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Abstract

Objective-Leukotriene B$_4$ (LTB$_4$), a potent leukocyte chemoattractant, is known to promote several inflammatory diseases, including atherosclerosis. We sought to determine mechanisms through which LTB $_4$ modulates atherosclerosis in cell lines expressing LTB$_4$ receptors, BLT-1, and in mice deficient in BLT-1 as well as macrophage cell lines derived from BLT-1$^{+/+}$ and BLT$^{-/-}$ mice. Methods and Results-Analysis of global changes in gene expression induced by LTB $_4$ in rat basophilic leukemia cells (RBL-2H3) expressing the human BLT-1 showed highest-fold increase in expression of fatty acid translocase/CD36 and the chemokine MCP1/JE/CCL2, which are critical in atherogenesis. To determine the importance of BLT-1 in atherogenesis, we crossed BLT-1-null mice with apolipoprotein (apo)-E-deficient mice, which develop severe atherosclerosis. Deletion of BLT-1 significantly reduced the lesion formation in apo-E$^{-/-}$ mice only during initiating stages (4 and 8 weeks) but had no effect on the lesion size in mice fed atherogenic diet for 19 weeks. Macrophage cell lines from BLT-1-deficient mice expressed the low-affinity LTB$_4$ receptor, BLT-2, and exhibited chemotaxis to LTB$_4$. Conclusions-The effects of LTB$_4$ in atherosclerosis are likely mediated through the high-affinity BLT-1 and the low-affinity BLT-2 receptors. LTB$_4$ promotes atherosclerosis by chemo-attracting monocytes, by providing an amplification loop of monocyte chemotaxis via CCL2 production, and by converting monocytes to foam cells by enhanced expression of CD36 and fatty acid accumulation.

Keywords

Atherosclerosis; Leukotriene B4 receptors; Monocyte/macrophages

Disciplines

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Methods and Results—Analysis of global changes in gene expression induced by LTB$_4$ in rat basophilic leukemia cells (RBL-2H3) expressing the human BLT-1 showed highest-fold increase in expression of fatty acid translocase/CD36 and the chemokine MCP1/JE/CCL2 , which are critical in atherogenesis. To determine the importance of BLT-1 in atherogenesis, we crossed BLT-1-null mice with apolipoprotein (apo)-E-deficient mice, which develop severe atherosclerosis. Deletion of BLT-1 significantly reduced the lesion formation in apo-E$^{-/-}$ mice only during initiating stages (4 and 8 weeks) but had no effect on the lesion size in mice fed atherogenic diet for 19 weeks. Macrophage cell lines from BLT-1-deficient mice expressed the low-affinity LTB$_4$ receptor, BLT-2, and exhibited chemotaxis to LTB$_4$.

Conclusions—The effects of LTB$_4$ in atherosclerosis are likely mediated through the high-affinity BLT-1 and the low-affinity BLT-2 receptors. LTB$_4$ promotes atherosclerosis by chemo-attracting monocytes, by providing an amplification loop of monocyte chemotaxis via CCL2 production, and by converting monocytes to foam cells by enhanced expression of CD36 and fatty acid accumulation. (*Arterioscler Thromb Vasc Biol*. 2004;24:369-375.)

Key Words: atherosclerosis ■ monocyte/macrophages ■ leukotriene B$_4$ receptors

Accumulation of monocytes in vascular subendothelial spaces and their conversion into lipid-laden "foam cells" is an early and important event in atherogenesis.[1] The molecular mechanisms that regulate the recruitment of monocyte/macrophages to the vessel wall and the signaling pathways underlying their conversion to foam cells are poorly understood. Several recent studies suggest that chemokines CCL2/MCP1/JE, interleukin-8 (IL-8), and fractalkine and their receptors CCR2, CXCR2, and CX3CR1 are critical mediators of atherosclerosis.[2–7] Mice lacking macrophage colony-stimulating factor have severely reduced atherosclerosis in experimental models.[8] Thus, macrophages play a central role in the development of atherosclerotic vascular disease, which is now considered a chronic inflammatory disease.[9]

