Insulin-Like Growth Factor-1 Receptor Deficiency in Macrophages Accelerates Atherosclerosis and Induces an Unstable Plaque Phenotype in Apolipoprotein E–Deficient Mice

Yusuke Higashi, PhD; Sergiy Sukhanov, PhD; Shaw-Yung Shai, PhD; Svitlana Danchuk, MS; Richard Tang, BS; Patricia Snarski, BS; Zhaohui Li, MD; Patricia Lobelle-Rich, MS; Meifang Wang, MS; Derek Wang; Hong Yu, BS; Ronald Korthuis, PhD; Patrice Delafontaine, MD

Background—We have previously shown that systemic infusion of insulin-like growth factor-1 (IGF-1) exerts anti-inflammatory and antioxidant effects and reduces atherosclerotic burden in apolipoprotein E (ApoE)–deficient mice. Monocytes/macrophages express high levels of IGF-1 receptor (IGF1R) and play a pivotal role in atherogenesis, but the potential effects of IGF-1 on their function are unknown.

Methods and Results—To determine mechanisms whereby IGF-1 reduces atherosclerosis and to explore the potential involvement of monocytes/macrophages, we created monocyte/macrophage–specific IGF1R knockout (MΦ-IGF1R-KO) mice on an ApoE−/− background. We assessed atherosclerotic burden, plaque features of stability, and monocyte recruitment to atherosclerotic lesions. Phenotypic changes of IGF1R-deficient macrophages were investigated in culture. MΦ-IGF1R-KO significantly increased atherosclerotic lesion formation, as assessed by Oil Red O staining of en face aortas and aortic root cross-sections, and changed plaque composition to a less stable phenotype, characterized by increased macrophage and decreased α-smooth muscle actin–positive cell population, fibrous cap thinning, and decreased collagen content. Brachiocephalic artery lesions of MΦ-IGF1R-KO mice had histological features implying plaque vulnerability. Macrophages isolated from MΦ-IGF1R-KO mice showed enhanced proinflammatory responses on stimulation by interferon-γ and oxidized low-density lipoprotein and elevated antioxidant gene expression levels. Moreover, IGF1R-deficient macrophages had decreased expression of ABCA1 and ABCG1 and reduced lipid efflux.

Conclusions—Our data indicate that macrophage IGF1R signaling suppresses macrophage and foam cell accumulation in lesions and reduces plaque vulnerability, providing a novel mechanism whereby IGF-1 exerts antiatherogenic effects. (Circulation. 2016;133:2263-2278. DOI: 10.1161/CIRCULATIONAHA.116.021805.)

Key Words: atherosclerosis • inflammation • insulin-like growth factor 1 • macrophages

Insulin-like growth factor -1 (IGF-1) is a pleiotropic factor that is produced and acts locally (ie, via autocrine or paracrine effects) or circulates in blood and exerts endocrine effects. Circulating IGF-1 levels reach a peak during the pubertal growth phase, eventually declining with the progression of age. Although its role in developmental and pubertal growth as the major mediator of the effects of growth hormone is well documented, the physiological roles of IGF-1 in aged subjects are not understood. Aging is a major independent risk factor for coronary heart disease; in fact, there is increased cardiovascular and coronary heart disease prevalence with age in both sexes. In light of the aging-dependent decline in circulating IGF-1 levels, a potential link between IGF-1 levels and the elevated prevalence of cardiovascular diseases has been suggested. Indeed, epidemiological data have suggested that low IGF-1 levels are an important predictor of coronary events in aged subjects.1-3 In an animal model of atherosclerosis, we have shown that low levels of circulating IGF-1 are associated with more atherosclerosis4 and, vice versa, that an increase in circulating IGF-1 decreases atherosclerotic burden.5

Clinical Perspective on p 2278

The pathogenesis of atherosclerosis is complicated, involving multiple cell types, including vascular endothelial cells, smooth muscle cells, and proinflammatory cells such as macrophages. To determine the potential target(s) whereby IGF-1...
reduces atherosclerosis, we previously investigated whether smooth muscle–specific overexpression of IGF-1 alters atherosclerosis.6 Intriguingly, overexpression of IGF-1 in smooth muscle cells did not alter atherosclerotic burden but increased features of plaque stability,6,7 suggesting that other cellular components were a potential target for the antiatherogenic effects of IGF-1. Macrophages play a pivotal role in the pathogenesis of atherosclerosis by modulating inflammatory status and by scavenging and accumulating excess lipid to become foam cells. Thus, regulation of macrophage functions in terms of inflammation and phagocytosis is key to comprehending the disease process. Because macrophages have a predominant role in the inflammatory status of atherosclerotic lesions, it is critical to determine whether IGF-1 regulates macrophage function, particularly inflammatory activation and phagocytic activity. Thus far, there is very limited information on the potential link between IGF-1 effects and macrophage function, particularly in relation to vascular disease. IGF-1 has been reported to enhance chemotactic macrophage migration,8 to stimulate tumor necrosis factor–α (TNFα) expression,8 and to enhance low-density lipoprotein (LDL) uptake and cholesterol esterification.9 There are also reports from clinical investigations providing indirect evidence of anti-inflammatory effects of IGF-1. For example, there is an inverse relation between serum interleukin (IL)-6 and IGF-1 levels10; IGF-1/insulin-like growth factor–binding protein 3 administration to patients with severe burn injury induced an anti-inflammatory effect and reduced IL-6 and TNFα11,12; and low IGF-1 and high IL-6 and TNFα levels are associated with higher mortality in elderly patients.13,14 In this study, we used a well-established animal model of atherosclerosis to examine the role of macrophage IGF-1 signaling in atherosclerosis development and progression.

Methods
A detailed Materials and Methods section is available in the online-only Data Supplement.

Animals
All animal experiments were performed according to protocols approved by the institutional animal care and use committee. Monocyte/macrophage–targeted IGF-1 receptor–null mice on Apoe−/− background (MΦ-IGF1R-KO) and control mice (IGF1R-flox) were generated as described in the online-only Data Supplement. Eight-week old mice were fed a high-fat diet for 8 weeks before atherosclerosis was assessed.

Atherosclerotic Burden and Plaque Composition
Atherosclerotic burden was quantified with the use of en face preparations of whole aorta stained with Oil Red O and in cross sections of aortic root. Plaque composition was assessed in cross sections of aortic root by immunostaining for Mac-3 (macrophage) and α-smooth muscle actin (smooth muscle cell), and Masson’s Trichrome staining was used for collagen. Brachiocephalic artery lesions were analyzed for collagen. Brachiocephalic artery lesions were analyzed for collagen. Brachiocephalic artery lesions were analyzed for collagen.

Tracing Recruitment of Circulating Monocytes
Circulating Ly6C+ or Ly6C− monocytes were labeled in vivo with polychromatic red microspheres as described elsewhere,15 and numbers of red microsphere–positive cells were counted in a plaque and normalized to the efficiency of labeling in circulating monocytes (percent labeled/total number) and assessed by flow cytometry.

Intravital Fluorescence Microscopy
Intravital fluorescence microscopy was performed as described elsewhere.15

Macrophage Culture, Western Blot Analysis, and Quantitative Real-Time Reverse Transcription–Polymerase Chain Reaction
Thioglycolate-elicited macrophages were obtained from MΦ-IGF1R-KO and IGF1R-flox mice.16 Western blot analysis,17 total RNA extraction,18 and real-time reverse transcription–polymerase chain reaction18 were performed as previously described.

Statistical Analysis
All numeric data are expressed as mean±SEM. Statistical analyses were performed with GraphPad PRISM (version 6.07) software. Data sets were first assessed for residuals distribution with the D’Agostino-Pearson omnibus normality test and for equal variances with the Levene test for equality of variances. Differences in outcomes were determined by ANOVA and Bonferroni multiple-comparisons test, Kruskal–Wallis test, unpaired Student t test with or without the Welch correction, or Mann–Whitney U test, accordingly with the normality of residuals distribution. Differences were considered significant at P<0.05. The Fisher’s exact test was used to compare frequency of observed indexes of plaque vulnerability (Table).

Results
Generation of MΦ-IGF1R-KO/Apoe−/− Mice
MΦ-IGF1R-KO were generated on Apoe−/− background (LyzCre+/Igf1rflox/flox/Apoe−/−: MΦ-IGF1R-KO) by crossing LyzCre+ mice into Igf1rflox/flox/Apoe−/− (IGF1R-flox, served as a control). Genotype segregation in the offspring followed the expected mendelian frequency, and we did not recognize any developmental/morphological abnormalities. IGF1R deficiency was confirmed by lack of IGF1R protein detection by Western blot and IGF-1–dependent phosphorylation on Akt in peritoneal macrophages (Figure IA in the online-only Data Supplement). Consistent with myeloid-selective Cre expression,18–22 we found that the exon 3 of Igf1r was also excised in neutrophils (Figure IB in the online-only Data Supplement). However, we were unable to detect IGF1R protein in IGF1R-flox neutrophils, indicating extremely low levels of IGF1R protein expression in neutrophils (Figure IC in the online-only Data Supplement).

Table. MΦ-IGF1R-KO-Induced Features of Ruptured Plaques in the Brachiocephalic Artery

<table>
<thead>
<tr>
<th></th>
<th>IGF1R-Flox</th>
<th>MΦ-IGF1R-KO</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrous cap disruption</td>
<td>0/22</td>
<td>1/17</td>
<td>0.44</td>
</tr>
<tr>
<td>Intraplaque hemorrhage</td>
<td>3/22</td>
<td>8/17</td>
<td>0.03</td>
</tr>
<tr>
<td>Fibrin deposition</td>
<td>0/22</td>
<td>0/17</td>
<td>ND</td>
</tr>
<tr>
<td>Medial elastin breaks</td>
<td>0/22</td>
<td>4/17</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Data indicate positive observations in a total number of samples. Statistical significance was tested by the Fisher’s exact test. IGF1R indicates insulin-like growth factor-1 receptor; MΦ-IGF1R-KO, monocyte/macrophage–specific insulin-like growth factor-1 receptor knockout; and ND, not determined.
IGF1R and insulin receptor (InsR) can form a hybrid receptor (heterotetramer consisting of α+β subunits of IGF1R and α+β subunits of InsR), which binds IGF-1 with high affinity but not insulin. Depletion of IGF1R in endothelial cells is reported to allow InsR to form a holotetramer, thereby enhancing insulin sensitivity. Because insulin signaling in macrophages has significant effects on atherosclerosis (there are contradictory reports showing antiatherogenic or proatherogenic effects), we assessed insulin signaling activity in MΦ-IGF1R-KO macrophages. IGF1R deficiency did not alter InsR expression levels (Figure ID in the online-only Data Supplement). In IGF1R-flox macrophages, immunoprecipitation of InsR pulled down 100% of IGF1R (Figure IE, left, in the online-only Data Supplement), whereas anti-IGF1R immunoprecipitation pulled down 50% of InsR (Figure IE, right, in the online-only Data Supplement), suggesting that the ratio of IGF1R/InsR-hybrid receptors to InsR-holoreceptors was 2:1 without the presence of IGF1R-holoreceptors. IGF1R deficiency did not alter insulin-induced dose-dependent phosphorylation of Akt (Figure IF in the online-only Data Supplement).

Animals were assessed for circulating leukocyte counts (Table I in the online-only Data Supplement), cholesterol levels, and cytokine levels (Table II in the online-only Data Supplement). As has been reported, Western diet feeding for 8 weeks was associated with elevated monocyte count; however, IGF1R deficiency did not result in a significant difference in white blood cell count or circulating monocyte count (Table I in the online-only Data Supplement). A subpopulation of circulating monocytes as defined by CD11b+/CD90−/B220−/CD49b−/NK1.1−/Ly6G−/Ly6Chigh cells (Ly6C hi monocytes) has been reported to be proinflammatory and to be increased under atherogenic conditions such as hyperlipidemia. We did not observe a significant difference in Ly6C hi monocyte levels between MΦ-IGF1R-KO and IGF1R-flox mice (Table I in the online-only Data Supplement). MΦ-IGF1R-KO did not significantly alter circulating IGF-1, proinflammatory cytokine (IL-6, TNFα, monocyte chemoattractant protein-1 [MCP-1]), or cholesterol levels (Table II in the online-only Data Supplement).

