Selective Interleukin-12 Synthesis Defect in 12/15-Lipoxygenase-deficient Macrophages Associated with Reduced Atherosclerosis in a Mouse Model of Familial Hypercholesterolemia*

Received for publication, June 10, 2002, and in revised form, July 15, 2002 Published, JBC Papers in Press, July 16, 2002 DOI 10.1074/jbc.M205738200

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Targeted gene disruption or overexpression of 12/15-lipoxygenase in mice on the genetic background of apolipoprotein E or low density lipoprotein-receptor (LDL-R) deficiency has implicated 12/15-lipoxygenase in atherogenesis. The data support indirectly a role for 12/15-lipoxygenase in the oxidative modification of low density lipoprotein. In this study we set out to explore other potential mechanisms for 12/15-lipoxygenase in atherosclerosis using apolipoprotein B mRNA editing catalytic polypeptide-1/LDL-R double-deficient mice, a model highly related to the human condition of familial hypercholesterolemia. 12/15-Lipoxygenase deficiency in this strain led to ~50% decrease in aortic lesions in male and female mice at 8 months on a chow diet in the absence of cholesterol differences. While studying 12/15-lipoxygenase-deficient macrophages in culture, we discovered a remarkable selective defect (75–90% decrease) in interleukin-12 production but not in tumor necrosis factor-α or nitric oxide release, in response to lipopolysaccharide in the presence or absence of interferon-γ priming. The lipopolysaccharide/interferon-γ response was associated with a 33–50% decrease in nuclear interferon consensus sequence-binding protein, which is consistent with interferon consensus sequence-binding protein containing protein complex-dependent regulation of the interleukin-12 p40 gene. The decrease in interleukin-12 production was recapitulated in vivo in mouse aortas of the triple knockout group and was reflected in a marked decrease in interferon-γ expression. The data provide support for a novel mechanism linking the 12/15-lipoxygenase pathway to a known immunomodulatory Th1 cytokine in atherogenesis.

Atherosclerosis, a complex, chronic inflammatory disease process progressing from fatty lesions to fibrous and unstable plaques, is the major underlying cause of most cases of coronary artery disease (1). During the early phase of fatty streak formation, monocytes are recruited and transformed into lipid-enriched foam cells, and a complex network of regulatory molecules and cellular interactions amplify the pathophysiological process. Substantial evidence supports an active role for Th1-derived cytokines like interleukin-12 (IL-12)

1 The abbreviations used are: IL-12, interleukin-12; 12/15-LO, 12/15-lipoxygenase; apobec-1, apolipoprotein B mRNA editing catalytic polypeptide-1; LDL-R, low density lipoprotein-receptor; NO, nitric oxide; TNF-α, tumor necrosis factor-α; apoE, apolipoprotein E; RPA, ribonuclease protection assay; ICSSBP, interferon consensus sequence-binding protein; IRF-1, IFN regulatory factor 1; iNOS, inducible NO synthase; LPS, lipopolysaccharide; LDL, low density lipoprotein; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; IFN-γ, interferon-γ; ELISA, enzyme-linked immunosorbent assay; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RT, reverse transcriptase; MIF, migration inhibitory factor.

* This work was supported by National Institutes of Health Grants HL53558 (to C. D. F.), AI45813, HL65507 (to E. P.), HL57811 (to C. D. F.), and a Deutsche Forschungsgemeinschaft Fellowship (to L. Z.) and Grants 030211N (to D. P.) and 60182U (to C. A. C.), and a Deutsche Forschungsgemeinschaft Fellowship (to L. Z.) and Grants 030211N (to D. P.) and 60182U (to C. A. C.), and a Deutsche Forschungsgemeinschaft Fellowship (to L. Z.) and Grants 030211N (to D. P.) and 60182U (to C. A. C.), and a Deutsche Forschungsgemeinschaft Fellowship (to L. Z.) and Grants 030211N (to D. P.) and 60182U (to C. A. C.).

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Printed in U.S.A.

