected i.v. via the lateral tail vein into 12-week-old apoE−/- and apoE−/-/LTbR−/- mice. For Ly6Chigh specific labelling, clodronate was injected 18 hours prior to i.v. injection of fluorescent microspheres in order to delete circulating monocytes. Labeling of circulating Ly6Clow and Ly6Chigh monocytes was verified one day after injection by flow cytometric analysis, as described above. Three days after injection of microspheres, mice were sacrificed, blood was collected for analysis of monocyte labelling, and hearts were harvested and fixed for immunohistochemical staining of mac2, as described above. The complete aortic root was cut into 30 to 40 sections, and every third section (20 µm thick) was stained for mac2 as described above; goat anti-rat Alexa Fluor® 647 (Life Technologies, Carlsbad, CA, USA) was used as a secondary antibody. Nuclei were counterstained with DAPI. Latex microsphere-positive macrophages were counted in all sections. The number of invading monocytes was calculated by dividing the number of lesional, microsphere-positive macrophages by the number of circulating labelled Ly6Clow or Ly6Chigh monocytes in the blood.

Carotid artery ligation

The carotid artery ligation model, as described by Kumar and Lindner, was used to evaluate the effect of LTbR-deficiency on neointimal hyperplasia. Briefly, male 10-week-old apoE−/-LTbR−/- mice and age-matched apoE−/- controls were anesthetized with ketamine (100 mg·kg−1) and xylazine (5 mg·kg−1), and the left common carotid artery was ligated near the carotid bifurcation. All animals were fed normal chow for 28 days after surgery. For morphometric analysis, left and right carotid arteries were excised after perfusion with 4% PFA. Carotid arteries were fixed in 4% PFA for 24 hours, dehydrated, and embedded in paraffin for sectioning. Vessels were serially sectioned (5 µm thick) more than 1000 µm proximal to the ligature. Sections were stained with hematoxylin and eosin (H&E) at 250 µm intervals, and morphometric analysis was performed to determine the areas of media, neointima, and lumen.
Supplemental References


Supplemental Figure I:

**Body mass index (BMI) and plasma lipids of apoE-/-/LTbR-/- mice compared to their apoE-/- littermates.** Determination of (A) BMI and (B) triglycerides, (C) cholesterol, (D) LDL/VLDL and (E) HDL cholesterol level in plasma of 12-week-old apoE-/- and apoE-/-/LTbR-/- mice. Data are presented as mean ± SEM; n=6-8 (A); n=6-7 (B-E).
Supplemental Figure II:

**No changes in plaque composition of apoE^-/-/LTbR^-/- mice at the aortic root at 23 weeks of age.**
Representative images and quantification of (A) Oil Red O (n=6, 8) and (B) α-SMA (n=6, 8) staining at the aortic root of apoE^-/- and apoE^-/-/LTbR^-/- mice. (C) Total collagen content and (D) glycosaminoglycan content evidenced by Sirius red (n=5,7) and alcian blue staining (n=7,8) of plaques at the aortic root of apoE^-/- and apoE^-/-/LTbR^-/- mice and their respective quantification. Immunohistochemical stainings of the proteoglycans (E) versican (n=8, 7), (F) biglycan (n=7, 6) and (G) perlecan (n=9). All pictures are taken at 100-fold magnification. Data are presented as mean ± SEM; *P<0.05.
Supplemental Figure III:
(A) Representative gating scheme for macrophages (CD11b+/F4/80+) in the aorta, their relative M1 (CD86+) and M2 (CD206+) distribution and (B) isotype control staining.
Supplemental Figure IV: Immune cell composition of aorta and aortic root. Flow cytometric analysis of immune cells in the aortic wall of apoE\(^{-/-}\) and apoE\(^{-/-}\)/LTbr\(^{-/-}\) mice at 12 weeks of age. Absolute numbers of (A,B) CD45\(^+\) leukocytes, (C) total lymphocytes, (D-F) B and T cells per aorta as well as the respective distribution of B- and T cells are shown. Representative plots gated on living cells (B) or CD45\(^+\) cells (F) are shown. Data are presented as mean ± SEM; n=5-7; *P<0.05 (Mann-Whitney test).
Supplemental Figure V:

(A) Representative gating scheme for B (CD45+/CD19+) and T (CD45+/CD3+) lymphocytes in the aorta and (B) isotype control staining.
**Supplemental Figure VI:**

**Immune cell composition of the spleen of apoE<sup>−/−</sup> and apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> mice.** Flow cytometric analysis of immune cells in the spleen of apoE<sup>−/−</sup> and apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> mice at 12 weeks of age. Absolute numbers of (A,B) CD45<sup>+</sup> leukocytes, (C) total lymphocytes, (D-F) B and T cells per mg tissue as well as (G) the respective distribution of B cells and T cells are shown. Analysis of (H) CD11b<sup>+</sup>/F4/80<sup>+</sup> macrophages and (I) Ly6C<sup>high</sup> and (J) Ly6C<sup>low</sup> monocyte subsets. Representative plots are gated on total cells (B), CD45<sup>+</sup> cells (F) or CD11b<sup>+</sup>/CD45<sup>+</sup> cells (K). Data are presented as mean ± SEM; n=5-6 in (A and B); n=4-5 in (C-K); *P<0.05 (Mann-Whitney test).
Supplemental Figure VII:

**Immune cells in the peritoneal cavity.** Flow cytometric analysis of immune cells in the peritoneal lavage fluid of apoE−/− and apoE−/−/LTbR−/− mice at 12 weeks of age. Absolute numbers of (A,B) CD45+ leukocytes, (C) total lymphocytes, (D-F) B and T cells per µl lavage fluid as well as the respective distribution of B cells and T cells (G) are shown. (H,I) Analysis of CD11b+/F4/80+ macrophages. Representative plots are gated on (B) living cells or (F,I) CD45+ cells. Data are presented as mean ± SEM; n=6-7 (A,B and D-G); n=4-7 in (H,I); *P<0.05.
Supplemental Figure VIII:

**Increased peritoneal influx of inflammatory cells after thioglycollate injection in apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> mice.** Influx of thioglycollate-elicited peritoneal cells isolated from the peritoneal exsudate on day 5 after thioglycollate injection in apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> mice and their apoE<sup>−/−</sup> littermates. (A) Total numbers of macrophages (CD11b<sup>+</sup>/F4/80<sup>+</sup>) per mouse as fold of apoE<sup>−/−</sup> and (B) calculation of the invading macrophages relative to the respective circulating monocyte numbers per ml blood are given. Data are presented as mean ± SEM; n=9; *P<0.05.
Supplemental Figure IX:

Absolute concentrations of circulating cytokines in apoE<sup>-/-</sup> and apoE<sup>-/-</sup>/LTbR<sup>-/-</sup> mice at 12 and 23 weeks of age. Determination of plasma concentrations of different chemokines/cytokines in male apoE<sup>-/-</sup>/LTbR<sup>-/-</sup> mice and their respective apoE<sup>-/-</sup> littermate controls at 12 and 23 weeks of age using a multiplex bead based immunoassay. Data are presented as mean ± SEM; 12 weeks, n=6-11; 23 weeks, n=6-9, *P<0.05 (One-way ANOVA followed by Holm-Sidak's multiple comparison test).
Supplemental Figure X

Supplemental Figure X:

Validation of bone marrow chimerism

Representative PCR for the deleted and wildtype LTbR allele on whole blood genomic DNA of apoE^-/- -> apoE^-/-/LTbR^-/- and apoE^-/-/LTbR^-/- -> apoE^-/- chimeras.
Supplemental Figure XI:

Flow cytometric analysis of circulating immune cells in 12-week-old apoE\(^{+}\) and apoE\(^{+}/LTbR^{-}\) mice. Proportions of (A) leukocytes, B and T cells determined in CD45\(^{+}\) cells. (B) Neutrophils, Ly6C\(^{\text{high}}\) and Ly6C\(^{\text{low}}\) monocytes were determined in the non-lymphoid cell population as CD11b\(^{+}/\text{Ly6G}\) cells (neutrophils) or CD11b\(^{+}/\text{CD115}/\text{MHCII}\) cells (monocytes). Shown are representative gating schemes.
Supplemental Figure XII:

No differences in monocytes, MDP and cMoP cells in apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> bone marrow.
Flow cytometric analysis of (A) mature Ly6C<sup>low</sup> and Ly6C<sup>high</sup> monocytes, (B) monocyte-macrophage dendritic cell progenitors (MDP) and (C) common monocyte progenitor (cMoP) in the bone marrow of male 12-week-old apoE<sup>−/−</sup>/LTbR<sup>−/−</sup>mice and apoE<sup>−/−</sup> controls. A representative gating scheme for MDP and cMoP is shown in (D). Data are presented as mean ± SEM; *P<0.05; n=4.
Supplemental Figure XIII:

**Proliferation and apoptotic cell death were not altered in lesions of apoE^{−/−}/LTbR^{−/−} mice.** (A) Analysis of macrophage proliferation in atherosclerotic plaques of the aortic root at 12 weeks of age and quantification of BrdU positive nuclei (green). Macrophages (mac2) are shown in magenta and nuclei were counterstained using DAPI (blue). Data are presented as mean ± SEM; n=5-6. (B) Sections of the aortic root of 23-week-old apoE^{−/−} (left) and apoE^{−/−}/LTbR^{−/−} (right) mice labeled by TUNEL assay. TUNEL-positive cells (red) colocalized with DAPI positive nuclei were observed in the intima and within the necrotic area (marked with arrowheads). For better orientation autofluorescence of elastin in the FITC channel is shown. Pictures are taken at 100-fold and 200-fold magnification, respectively. Data are presented as mean ± SEM; n=7-8.
Supplemental Figure XIV:

Volcano plots and hierarchical clustering of differentially regulated genes in aortas and isolated CD115+ cells of apoE−/− and apoE−/−/LTbR−/− mice. Detection of regulated genes in isolated peripheral blood CD115+ cells (A,B) and the aortas (C,D) of apoE−/− and apoE−/−/LTbR−/− mice at the age of 12 weeks. 267 and 678 differentially expressed transcripts (P<0.01) were detected in CD115+ cells and in the aorta, respectively. (A,C) Volcano plots showing fold changes and significances for the detected genes. Significantly (P<0.01) regulated genes are shown in red. (B,D) Hierarchical clustering showing the grouping of apoE−/− and apoE−/−/LTbR−/− mice for isolated CD115+ cells (n=3) and the aorta (n=4).
Supplemental Figure XV:

**LTbR deficiency does not alter neointima hyperplasia in carotid arteries.**

(A) Representative pictures of H&E-stained sections of carotid arteries after ligation (left) and uninjured carotid arteries (right) in apoE−/− and apoE−/−/LTbR−/− mice taken 250 µm proximal to ligation are shown (200x magnification). (B) The neointimal area is plotted as function of the distance to the suture. (C) Mean intima/media ratio across all analysed sections over 1000 µm. (D) Neointimal, (E) medial and (F) luminal areas. (G) Mean circumference of *lamina elastica externa*. Data are presented as mean ± SEM; n=7.
Supplemental Figure XVI:

**Schematic overview of the proposed mechanism of the antiatherosclerotic effect in apoE<sup>−/−</sup>/LtbR<sup>−/−</sup> mice.** LTbR deficiency in apoE<sup>−/−</sup> mice causes monocytosis characterized by elevated Ly6<sub>Clow</sub> monocytes and decreased recruitment of these cells. It is generally believed that Ly6<sub>Clow</sub> monocytes adhere via LFA-1 and ICAM-1 to the atherosclerotic vessel wall. The current data point toward a role of especially Ccl5 known to be critical for arrest, transendothelial migration and activation of lesional monocytes/macrophages in atherosclerotic lesion formation. In line it is shown here that activation of LTbR by its ligands induces release of Ccl5 and other cytokines by monocytes/macrophages.
**Supplemental Table I**

### A

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Five most regulated pathways in isolated CD115⁺ cells of apoE⁺/⁺/L Tb R⁻⁻ mice. Total RNA was isolated from CD115⁺ cells at 12 weeks of age and subjected to whole genome gene array analysis. Indicated are the transcriptional pathways that were regulated in apoE⁺/⁺/L Tb R⁻⁻ mice compared to apoE⁻⁻. 

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*Supplemental Table II.*
Supplemental Table III

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Supplemental Table III.