LTB$_4$, a potent leukocyte chemoattractant, is known to promote a number of chronic inflammatory diseases.[10] G-protein coupled receptors BLT-1 and BLT-2 and the peroxisome proliferator activator receptor α (PPARα) are the currently known LTB$_4$ receptors.[11–13] While BLT-1 and BLT-2 likely mediate the proinflammatory responses of LTB$_4$, PPARα, a transcription factor, might serve as a mediator of the anti-inflammatory effects of LTB$_4$. Studies on mouse models and antagonists of LTB$_4$ suggested a role for BLT-1 in rheumatoid arthritis, acute septic peritonitis, and atherosclerosis.[14–16] To determine the role of BLT-1 in chronic inflammatory diseases, we analyzed LTB$_4$-induced changes in global gene expression in cells expressing BLT-1 and atherosclerotic lesion development in BLT-1 and apolipoprotein-E (apo-E) double-deficient mice. We observed that several genes with known functions in the development of atherosclerosis are upregulated by LTB$_4$, and deletion of BLT-1 gene offered early protection against development of atherosclerotic lesions in mice. Macrophage cell lines derived from BLT-1-deficient mice expressed a second LTB$_4$ receptor, BLT-2. These cells also showed chemotaxis to higher LTB$_4$ concentrations that are likely to occur in established atherosclerotic plaques. These results suggest that LTB$_4$ is an important mediator and its receptors, BLT-1 and BLT-2, play critical and sequential roles during atherogenesis.

Methods

Analysis of LTB$_4$-Induced Changes in Gene Expression

Global changes in LTB$_4$-induced gene expression were determined in a rat basophilic leukemia cell line (RBL-2H3) expressing the human BLT-1 (hBLT-1) using the standard Affymetrix protocols and rat genome chip U34A (Affymetrix) containing probe sets for 8740 known rat genes. Changes in individual mRNAs were determined in real-time PCR experiments and individual proteins by flow cytometry or ELISA as detailed in supplemental data (see www.ahajournals.org).

Generation of BLT-1$^{-/-}$ and Apo-E$^{-/-}$ Mice

BLT-1$^{-/-}$ mice[17,18] were backcrossed onto C57BL/6 background to the 8th generation. These mice were mated with apoE$^{-/-}$ mice also on the C57BL/6 background (Jackson Labs). The resulting offspring (BLT-1$^{+/-}$ and apoE$^{+/-}$) were crossed with apo-E$^{-/-}$ mice and all the offspring were genotyped by standard PCR methods to select apo-E$^{-/-}$ and BLT-1$^{+/-}$ animals. These animals were setup as breeders and all 3 types of offspring, ie, BLT-1$^{+/+}$, BLT-1$^{+/-}$, and BLT-1$^{-/-}$ mice, in apo-E$^{-/-}$ background served as experimental animals. The double-knockout (apoE$^{-/-}$ BLT-1$^{-/-}$) mice were born at the expected Mendelian ratios, developed normally, and were disease-free. Mice were weaned at 4 weeks, fed normal rodent chow (4.5% fat; Ralston Purina) for 3 more weeks, and switched to the western diet (21% fat, 0.15% cholesterol; Harlan Tekland no. 88137) at age 7 weeks.

Quantification of Atherosclerotic Lesions

Atherosclerotic lesions were analyzed using standard protocols with minor modifications.[19,20] After collecting 0.5 mL whole blood, the anesthetized mice were transcardially perfused with saline followed by 4% buffered formalin. Aorta tree beginning at the aortic valve to the brachiocephalic artery, which is ≈2.5 mm, was removed and embedded in cryostat molds. Samples were snap-frozen by immersion in liquid nitrogen and stored at −20°C. The sectioning strategy was modified from that of Paigen et al[20] as follows. The ascending segment from the appearance of aortic valve leaflets to the junction of the brachiocephalic artery, which is ≈1.5 mm, was sectioned. Then, 10-μm cryosections were serially made such that 2 contiguous sections were placed on 2 different slides. The next 5 sections were discarded. The cycle was repeated until the end of this segment. This method gave us at least 10 and up to 25 sections on each slide. One of the 2 slide sets was stained with Oil Red O stain as described.[21] Images were examined in bright field using Nikon fluorescence microscope TE 300 and captured with digital color camera. Morphometric image analysis of the foamy–cell-laden atherosclerotic plaque was performed with Metamorph software version 5.0. For each animal, the average lesion area of 10 to 20 sections was determined and the data expressed as percent lesion area±SD. Representative sections from the other set were stained with MOMA-2 immunostain of the monocytes as described[4], and the number of macrophages in lesions were counted (cells per high-power field). The remainder of the aorta was divided into the aortic arch, the thoracic aorta, and abdominal aorta for en face quantitative analysis of the atherosclerotic lesion by Sudan IV staining.[16] The lesion area was expressed as percent lesion area of total area of pinned out arteries measured by digital morphometric analysis. Total plasma cholesterol and triglyceride levels were measured using commercially available kits (Sigma). Mice were fasted overnight before the collection of blood samples for lipid analysis. Statistical analyses of the lesion size data were performed using the nonparametric Mann-Whitney *U* test.