Atherosclerosis Was Enhanced by MΦ-IGF1R-KO
Atherosclerotic lesion formation was assessed after 8 weeks of high-fat diet feeding. En face Oil Red O staining of aortas revealed a significant ≈64% increase in Oil Red O–positive lesion area, and there was a consistent ≈34% increase in plaque size at the aortic root in MΦ-IGF1R-KO mice (Figure 1). The effect was confirmed in each sex, indicating that there was no sex-specific effect of IGF1R deficiency (Figure II in the online-only Data Supplement). Plaque composition with
regard to Mac3 (macrophage)-positive cells, α-smooth muscle actin–positive cells, and collagen content (Masson’s trichrome stain) was significantly altered by MΦ-IGF1R-KO (Figure 2); there was a 49% increase in Mac3 detection (Figure 2A) and a 31% decrease in α-smooth muscle actin detection (Figure 2B). Plaques in MΦ-IGF1R-KO mice had a thinner smooth muscle cap than IGF1R-flox mice (Figure 2B) and decreased collagen content (Figure 2C). Because we observed a decrease in collagen content in plaques from MΦ-IGF1R-KO mice, we hypothesized that this could be attributable to enhanced collagen degradation by matrix metalloproteinases (MMPs). Cultured peritoneal macrophages from MΦ-IGF1R-KO animals expressed higher levels of MMP-1, -2, -8, -9, -12, -13, and -14 than those from IGF1R-flox animals, suggesting enhanced MMP activity (Figure 2D). The MMP protein levels in tissue lysates of ascending aortas after 2 months of Western diet feeding (Figure IIIA and IIIB in the online-only Data Supplement) showed a significant increase in the expression of MMP-1, -2, -8, and -9 in MΦ-IGF1R-KO animals. These observations are consistent with enhanced lesion formation in MΦ-IGF1R-KO mice with a phenotypic shift toward increased features of plaque vulnerability.

**Plaque Destabilization in MΦ-IGF1R-KO Mice**

Because our findings suggested plaque destabilization in MΦ-IGF1R-KO mice, we assessed indicators of vulnerability in brachiocephalic artery plaques (Figure IV in the online-only Data Supplement) by staining cross sections of brachiocephalic artery with the Carstairs34 and Verhoeff–Van Gieson methods.35 Cross sections (5 μm thick) were made every 50 μm along the artery, for a total of 10 sections per artery. If 3 consecutive sections indicated a fibrous cap disruption, intraplaque hemorrhage, fibrin deposition, or medial elastin break, the artery was considered positive for signs of plaque vulnerability. After 8 weeks on a high fat diet, MΦ-IGF1R-KO animals had increased features of vulnerable plaques compared with IGF1R-flox animals, as determined by the presence of intraplaque hemorrhage (IGF1R-flox, 3 positive in 22 animals versus MΦ-IGF1R-KO, 8 positive in 17 animals; P=0.03; Table) and medial elastin breaks (IGF1R-flox, no positive in...
Figure 3. Proinflammatory cytokine and chemokine production by cultured macrophages isolated from insulin-like growth factor-1 receptor (IGF1R)-flox and monocyte/macrophage-specific (MΦ)–IGF1R knockout (KO) mice. Interleukin (IL)-1α (A), IL-6 (B), tumor necrosis factor-α (TNFα; C), monocyte chemoattractant protein-1 (MCP-1; D), and fractalkine (E) secretion by peritoneal macrophages was assessed by respective ELISA. Macrophages were isolated by peritoneal lavage from IGF1R-flox (open column) and MΦ–IGF1R-KO (closed column) mice and allowed to adhere on culture plates overnight. After removal of nonadhering cells, cells were primed by interferon-γ (IFNγ), and conditioned media were collected after 24 hours. *P<0.05, **P<0.01 vs IGF1R-flox; ##P<0.01 vs IGF1R-flox+IFNγ; $P<0.01 vs MΦ–IGF1R-KO. Statistical significance was assessed by 2-way ANOVA and subsequent post hoc analysis with the Bonferroni multiple-comparisons test; n=6. F, Nuclear factor-κB (NFκB) DNA binding activity was assessed (expressed as relative light units [RLU]) in cell lysates of IGF1R-flox and MΦ–IGF1R-KO macrophages with or without priming by IFNγ for 3 hours. *P<0.05 vs IGF1R-flox by 2-way ANOVA; n=3. G through J, Effects of NFκB inhibitors on TNFα (G and H) and IL-6 (I and J) secretion by peritoneal macrophages. IGF1R-flox (open column) and MΦ–IGF1R-KO (closed column) macrophages were exposed to the indicated dose of BMS-345541 or parthenolide for 1 hour before activation by IFNγ or lipopolysaccharide (LPS). Conditioned media were collected after 24 hours and assessed for cytokine concentration with the respective ELISA kit (R&D Systems). ##P<0.05 vs IGF1R-flox+IFNγ, #P<0.05 vs IGF1R-flox+LPS by the Mann–Whitney U test; n=4.
22 animals versus MΦ-IGF1R-KO, 4 positive in 17 animals; \( P=0.03 \); Table), suggesting that plaques in MΦ-IGF1R-KO mice are more unstable than in IGF1R-flox mice.

Proinflammatory Responses Are Enhanced in IGF1R-Deficient Macrophages

Thioglycolate-elicited peritoneal macrophages from the MΦ-IGF1R-KO mice were assessed for proinflammatory cytokine and chemokine secretion (Figure 3). Among tested cytokines and chemokines, IL-1\(\beta\) production was below detectable levels, whereas IL-1\(\alpha\), IL-6, TNF\(\alpha\), MCP-1, and fractalkine were detected (Figure 3A–3F). IGF1R-deficient macrophages secreted significantly higher levels of all the tested cytokines and chemokines except fractalkine, which showed a strong trend toward an increase \( (P=0.0508) \). Interferon-\(\gamma\) (IFN\(\gamma\)) significantly enhanced TNF\(\alpha\) and MCP-1 production in IGF1R-flox cells but not in MΦ-IGF1R-KO cells. Intriguingly, IFN\(\gamma\) did not enhance fractalkine production in IGF1R-flox macrophages, whereas in MΦ-IGF1R-KO cells, it decreased fractalkine production (Figure 3E). In a setting of exposure to IFN\(\gamma\), MΦ-IGF1R-KO cells secreted higher levels of IL-1\(\alpha\), IL-6, and MCP-1 compared with IGF1R-flox. We tested nuclear factor-\(\kappa\)B (NF\(\kappa\)B) DNA binding activity in these cells (Figure 3F). There was significantly higher NF\(\kappa\)B DNA binding activity in MΦ-IGF1R-KO, which was further enhanced by IFN\(\gamma\), consistent with higher cytokine/chemokine production. To further assess NF\(\kappa\)B involvement, we exposed cells to NF\(\kappa\)B inhibitors (BMS-345541 and parthenolide, Figure 3G–3J). BMS-345541 completely abolished enhanced TNF\(\alpha\) and IL-6 production in MΦ-IGF1R-KO cells (Figure 3G and 3H), and parthenolide recapitulated the BMS-345541 effect (Figure 3I and 3J), supporting the importance of NF\(\kappa\)B-dependent cytokine production in MΦ-IGF1R-KO macrophages.

Macrophage Polarization

Because IGF1R-deficient macrophages manifested enhanced proinflammatory responses, we examined whether IGF1R
deficiency influenced macrophage polarization. Classic activation (M1) was induced by exposure to IFNγ and subsequently to lipopolysaccharide (Figure 4A–4E). M1 marker gene (Tnf, Nos2, Il6, Ccl2, and Ccl5) expression levels were highly induced by exposure to IFNγ-LPS, and IGF1R deficiency further enhanced the expression, implying enhanced

Figure 5. Insulin-like growth factor-1 receptor (IGF1R) deficiency skews macrophage activation induced by oxidized low-density lipoprotein (oxLDL). Macrophages isolated from IGF1R-flox (open column) and monocyte/macrophage-specific (MΦ)-IGF1R knockout (KO; closed column) mice were primed with interferon-γ (IFNγ) for 6 hours and exposed to oxLDL for 18 hours. Total RNA was extracted and subjected to quantitative reverse transcription-polymerase chain reaction, testing oxidized lipid-induced activation (Mox) marker (Hmox1, A; Txnrd1, B), M1 activation marker (Tnf, C; Nos2, D; Il6, E; Ccl2, F; Ccl5, G), and M2 activation marker (Arg1, H; Mrc1, I; Pparg, J) mRNA levels. Representative data from 3 independent experiments are shown. #P<0.01 vs respective condition without oxLDL, $P<0.01 vs IGF1R-flox of the respective condition, ¶P<0.01 vs respective condition without IFNγ, **P<0.01 vs IFNγ-treated MΦ-IGF1R-KO, #P<0.05 vs IL-4- and oxLDL-treated IGF1R-flox, *P<0.05 vs respective condition without oxLDL by 2-way ANOVA and Bonferroni multiple-comparisons test; n=3.
M1 activation in MΦ-IGF1R-KO macrophages. On the other hand, IL-4–induced gene expression levels (ie, M2 activation markers; Arg1, Mrc1, and Pparg) were not influenced by IGF1R deficiency (Figure 4F–4H). It has been reported that oxidatively modified LDL (oxLDL) and that oxidized lipid components of oxLDL alter macrophage activation status or induce a distinctive activation status.36–39 Hemeoxygenase-1 (Hmox1) and thioredoxin reductase-1 (Txnrd1) are signature genes that have been shown to be upregulated in macrophages exposed to oxidized phospholipid, leading to a polarization status of Mox.39 In fact, oxLDL exposure enhanced Hmox1 and Txnrd1 expression significantly (Figure 5A and 5B). Intriguingly, IGF1R deficiency did not influence Txnrd1 mRNA levels (Figure 5B) but significantly upregulated Hmox1 mRNA levels (Figure 5A). We further assessed whether IGF1R deficiency influences the effect of oxLDL on expression levels of macrophage activation marker genes (Figure 5). With regard to M1 activation markers, oxLDL by
itself did not alter mRNA levels in IGF1R-flox cells (Tnf, Nos2, Il6, Ccl2, and Ccl5: Figure 5C–5G); however, in MΦ-IGF1R-KO cells, in which these mRNA levels were significantly elevated compared with IGF1R-flox cells, oxLDL significantly further upregulated Tnf, Nos2, Il6, and Ccl2 mRNA levels (Figure 5C–5F), whereas Ccl5 mRNA was suppressed (Figure 5G). On the other hand, of the M2 activation markers, Mrc1 mRNA levels were moderately upregulated by oxLDL (Figure 5I), whereas Arg1 and Pparg were not affected by oxLDL (Figure 5H and 5J). Although there were modestly lower Arg1 mRNA levels in IL-4/oxLDL–treated MΦ-IGF1R-KO cells than in IGF1R-flox cells, overall, oxLDL or IGF1R deficiency did not robustly alter M2 marker levels. These data suggested that IGF1R deficiency influenced macrophage polarization, namely enhancing the proinflammatory M1 phenotype as evoked by IFNγ and oxLDL stimulation; however, the effect is not entirely classic activation, as implicated by Ccl5 downregulation (Figure 5G). These results prompted us to examine whether Mox marker gene expression was altered by IGF1R deficiency in IFNγ-primed macrophages. IFNγ suppressed Hmox1 and Txnrld mRNA levels in IGF1R-flox macrophages (Figure 5A and 5B); however, in MΦ-IGF1R-KO cells, IFNγ exerted the opposite effect, leading to an upregulation of Hmox1 mRNA (Figure 5A). IFNγ suppressed Txnrld mRNA levels in IGF1R-deficient macrophages but to a lesser extent than in IGF1R-flox cells, resulting in higher expression levels compared with IGF1R-flox cells (Figure 5B).

Figure 7. Insulin-like growth factor-1 receptor (IGF1R) deficiency downregulated ABCG1 expression levels in acetylated low-density lipoprotein (acLDL)–loaded macrophages. Peritoneal macrophages were not activated (NA) or were activated to M1 and M2 and then exposed to acLDL for 24 hours. ABCA1 (A), ABCG1 (B), and SRB1 (C) expression levels were assessed by Western blot analysis (representative measurements and blots [D] from 3 independent experiments are shown). IGF1R-flox, open column; monocyte/macrophage–specific (MΦ)-IGF1R knockout (KO), closed column. *P<0.05 vs IGF1R-flox cells by 2-way ANOVA. E, Western blot analysis for ABCG1 expression, assessing effects of LXR agonists. AcLDL–loaded macrophages were exposed to LXR agonists (1 μmol/L GW3965, 1 μmol/L T0901317) for 48 hours and assessed for ABCG1 expression levels. Representative results from 3 independent experiments are shown. *P<0.05 vs IGF1R-flox, **P<0.01 vs IGF1R-flox by the Student t test; n=3.
Efferocytosis, a process by which apoptotic cells are removed by phagocytosis, is considered a significant mechanism involved in the resolution of inflammation. We evaluated expression levels of genes involved in efferocytosis (Figure V in the online-only Data Supplement). M1 activation suppressed the expression of efferocytosis-related genes (Anxa1, Gas6, C1qa, and Mfge8), and M2 activation had no effect (Anxa1, C1qa, Merk; Figure V in the online-only Data Supplement) or downregulated (Gas6, Mfge8) gene expression levels. IGF1R deficiency did not alter these mRNA levels in either activated or nonactivated cells. OxLDL by itself did not induce obvious effects except for an upregulation of Mertk mRNA levels (Figure VD in the online-only Data Supplement). IGF1R deficiency upregulated Anxa1 and Mfge8 in the presence of oxLDL (Figure VE and VF in the online-only Data Supplement), but it caused a trend to downregulation of C1qa (Figure VC in the online-only Data Supplement). Thus, IGF1R deficiency did not induce changes suggesting enhanced/reduced efferocytosis.