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**EXPERIMENTAL PROCEDURES**

**Mice**—Apobec-1⁻/⁻/LDL-R⁻/⁻ mice (15) backcrossed six times to the C57BL/6 genetic background were kindly provided by L. Powell-Braxton. The generation of 12/15-LO-deficient mice (backcrossed 7 times to C57BL/6 background) was described previously (9, 17). Generation of apobec-1⁻/⁻/LDL-R⁻/⁻/12/15-LO⁻/⁻ triple knockout mice was achieved by cross-breeding, and mice were genotyped individually by PCR analysis for each of the three genes as described at The Jackson Laboratory website (www.jax.org). For macrophage experiments, age- and gender-matched C57BL/6 (Taconic Farms) and 12/15-LO⁻/⁻ mice were used.

**Tissue Preparation for Morphometric Determination of Atherosclerotic Lesions**—Mice were anesthetized and bled via cardiac puncture. Aorta en face preparations/images were performed as described (18). Measurement of Urinary Isoprostanes and Plasma Lipids—Urine collection and isoprostane 8,12-iso-iPF2α-VI measurements were performed as described (18). Plasma total cholesterol levels were determined by an automated enzymatic technique on a Cobas Fara II autoanalyzer.

**Peritoneal Macrophages Cultures**—C57BL/6 and 12/15-LO⁻/⁻ mice were injected intraperitoneally with 2 ml of sterile 3% Brewer's thiglycollate broth. After 4 days, macrophages were harvested by peritoneal lavage with Ca²⁺/Mg²⁺-free phosphate-buffered saline, adherence-purified, and cultured in RPMI supplemented with 10% fetal calf serum, 50 μM β-mercaptoethanol, 10 μg/ml polymyxin B, 1% penicillin, streptomycin, and fungizone with 5% CO₂. Cells were treated with 10 ng/ml lipopolysaccharide (LPS) (Sigma) for 24 h, and supernatants were stored at −20°C for future analysis. In some experiments cells were pretreated with 100 units/ml IFN-γ (Genzyme) for 16 h prior to stimulation with LPS. For RNA analysis, cells were stimulated with LPS for 6 h prior to RNA extraction. For nuclear transcription factor analysis, cells were treated with IFN-γ (100 units/ml) and LPS (1 μg/ml) for 4 h. All cultures were analyzed for viability by the metabolic MTT assay as described previously (19, 20).

**RT-PCR and Southern Blot Hybridization of PCR Products**—Total RNA was extracted from mouse whole aorta using Trizol Reagent (Invitrogen). cDNA was synthesized from 2 μg of total RNA by reverse transcription using the Superscript™ First-strand Synthesis System for RT-PCR (Invitrogen), and defined aliquots were used for PCR. The primers and reaction conditions for analysis of β-actin, IL-12 p40, and IFN-γ were as described (2) except the number of PCR cycles used for β-actin and IFN-γ were 25 and 35, respectively, and conditions for IL-12 p40 proceeded for 40 cycles at 94°C for 30 s, 62°C for 30 s, and 72°C for 45 s, followed by an extension at 72°C for 7 min using Advantage™ cDNA PCR kit (CLONTECH, Palo Alto, CA). PCR products were electrophoresed and transferred to Hybond-N nylon membranes (Amersham Biosciences). Specific oligonucleotides internal to the PCR amplification primers were used as probe for hybridization. The sequences of the oligonucleotides are as follows: β-actin, 5′-CCATGTACCCAGGCA- TTGCTGACAGGATGC-3′; IL-12 p40, 5′-CTTGTCAGACAGCTGAGCATCA- TTGATCAGGATGC-3′; IFN-γ, 5′-CAGCGCTTTAACAGCAGGCCA- TCAGATCATC-3′; and IFN-γ, 5′-CAGCGCTTTAACAGCAGGCCA- TCGATCATC-3′. The probe was 5′-end-labeled using T4 polynucleotide kinase (New England Biolabs) and [γ-³²P]ATP (3,000 Ci/mmol) (PerkinElmer Life Sciences). Blots were pre-hybridized in Rapid-Hyb buffer (Amersham Biosciences) at 42°C for 1 h, followed by probe hybridization for 4 h. Blots were washed in 2× SSC and 0.1% SDS at room temperature for 2–5 min and then exposed to film.