Generation and Analysis of Immortalized Macrophage Cell Lines

Immortalized wild-type and BLT-1-deficient murine macrophage cell lines were generated by J2 retroviral transformation as previously described.[22] Details of phenotype, mRNA expression analysis, and methods for analysis of chemotaxis are provided in supplemental data (see www.ahajournals.org).

Results

LTB$_4$-Induced Genes Involved in Atherosclerosis

Previous studies have shown that RBL cells expressing the hBLT-1 activate actin polymerization, pseudopod extension, chemotaxis, calcium mobilization, and degranulation in response to LTB$_4$.[23] To further assess the extent of cellular responses to LTB$_4$, we analyzed changes in mRNA expression on Affymetrix rat U34A microarrays. Gene expression profiles in vehicle-treated hBLT-1-RBL cells or parental RBL cells treated with LTB$_4$ were compared with LTB$_4$-treated hBLT-1-RBL cells. The application of the double filter to data analysis resulted in a total of 17 upregulated genes, and the most striking observation from this analysis is that genes with well-established functions in the development of atherosclerosis showed highest-fold increases (Table 1). Fatty acid translocase/CD36 is a transmembrane protein and a specific receptor for oxidized LDL that transports LDL cholesterol into monocytes, thereby converting them to foam cells. Deletion of the CD36 gene in mice was protective against development of atherosclerotic lesions in otherwise susceptible mice.[24] Rat JE (MCP-1/CCL2) is a ligand for the chemokine receptor CCR2 and a major regulator of monocyte/macrophage functions. CCL2, presumably produced at the site of vascular lesions, attracts monocytes to the area, and deletion of either CCL2 gene or its receptor CCR2 gene protected mice from developing atherosclerosis.[2,4] Conversely, overexpression of CCL2 resulted in enhanced atherosclerosis in mice.[25]

Among other genes specifically induced by LTB$_4$ were urokinase plasminogen activator (uPA), colony-stimulating factor (CSF-1), and osteopontin. Although known to reduce vascular thrombosis, uPA was recently shown to significantly enhance experimental atherogenesis.[26] CSF-1, the gene mutated in osteopetrotic mice, is essential for the development of atherosclerosis.[27] A close examination of the microarray data also showed that osteopontin mRNA was upregulated ≈40-fold from parental RBL cells treated with LTB$_4$ versus hBLT-1 cells treated with LTB$_4$. Together, CSF-1 and osteopontin may be critical in promoting calcification and development of clinically significant aortic lesions.[27,28] In addition, a number of other upregulated genes such as CD44, Src-like adapter protein (SLAP), and protein tyrosine phosphatase SHP-1, may have important functions in leukocyte recruitment and signal transduction. These results suggest that LTB$_4$ is likely to have a major role in atherogenesis and provide targets for further analysis.

LTB$_4$-induced upregulation of individual mRNAs and the corresponding proteins were tested in real-time PCR and protein expression experiments. The data (Figure 1) demonstrate that LTB$_4$ enhanced the expression of mRNA and protein levels of CD36 (Figure 1A, 1C, and 1E) and CCL2 (Figure 1B, 1D, and 1F). Incubation of parental RBL cells with LTB$_4$ did not result in any induction of either CD36 or CCL2. Moreover, the expression of both mRNAs and the corresponding proteins were dependent on Gα_i-protein signaling, as evidenced by near-complete inhibition of induction

Microarray Analysis of Changes in LTB_4-induced Gene Expression

Probe Set Name	Gene	Fold Change hBLT-1 +LTB_4 vs hBLT-1 −LTB_4	*P*-Value	Fold Change hBLT-1 +LTB_4 vs RBL +LTB_4	*P*-Value
AF072411_g_at	Fatty acid translocase/CD36	11.2	0.000047	7.3	0.000043
X17053cds_s_at	Monocyte chemoattractant protein-1 (MCP-1) /CCL2/JE	8.7	0	10.0	0
rc_AI639338_at	Src Like Adapter Protein-1 (SLAP-1)	5.7	0	12.6	0.000001
X63434_at	Urokinase-type plasminogen activator	4.1	0.000003	2.2	0.000005
M61875_s_at	CD44	4.1	0.000005	3.7	0.000001
U63740_at	Synaptotagmin binding zyginI mRNA	4.0	0.000011	4.0	0.000001
rc_AI235890_s_at	Major histocompatibility complex protein class I	2.9	0.000001	4.1	0.000001
U77038_at	Protein Tyrosine phosphatase (SHP-1)	2.8	0	4.0	0
X06916_at	Protein p9Ka homologous to CaBP	2.5	0.000005	3.9	0.000955
D50436_at	Adrenodoxin	2.5	0	3.1	0
M84361_at	CSF-1	2.3	0.000079	2.5	0.00001
L09119_g_at	Rattus norvegicus C kinase substrate calmodulin-binding protein (RC3) mRNA	2.3	0.000004	2.1	0
U10995_g_at	Wistar orphan receptor COUP-TFI	2.2	0.000001	5.2	0.000001
rc_AA875043_at	Rattus norvegicus testis specific protein kinase1	2.1	0	3.2	0.000002
AF009511_at	Rattus norvegicus NKR-P2 (Nkrp2) mRNA	2.1	0.000004	3.2	0.00022
AF002251_at	Rattus norvegicus Maxp1 mRNA	2.0	0.000001	2.0	0.000001
M14656_at*	Rat Osteopontin mRNA	1.5	0	41.4	0