**Five most regulated pathways in aortas of apoE−/−/LTbR−/− mice.** Total RNA was isolated from CD115+ cells at 12 weeks of age and subjected to whole genome gene array analysis. Indicated are the transcriptional pathways that were regulated in apoE−/−/LTbR−/− mice compared to apoE−/−.
### Supplemental Table IV

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<td>Oas1g</td>
<td>Mm.389688///Mm.389688///Mm.389688///Mm.389688///Mm.389688///Mm.389688</td>
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<td>0,0001</td>
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<tr>
<td>T cell receptor beta, joining region</td>
<td>Tcrb-J</td>
<td>Mm.333026///Mm.389744///Mm.429394</td>
<td>-2,39</td>
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<td>2'-5' oligoadenylate synthetase 2</td>
<td>Oas2</td>
<td>Mm.260926///Mm.260926///Mm.260926///Mm.260926///Mm.260926 ///Mm.260926</td>
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<td>receptor transporter protein 4</td>
<td>Rtp4</td>
<td>Mm.475107///Mm.475107///Mm.475107</td>
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<td>0,0055</td>
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<tr>
<td>RIKEN cDNA C330006A16 gene</td>
<td>C330006A16Rik</td>
<td>Mm.490426///Mm.490529</td>
<td>-1,79</td>
<td>0,0054</td>
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<tr>
<td>membrane-spanning 4-domains, subfamily A, member 6D</td>
<td>Ms4a6d</td>
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<td>Ltbr</td>
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<td>Oas1a</td>
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<td>hydroxymethylbilane synthase</td>
<td>Hmbs</td>
<td>Mm.247676///Mm.247676///Mm.247676///Mm.247676///Mm.247676 ///Mm.247676</td>
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<td>0,0055</td>
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<tr>
<td>Rho GTPase activating protein 42</td>
<td>Arhgap42</td>
<td>Mm.334955///Mm.334955///Mm.334955///Mm.334955///Mm.334955 ///Mm.334955</td>
<td>-1,48</td>
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<tr>
<td>ubiquitin specific peptidase18</td>
<td>Usp18</td>
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<td>major urinary protein 20</td>
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<td>transmembrane protein 106A</td>
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</tbody>
</table>

**15 most downregulated genes in isolated CD115+ cells from apoE−/−/LTbR−/− mice.** Total RNA was isolated from CD115+ cells at 12 weeks of age and subjected to whole genome gene array analysis. Indicated are the differentially regulated genes in apoE−/−/LTbR−/− mice compared to apoE−/−. Genes without annotation were excluded from the table. FC = fold change.
### Supplemental Table V

<table>
<thead>
<tr>
<th>Gene description</th>
<th>Gene symbol</th>
<th>UnigeneID</th>
<th>FC</th>
<th>p-value</th>
</tr>
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<tbody>
<tr>
<td>AF4/FMR2 family, member3</td>
<td>Aff3</td>
<td>Mm.336679///Mm.336679///Mm.336679///Mm.336679///Mm.336679</td>
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<tr>
<td>interleukin 9 receptor</td>
<td>Il9r</td>
<td>Mm.384///Mm.384///Mm.384///Mm.384///Mm.384</td>
<td>+2.08</td>
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<td>stathmin1, related sequence 1</td>
<td>Stmn1-rs1</td>
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<td>predicted gene 2178</td>
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<td>RIKEN cDNA 1700021K19 gene</td>
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<tr>
<td>myosin IE</td>
<td>Myo1e</td>
<td>Mm.249311///Mm.249311///Mm.249311///Mm.249311</td>
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<td>synaptic nuclear envelope 1</td>
<td>Syne1</td>
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<tr>
<td>RIKEN cDNA 4930478L05 gene</td>
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<td>CDK5 and Abl enzyme substrate 2</td>
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<td>histocompatibility 2, Q region locus 5</td>
<td>histocompatibility 2, Q region locus 6-like</td>
<td>H2-Q5</td>
<td>LOC68395</td>
<td>Mm.482320///Mm.482320</td>
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<tr>
<td>phosphoinositide-3-kinase interacting protein 1</td>
<td>Pik3ip1</td>
<td>Mm.390323///Mm.390323///Mm.390323///Mm.390323///Mm.390323///Mm.390323///Mm.390323///Mm.390323</td>
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<tr>
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<tr>
<td>prickle homolog 3 (Drosophila)</td>
<td>Prickle3</td>
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<tr>
<td>guaninenucleotide-binding protein G(I)/G(S)/G(O) subunit gamma 5-like</td>
<td>LOC100048410</td>
<td>Gm15776</td>
<td>Gm13342</td>
<td>Gng5</td>
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<tr>
<td>glutathione S-transferase, pi 2</td>
<td>Gstp2</td>
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<td>+1.37</td>
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</tr>
</tbody>
</table>