Lipid Internalization and Efflux
Macrophage internalization and accumulation of modified LDL lead to foam cell formation, which is a hallmark of atheroma formation. Macrophage lipid internalization was assessed by exposing cells to oxLDL and acetylated LDL (Figure 6A and 6B). Macrophages were exposed to IFNγ/lipopolysaccharide (M1 activation) or IL-4 (M2 activation) and then tested for lipid incorporation after exposure to modified LDL for 48 hours. M1 cells internalized far smaller amounts of lipid compared with the nonactivated cells or M2 cells (Figure 6A and 6B), whereas M2-activated cells incorporated more lipid than untreated cells when they were exposed to acetylated LDL (Figure 6A). IGF1R deficiency did not alter lipid incorporation (Figure 6A and 6B), regardless of activation status and exposure to acetylated LDL or oxLDL.

Cholesterol efflux from cells to extracellular lipid acceptors also contributes to lipid accumulation and thus foam cell formation. Thus, activated and lipid-laden macrophages were tested for cholesterol efflux activity (Figure 6C and 6D). IGF1R deficiency markedly reduced apolipoprotein AI–dependent cholesterol efflux in M1-activated cells (Figure 6C). We measured HDL-dependent cholesterol efflux in nonactivated, M1-activated, and M2-activated cells (Figure 6D) and found that IGF1R deficiency caused a small but significant reduction in cholesterol efflux to HDL (Figure 6D). To gain insights into potential mechanisms, we assessed expression levels of ABCA1, ABCG1, and SRB1, which are major lipid transporters responsible for cholesterol efflux. They were differentially regulated by IGF1R deficiency (Figure 7); ABCG1 expression levels were downregulated by ≈50% in IGF1R-deficient macrophages, regardless of activation status (Figure 7B and 7D), whereas ABCA1 and SRB1 were not regulated. To investigate ABCG1 expression in MΦ-IGF1R-KO cells, we exposed the cells to ligands of LXR, which is a major regulator of ABCG1.
gene expression.\(^{40}\) LXR agonist GW3965 or T0901317 induced ABCG1 and ABCA1 expression in IGF1R-flox macrophages but not in MΦ-IGF1R-KO cells (Figure 7E). Thus, LXR-dependent regulation of ABCG1 expression is compromised by IGF1R deficiency, potentially accounting for down-regulation of ABCG1 in MΦ-IGF1R-KO cells.

**Monocyte Recruitment, Macrophage Proliferation, and Apoptosis in MΦ-IGF1R-KO Mice**

Because MΦ-IGF1R-KO caused an increase in Mac3 positivity within plaques, we evaluated monocyte recruitment to lesions, macrophage proliferation,\(^{41}\) and macrophage apoptosis,\(^{3,}\) 3 major determinants of macrophage number in lesions, in plaques.\(^{42}\) To evaluate monocyte recruitment, we labeled circulating monocytes in vivo by intravenous administration of fluorescent microspheres.\(^{3,}\) The microspheres are not capable of penetrating into tissue interstitial space; thus, red fluorescence-positive cells (identified by DAPI positivity) within plaque are considered recruited and infiltrated cells. We evaluated red microsphere labeling by flow cytometry. As described previously,\(^{44}\) intravenous injections of red microspheres labeled the Ly6C\(^{\text{hi}}\) population among circulating monocytes (Figure VIA in the online-only Data Supplement), whereas clodronate administration 1 day before the injection of red microspheres introduced the label to Ly6C\(^{\text{lo}}\) monocytes (Figure VIB in the online-only Data Supplement). As shown in Figure 8, we detected a significantly larger number of red fluorescence-positive cells in plaques from MΦ-IGF1R-KO mice both without (Figure 8C) and with (Figure 8D) clodronate administration. It is noteworthy that more red microsphere-positive cells are detected after clodronate administration, indicating that Ly6C\(^{\text{lo}}\) monocytes are the dominant subpopulation to be recruited to lesions.\(^{3,}\) To assess leukocyte adhesion and rolling on the luminal side of the endothelium in vivo, we performed intravital microscopy to detect CD11b-positive cells in the mesenteric circulation. MΦ-IGF1R-KO significantly increased CD11b\(^{\text{+}}\) leukocyte adhesion (Figure 8E) and rolling (Figure 8F) on the luminal surface of the endothelium, consistent with increased recruitment of CD11b\(^{\text{+}}\) cells, that is, monocytes and neutrophils. Because IGF1R expression was undetectable in neutrophils (Figure 1 in the online-only Data Supplement), it is unlikely that IGF1R gene deletion in neutrophils contributed significantly to these findings.

To assess proliferation activity of macrophages in plaques, we assessed Ki67 and proliferating cell nuclear antigen gene expression levels\(^{44–46}\) (Figure VIC in the online-only Data Supplement). Macrophage Marker (SC-101447; a monoclonal antibody raised against isolated macrophages of mouse origin; Santa Cruz)\(^{47,48}\) –positive plaque area was laser dissected from aortic root for RNA isolation, followed by quantitative reverse transcription–polymerase chain reaction. Equal amplification of CD68 was confirmed between IGF1R-flox and MΦ-IGF1R-KO lesions. Macrophage-rich regions from MΦ-IGF1R-KO plaques expressed the same levels of Ki67 or proliferating cell nuclear antigen compared with IGF1R-flox (Figure VIC in the online-only Data Supplement), suggesting no difference in macrophage proliferation in plaques. Terminal deoxynucleotidyl transferase dUTP nick-end labeling/Mac3 double-positive cell numbers were not different (Figure VID in the online-only Data Supplement). Taken together, our data suggest that IGF1R deficiency enhances monocyte recruitment to lesions, thereby increasing the macrophage cell population.

**OxLDL Downregulates IGF1R in Macrophages**

OxLDL plays a critical role in atherogenesis, and we have previously shown that oxLDL downregulates IGF1R in vascular smooth muscle cells.\(^{49}\) To determine potential regulation of macrophage IGF1R expression by oxLDL, we used the human monocyte cell line THP-1, which was differentiated into macrophages and exposed to 100 μg/mL oxLDL (Figure VII in the online-only Data Supplement). OxLDL exposure for 24 hours downregulated IGF1R levels by 80%.

**Discussion**

IGF-1 production has been documented in macrophages\(^{8,9,50,51}\); however, precise effects of IGF-1 in macrophages in relation to the pathogenesis of atherosclerosis have not been elucidated. In this study, we generated macrophage/monocyte–specific IGF1R-deficient mice and discovered pivotal roles of IGF-1 in the regulation of inflammatory responses and lipid handling in macrophages, which are relevant to the antiatherogenic effects of IGF-1. Our data indicate that IGF1R deficiency in macrophages enhances proinflammatory activation (ie, M1 polarization), thereby promoting proinflammatory cytokine production (Figures 3 and 4), and enhances lipid accumulation as a result of reduced efflux (Figures 6 and 7). These phenotypic changes resulted in increased recruitment of macrophages to atherosclerotic plaques and increased atherosclerotic burden in MΦ-IGF1R-KO mice (Figures 1 and 2). Moreover, histological evaluation of brachiocephalic arteries indicated that the MΦ-IGF1R-KO induced features of unstable plaques (Table).

There is growing interest in the role of IGF-1 in cardiovascular disease. Low circulating IGF-1 levels have been associated with cardiovascular disease risk factors,\(^{52–56}\) and in particular, there is growing evidence for a role for IGF-1 deficiency in the pathogenesis of metabolic syndrome.\(^{57}\) Acromegaly (ie, excessive growth hormone and IGF-1) or otherwise growth hormone and IGF-1 deficiency have been linked to cardiovascular complications.\(^{58,59}\) However, epidemiological studies linking IGF-1 with cardiovascular disease report mixed results. Some cross-sectional and prospective studies\(^{60–65}\) suggest a positive association between IGF-1 and in some cases insulin-like growth factor–binding protein 3) and atherosclerosis, but others have found that low IGF-1 is a predictor of ischemic heart disease and mortality, consistent with the potential anti-apoptotic, antioxidant, and plaque stabilization effects of IGF-1.\(^{1,3,11,66–72}\) Methodological constraints could explain these contradictions because measurement of total IGF-1 levels represents only a crude estimate of the biologically active IGF-1. Thus, an IGF-1–specific kinase receptor activation assay may better reflect IGF-1 bioactivity.\(^{73}\) In fact, it has been reported that higher IGF-1 bioactivity is associated with significantly longer survival in subjects with a high inflammatory risk profile or history of cardiovascular disease.\(^{74}\) Additionally, polymorphisms in the IGF-1 gene promoter region that influence circulating IGF-1 levels have been
The alleles that indicate lower circulating IGF-1 levels have been associated with increased risk for type 2 diabetes mellitus, myocardial infarction, left ventricular hypertrophy, higher carotid intima-media thickness, higher aortic pulse-wave velocity, and lower endothelium-dependent vasodilation. Intriguingly, IGF-1 resistance in the endothelium was reported in an animal model of obesity, thereby blunting the vasodilatory response to IGF-1 via attenuated endothelial nitric oxide synthase phosphorylation and nitric oxide production. Our present finding that reduced macrophage IGF-1 signaling is highly proinflammatory and increases atherosclerotic burden is consistent with the growing evidence that decreased IGF-1 action may be a significant contributor to the pathogenesis of atherosclerosis. Of note, we found that oxLDL downregulates IGF1R levels in human-derived THP1 macrophage (Figure VII in the online-only Data Supplement), which is consistent with our previous reports that both IGF-1 and IGF1R expression were significantly lower in human atherosclerotic plaque intimal regions with macrophage infiltration, where oxLDL was highly detected.

We used MΦ-IGF1R-KO mice, which have 1 allele of Lys2 ablated; the IGF1R-flox mice (control) have both alleles intact. Previous studies in LysCre+/ mice with regard to potential alterations in monocyte/macrophage biology caused by the hemizygous deficiency of Lys2 showed no evidence of a heterozygous phenotype. In addition, complete ablation of both alleles of Lys2 does not influence atherosclerosis in Apoe−/− mice. Lys2-cre–mediated gene excision occurs in monocytes and neutrophils. We confirmed deletion of exon 3 of the IGF1r gene in both macrophages and neutrophils isolated from the MΦ-IGF1R-KO mouse, but we were unable to detect IGF1R protein even in IGF1R-normal neutrophils (ie, IGF1R-flox neutrophils), indicating extremely low expression levels. Thus, although contribution of neutrophils to the phenotype of MΦ-IGF1R-KO mice cannot be excluded, it appears much more likely that macrophages, which express significant levels of IGF1R, play the predominant role.

IGF1R and InsR are structurally similar and form a heteromeric hybrid receptor consisting of α+β subunits of IGF1R and α+β subunits of InsR. The hybrid receptor binds IGF-1 with high affinity but does not bind insulin at physiological ranges. To the best of our knowledge, this is the first study evaluating the expression ratio between IGF1R and InsR in macrophages, showing predominant expression of InsR. Thus, IGF1R-flox macrophages (expressing both IGF1R and InsR) responded to a physiological dose of insulin (Figure I in the online-only Data Supplement). Intriguingly, IGF1R deficiency in macrophages, although it should free up InsR hemidimers to form holoreceptors, did not increase insulin signaling. This finding is relevant because macrophage InsR deficiency has been shown to modulate atherosclerosis development (although results have been contradictory). Because we found that MΦ-IGF1R-KO did not alter insulin signaling in macrophages, it is unlikely that changes in insulin action on macrophages plays a significant role in the phenotype of MΦ-IGF1R-KO mice.

Macrophages become activated as they infiltrate into a target tissue and are exposed to stimuli, expressing a highly proinflammatory phenotype or a less inflammatory but phagocytic and antigen-presenting phenotype. The former status was called classic activation or M1 activation; the latter was referred to as alternative activation or M2 activation. Recent investigations indicate, however, that macrophage activation status likely encompasses a broad spectrum in which M1 and M2 activation are likely 2 extremes. Recognizing that macrophage activation represents a continuum, we tested M1 and M2 activation, as well as Mox activation, which was described as a unique activation status found in plaque macrophages, to provide insights into the effects of IGF1R deficiency. Our results indicate that IGF1R deficiency resulted in macrophages acquiring a highly inflammatory (ie, M1) phenotype (Figure 3), whereas M2 marker gene expression levels were not altered (Figure 4F–4H). However, MΦ-IGF1R-KO macrophages are not simply skewed to a more inflammatory phenotype; these cells were also shifted to a phenotype induced by oxLDL, characterized by upregulation of the antioxidant genes Hmox1 and Txnrd1, described as Mox activation (Figure 5A and 5B). Reports of the effects of oxLDL and its specific lipid moieties on macrophage activation are variable and include enhancement of the inflammatory phenotype, induction of M2 activation, or a unique activation status. In summary, our results suggest that IGF1R deficiency skews macrophages to a unique activation state that can be characterized by enhanced proinflammatory response and elevated antioxidant system.