Affymetrix Rat U34A gene chips were hybridized with cRNA generated from control and sample RNAs and analyzed as described in *Methods*, and the genes displaying more than two-fold upregulation with the double filter are shown in order.

*Rat osteopontin mRNA was included in this table based on its highest fold induction difference in comparison with RBL cells treated with LTB_4 vs hBLT-1 cells treated with LTB_4. The significance (*P*) for each call of change in mRNA level as determined by the Affymetrix software is indicated.

in cells treated with pertussis toxin. Pertussis toxin also inhibited the LTB_4-induced upregulation of SLAP and uPA mRNAs (data not shown). A major difference between the expression of CD36 and CCL2 genes was that CCL2 induction appears to reach maximum levels both at lower doses of LTB_4 and at earlier time points. This raises the possibility that some of the products of the genes induced early might promote upregulation of CD36. CSF-1 was one of the LTB_4-induced genes (Table 1) and was known to induce the expression of CD36.[29] However, addition of the medium from LTB_4-treated hBLT1 cells (6 hours with 1 μmol/L) to RBL cells did not result in any detectable CD36 expression after an additional 18 hours, suggesting that LTB_4-induced CD36 expression may be a direct effect of LTB_4 on BLT-1 (data not shown). Therefore, LTB_4 presumably activates distinct signaling pathways for the coordinated regulation of several genes involved in the development of atherosclerosis.

BLT-1 Promotes Early Atherosclerotic Lesions in Apo-E$^{-/-}$-Deficient Mice

Given the dramatic induction of genes involved in atherogenesis by LTB_4, we examined the involvement of BLT-1 in the development of atherosclerotic lesions in BLT-1-deficient mice. Deletion of BLT-1 is known to reduce LTB_4-induced leukocyte adhesion under flow and inflammatory responses to thioglycolate or zymosan.[17,18] Apo-E$^{-/-}$ mice spontaneously have lesions in the aortic valve and throughout arterial tree.[4,30,31] Therefore, we bred BLT-1$^{-/-}$ mice with apo-E$^{-/-}$ mice and generated BLT-1 and apo-E double-deficient mice. Quantitative analysis of atherosclerotic lesion development in