**Cytokine Analysis**—Macrophage supernatants were analyzed for IL-12 p40 production by radioimmunoassay as described previously (19, 20). Tumor necrosis factor-α (TNF-α) levels were measured by ELISA kit (BD Pharmingen) and nitric oxide with Greiss reagents and spectrophotometric assay with sodium nitrite as standard (21). All values were normalized for cell viability by the MTT assay. Aorta IL-12 p40 levels were measured by ELISA, Monoclonal antibody C17.8 (BD Pharmingen) was used as a capture antibody and biotinylated C15.6 (BD Pharmingen) as a detecting antibody.

**RNase Protection Assay (RPA)**—RNA was extracted from peritoneal macrophage cultures using Trizol reagent. mRNA levels of IL-12 p40 and MIF, as well as the housekeeping genes L32 and glyceraldehyde-3-phosphate dehydrogenase were analyzed using BD RiboQuant™ Multiprobe Template Set (BD Pharmingen) and RPA Assay kit (Ambion). Relative levels of mRNA expression were quantified using a PhosphorImager. Data were normalized to the level of L32 detected in each sample as control for variation in sample loading.

**Western Blot Analysis**—Nuclear proteins were extracted with NE-PER™ nuclear and cytoplasmic extraction reagents (Fierce). Protein concentrations were determined by the Bradford method (Bio-Rad). The generation of 12/15-LO-deficient mice (backcrossed 7 times to C57BL/6 genetic background) was kindly provided by L. Powell-Braxton. The generation of apobec-1⁻/⁻/LDL-R⁻/⁻/12/15-LO⁻/⁻ triple knockout mice was achieved by cross-breeding, and mice were genotyped individually by PCR analysis for each of the three genes as described at The Jackson Laboratory website (www.jax.org). For macrophage experiments, age- and gender-matched C57BL/6 (Taconic Farms) and 12/15-LO⁻/⁻ mice were used.

**RESULTS**

**Correlation of 8,12-iso-iPF2α-VI Excretion with Lesion Size in 12/15-LO⁻/⁻ Mice**—Mice—We assessed whether an indirect measure of oxidative stress, urinary isoprostane levels, was diminished as a result of 12/15-LO deficiency in the apobec-1⁻/⁻/LDL-R⁻/⁻ mouse model. At 8 months, urinary 8,12-iso-iPF2α-VI from apobec-1⁻/⁻/LDL-R⁻/⁻/12/15-LO⁻/⁻ male mice (0.88 ± 0.19 ng/mg creatinine, n = 4) was significantly lower (p < 0.05) compared with levels from apobec-1⁻/⁻/LDL-R⁻/⁻ mice (1.50 ± 0.06 ng/mg creatinine, n = 3) (Fig. 2A). A similar significant decrement was also observed in female mice (0.58 ± 0.11 ng/mg creatinine, n = 4, versus 1.20 ± 0.16 ng/mg creatinine, n = 3, p < 0.05) (Fig. 2B).

**Mouse Model**—Based on cytokine assays with 12/15-LO-deficient macrophages (see below), we measured...
IL-12 levels in mouse aortas. IL-12 p40 levels were significantly lower in 8-month-old apobec-1−/−/LDL-R−/−/12/15-LO−/− mouse aortas (1.0 ± 0.2 ng/mg tissue, n = 4) than in apobec-1−/−/LDL-R−/−/12/15-LO−/− aortas (3.8 ± 0.1 ng/mg tissue, n = 4) (Fig. 3A). To determine whether the decrease of IL-12 in apobec-1−/−/LDL-R−/−/12/15-LO−/− mouse aortas was due to decreased mRNA expression in lesions, aortic IL-12 p40 cDNA prepared from 8-month mice of both genotypes was analyzed by RT-PCR and Southern blot hybridization (Fig. 3B). Quantitation of the signals after normalization to β-actin expression indicated a significant decrease in IL-12 p40 levels in apobec-1−/−/LDL-R−/−/12/15-LO−/− aortas compared with apobec-1−/−/LDL-R−/−/12/15-LO−/− aortas (0.92 ± 0.11, n = 6, versus 1.46 ± 0.08, n = 5, p < 0.005) (Fig. 3C). IL-12 plays an essential role in the induction of IFN-γ production by Th1 cells, and IFN-γ has been shown to be pro-atherogenic in mice (22, 23). Therefore, we investigated the impact of the reduced IL-12 levels in aortas of 12/15-LO−/− mice on the IFN-γ expression in the respective double knockout group. To determine whether the defect in IL-12 production in 12/15-LO−/− macrophages was also detected in the presence of IFN-γ, whereas IFN-γ increased the level of LPS-induced IL-12 production, the 12/15-LO−/− macrophages still made 75–90% less IL-12 compared with 12/15-LO−/− cells (Fig. 4B). To determine whether the deficit in IL-12 production in 12/15-LO−/− macrophages was indicative of a general defect in macrophage production of...
inflammatory mediators, we investigated the capacity of 12/15-LO/Hi1002 macrophages to produce TNF-α and nitric oxide (NO). 12/15-LO/Hi1002 macrophages produced comparable levels of TNF-α and NO (Fig. 4C and Ref. 17) suggesting that 12/15-LO may play a specific role in regulating production of IL-12.