The effect of IGF1R deficiency on macrophage activation status prompted us to examine how IGF1R deficiency influences uptake of modified LDL, cholesterol efflux, and effecrocytosis. It has been shown that M1-activated macrophages demonstrate reduced foam cell formation in response to oxLDL and that Mox–activated macrophages have attenuated phagocytosis and effecrocytosis. It would thus have been reasonable to speculate that IGF1R deficiency, which enhances the proinflammatory phenotype and Mox marker expression levels, could lead to reduced foam cell formation. However, our results showed no evidence of altered acetylated LDL or oxLDL uptake by IGF1R-deficient macrophages (Figure 6A and 6B). We also assessed expression levels of genes that are functional in effecrocytosis, but there was no apparent alteration caused by IGF1R deficiency (Figure V in the online-only Data Supplement). Thus, despite enhanced inflammatory responses and redox gene expression, IGF1R-deficient macrophages do not appear to have impairment in modified lipid uptake or effecrocytosis. However, further assessment of lipid handling by IGF1R-deficient macrophages indicated that cholesterol efflux is impaired by IGF1R deficiency (Figure 6C and 6D), potentially promoting foam cell formation. Cholesterol efflux is mediated by the lipid transporters ABCA1, ABCG1, and SRB1. IGF1R deficiency caused lower expression levels of ABCG1 (Figure 7), which should, at least in part, account for lowered cholesterol efflux. Intriguingly, the LXR-dependent regulation of ABCG1 expression was compromised in MΦ-IGF1R-KO macrophages. LXR is a major regulator of ABCG1 expression. In fact, LXR drives ABCG1 expression on lipid loading by modified LDL (which causes accumulation of oxysterols). Thus, impaired LXR regulation of ABCG1 expression could be an important mechanism underlying lowered cholesterol efflux in MΦ-IGF1R-KO macrophages.
MΦ-IGF1R-KO increased atherosclerosis burden as assessed by Oil Red O staining of en face aortas and by histological analysis of aortic root cross sections (Figure 1). These mice had an increase in lesional macrophages and in recruitment of monocytes to atherosclerosis plaques (Figure 8). Consistent with the increased recruitment of monocytes, chemokine expression levels were upregulated in MΦ-IGF1R-KO macrophages (ie, MCP-1 and fractalkine; Figures 3–5). Moreover, MΦ-IGF1R-KO increased features of plaque vulnerability, as evidenced by histological features of intra-plaque hemorrhage and medial elastin breaks in lesions from brachiocephalic artery (Figure IV in the online-only Data Supplement and Table). These findings were in accordance with the changes in plaque composition, notably the increased population of macrophages, the reduced population of smooth muscle cells particularly within the plaque cap, and decreased collagen (Figure 2). To obtain insights into the underlying mechanisms, we assessed MMP levels in peritoneal macrophages from MΦ-IGF1R-KO mice and showed significant upregulation of MMPs (Figure 2D), which have previously been shown to be relevant to atherogenesis and plaque vulnerability.51,92 In addition, we found significant upregulation of MMP-1, -2, -8, and -9 in lysates of ascending aorta from MΦ-IGF1R-KO animals, consistent with data obtained with peritoneal macrophages. A major source of collagen matrix synthesis and deposition in atherosclerotic plaques is smooth muscle cells, and thus, one can speculate that the enhanced inflammatory milieu induced by MΦ-IGF1R-KO altered smooth muscle cell homeostasis. In fact, it is noteworthy that MΦ-IGF1R-KO robustly enhanced macrophage production of proinflammatory cytokines such as IL-1α, TNFα, and IL-6 (Figure 3), and it has been reported that cytokines such as TNFα reduce IGF-1 and increase IGF binding protein-3 in vascular smooth muscle cells, leading to a reduction in bioactive IGF-1.93 IGF-1 positively regulates collagen synthesis by smooth muscle cells.94 Indeed, we have recently shown that increased IGF-1 signaling in vascular smooth muscle cells increases features of plaque stability, as determined by increased fibrous cap area, α-smooth muscle actin–positive smooth muscle cells, and collagen content, without affecting plaque burden,6 potentially mediated by IGF-1 induction of smooth muscle differentiation6 and collagen synthesis.94 Thus, it seems reasonable to speculate that increased production of inflammatory cytokines in MΦ-IGF1R-KO mice disrupts normal IGF-1 signaling in smooth muscle cells and reduces collagen deposition in plaques of MΦ-IGF1R-KO mice. Taken together with our previous results, the present study provides insights into potential interactions between macrophages and smooth muscle cells; that is, the enhanced inflammatory milieu by macrophages suppresses IGF-1 signaling in smooth muscle cells, leading to a reduction of plaque stability.

Our results are consistent with the growing body of experimental evidence that IGF1 has antiatherogenic effects. Infusion of IGF-1 in ApoE−/− mice reduces atherosclerotic burden,1 and overexpression of IGF-1 in vascular smooth muscle cells increases plaque collagen content and smooth muscle cell levels and reduces necrotic core size.6 IGF-1 has been shown to have antioxidant effects on the endothelium via upregulation of glutathione peroxidase levels.95 Our current findings demonstrate that IGF-1 signaling has a major effect on macrophage biology that is critical for atherogenesis. However, a limitation of experimental studies to date has been that they have been performed in murine models and that studies in larger animals phylogenetically closer to humans are lacking. Such studies will be important for the development of IGF-1–based therapeutic strategies.

Conclusions

We have shown that IGF1R deficiency in macrophages of ApoE−/− mice increases atherosclerotic burden and changes plaque composition to one of lowered smooth muscle cell and collagen content. Our data suggest that the loss of IGF-1 signaling skews macrophage activation to a proinflammatory status and promotes lipid accumulation in macrophages by lowering lipid efflux. There is increasing evidence linking low IGF-1 to multiple cardiovascular risk factors, including metabolic syndrome and aging.57,58 In fact, it has been reported that decreased IGF-1 bioavailability is an adverse prognostic factor for coronary heart disease (reviewed elsewhere97,98). Our findings herein suggesting that IGF-1 regulates macrophage inflammatory responses and lipid metabolism may provide the basis for a novel therapeutic approach for the treatment of atherosclerotic vascular disease development and progression.

Acknowledgments

We thank Chelsea Deroche, PhD (Biostatistics and Research Design Unit in the Office of Medical Research and Health Management and Informatics, University of Missouri School of Medicine), for her expert advice on statistical assessments.

Sources of Funding

This work was supported by National Institutes of Health grants R01-HL070241 (Dr Delafontaine), R01-HL080682 (Dr Delafontaine), R21-HL113705 (Dr Sukhanov), R01-HL059976 (Dr Korthuis), R01-HL095486 (Dr Korthuis), and R01-AA022108 (Dr Korthuis), as well as American Heart Association Grant-in-Aid 13GRNT17230069 (Dr Sukhanov).

Disclosures

None.

References

Insulin-like growth factor I and interleukin-6 contribute synergistically to inflammatory signaling.

**References**


58. Schut AF, Groenewoud J, den Hertogh J, wieland EM, van Duijn CM, Hofman A, Stricker BH. A promoter polymorphism of the insulin-like growth factor I gene is related to carotid intima-media thickness and aortic pulse wave velocity in


**CLINICAL PERSPECTIVE**

Atherosclerosis is an inflammatory disease, and acute cardiovascular events result largely from erosion or rupture of unstable plaques with increased inflammatory cells and a relative reduction in vascular smooth muscle cells. Macrophages play a major role in atherogenesis by scavenging and accumulating lipids to become lipid-laden foam cells. Furthermore, proinflammatory macrophages induce smooth muscle cell death by secreting cytokines and degrade extracellular matrix by producing enzymes such as matrix metalloproteinases, weakening the tensile strength of plaques and predisposing them to rupture. In the past decade, insulin-like growth factor-I (IGF-1) has demonstrated antiatherogenic effects in experimental models, but the mechanisms are poorly elucidated. In this study, we investigated macrophage–IGF-1 receptor deficiency in a murine model of atherosclerosis. We found that IGF-1 receptor deficiency increased monococyte/macrophage recruitment to lesions, skewed macrophage activation to a proinflammatory status, promoted lipid accumulation in macrophages by lowering lipid efflux, and upregulated matrix metalloproteinase production, resulting in an increase in atherosclerotic burden and a decrease in features of plaque stability. Our findings are consistent with epidemiological studies suggesting that low circulating IGF-1 is a predictor of ischemic heart disease and mortality. There is also evidence linking low IGF-1 to multiple cardiovascular risk factors, including metabolic syndrome. Our findings suggest that IGF-1 regulation of macrophage inflammatory responses and lipid metabolism may be the basis for novel approaches to reduce atherosclerotic lesion progres- sion and to promote plaque stability.
Insulin-Like Growth Factor-1 Receptor Deficiency in Macrophages Accelerates Atherosclerosis and Induces an Unstable Plaque Phenotype in Apolipoprotein E–Deficient Mice

Yusuke Higashi, Sergiy Sukhanov, Shaw-Yung Shai, Svitlana Danchuk, Richard Tang, Patricia Snarski, Zhaohui Li, Patricia Lobelle-Rich, Meifang Wang, Derek Wang, Hong Yu, Ronald Korthuis and Patrice Delafontaine

_Circulation_. 2016;133:2263-2278; originally published online May 6, 2016; doi: 10.1161/CIRCULATIONAHA.116.021805

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2016 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/133/23/2263

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2016/05/06/CIRCULATIONAHA.116.021805.DC1
http://circ.ahajournals.org/content/suppl/2017/07/10/CIRCULATIONAHA.116.021805.DC2

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org/subscriptions/
Supplemental Materials and Methods

Materials

IGF-1 ELISA kits were obtained from Diagnostic Systems Laboratories (Webster, TX). TNFα, IL-1α, IL-6, MCP-1, and Fractalkine ELISA kits were obtained from Ray Biotech (Norcross, GA). Cholesterol assay kit was from Cell Biolab (San Diego, CA). Apolipoprotein AI (ApoAI) and lipopolysaccharide (LPS) were obtained from Sigma-Aldrich (St. Louis, MO). Recombinant mouse interferon γ (IFN γ) and mouse interleukin 4 (IL4) were obtained from BioLegend (San Diego, CA). Human high-density lipoprotein (HDL), low-density lipoprotein (LDL), acetylated LDL, and oxidized LDL were obtained from Kalen Biomedical (Montgomery Village, MD). The antibodies used were anti-smooth muscle actin (SMA) and anti-ABCA1 antibodies from Abcam; Mac-3 monoclonal antibody from BD Pharmingen; anti-ABCG1 antibody from Santa Cruz Biotechnology; and anti-SRB1 antibody from R&D Systems; anti-MMP-1, -2, -9, -12, -13, and -14 antibodies from Novus Biologicals; anti-MMP-8 antibody from R&D Systems; anti-MMP-2 antibody (for Western blot analysis of macrophage lysates) from Santa Cruz Biotechnology.

Animals

All animal experiments were performed according to protocols approved by the institutional animal care and use committee. Igf1r^flox/flox/Apoe^−/− mice (referred to as IGF1R-flox mice on C57Bl/6 genetic background) were generated by cross breeding Apoe^−/− mice (C57Bl/6 background, Jackson Laboratory) with Igf1R^flox/flox mice (C57Bl/6 background, a generous gift
from Dr. Brüning, University of Leipzig, Germany1, 2). We generated monocyte/macrophage targeted IGF-1 receptor null mice (referred to as MΦ-IGF1R-KO mice on C57Bl/6 genetic background) on Apoe−/− background (LyzCre/+/Igf1rflox/flox/Apoe−/−) by crossing LyzCre/+ mice (C57Bl/6 background, Jackson Laboratory, B6.129P2-Lyz2tm1(cre)Ifm/J, Stock number 004781) into Igf1rflox/flox/Apoe−/− mice. Successful excision of the exon 3 of Igf1r was confirmed in peritoneal macrophages by RT-PCR, using an Igf1r exon 2 (forward) and 4 (reverse) specific primer set (forward: CGTCATCACTGAGTACTTGCTGCTC, reverse: CGAATCGATGGTTTTCGTTTTCTTC). Further, IGF-1 receptor deficiency was confirmed by lack of IGF1R protein detection by western blot, and IGF-1-dependent phosphorylation on Akt in peritoneal macrophages. Eight-week old mice were fed a high-fat diet (42% of total calories from fat, 0.15% cholesterol, Harlan, Indianapolis, IN) for 8 weeks.