these mice fed a high fat western diet showed significantly reduced lesions in BLT-1$^{-/-}$ mice compared with BLT-1$^{+/+}$ mice (Figures 2 and 3). First, we examined the lesion development along the aorta in whole-mount en face preparations stained with Sudan IV (Figure 1). In mice fed western diet for 4 weeks, the average lesions were 5.6±2.3% of the total area in BLT-1$^{+/+}$ and 3.5±1.7% of the total area in BLT-1$^{-/-}$ mice (*P*=0.019, Mann-Whitney test). Although a 30% reduction in mean lesion area was also observed in BLT-1$^{-/-}$ mice at 8 weeks, the data are not statistically significant (data not shown). Aortic lesions were also measured by two other independent methods, a quantitative Oil Red O staining[21] of the fatty lesions in aortic cross sections and a qualitative immunohistochemical staining of macrophages with MOMA-2 antibody in the same areas.[4] Staining with Oil Red O confirmed the significant decrease in the total lesion area in BLT-1$^{-/-}$ mice after 4 weeks on the high-fat diet (Figure 3A–C). BLT-1$^{+/-}$ showed intermediate lesions both in en face and aortic cross-section analyses, suggesting a gene dosage effect. Typical MOMA-2 staining shown in Figure 3D and 3E indicates reduced monocyte infiltration in the lesions of the BLT-1$^{-/-}$ mice relative to BLT-1$^{+/+}$ mice. However, when the mice were continued on the high-fat diet for 19 weeks, the lesion size was similar in BLT-1$^{+/+}$ and BLT-1$^{-/-}$ mice as determined both in en face and aortic cross-section analyses (Figure I, available at http://atvb.ahajournals.org). Cholesterol and triglyceride levels in plasma samples of these mice were measured (Table I, available at http://atvb.ahajournals.org). While some variation was ob-
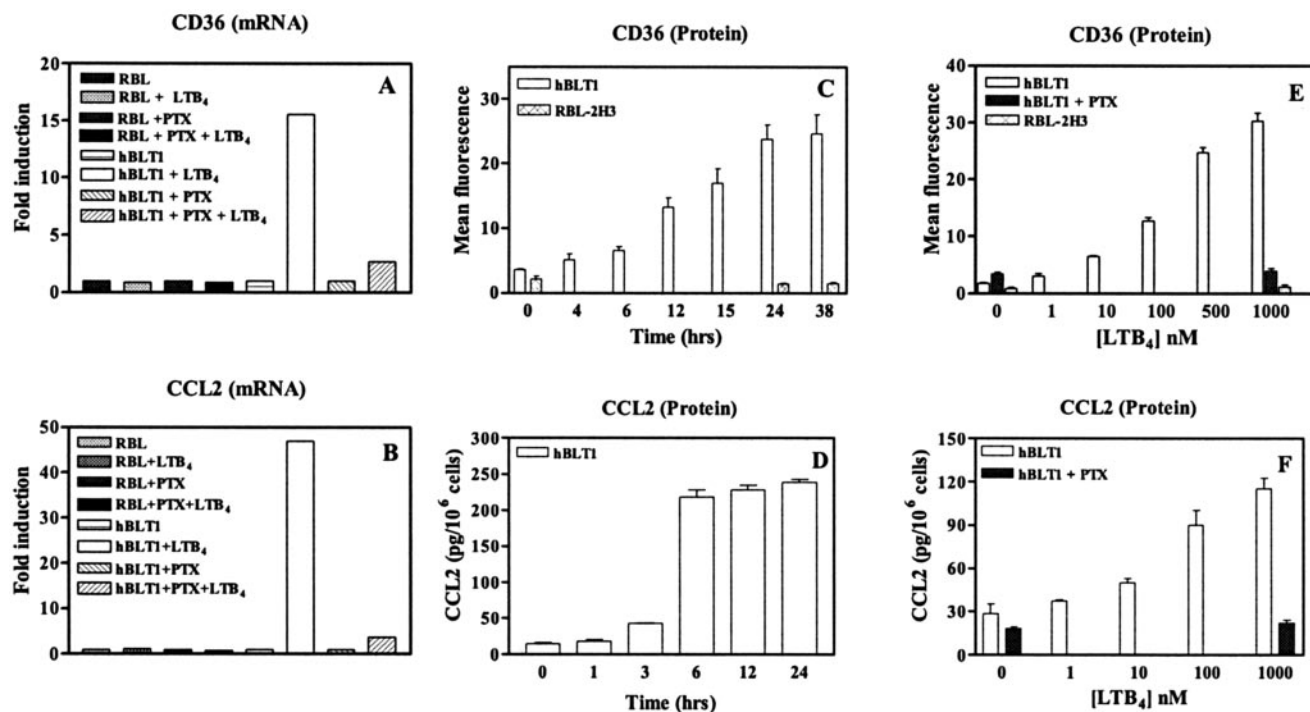
Figure 1. Analysis of LTB$_4$-induced gene expression. Parental RBL cells or cells expressing human BLT-1 (hBLT-1) were treated as indicated and total RNA was isolated. Real-time PCR analysis was performed as described in *Methods*, and the data were expressed as fold induction over the levels in vehicle-treated versus LTB$_4$-treated (1 μmol/L) cells for CD36 (A) and CCL2 (B) mRNAs. Cells were incubated with Pertussis toxin at 100 ng/mL overnight as indicated before the addition of LTB$_4$. Time–course and dose–response of gene induction by LTB$_4$ were determined in RBL or hBLT-1 cells. The indicated cell lines were treated with LTB$_4$ (1.0 μmol/L) for different times (C and D) or different concentrations of LTB$_4$ for 16 hours (E) or 6 hours (F), and the protein expression of CD36 (C and E) or CCL2 (D and F) were determined as described in *Methods*. The data in A–F are representative of at least 3 independent experiments, each with triplicate measurements.

served in these values, there is no clear pattern of changes in plasma lipid levels between BLT-1$^{+/+}$ and BLT-1$^{-/-}$ animals.