**Fig. 3.** IL-12 and IFN-γ expression are markedly reduced in aortas of apobec-1-/-/LDL-R-/-/12/15-LO-/- mice. A, IL-12 p40 levels were examined by ELISA, and results are presented as mean ± S.E. of duplicate determinations; *, p < 0.0001, n = 4. B, IL-12 p40, IFN-γ, and β-actin gene expression in mouse aortas was examined by RT-PCR followed by hybridization with an oligonucleotide internal to the PCR primers. Densitometric analysis of relative IL-12 p40 mRNA levels (C) and IFN-γ mRNA levels (D) in mouse aortas. The results are presented as mean ± S.E. *, p < 0.001, n = 5 for apobec-1-/-/LDL-R-/- group and 6 for apobec-1-/-/LDL-R-/-/12/15-LO-/-.

**Fig. 4.** Selective defect of IL-12 production by macrophages derived from 12/15-LO knockout mice. IL-12 p40 in supernatants of peritoneal macrophage incubations stimulated with LPS without (A) or with (B) IFN-γ priming. Significantly, less IL-12 p40 is observed in 12/15-LO/Hi1002 macrophages (*, p < 0.05, n = 4). C, TNF-α levels in supernatants of peritoneal macrophage incubations stimulated with LPS. There is no difference in TNF-α levels between 12/15-LO/Hi1001 and 12/15-LO/Hi1002 macrophages (n = 5). Data in A-C were all normalized to viable cell recovery based on MTT. D, RNase protection analysis for IL-12 p40 and MIF mRNA transcripts in 12/15-LO/Hi1001 (lanes 1 and 2) and 12/15-LO-/- (lanes 3 and 4) macrophages either untreated (lanes 1 and 3) or stimulated with IFN-γ plus LPS (lanes 2 and 4). E, quantitation of IL-12 p40 (a) and MIF mRNA (b) expression. Data are normalized to the mRNA level of L32 as control.
phage cultures were not affected by transfer of conditioned media from 12/15-LO−/− macrophages receiving fresh media, conditioned media from 12/15-LO−/−, or 12/15-LO+−/− macrophage cultures are compared. IL-12 p40 levels are not affected by conditioned media from macrophages of either genotype (n = 3). Co-cultures containing 50% each of 12/15-LO−/− and 12/15-LO+−/− macrophages receiving fresh media produced an intermediate level of IL-12 (p < 0.0001, one-way analysis of variance, n = 3). CM, conditioned media.

To investigate further the mechanisms by which 12/15-LO regulates IL-12 production, we performed RPA for IL-12 p40 mRNA transcript in macrophage cultures stimulated with IFN-γ and LPS. Stimulation of 12/15-LO+−/− macrophages with IFN-γ + LPS induced a 3-fold increase of IL-12 p40 mRNA levels above the level detected in untreated cultures. However, the induction of IL-12 p40 by IFN-γ + LPS was dramatically reduced in 12/15-LO−/− macrophages (Fig. 4, D and E(a)). Expression of the macrophage MIF cytokine gene by IFN-γ + LPS was not significantly different between 12/15-LO+−/− and 12/15-LO−/− macrophages (Fig. 4, D and E(b)) further indicating that 12/15-LO+−/− macrophages do not exhibit a general defect in cytokine gene expression.