**15 most upregulated genes in isolated CD115+ cells from apoE−/−/LTbR−/− mice.** Total RNA was isolated from CD115+ cells at 12 weeks of age and subjected to whole genome gene array analysis. Indicated are the differentially regulated genes in apoE−/−/LTbR−/− mice compared to apoE−/−. Genes without annotation were excluded from the table. FC = fold change.
<table>
<thead>
<tr>
<th>Gene description</th>
<th>Gene symbol</th>
<th>UnigeneID</th>
<th>FC</th>
<th>p -value</th>
</tr>
</thead>
<tbody>
<tr>
<td>glycosylation-dependent cell adhesion molecule 1</td>
<td>Glycam1</td>
<td>Mm.219621///Mm.219621///Mm.219621</td>
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<td>4,85E-04</td>
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<tr>
<td>immunoglobulin heavy chain (gamma polypeptide)</td>
<td>Ighg</td>
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<td>- 14,03</td>
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<tr>
<td>lymphocyte antigen 6 complex, locusD</td>
<td>Ly6d</td>
<td>Mm.878///Mm.878///Mm.878</td>
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<td>3,01E-06</td>
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<tr>
<td>immunoglobulin heavy constant gamma 3</td>
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<tr>
<td>histocompatibility 2, class II, locus Mb2</td>
<td>H2-DMb2</td>
<td>Mm.195060///Mm.195060///Mm.195060///Mm.195060</td>
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<tr>
<td>immunoglobulin kappa chain variable 28 (V28)</td>
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<tr>
<td>BE692007</td>
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<td>- 6,50</td>
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<tr>
<td>lymphotoxin B</td>
<td>Ltb</td>
<td>Mm.1715///Mm.1715///Mm.1715///Mm.1715///Mm.1715</td>
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<tr>
<td>selectin, lymphocyte</td>
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<td>recombination activating gene 1</td>
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<tr>
<td>histone cluster 1, H1b</td>
<td>Hist1h1b</td>
<td>Mm.221314///Mm.221314</td>
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<tr>
<td>CD79A antigen (immunoglobulin-associated alpha)</td>
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<tr>
<td>CD3 antigen, gamma polypeptide</td>
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<td>- 4,51</td>
<td>0,008</td>
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</table>

**Supplemental Table VI.**

**15 most downregulated genes in aortas from apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> mice.** Total RNA was isolated from aortas at 12 weeks of age and subjected to whole genome gene array analysis. Indicated are the differentially regulated genes in apoE<sup>−/−</sup>/LTbR<sup>−/−</sup> mice compared to apoE<sup>−/−</sup>. Genes without annotation were excluded from the table. FC = fold change
### Supplemental Table VII

<table>
<thead>
<tr>
<th>Gene description</th>
<th>Gene symbol</th>
<th>UnigenelID</th>
<th>FC</th>
<th>p -value</th>
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<tbody>
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<td>inter alpha-trypsin inhibitor, heavy chain 4</td>
<td>Itih4</td>
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<td>predicted pseudogene 6654</td>
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<tr>
<td>AHNAK nucleoprotein 2</td>
<td>Ahnak2</td>
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<tr>
<td>per-hexamer repeat gene 4</td>
<td>Phxr4</td>
<td>Mm.41972///Mm.41972</td>
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<tr>
<td>synaptic nuclear envelope 1</td>
<td>Syne1</td>
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<td>+ 2.28</td>
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<tr>
<td>lectin, galactosebinding, soluble 4</td>
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<td>Lgals4</td>
<td>Lgals6</td>
<td>Mm.210336///Mm.210336///Mm.210336///Mm.210336///Mm.210336///Mm.210336///Mm.210336 ///Mm.210336</td>
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<td>cell adhesion molecule with homology to L1CAM</td>
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<td>cadherin 19, type2</td>
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</tr>
</tbody>
</table>

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