Macrophages From BLT-1$^{-/-}$ Mice Express a Functional BLT-2 Receptor

To analyze the expression of different leukotriene receptors and their signal transduction pathways in macrophages, the predominant leukocyte implicated in atherosclerosis, we generated immortalized macrophage cell lines from both BLT-1$^{+/+}$ and BLT-1$^{-/-}$ cells.[22] J2 retroviral transformation of murine bone marrow has been shown to result in the selective immortalization of cell lines that display the phenotypic and functional characteristics of macrophages, including LPS responsiveness and production of inflammatory cytokines, and nitric oxide production.[32] Phenotypic analysis showed both of these cell lines expressed the typical macrophage marker CD11b and no T and B cell markers (Figure II, available at http://atvb.ahajournals.org). Real-time PCR analyses showed that both BLT-1 and BLT-2 are expressed in macrophages derived from BLT-1$^{+/+}$ mice. More importantly, macrophages from BLT-1$^{-/-}$ mice showed the absence of BLT-1 transcripts but normal BLT-2 expression (Figure 4A). BLT-1$^{+/+}$ macrophages showed robust chemotactic response with a maximum response at 1.0 nM LTB$_4$. The BLT-1$^{-/-}$ cell line also showed chemotaxis but required a 100-fold higher LTB$_4$ concentration for maximal response (Figure 4B and 4C). Both cell lines showed identical chemo-

tactic responses to platelet activating factor (Figure III, available at http://atvb.ahajournals.org).

Discussion

The results presented here point to a complex role for LTB$_4$ and the two G-protein coupled receptors, BLT-1 and BLT-2, in atherosclerosis. Several recent reports support the view that LTB$_4$ may play a critical role in atherosclerosis. High levels of all components involved in LTB$_4$ biosynthesis, ie, 5-lipoxygenase, 5-lipoxygenase activating protein, and LTA$_4$ hydrolase, were detected in human atherosclerotic lesions.[33] Antagonists of LTB$_4$ blocked the development of atherosclerosis in apo-E–deficient and LDLR-deficient mice,[16] and mice deficient in 5-lipoxygenase showed greatly reduced lesions in LDLR$^{-/-}$ background, suggesting leukotrienes may play a dominant role in atherogenesis.[34] However, the mechanisms through which LTB$_4$ could bring about such dramatic differences are unclear. The data presented here suggest several mechanisms through which LTB$_4$ might regulate atherogenesis (Figure IV, available at http://atvb.ahajournals.org). First, as a chemoattractant of monocytes, LTB$_4$ could attract monocytes to the lipid-rich subendothelial spaces where it might be produced through the activation of 5-lipoxygenase. Alternatively, non-enzymatic lipid peroxidation produces isoleukotrienes that modulate biological processes through the activation of leukotriene receptors.[35] Second, after the recruitment of monocytes to the developing atherosclerotic lesion, LTB$_4$ can upregulate CCL2, CD36, uPA, CSF-1, and
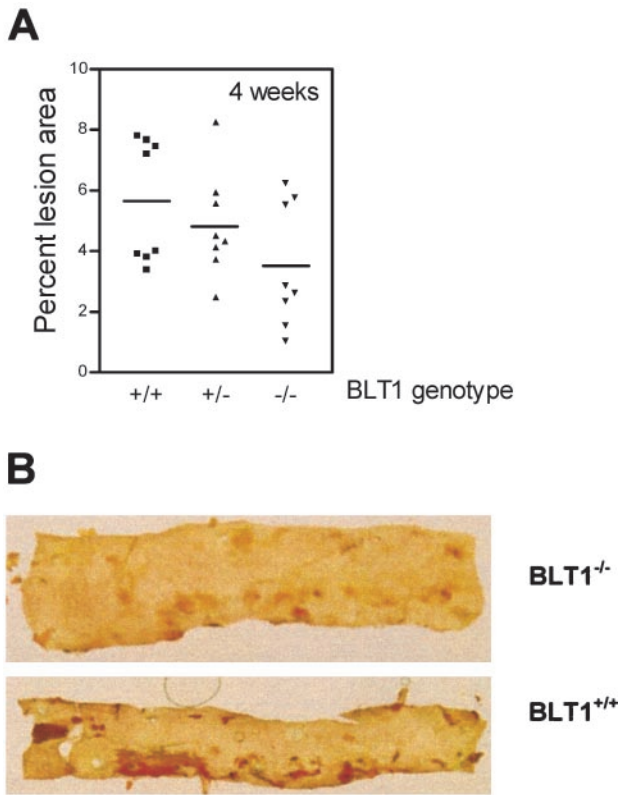
Figure 2. Total lesion coverage in aortas of mice. A, The whole aorta divided into the aortic arch, the thoracic aorta, and abdominal aortas from mice fed high-fat diet for 4 or 8 weeks were mounted en face and stained for lipid with Sudan IV. Total lesion area in each of these segments was measured by digital morphometry. Each symbol represents a single animal and the bar represents means. The percentage of the lesion areas in BLT-1$^{+/+}$ mice (n=8) at 4 weeks is significantly higher than in BLT-1$^{-/-}$ (n=8) mice (P=0.019, Mann–Whitney test; P=0.005, ANOVA). B, Representative sections of en face preparations stained with Sudan IV from BLT-1$^{-/-}$ and BLT-1$^{+/+}$ mice.