The Defect of IL-12 Production by 12/15-LO-deficient Macrophages Is Not Mediated by Soluble Factors—Several soluble factors have been identified that can negatively regulate IL-12 production including IL-10, prostaglandin E2, and TNF-α (24–26). To determine whether 12/15-LO regulates IL-12 production through one of these or other unidentified soluble factor(s), we performed a series of supernatant transfer experiments. Comparable levels of IL-12 were produced by 12/15-LO−/− macrophages regardless of the presence of fresh media or conditioned media from stimulated macrophages from either 12/15-LO+−/− or 12/15-LO−/− mice. Furthermore, the higher IL-12 levels detected in 12/15-LO+−/− macrophage cultures were not affected by transfer of conditioned media from 12/15-LO−/− cells (Fig. 5). Together these data suggest that the regulation of IL-12 by 12/15-LO is not mediated through a stable soluble factor. However, these experiments do not eliminate the possibility that the regulation of IL-12 expression by 12/15-LO may be mediated through a soluble mediator with a short half-life or one that is consumed quickly by the macrophage cultures. To address this question, we also set up co-cultures that contained 50% each of 12/15-LO−/− and 12/15-LO+−/− macrophages. These incubations produced an intermediate level of IL-12 providing further evidence that the nature of the defect in IL-12 production is intrinsic to the metabolism of the 12/15-LO−/− macrophages rather than mediated by production of a soluble transferable mediator (Fig. 5).

12/15-LO−/− Macrophages Exhibit Defective Nuclear Expression of ICSBP—ICSBP acts as a principal activator of IL-12 p40 transcription in RAW 264.7 cells (27). Together with c-Rel and PU.1, ICSBP has been shown to form a multiprotein complex, which binds to the Ets-2 site (5’-TTTCCG-3’; −210 to −205 for human and −218 to −213 for murine) on the IL-12 p40 promoter. The inhibition of this complex is responsible for the selective decrease of IL-12 p40 in macrophages following Fcγ receptor ligation (28). To examine further the mechanism involved in the selective decrease of IL-12 p40 in 12/15-LO-deficient macrophages, we performed Western blot analysis of several transcription factors that have been reported to interact with the Ets-2 site on the IL-12 p40 gene promoter. In the nuclei of unstimulated wild type macrophages, minimal levels of Ets-2, ICSBP, IRF-1, and c-Rel were detected. Stimulation of the macrophages with IFN-γ + LPS significantly induced Ets-2, ICSBP, IRF-1, and c-Rel. ICSBP induction was markedly diminished, whereas no obvious changes were observed in Ets-2, IRF-1, and c-Rel in 12/15-LO−/− peritoneal macrophages compared with 12/15-LO+−/− macrophages (Fig. 6).

**DISCUSSION**

Our studies conclusively demonstrate that 12/15-LO gene disruption markedly attenuates atherosclerotic lesion development in 8-month-old apoe−/−/LDL-R−/− mice on a normal chow diet. These observations are consistent with studies performed in apoE−/− mice (9, 10) and LDL-R−/− mice (11, 12). The apoE-deficient mouse develops typical lesions on a normal chow diet that faithfully mimics human disease progression from monocyte adhesion to foamy macrophages, fatty streaks, and advanced fibrosis (6, 7). The LDL-R-deficient mouse model (8) is also well established but requires a high fat diet to induce elevated LDL cholesterol levels and lesion development. It should be noted that the current model, chow-fed apoe−/−/LDL-R−/− mice, is the most similar to human lipoprotein physiology and is an excellent model for familial hypercholesterolemia. The advantage of the apoe−/−/LDL-R−/− mouse model over the apoE−/− and LDL-R−/− models lies in two points: 1) atherosclerosis in the apoe−/−/LDL-R−/− mouse model is much more pronounced in males than in females, whereas neither apoE−/− nor LDL-R−/− mouse model exhibits the clear gender-based distinction in expression of the disease so evident in humans (men are far more susceptible to atherosclerosis than premenopausal women); 2) familial hypercholesterolemia in humans is characterized by increased LDL cholesterol levels. The apoe−/−/LDL-R−/− mouse model exhibits elevated plasma levels of LDL cholesterol. In the apoE−/− mouse model, the elevated cholesterol is in very low density lipoprotein which is not the case in humans. Thus, a
pro-atherogenic role for 12/15-LO is consistently detected in three different atherosclerotic mouse models. In each, a ~50% decrease in lesion size is observed at varying time points throughout the lifespan of the mice. At early time points (10 and 15 weeks) in apoE<sup>−/−</sup> mice there is a remarkable lag in lesion initiation (9, 10). In the current study, decreased lesions were evident in male apobec-1<sup>−/−</sup>/LDL-R<sup>−/−</sup>12/15-LO<sup>−/−</sup> aortas at 15 weeks but not in females. The average lesion size in females is always lower than males in this model (15) and, due to normal variation and limitations of the assay, may have been too early for detection of significant differences in females. Alternatively, hormonal status may be an important factor in early atherosogenesis in apobec-1<sup>−/−</sup>/LDL-R<sup>−/−</sup> female mice. The paradoxical results showing that overexpression of 15-LO from the lysozyme promoter in transgenic rabbits decreased aortic lesion formation remains an enigma, potentially indicating strong species differences in the roles of 12/15-LO (29).