**Atherosclerosis Quantification**

Atherosclerotic burden was quantified by measuring the surface area of Oil Red O–positive lesions on en face preparations of whole aortas as previously described3. In addition, serial sections (6 μm) were taken throughout the entire aortic valve area and stained with H&E for quantitation of plaque cross-sectional area as previously described3. The area of the smooth muscle cell fibrous cap was measured using anti-SMA-stained aortic valve sections, with the cap identified as the immunopositive part of the plaque covering the necrotic core. The areas of the necrotic core and fibrous cap were measured using Image-Pro, and data are presented as a percentage of total plaque area.
Atherosclerotic plaque composition

Serial 6 μm paraffin-embedded cross-sections were taken throughout the entire aortic valve area, and 3 sets of serial sections obtained at 60 μm intervals were used for measurement of macrophage positive lesion area using rat anti-mouse Mac-3 monoclonal antibody as previously described\(^3\). Serial sections were also stained for SMA. Sections were also stained with Masson’s Trichrome (Richard-Allan Scientific, Kalamazoo, MI) method.

Analysis of lesions in the brachiocephalic artery

One hundred 5-μm sections were cut and lesion characteristics analyzed every 50 μm following histological staining by Carstairs’ method to detect fibrin (orange-red), red blood cells (yellow-red), smooth muscle cells (dark red), and collagen (bright blue)\(^4\). Features of plaque vulnerability were assessed according to the criteria proposed by Gough et al\(^4\): An animal was scored positive for fibrous cap disruption when at least 3 sections separated by 50 μm showed a discontinuity or disruption in the elastin layer in the cap while a break was defined as a clear break of the fibrous cap into the lumen. Intraplaque hemorrhage was classified as the presence of red blood cells in at least 3 sections separated by 50 μm, and medial elastin breaks were scored if there were discontinuities in any of the lamellae of the medial elastin wall. Fibrin deposition was defined as the presence of red-orange Carstairs staining and positive anti-fibrin/fibrinogen immunostaining in serial sections from at least 3 sections of the brachiocephalic artery separated by 50 μm.

Blood cell count
Total white blood cell counts were determined by peripheral blood smear. To count circulating monocytes and Ly6C\textsuperscript{hi} monocytes, we followed the procedure reported by others. In brief, whole blood was mixed with a cocktail of monoclonal antibodies against T cells (CD90-PE, 53-2.1), B cells (B220-PE, RA3-6B2), NK cells (CD49b-PE, DX5 and NK1.1-PE, PK136), granulocytes (Ly-6G-PE, 1A8), myeloid cells (CD11b-APC, M1/70) and monocyte subsets (Ly-6C-FITC, AL-21). Using flow-cytometer (Gallios analyzer, Beckman-Coulter), monocytes were identified as CD11\textsubscript{b}hi/CD90lo/B220lo/CD49blo/NK1.1lo/Ly-6Glo cells. Monocyte numbers were calculated as total leukocytes (as determined on blood smears) multiplied by percent cells within the monocyte gate of the mononuclear cell fraction. Within this population, subsets were identified as Ly-6C\textsuperscript{hi} monocyte.

**Tracing recruitment of circulating monocytes**

Circulating monocytes were labeled with polychromatic red microspheres by following the method described elsewhere. Animals were fed on a high-fat diet for 12 weeks; To label Ly6C\textsuperscript{lo} monocytes, 0.5-\textmu m Fluoresbrite polychromatic red microspheres (2.5% solids [wt/vol]; Polysciences Inc.) were diluted 1:25 in PBS and 250 \textmu l of the solution was injected into the lateral tail vein of mice twice, at 3 and 7 days before sacrifice. To label Ly6C\textsuperscript{hi} monocytes, 0.2 ml of dichloromethylene-bisphosphonate (clodronate) liposomes (Sigma-Aldrich) or control liposomes were injected i.v., 24 hours prior to administration of the red microspheres. Serial sections were obtained at the aortic root and stained with Oil Red O to visualize plaque, Mac3 antibody (marker of macrophages) or DAPI. Red microsphere-labeled cells were also Mac3-immunopositive, confirming labeling specificity. Numbers of red microsphere-positive cells were counted in a
plaque and normalized to the efficiency of labeling in circulating monocytes (% labeled/total number), assessed by flow cytometry.

_Intravital Fluorescence Microscopy_

Intravital fluorescence microscopy was performed as described elsewhere⁸. Mice were administered i.v. with Alexa Fluor 488-labeled anti-CD11b antibody (clone M1/70, ABLab, Vancouver, B.C., Canada) via jugular vein, 1 hour prior to assessment. Mice were placed on a Plexiglas board, and a section of the small intestine was exteriorized over a glass coverslip and superfused with a bicarbonate-buffered saline (37°C, pH 7.4). Body temperature was maintained between 36.5 and 37.5°C by means of a thermostatically controlled heat lamp. The Plexiglas board was mounted on the stage of an inverted microscope (Eclips TE2000, Nikon), and the intestinal microcirculation was observed through a x20 objective lens. Fluorescent images (excitation: 420–490 nm and emission: 520 nm) were detected with a charge-coupled device camera (Photometrics COOLSNAP ES). Images were projected onto a television monitor (PVM-1953MD, Sony) and recorded on a DVD recorder (DMR-E50, Panasonic). A time-date generator (WJ810, Panasonic) displayed this function on the monitor. The intestinal segment was scanned, and 10 single unbranched venules (diameter: 20–50 µm and length: 100 µm) were observed for at least 1 min. Monocyte-endothelial interactions (number of rolling monocytes and number of firmly attached monocytes) were quantified in each venule followed by calculation of the mean for 10 venules. Monocytes were considered to be adherent if they did not move for at least 30 s. Rolling cells were defined to be those passing a cross line at a velocity significantly slower than
the centerline velocity and are expressed as rolling cells per minute. Numbers of adherent cells were normalized in terms of millimeter squared surface area.

*Laser Capture Microdissection (LCM)*

OCT-embedded frozen hearts were cut for 10 micron cross-sections and placed on PEN membrane glass slides (LCM0522, Thermo Fisher). Slides were immediately processed with Histogene® LCM Frozen Section Staining Kit (Thermo Fisher). Slides were fixed with 75% ethanol, stained with Histogene staining solution, dehydrated and dried as per kit’s instructions. Serial sections were stained with rat macrophage marker antibody (sc-101447)⁹,¹⁰ and developed with anti-rat Alexa488 secondary antibody (Invitrogen) plus DAPI and used as a reference for plaque macrophage dissection with LCM. LCM was performed with the Applied Biosystems® ArcturusXT™ LCM System by using a Nikon Eclipse Ti-E inverted research microscope and a combination of IR and UV lasers to provide laser-capture and laser cutting into one modular platform. LCM dissected tissue was kept in dry ice and used for RNA isolation with Arcturus® PicoPure® Frozen RNA Isolation Kit (Thermo Fisher). RNA quality/concentration was evaluated with Take3 plates on Cytation5 imager (Bio-Tek). In addition, quality of RNA isolated from LCM dissected tissue was evaluated with RT² PCR Array Mouse RNA QC (Qiagen). Eight ng of RNA was used for cDNA synthesis with Arcturus® RiboAmp® HS Plus cDNA kit (Thermo Fisher) according to the manufacturer’s instructions. RT primers for CD68, PCNA, Ki67 and β-actin were purchased from SABiosciences (Qiagen).

*Macrophage culture*
Thioglycolate elicited macrophages were obtained from MΦ-IGF1R-KO and IGF1R-flox mice. Four days after i.p. administration of sterile thioglycolate medium, peritoneal lavage was performed to collect peritoneal cells. Recovered cells were inoculated on to culture dishes and allowed to adhere overnight in RPMI 1640 medium supplemented with 10% fetal bovine serum. Floating cells were washed away using phosphate buffered saline, and the cell culture was continued overnight. Macrophage purity was confirmed to be more than 95% by immunological staining using anti-F4/80 antibody. To polarize macrophages, cells were exposed to 150 U/mL IFNγ for 6 hours followed by exposure to 50 ng/mL LPS for 18 hours to induce M1 activation, and exposed to 20 U/mL IL4 for 24 hours to induce M2 activation. In order to test effects of oxLDL, macrophages were primed with IFNγ for 6 hours followed by an exposure to 50 μg/mL oxLDL for 18 hours.

**Western Blot Analysis**

Western blot analysis was performed as described previously. In brief, cells were washed with PBS and lysed in RIPA buffer, containing 150 mmol/L NaCl, 20 mmol/L Tris-Cl, pH 7.2, 1 mmol/L EDTA, 1% Nonidet P-40, 5 mmol/L dithiothreitol, 0.1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 0.1 mol/L okadaic acid, 0.1 mol/L aprotinin, 10 g/mL leupeptin, and 10 mmol/L NaF. Lysates were subjected to 10% SDS-PAGE and Western blotting analysis. Immunopositive bands were visualized by enhanced chemiluminescence (Amersham). Blots were stripped and reprobed with monoclonal anti-β-actin antibody as a control for equal loading.

**Quantitative Real-Time RT-PCR**
Total RNA extraction and real-time PCR was performed as previously described\textsuperscript{13}. Briefly, total aortic RNA was isolated using the TriPure Isolation Reagent (Roche) followed by purification with the RNeasy mini kit (Qiagen). Complementary DNA was synthesized using the First Strand cDNA Synthesis kit (Amersham) and used for 40-cycle 2-step PCR with sequence-specific primer pairs in the iCycler IQ Real-Time Detection System (Bio-Rad). All the primers used were obtained from Qiagen (RT\textsuperscript{2} qPCR Primer Assay).

 Statistical Analysis

All numeric data are expressed as mean ± SEM. Statistical analyses were performed using GraphPad PRISM (version 6.07) software. Data sets were first assessed for residuals distribution using D’Agostino-Pearson omnibus normality test and for equal variances using Levene’s Test for Equality of Variances. Differences in outcomes were determined by ANOVA and Bonferroni’s multiple comparisons test, Kruskal-Wallis test, unpaired Student’s t-test with or without Welch’s correction, or Mann-Whitney U test, accordingly with the normality of residuals distribution. Differences were considered significant at P<0.05. Fisher’s exact test was used to compare frequency of observed indices of plaque vulnerability (Table 1).
Supplemental Table 1. Circulating leukocyte and monocyte counts

<table>
<thead>
<tr>
<th></th>
<th>IGF1R-flox</th>
<th></th>
<th>MΦ-IGF1R-KO</th>
<th></th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N.C.</td>
<td>W.D.</td>
<td>N.C.</td>
<td>W.D.</td>
<td></td>
</tr>
<tr>
<td>White blood cell</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>count (x10^3/mm^3)</td>
<td>3.76±0.30</td>
<td>4.36±0.63</td>
<td>5.75±0.97</td>
<td>3.66±0.50</td>
<td>Diet: P=0.11</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IGF1R-KO: P=0.20</td>
</tr>
<tr>
<td>Monocyte (/mm^3)</td>
<td>58±3</td>
<td>224±54</td>
<td>119±31</td>
<td>186±42</td>
<td>Diet: P=0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IGF1R-KO: P=0.82</td>
</tr>
<tr>
<td>Ly6C^hi^-monocyte</td>
<td>64±10</td>
<td>73±7</td>
<td>67±6</td>
<td>76±5</td>
<td>Diet: P=0.24</td>
</tr>
<tr>
<td>(%-monocyte)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IGF1R-KO: P=0.66</td>
</tr>
</tbody>
</table>

Mean ± sem; IGF1R-flox, n = 11 (N.C., 4 mice; W.D., 7 mice); MΦ-IGF1R-KO, n = 13 (N.C., 4 mice; W.D., 9 mice). Two-way ANOVA results are shown.
Supplemental Table 2. Plasma cholesterol and cytokine levels

<table>
<thead>
<tr>
<th></th>
<th>IGF1R-flox</th>
<th>MΦ-IGF1R-KO</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>3345 ± 178</td>
<td>3234 ± 126</td>
<td>0.61</td>
</tr>
<tr>
<td>IGF-1 (ng/mL)</td>
<td>297.9 ± 9.9</td>
<td>266.4 ± 15.4</td>
<td>0.13</td>
</tr>
<tr>
<td>TNFα (pg/mL)</td>
<td>7.1 ± 1.6</td>
<td>8.0 ± 1.8</td>
<td>0.72</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>8.6 ± 2.7</td>
<td>2.9 ± 1.5</td>
<td>0.06</td>
</tr>
<tr>
<td>MCP-1 (pg/mL)</td>
<td>153.7 ± 50.8</td>
<td>96.2 ± 11.7</td>
<td>0.21</td>
</tr>
</tbody>
</table>

Mean ± sem; Student’s t-test results are shown; IGF1R-flox, n = 13; MΦ-IGF1R-KO, n = 18
Supplemental Figure 1