Figure 3. Effect of BLT-1 deletion on lesions. A, Aortic cross sections (10 μm) were stained for lipid with Oil Red O and quantitated by digital morphometry. Each symbol represents an animal and the bars represent means for comparisons of BLT-1$^{-/-}$ and BLT-1$^{+/+}$ mice at 4 weeks (P=0.036) and at 8 weeks (P=0.085, Mann–Whitney test). The difference in lesions between BLT-1$^{+/+}$ and BLT-1$^{+/-}$ mice at 8 weeks is significant (P=0.041 Mann–Whitney test). Representative Oil Red O-stained sections of BLT-1$^{+/-}$ (B) and BLT-1$^{+/+}$ (C), original magnification ×200. Sections of aortic sinus stained for macrophages with MOMA-2 antibody (brown) and counter stained with hematoxylin from BLT-1$^{-/-}$ (D) and BLT-1$^{+/+}$ (E) mice.

osteopontin, all of which have well-established roles at various stages during the development of atherosclerosis.[2,4,24,26–28] In particular, the induction of CCL2 by LTB₄ provides a positive feedback loop to recruit macrophages and further generation of LTB₄ by CCL2-mediated activation of arachidonic acid metabolism.[15] Likewise, the induction of CD36 by LTB₄ provides yet another positive feedback loop via OxLDL uptake, conversion of macrophages to foam cells, and to the production of additional chemokines by OxLDL. Although multiple regulatory mechanisms for the expression of these individual genes are known, the coordinated regulation of all of these molecules through the activation of BLT-1 by LTB₄ offers a clear initiating step in atherogenesis. We have not detected changes in CD36 and/or CCL2 mRNA levels in response to LTB₄ in primary macrophages or macrophage lines. This could be because of the activation state and expression levels of BLT-1 in these cells. Resting macrophages express low levels of BLT-1, and activation was shown to increase BLT-1 mRNA levels.[36] In addition, heterogeneous nature of monocyte/macrophages with subsets of cells expressing distinct markers was also reported.[37] The ma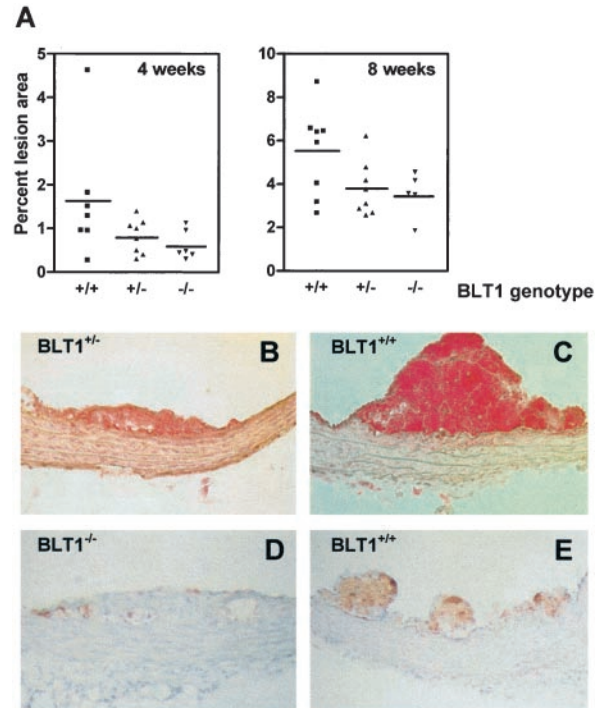crophages in the atherosclerotic plaque are likely to be different from those in circulation or elicited by inflammatory mediators in peritoneal exudates.