In addition to the effects of 12/15-LO gene disruption on IL-12 production, we also observed a decrease of oxidative stress as detected by urinary isoprostane measurement. Isoprostanes are chemically stable prostaglandin isomers that result from oxidative modification of arachidonic acid through a mechanism catalyzed by free radicals (30). They are associated with atherosclerotic lesion development and are regarded as reliable markers of oxidative stress (31). A strong correlation of urinary 8, 12-iso-iPF<sub>2α-2</sub>-Vi levels with lesion formation in apobec-1<sup>−/−</sup>/LDL-R<sup>−/−</sup> mice was observed. Despite no significant difference in total cholesterol between age- and gender-matched apobec-1<sup>−/−</sup>/LDL-R<sup>−/−</sup> and apobec-1<sup>−/−</sup>/LDL-R<sup>−/−</sup>/12/15-LO<sup>−/−</sup> mice, disruption of 12/15-LO in the double knockout mice caused a parallel decrease of 8,12-iso-iPF<sub>2α-2</sub>-Vi levels and lesion formation. These results are consistent with our recent report (10) that 8,12-iso-iPF<sub>2α-2</sub>-VI levels decreased in parallel with decreased lesion size in apoE<sup>−/−</sup>/12/15-LO<sup>−/−</sup> mice. Although the mechanism involved in this pathway still needs to be investigated, one could speculate that 12/15-LO enzymatic activity on macrophage membranes may initiate a subsequent series of non-enzymatic lipid peroxidations that lead to the formation of isoprostanes in oxidized LDL.

We have unveiled a previously unrecognized connection between a lipoxygenase pathway and IL-12 production in macrophages stimulated in vitro and in a chow-fed mouse model of hypercholesterolemia and atherosclerosis in vivo. Lipid-laden macrophages or foam cells are the predominant cell type in atherosclerotic lesions. IL-12 is a heterodimeric cytokine mainly produced by monocytes/macrophages. The production of IL-12 heterodimer usually depends on the inductive transcription of the p40 subunit; however, pre-formed stores can be mobilized quickly in some cases of parasite infection (22, 32). We detected a decreased production of IL-12 that was associated with decreased steady-state IL-12 mRNA levels suggesting that 12/15-LO regulates directly or indirectly with the transcription of the IL-12 p40 gene or the stability of IL-12 p40 mRNA. This reduction in IL-12 production was evident in 12/15-LO-deficient macrophages that were stimulated through the LPS-Toll receptor pathway. IL-12 promotes the generation of Th1 type responses by playing an essential role in the induction of IFN-γ production by Th1 cells. Both IL-12 and IFN-γ are present in human and murine atherosclerotic lesions, and recombinant IL-12 administration accelerates progression of atherosclerotic disease in these mice (2, 3). Furthermore, atherosclerotic lesion area is reduced in IFN-γ receptor null mice (23). Together these findings support an active role for Th1 cytokines in modulating atherogenesis in apoE-deficient mice (2–4). The decreased IL-12 production in atherosclerotic lesions in turn resulted in a marked reduction of IFN-γ mRNA expression in apobec-1<sup>−/−</sup>/LDL-R<sup>−/−</sup>/12/15-LO<sup>−/−</sup> mouse aortas compared with apobec-1<sup>−/−</sup>/LDL-R<sup>−/−</sup>aortas. The lower expression of IFN-γ implies a weaker Th1-mediated immune response at the aortic macrophage level in 12/15-LO<sup>−/−</sup> mice. A complete block in Th1-mediated responses as a result of 12/15-LO deficiency is not evident because the mice still mount normal responses to infection with Listeria monocytogenes and Toxoplasma gondii (17).<sup>2</sup> Taken together, these data suggest that the pro-atherogenic contribution of 12/15-LO may function partly via an effect on the IL-12/IFN-γ pathway.