(A) IGF1R-flox \_MΦ-IGF1R-KO

+Ins +IGF \quad +Ins +IGF

IGF1R

P-Ser473-Akt

Akt

B-actin

(B) Ex2Fw

Ex2 \rightarrow Ex3 \rightarrow Ex4

Ex2Fw

Ex2 \rightarrow Ex4

Ex4Rev

Exon2+3+4

Exon2+4 △

macrophage

neutrophil

Flox Cre+

Ex2-4: Anticipated PCR products

Flox: = 796 bp

Cre\(^+\)/Flox: = 483 bp
Supplemental Figure 1

(C) Protein loading: 100 ug

(D)
Supplemental Figure 1

(E) 

IP: IGF1R  InsR  
IB: IGF1R  

<table>
<thead>
<tr>
<th></th>
<th>IGF1R</th>
<th>InsR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 : 1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

IB: InsR  

<table>
<thead>
<tr>
<th></th>
<th>IGF1R</th>
<th>InsR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 : 2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

IGF1R : IGF1R/InsR : InsR = 0 : 2 : 1

(F) 

Insulin (ng/mL)  0  2.5  50  0  2.5  50  
IGF1R-flox  MΦ-IGF1R-KO  
P-Ser473-Akt  
Akt  

p-AKT/AKT  

Insulin (ng/mL)  0  10  20  30  40  50  
IGF1R-flox  MΦ-IGF1R-KO  

13
Supplemental Figure 3

<table>
<thead>
<tr>
<th>(A)</th>
<th>IGF1R-flox</th>
<th>MΦ-IGF1R-KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP 1</td>
<td><img src="MMP_1.png" alt="Image" /></td>
<td><img src="MMP_1.png" alt="Image" /></td>
</tr>
<tr>
<td>MMP 2</td>
<td><img src="MMP_2.png" alt="Image" /></td>
<td><img src="MMP_2.png" alt="Image" /></td>
</tr>
<tr>
<td>MMP 8</td>
<td><img src="MMP_8.png" alt="Image" /></td>
<td><img src="MMP_8.png" alt="Image" /></td>
</tr>
<tr>
<td>MMP 9</td>
<td><img src="MMP_9.png" alt="Image" /></td>
<td><img src="MMP_9.png" alt="Image" /></td>
</tr>
<tr>
<td>MMP 12</td>
<td><img src="MMP_12.png" alt="Image" /></td>
<td><img src="MMP_12.png" alt="Image" /></td>
</tr>
<tr>
<td>MMP 13</td>
<td><img src="MMP_13.png" alt="Image" /></td>
<td><img src="MMP_13.png" alt="Image" /></td>
</tr>
<tr>
<td>MMP 14</td>
<td><img src="MMP_14.png" alt="Image" /></td>
<td><img src="MMP_14.png" alt="Image" /></td>
</tr>
<tr>
<td>βActin</td>
<td><img src="%CE%B2Actin.png" alt="Image" /></td>
<td><img src="%CE%B2Actin.png" alt="Image" /></td>
</tr>
</tbody>
</table>
Supplemental Figure 4

(A) IGF1R-flox

(B) ΜΦ-IGF1R-KO

(C)

[Images and annotations]
Supplemental Figure 6

(C) Bar graph showing relative expression (normalized to β-actin) of genes Pcn, Ki67, and CD68.

(D) Images of IGF1R-flox and MΦ-IGF1R-KO samples. TUNEL+/Mac3+ count per plaque is also shown.
Supplemental Figure 7

IGF1R

β-actin

Sf  nLDL  oLDL

21
Supplemental Figure legends

Supplemental Figure 1. IGF1R and InsR expression in peritoneal macrophages

(A) (Left panel): Western blot analysis of peritoneal macrophages isolated from IGF1R-flox and MΦ-IGF1R-KO mice. Cells were exposed to 7.6 ng/mL insulin (+Ins) or 10 ng/mL IGF-1 (+IGF) for 15 minutes and then assessed for IGF1R β-chain (IGF1R), phospho-Ser473-Akt, Pan-Akt (Akt), and β-actin protein levels. (Right panel): Quantification of western blot results of phospho-Ser473-Akt, normalized to Pan-Akt levels. Each bar is an average value of duplicates. Shown is a representative result from 5 independent experiments. (B) Confirmation of excision of the floxed exon 3 of Igf1r. A schematic for exon 3 excision from Igf1r gene is illustrated. Total RNA was purified from the cultured peritoneal macrophages of IGF1R-flox (Flox) or MΦ-IGF1R-KO (Cre+) mice, and was used for RT-PCR to amplify a cDNA fragment corresponding to a part of exon2 + exon 3 + a part of exon 4. (C) Western blot analysis of peritoneal neutrophils and macrophages for IGF1R expression. Peritoneal neutrophils were isolated by peritoneal lavage after 24 hours of i.p. administration of thioglycolate, followed by a centrifugal isolation using Percoll (63%). Purity of neutrophils was confirmed by flow cytometry by Ly6G and CD11b double-positivity (>98 %). 100 µg (neutrophil) or 10 µg (macrophage) of protein was used for IGF1R β-chain detection. (D) Western blot analysis of peritoneal macrophages isolated from IGF1R-flox or MΦ-IGF1R-KO mice for InsR expression levels. Right panel: quantification of InsR expression levels normalized to β-actin. Each bar is an average of duplicates ± sem. Representative result from 3 independent experiments is shown. (E) Assessment of the presence of IGF1R/InsR hybrid receptor. Immunoprecipitation was performed to pull down IGF1R (Santa Cruz Biotech, C-20) and InsR
(Santa Cruz Biotech, C-19) from cell lysates of IGF1R-flox macrophage. Precipitates were resolved by SDS-PAGE, detected by western blotting. The efficiency of immunoprecipitation (~100%) was confirmed by an equivalent detection in lanes loaded with the same amount of lysates. A representative result from 3 independent experiments is shown. (F) Insulin-dose response for Akt-phosphorylation in peritoneal macrophages. Peritoneal macrophages isolated from IGF1R-flox mice or MΦ-IGF1R-KO mice were exposed to 0, 2.5, 5, 10, 25, and 50 ng/mL insulin for 15 minutes and then assessed for phospho-Ser473-Akt levels by Western blot analysis. Right panel: a quantification of the Western blot analysis. Each data point is an average of duplicates, and error bars represents SEM. A representative result from 3 independent experiments is shown.

Supplemental Figure 2. Monocyte/macrophage IGF1R deficiency aggravated atherosclerosis
Atherosclerotic lesion formation was assessed in MΦ-IGF1R-KO and IGF1R-flox mice (control) fed on a high-fat diet for 2 months. Plaque development was assessed using oil red o staining of en face aorta; plaque covered area was determined and expressed as % of total area. Data was separately plotted for each gender, showing the effect of MΦ-IGF1R-KO was gender independent. Open circle: IGF1R-flox control, closed circle: MΦ-IGF1R-KO. *P<0.01 by non-parametric 2-way ANOVA, and these statistical results were further confirmed by Kruskal-Wallis test.

Supplemental Figure 3. Matrix Metalloproteinase expression in ascending aorta.
(A) Western blot analysis of tissue lysates of ascending aorta dissected from IGf1R-flox and MΦ-IGF1R-KO mice fed on a high-fat diet for 2 months. Representative results from 2 independent experiments were shown. (B) Quantification of the Western blot analysis of MMPs expression in
lysates of ascending aorta. N=6 for IGF1R-flox and N=4 for MΦ-IGF1R-KO. *P<0.05 and **P<0.01 by Student’s t-test.

Supplemental Figure 4. Representative lesions from IGF1R-flox and MΦ-IGF1R-KO mice demonstrate features of vulnerable plaques.

Carstairs stain of brachiocephalic lesions from IGF1R-flox (A) and M-IGF1R-KO (B) mice. Note thinner blue staining within a lesion of MΦ-IGF1R-KO mice (B) compared to IGF1R-flox mice (A), demonstrating diminished collagen deposition, consistent with Fig 2C. Red blood cells were observed within lesions implying plaque vulnerability (B, inset; higher magnification is shown in lower panel with arrowheads pointing to red blood cells). (C) Representative staining result of Verhoeff–Van Gieson stain. Medial elastin breaks and fibrous cap disruption are observed (arrowheads). Scale bar: 200 µm.

Supplemental Figure 5. Efferocytosis gene expression levels in MΦ-IGF1R-KO macrophages

After isolation and adhesion to culture plates, macrophages were activated to M1 (IFNγ+LPS) or M2 (IL-4), or exposed to IL-4 and oxLDL for 24 hours. Total RNA was extracted and subjected to quantitative RT-PCR. IGF1R-flox, open column; MΦ-IGF1R-KO, closed column. Representative results are shown from 3 independent experiments. *P<0.01 vs not-activated cells. aP<0.01 vs. IGF1R-flox, oxLDL-treated. bP<0.01 vs. IGF1R-flox, IL-4- and oxLDL-treated. cP<0.01 vs. oxLDL-treated cells. dP<0.01 oxLDL-treatment vs. not-activated or IL-4-treated cells. #P<0.05 vs. IGF1R-flox, IL-4-treated, by 2-way ANOVA and Bonferroni’s multiple comparisons test, N=3.
Supplemental Figure 6. Plaque macrophage recruitment, proliferation, and apoptosis.

(A) and (B) Flow cytometry of circulating monocytes in IGF1R-flox mice administered with red microspheres. Blood leukocytes were stained with anti-CD11b-AlexaFluor488, anti-CD115-biotin-avidin-eFluor450, and anti-Ly6C-AlexaFluor700, and a monocyte-gating was applied. Ly6C-positivity (left panel, histogram) and red microsphere-positivity (right panel y-axis: detected using PE-filter) were assessed. Each population size within the monocyte gating (%) is indicated in graphs. (A) Red microspheres were administered (i.e. labeling Ly6C\textsuperscript{lo} population), (B) red microspheres and clodronate were administered (labeling Ly6\textsuperscript{hi} and Ly6C\textsuperscript{lo} monocyte populations). (C) PCNA and Ki67 mRNA levels assessed by quantitative RT-PCR in the region within plaques, which is positively stained by the monoclonal antibody raised against macrophages of mouse origin. Macrophage-rich areas within atherosclerotic plaques were located by immunostaining and dissected by laser-capture microdissection. Total RNA was extracted and subjected to quantitative RT-PCR. CD68 mRNA levels were quantified to confirm successful and equivalent dissection of macrophage-rich sections. There were no statistically significant differences between IGF1R-flox (open column) and MΦ-IGF1R-KO (closed column) by Student’s t-test, N=8. (D) Assessment of apoptotic macrophages within plaques. PFA-fixed, paraffin embedded sections of aortic root were stained for Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) positivity (red), Mac3 (green) and DAPI (blue). Co-localization of TUNEL- and Mac3- positivity within a plaque was counted. There were no statistically significant differences between IGF1R-flox (open column) and MΦ-IGF1R-KO (closed column) by Student’s t-test: IGF1R-flox, n=4; M-IGF1R-KO, n=9.
Supplemental Figure 7. Oxidized LDL downregulates IGF1R levels in human derived THP1 macrophages. THP1 cells were differentiated to macrophages by exposing to 50 ng/mL phorbol 12-myristate 13-acetate for 24 hours. Cells were washed in serum-free RPMI, and then exposed to 100 µg/mL oxidized LDL, 100 µg/mL native LDL (both from Kalen Biomedical), or in serum-free RPMI for 24 hours. Cell lysates were analyzed for IGF1R β-chain expression by Western blot. A representative result from 5 independent experiments is shown.
Supplemental References

대식세포의 Insulin-Like Growth Factor-1 수용체가 활성화되면 Form Cell의 형성을 억제할 수 있다

한 기훈 교수 서울아산병원 심장내과

초록

배경

연구진은 이전에 IGF-1(insulin-like growth factor-1)을 체내 주입하면 혈액중 및 혈관내 작용을 일으켜 축성경화를 경감시킨다는 결과를 Apoe(apolipoprotein E) 급립 죽을 통해 보여주었다. 단백질/대식세포는 IGF-1 수용체(IGF-1 receptor, IGF1R)를 높은 발현도로 보이기 때문에 축성경화의 발생에 역할을 할 것이라 추정되지만, 증명된 바는 없다.