The partial protection against lesion development in BLT-1-deficient mice at early times and no reduction in lesions in mice on prolonged high-fat diet is in contrast to the significant protection observed in mice treated with BLT-1 antagonist.[16] This suggests that compensatory mechanisms for the loss of BLT-1 might have occurred in BLT-1-deficient mice. These may include upregulation of other chemokines, like MIP-2, KC, CX3CL1, and possibly CCL-2, acting through CXCR2, CX3CR1, and CCR2 receptors (all demonstrated to be involved in macrophage influx during atherogenesis).[2–4] In addition, the nearly complete protection against atherogenesis offered by reduced 5-lipoxygenase activity[34] suggests that other leukotrienes (LTC₄ and LTD₄) and leukotriene receptors (Cys LTs and BLT-2) are likely to be critical mediators of lesion development. Indeed, the results presented here showed that macrophages from BLT-1-deficient mice expressed the low-affinity LTB₄ receptor BLT-2 and chemotactic response to LTB₄. Cys LT and BLT-2 were also shown to be expressed on macrophages as well as in endothelial cells.[38] The studies with LTB₄ antagonists are also in agreement with a late role for BLT-2, because there was a greater protection at early times (< 70%) but reduced effectiveness at later
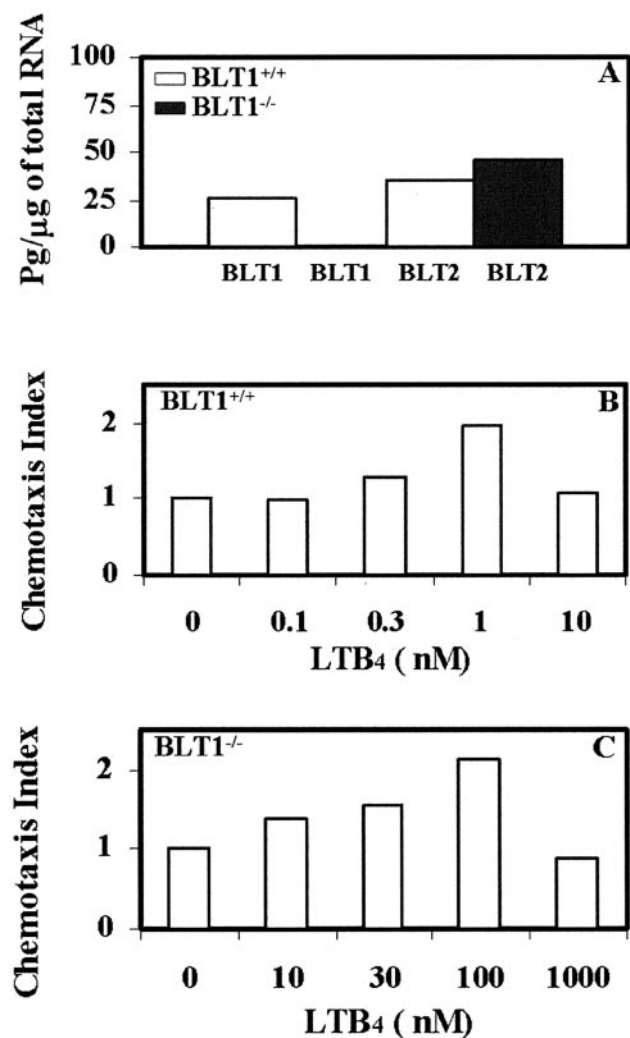
Figure 4. Expression and function of BLT-1 and BLT-2 in murine macrophages: A, Levels of BLT-1 and BLT-2 mRNAs in murine macrophage cell lines from BLT-1$^{+/+}$ and BLT-1$^{-/-}$ mice were measured as described in *Methods*. The chemotaxis of BLT-1$^{+/+}$ (B) and BLT-1$^{-/-}$ (C) macrophages to LTB$_4$ is determined in transmembrane chemotaxis assays. The data shown are representative of three independent experiments.

times ($>$25%).[16] Because established atherosclerotic plaques have very high levels of 5-lipoxygenase pathway components,[33] it is likely that high local LTB$_4$ concentrations that are inhibitory for chemotaxis through BLT-1 will be generated at these sites. Presence of a low-affinity BLT-2 on monocytes will allow these cells to migrate to the lesions at high LTB$_4$ concentrations. Such tandem of high- and low-affinity chemoattractant receptors have been shown to mediate chemotaxis at different concentrations of the ligand.[39] Demonstration of a more direct role for BLT-2 in atherosclerosis requires the development of BLT-2-specific antagonists and generation of BLT-2$^{-/-}$ mice.

The results presented herein suggest that LTB$_4$ and its receptors BLT-1 and BLT-2 might play distinct roles in the initiation and progression of atherosclerotic disease. Selective interference with either LTB$_4$ synthesis or function of BLT-1 and BLT-2 offers attractive targets for the development of pharmacological agents to block the progression of atherosclerotic vascular disease.

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