Data from RPA provide evidence that 12/15-LO deficiency may contribute to IL-12 p40 gene down-regulation at the transcription level. Reportedly, IL-12 p40 gene expression is regulated by soluble factors and transcription factors that bind to regulatory elements on the IL-12 p40 promoter including Ets-2, NF-κB, C/EBP, and GA-12 sites (24–27, 33–35). The possibility of IL-12 p40 dysregulation by soluble transferable mediators was ruled out by supernatant transfer and co-culture experiments. Should any of the transcription factors be responsible for the selective IL-12 p40 gene dysregulation in 12/15-LO-deficient macrophages, then it should reveal the following features: 1) functional uniqueness to the IL-12 p40 gene (i.e. responsible for selective alterations in IL-12 p40 gene and not for genes controlling TNF-α and NO synthesis that were unchanged in 12/15-LO-deficient macrophages), and 2) should be induced by LPS and its transcriptional regulatory effect should be enhanced by IFN-γ priming. NF-κB is required for the induction of both TNF-α and inducible NO synthase genes. Furthermore, NF-κB was shown not to account for the selective suppression of IL-12 transcription in macrophages following Fcγ receptor ligation (28). Thus, although additional experiments will be required to formally exclude a role for NF-κB, we do not consider it likely that NF-κB is a critical factor in regulating the IL-12 p40-selective decrease in 12/15-LO-deficient macrophages.

A multiprotein complex that could bind to the Ets-2 site fits the two criteria mentioned above. It has been shown to be responsible for the selective IL-12 down-regulation following Fcγ receptor ligation (28), and its components, including IRF-1, c-Rel, and ICSBP, are highly induced by either IFN-γ or LPS. Based on the reported difficulties in quantitating protein complexes in primary macrophages (26), we instead examined the nuclear levels of the individual protein complex components. Markedly attenuated ICSBP nuclear levels were detected in 12/15-LO<sup>−/−</sup> macrophages in response to IFN-γ + LPS. This observation is in accordance with previous findings (36) that ICSBP knockout mice display defective IL-12 production. Our data provide evidence that the defect of the ICSBP-containing protein complex correlates with the selective IL-12 defect in LPS + IFN-γ-stimulated 12/15-LO<sup>−/−</sup> macrophages. The nature of the decreased ICSBP nuclear accumulation in LPS + IFN-γ-stimulated 12/15-LO<sup>−/−</sup> macrophages remains unknown. Several factors may be responsible for this ICSBP attenuation, and these factors include the following: 1) the products of 12/15-LO, such as 12-hydroxyeicosatetraenoic acid, 15-hydroxyeicosatetraenoic acid, 13-hydroxyoctadecadienoic acid, 12-hydroperoxyeicosatetraenoic acid, 15-hydroperoxyeicosatetraenoic acid, and 13-hydroxyoctadecadienoic acid; 2) an undetermined protein that is down-regulated or functionally deficient in 12/15-LO-disrupted macrophages. The function of this protein may be closely related to the ICSBP nuclear

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<sup>2</sup>L. Zhao, U. Wille, C. A. Hunter, E. Puré, and C. D. Funk, unpublished data.
expression; 3) oxidative stress-related factors that influence gene expression, such as reactive oxygen species (37–38). Further study to examine the contribution of these factors on IC15B attenuation is needed for a complete understanding of the mechanism of IL-12 synthesis defect in 12/15-LO-deficient macrophages.

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