방법 및 결과

IGF-1이 축성경화를 경감시키고, 이 과정에서 단백질/대식세포가 관여하는지를 증명하기 위하여, Apoe 급립 죽에서 선택적으로 단백질/대식세포의 IGF1R을 제거한(MΦ-IGF1R-KO) 죽을 만들었다. MΦ-IGF1R-KO 죽에서 축성경화의 증가 현상이 예상대로 관찰되었으며, 이는 대동맥의 en face 및 대동맥 핀막 수준의 축성경화반 부하로 증명되었다. 또한, 대식세포의 증가, actin 양성 및 평활근세포의 감소, fibrous cap의 얇아짐(thinning)과 콜라겐의 감소 등 축성경화반의 불안정화가 관찰되었다. Brachiocephalic artery의 조직학적 소견을 보면 폴리프의 불안정화가 관찰되었다. MΦ-IGF1R-KO 죽에서 대식세포를 분리 배양하여 분석하면, interferon-γ 및 oxidized low-density lipoprotein에 반응하는 염증성 변화가 증가되고, 혈관내 유전자
Insulin-like Growth Factor-1 Receptor Deficiency in Macrophages Accelerates Atherosclerosis and Induces an Unstable Plaque Phenotype in Apolipoprotein E–Deficient Mice

Yusuke Higashi, PhD; Sergiy Sukhanov, PhD; Shaw-Yung Shai, PhD; Svitlana Danchuk, MS; Richard Tang, BS; Patricia Snarski, BS; Zhaohui Li, MD; Patricia Lobelle-Rich, MS; Meifang Wang, MS; Derek Wang; Hong Yu, BS; Ronald Korthuis, PhD; Patrice Delafontaine, MD

Background—We have previously shown that systemic infusion of insulin-like growth factor-1 (IGF-1) exerts anti-inflammatory and antioxidant effects and reduces atherosclerotic burden in apolipoprotein E (ApoE)–deficient mice. Monocytes/macrophages express high levels of IGF-1 receptor (IGF1R) and play a pivotal role in atherogenesis, but the potential effects of IGF-1 on their function are unknown.

Methods and Results—To determine mechanisms whereby IGF-1 reduces atherosclerosis and to explore the potential involvement of monocytes/macrophages, we created monocyte/macrophage–specific IGF1R knockout (MΦ-IGF1R-KO) mice on an Apoe−/− background. We assessed atherosclerotic burden, plaque features of stability, and monocyte recruitment to atherosclerotic lesions. Phenotypic changes of IGF1R-deficient macrophages were investigated in culture. MΦ-IGF1R-KO significantly increased atherosclerotic lesion formation, as assessed by Oil Red O staining of en face aortas and aortic root cross-sections, and changed plaque composition to a less stable phenotype, characterized by increased macrophage and decreased α-smooth muscle actin–positive cell population, fibrous cap thinning, and decreased collagen content. Brachiocephalic artery lesions of MΦ-IGF1R-KO mice had histological features implying plaque vulnerability. Macrophages isolated from MΦ-IGF1R-KO mice showed enhanced proinflammatory responses on stimulation by interferon-γ and oxidized low-density lipoprotein and elevated antioxidant gene expression levels. Moreover, IGF1R-deficient macrophages had decreased expression of ABCA1 and ABCG1 and reduced lipid efflux.

Conclusions—Our data indicate that macrophage IGF1R signaling suppresses macrophage and foam cell accumulation in lesions and reduces plaque vulnerability, providing a novel mechanism whereby IGF-1 exerts antiatherogenic effects.

(Circulation. 2016;133:2263-2278. DOI: 10.1161/CIRCULATIONAHA.116.021805.)

Key Words: atherosclerosis ▪ inflammation ▪ insulin-like growth factor 1 ▪ macrophages

Insulin-like growth factor -1 (IGF-1) is a pleiotropic factor that is produced and acts locally (ie, via autocrine or paracrine effects) or circulates in blood and exerts endocrine effects. Circulating IGF-1 levels reach a peak during the pubertal growth phase, eventually declining with the progression of age. Although its role in developmental and pubertal growth as the major mediator of the effects of growth hormone is well documented, the physiological roles of IGF-1 in aged subjects are not understood. Aging is a major independent risk factor for coronary heart disease; in fact, there is increased cardiovascular and coronary heart disease prevalence of age in both sexes. In light of the aging-dependent decline in circulating IGF-1 levels and the elevated prevalence of cardiovascular diseases has been suggested. Indeed, epidemiological data have suggested that low IGF-1 levels are an important predictor of coronary events in aged subjects.1-3 In an animal model of atherosclerosis, we have shown that low levels of circulating IGF-1 are associated with more atherosclerosis4 and, vice versa, that an increase in circulating IGF-1 decreases atherosclerotic burden.5 The pathogenesis of atherosclerosis is complicated, involving multiple cell types, including vascular endothelial cells, smooth muscle cells, and proinflammatory cells such as macrophages. To determine the potential target(s) whereby IGF-1...
reduces atherosclerosis, we previously investigated whether smooth muscle–specific overexpression of IGF-1 alters atherosclerosis. Intriguingly, overexpression of IGF-1 in smooth muscle cells did not alter atherosclerotic burden but increased features of plaque stability suggesting that other cellular components were a potential target for the antiatherogenic effects of IGF-1. Macrophages play a pivotal role in the pathogenesis of atherosclerosis by modulating inflammatory status and by scavenging and accumulating excess lipid to become foam cells. Thus, regulation of macrophage functions in terms of inflammation and phagocytosis is key to comprehending the disease process. Because macrophages have a predominant role in the inflammatory status of atherosclerotic lesions, it is critical to determine whether IGF-1 regulates macrophage function, particularly inflammatory activation and phagocytic activity. Thus far, there is very limited information on the potential link between IGF-1 effects and macrophage function, particularly in relation to vascular disease. IGF-1 has been reported to enhance chemotactic macrophage migration,8 to stimulate tumor necrosis factor (TNFα) expression,8 and to stimulate low-density lipoprotein (LDL) uptake and cholesterol esterification.9 There are also reports from clinical investigations providing indirect evidence of anti-inflammatory effects of IGF-1. For example, there is an inverse relation between serum interleukin (IL)-6 and IGF-1 levels10; IGF-1/insulin-like growth factor–binding protein 3 administration to patients with severe burn injury induced an anti-inflammatory effect and reduced IL-6 and TNFα levels; and low IGF-1 and high IL-6 and TNFα levels are associated with higher mortality in elderly patients.13,14 In this study, we used a well-established animal model of atherosclerosis to examine the role of macrophage IGF-1 signaling in atherosclerosis development and progression.

Methods

A detailed Materials and Methods section is available in the online-only Data Supplement.

Animals

All animal experiments were performed according to protocols approved by the institutional animal care and use committee. Monocyte/macrophage–targeted IGF-1 receptor–null mice on Apoe+− background (MΦ-IGF1R-KO) and control mice (IGF1R-flox) were generated as described in the online-only Data Supplement. Eight-week old mice were fed a high-fat diet for 8 weeks before atherosclerosis was assessed.

Atherosclerotic Burden and Plaque Composition

Atherosclerotic burden was quantified with the use of en face preparations of whole aorta stained with Oil Red O and in cross sections of aortic root. Plaque composition was assessed in cross sections of aortic root by immunostaining for Mac-3 (macrophage) and β3 integrin. Brachiocephalic artery lesions were analyzed in the aortic root by immunostaining for Mac-3 (macrophage) and Masson’s Trichrome staining was used for collagen. Brachiocephalic artery lesions were analyzed by the Carstairs method as described by Gough et al.

Tracing Recruitment of Circulating Monocytes

Circulating Ly6C+ or Ly6C0 monocytes were labeled in vivo with polychromatic red microspheres as described elsewhere,16 and numbers of red microsphere–positive cells were counted in a plaque and normalized to the efficiency of labeling in circulating monocytes (percent labeled/total number) and assessed by flow cytometry.

Intravital Fluorescence Microscopy

Intravital fluorescence microscopy was performed as described elsewhere.

Macrophage Culture, Western Blot Analysis, and Quantitative Real-Time Reverse Transcription–Polymerase Chain Reaction

Thioglycolate-elicited macrophages were obtained from MΦ-IGF1R-KO and IGF1R-flox mice. Western blot analysis,19 total RNA extraction,20 and real-time reverse transcription–polymerase chain reaction20 were performed as previously described.

Statistical Analysis

All numeric data are expressed as means±SEM. Statistical analyses were performed with GraphPad PRISM (version 6.07) software. Data sets were first assessed for residuals distribution with the D’Agostino-Pearson omnibus normality test and for equal variances with the Levene test for equality of variances. Differences in outcomes were determined by ANOVA and Bonferroni multiple-comparisons test, Kruskal–Wallis test, unpaired Student t test with or without the Welch correction, or Mann–Whitney U test, according with the normality of residuals distribution. Differences were considered significant at P<0.05. The Fisher’s exact test was used to compare frequency of observed indexes of plaque vulnerability (Table).

Results

Generation of MΦ-IGF1R-KO/Apoe+− Mice

MΦ-IGF1R-KO were generated on Apoe+− background (LyzCre+/Igf1rflox/flox/Apoe+−; MΦ-IGF1R-KO) by crossing LyzCre+ mice into Igf1rflox/flox/Apoe+− (IGF1R-flox, served as a control). Genotype segregation in the offspring followed the expected mendelian frequency, and we did not recognize any developmental/morphological abnormalities. IGF1R deficiency was confirmed by lack of IGF1R protein detection by Western blot and IGF-1–dependent phosphorylation on Akt.

Intravital Fluorescence Microscopy

Intravital fluorescence microscopy was performed as described elsewhere.

Table. MΦ-IGF1R-KO-Induced Features of Ruptured Plaques in the Brachiocephalic Artery

<table>
<thead>
<tr>
<th></th>
<th>MΦ-IGF1R-KO</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrous cap disruption</td>
<td>0/22</td>
<td>1/17</td>
</tr>
<tr>
<td>Intraplaque hemorrhage</td>
<td>3/22</td>
<td>8/17</td>
</tr>
<tr>
<td>Fibrin deposition</td>
<td>0/22</td>
<td>0/17</td>
</tr>
<tr>
<td>Medial elastin breaks</td>
<td>0/22</td>
<td>4/17</td>
</tr>
</tbody>
</table>

Data indicate positive observations in a total number of samples. Statistical significance was tested by the Fisher’s exact test. IGF1R indicates insulin-like growth factor-1 receptor; MΦ-IGF1R-KO, monocyte/macrophage–specific insulin-like growth factor-1 receptor knockout; and ND, not determined.
IGF1R and insulin receptor (InsR) can form a hybrid receptor (heterotetramer consisting of α+β subunits of IGF1R and α+β subunits of InsR),24–26 which binds IGF-1 with high affinity but not insulin.27,28 Depletion of IGF1R in endothelial cells is reported to allow InsR to form a holotetramer, thereby enhancing insulin sensitivity.29 Because insulin signaling in macrophages has significant effects on atherosclerosis (there are contradictory reports showing antiatherogenic30 or proatherogenic31 effects), we assessed insulin signaling activity in MΦ-IGF1R-KO macrophages. IGF1R deficiency did not alter InsR expression levels (Figure ID in the online-only Data Supplement). In IGF1R-flox macrophages, immunoprecipitation of InsR pulled down 100% of IGF1R (Figure IE, left, in the online-only Data Supplement), whereas anti-IGF1R immunoprecipitation pulled down 50% of InsR (Figure IE, right, in the online-only Data Supplement), suggesting that the ratio of IGF1R/InsR-hybrid receptors to InsR-holoreceptors was 2:1 without the presence of IGF1R-holoreceptors. IGF1R deficiency did not alter insulin-induced dose-dependent phosphorylation of Akt (Figure IF in the online-only Data Supplement).

Animals were assessed for circulating leukocyte counts (Table I in the online-only Data Supplement), cholesterol levels, and cytokine levels (Table II in the online-only Data Supplement). As has been reported,32 Western diet feeding for 8 weeks was associated with elevated monocyte count; however, IGF1R deficiency did not result in a significant difference in white blood cell count or circulating monocyte count (Table I in the online-only Data Supplement). A subpopulation of circulating monocytes as defined by CD11b+/CD90−/B220−/CD49b−/NK1.1−/Ly6G−/Ly6Chigh cells (Ly6C hi monocytes) has been reported to be proinflammatory and to be increased under atherogenic conditions such as hyperlipidemia.33 We did not observe a significant difference in Ly6C hi monocyte levels between MΦ-IGF1R-KO and IGF1R-flox mice (Table I in the online-only Data Supplement). MΦ-IGF1R-KO did not significantly alter circulating IGF-1, proinflammatory cytokine (IL-6, TNFα, monocyte chemoattractant protein-1 [MCP-1]), or cholesterol levels (Table II in the online-only Data Supplement).

**Atherosclerosis Was Enhanced by MΦ-IGF1R-KO**

Atherosclerotic lesion formation was assessed after 8 weeks of high-fat diet feeding. En face Oil Red O staining of aortas revealed a significant ≈64% increase in Oil Red O–positive lesion area, and there was a consistent ≈34% increase in plaque size at the aortic root in MΦ-IGF1R-KO mice (Figure 1). The effect was confirmed in each sex, indicating that there was no sex-specific effect of IGF1R deficiency (Figure II in the online-only Data Supplement). Plaque composition with
regard to Mac3 (macrophage)-positive cells, α-smooth muscle actin–positive cells, and collagen content (Masson’s trichrome stain) was significantly altered by MΦ-IGF1R-KO (Figure 2); there was a 49% increase in Mac3 detection (Figure 2A) and a 31% decrease in α-smooth muscle actin detection (Figure 2B).

Plaques in MΦ-IGF1R-KO mice had a thinner smooth muscle cap than IGF1R-flox mice (Figure 2B) and decreased collagen content (Figure 2C). Because we observed a decrease in collagen content in plaques from MΦ-IGF1R-KO mice, we hypothesized that this could be attributable to enhanced collagen degradation by matrix metalloproteinases (MMPs).

Cultured peritoneal macrophages from MΦ-IGF1R-KO animals expressed higher levels of MMP-1, -2, -8, -9, -12, -13, and -14 than those from IGF1R-flox animals, suggesting enhanced MMP activity (Figure 2D). The MMP protein levels in tissue lysates of ascending aortas after 2 months of Western diet feeding (Figure IIIA and IIIB in the online-only Data Supplement) showed a significant increase in the expression of MMP-1, -2, -8, and -9 in MΦ-IGF1R-KO animals. These observations are consistent with enhanced lesion formation in MΦ-IGF1R-KO mice with a phenotypic shift toward increased features of plaque vulnerability.

**Plaque Destabilization in MΦ-IGF1R-KO Mice**

Because our findings suggested plaque destabilization in MΦ-IGF1R-KO mice, we assessed indicators of vulnerability in brachiocephalic artery plaques (Figure IV in the online-only Data Supplement) by staining cross sections of brachiocephalic artery with the Carstairs 34 and Verhoeff–Van Gieson methods. Cross sections (5 μm thick) were made every 50 μm along the artery, for a total of 10 sections per artery. If 3 consecutive sections indicated a fibrous cap disruption, intraplaque hemorrhage, fibrin deposition, or medial elastin break, the artery was considered positive for signs of plaque vulnerability. After 8 weeks on a high fat diet, MΦ-IGF1R-KO animals had increased features of vulnerable plaques compared with IGF1R-flox animals, as determined by the presence of intraplaque hemorrhage (IGF1R-flox, 3 positive in 22 animals versus MΦ-IGF1R-KO, 8 positive in 17 animals; P=0.03; Table) and medial elastin breaks (IGF1R-flox, no positive in...
Figure 3. Proinflammatory cytokine and chemokine production by cultured macrophages isolated from insulin-like growth factor-1 receptor (IGF1R)-flox and monocyte/macrophage-specific (MΦ)-IGF1R knockout (KO) mice. Interleukin (IL)-1α (A), IL-6 (B), tumor necrosis factor-α (TNFα; C), monocyte chemoattractant protein-1 (MCP-1; D), and fractalkine (E) secretion by peritoneal macrophages was assessed by respective ELISA. Macrophages were isolated by peritoneal lavage from IGF1R-flox (open column) and MΦ-IGF1R-KO (closed column) mice and allowed to adhere on culture plates overnight. After removal of nonadhering cells, cells were primed by interferon-γ (IFNγ), and conditioned media were collected after 24 hours. *P<0.05, **P<0.01 vs IGF1R-flox; ##P<0.01 vs IGF1R-flox+IFNγ; $P<0.01 vs MΦ-IGF1R-KO. Statistical significance was assessed by 2-way ANOVA and subsequent post hoc analysis with the Bonferroni multiple-comparisons test; n=6.

F, Nuclear factor-κB (NFκB) DNA binding activity was assessed (expressed as relative light units [RLU]) in cell lysates of IGF1R-flox and MΦ-IGF1R-KO macrophages with or without priming by IFNγ for 3 hours. *P<0.05 vs IGF1R-flox by 2-way ANOVA; n=3. G through J, Effects of NFκB inhibitors on TNFα (G and H) and IL-6 (I and J) secretion by peritoneal macrophages. IGF1R-flox (open column) and MΦ-IGF1R-KO (closed column) macrophages were exposed to the indicated dose of BMS-345541 or parthenolide for 1 hour before activation by IFNγ or lipopolysaccharide (LPS). Conditioned media were collected after 24 hours and assessed for cytokine concentration with the respective ELISA kit (R&D Systems). ##P<0.05 vs IGF1R-flox+IFNγ; #P<0.05 vs IGF1R-flox+LPS by the Mann–Whitney U test; n=4.
22 animals versus MΦ-IGF1R-KO, 4 positive in 17 animals; P=0.03; Table), suggesting that plaques in MΦ-IGF1R-KO mice are more unstable than in IGF1R-flox mice.

**Proinflammatory Responses Are Enhanced in IGF1R-Deficient Macrophages**

Thioglycolate-elicited peritoneal macrophages from the MΦ-IGF1R-KO mice were assessed for proinflammatory cytokine and chemokine secretion (Figure 3). Among tested cytokines and chemokines, IL-1β production was below detectable levels, whereas IL-1α, IL-6, TNFα, MCP-1, and fractalkine were detected (Figure 3A–3F). IGF1R-deficient macrophages secreted significantly higher levels of all the tested cytokines and chemokines except fractalkine, which showed a strong trend toward an increase (P=0.0508). Interferon-γ (IFNγ) significantly enhanced TNFα (Figure 3C) and MCP-1 (Figure 3D) production in IGF1R-flox cells but not in MΦ-IGF1R-KO cells. Intriguingly, IFNγ did not enhance fractalkine production in IGF1R-flox macrophages, whereas in MΦ-IGF1R-KO cells, it decreased fractalkine production (Figure 3E). In a setting of exposure to IFNγ, MΦ-IGF1R-KO cells secreted higher levels of IL-1α, IL-6, and MCP-1 compared with IGF1R-flox. We tested nuclear factor-kB (NFkB) DNA binding activity in these cells (Figure 3F). There was significantly higher NFkB DNA binding activity in MΦ-IGF1R-KO, which was further enhanced by IFNγ, consistent with higher cytokine/chemokine production. To further assess NFkB involvement, we exposed cells to NFkB inhibitors (BMS-345541 and parthenolide, Figure 3G–3J). BMS-345541 completely abolished enhanced TNFα and IL-6 production in MΦ-IGF1R-KO cells (Figure 3G and 3H), and parthenolide recapitulated the BMS-345541 effect (Figure 3I and 3J), supporting the importance of NFkB-dependent cytokine production in MΦ-IGF1R-KO macrophages.

**Macrophage Polarization**

Because IGF1R-deficient macrophages manifested enhanced proinflammatory responses, we examined whether IGF1R
deficiency influenced macrophage polarization. Classic activation (M1) was induced by exposure to IFNγ and subsequently to lipopolysaccharide (Figure 4A–4E). M1 marker gene (Tnf, Nos2, Il6, Ccl2, and Ccl5) expression levels were highly induced by exposure to IFNγ-LPS, and IGF1R deficiency further enhanced the expression, implying enhanced
M1 activation in MΦ-IGF1R-KO macrophages. On the other hand, IL-4–induced gene expression levels (ie, M2 activation markers; Arg1, Mrc1, and Pparg) were not influenced by IGF1R deficiency (Figure 4F–4H). It has been reported that oxidatively modified LDL (oxLDL) and that oxidized lipid components of oxLDL alter macrophage activation status or induce a distinctive activation status. Hemeoxygenase-1 (Hmox1) and thioredoxin reductase-1 (Txnrd1) are signature genes that have been shown to be upregulated in macrophages exposed to oxidized phospholipid, leading to a polarization status of Mox. In fact, oxLDL exposure enhanced Hmox1 and Txnrd1 expression significantly (Figure 5A and 5B). Intriguingly, IGF1R deficiency did not influence Txnrd1 mRNA levels (Figure 5B) but significantly upregulated Hmox1 mRNA levels (Figure 5A). We further assessed whether IGF1R deficiency influences the effect of oxLDL on expression levels of macrophage activation marker genes (Figure 5). With regard to M1 activation markers, oxLDL by

M1 activation in MΦ-IGF1R-KO macrophages. On the other hand, IL-4–induced gene expression levels (ie, M2 activation markers; Arg1, Mrc1, and Pparg) were not influenced by IGF1R deficiency (Figure 4F–4H). It has been reported that oxidatively modified LDL (oxLDL) and that oxidized lipid components of oxLDL alter macrophage activation status or induce a distinctive activation status. Hemeoxygenase-1 (Hmox1) and thioredoxin reductase-1 (Txnrd1) are signature genes that have been shown to be upregulated in macrophages exposed to oxidized phospholipid, leading to a polarization status of Mox. In fact, oxLDL exposure enhanced Hmox1 and Txnrd1 expression significantly (Figure 5A and 5B). Intriguingly, IGF1R deficiency did not influence Txnrd1 mRNA levels (Figure 5B) but significantly upregulated Hmox1 mRNA levels (Figure 5A). We further assessed whether IGF1R deficiency influences the effect of oxLDL on expression levels of macrophage activation marker genes (Figure 5). With regard to M1 activation markers, oxLDL by
itself did not alter mRNA levels in IGF1R-flox cells (Tnf, Nos2, Il6, Ccl2, and Ccl5; Figure 5C–5G); however, in MΦ-IGF1R-KO cells, in which these mRNA levels were significantly elevated compared with IGF1R-flox cells, oxLDL significantly further upregulated Tnf, Nos2, Il6, and Ccl2 mRNA levels (Figure 5C–5F), whereas Ccl5 mRNA was suppressed (Figure 5G). On the other hand, of the M2 activation markers, Mrc1 mRNA levels were moderately upregulated by oxLDL (Figure 5I), whereas Arg1 and Pparg were not affected by oxLDL (Figure 5H and 5J). Although there were modestly lower Arg1 mRNA levels in IL-4/oxLDL–treated MΦ-IGF1R-KO cells than in IGF1R-flox cells, overall, oxLDL or IGF1R deficiency did not robustly alter M2 marker levels. These data suggested that IGF1R deficiency influenced macrophage polarization, namely enhancing the proinflammatory M1 phenotype as evoked by IFNγ and oxLDL stimulation; however, the effect is not entirely classic activation, as implicated by Ccl5 downregulation (Figure 5G).

These results prompted us to examine whether Mox marker gene expression was altered by IGF1R deficiency in IFNγ-primed macrophages. IFNγ suppressed Hmox1 and Txnrd1 mRNA levels in IGF1R-flox macrophages (Figure 5A and 5B); however, in MΦ-IGF1R-KO cells, IFNγ exerted the opposite effect, leading to an upregulation of Hmox1 mRNA (Figure 5A). IFNγ suppressed Tnnd1 mRNA levels in IGF1R-deficient macrophages but to a lesser extent than in IGF1R-flox cells, resulting in higher expression levels compared with IGF1R-flox cells (Figure 5B).
Efferocytosis, a process by which apoptotic cells are removed by phagocytosis, is considered a significant mechanism involved in the resolution of inflammation. We evaluated expression levels of genes involved in efferocytosis (Figure V in the online-only Data Supplement). M1 activation suppressed the expression of efferocytosis-related genes \( \text{Anxa1}, \text{Gas6}, \text{C1qa}, \text{and Mfge8} \), and M2 activation had no effect \( \text{Anxa1, C1qa, Mertk} \); Figure V in the online-only Data Supplement) or downregulated \( \text{Gas6, Mfge8} \) gene expression levels. IGF1R deficiency did not alter these mRNA levels in either activated or nonactivated cells. OxLDL by itself did not induce obvious effects except for an upregulation of \( \text{Mertk} \) mRNA levels (Figure VD in the online-only Data Supplement). IGF1R deficiency upregulated \( \text{Anxa1} \) and \( \text{Mfge8} \) in the presence of oxLDL (Figure V A and VE in the online-only Data Supplement), but it caused a trend to downregulation of \( \text{C1qa} \) (Figure VC in the online-only Data Supplement). Thus, IGF1R deficiency did not induce changes suggesting enhanced/reduced efferocytosis.

**Lipid Internalization and Efflux**

Macrophage internalization and accumulation of modified LDL lead to foam cell formation, which is a hallmark of atheroma formation. Macrophage lipid internalization was assessed by exposing cells to oxLDL and acetylated LDL (Figure 6A and 6B). Macrophages were exposed to IFN\( \gamma \)/lipopolysaccharide (M1 activation) or IL-4 (M2 activation) and then tested for lipid incorporation after exposure to modified LDL for 48 hours. M1 cells internalized far smaller amounts of lipid compared with the nonactivated cells or M2 cells (Figure 6A and 6B), whereas M2-activated cells incorporated more lipid than untreated cells when they were exposed to acetylated LDL (Figure 6A). IGF1R deficiency did not alter lipid incorporation (Figure 6A and 6B), regardless of activation status and exposure to acetylated LDL or oxLDL.

Cholesterol efflux from cells to extracellular lipid acceptors also contributes to lipid accumulation and thus foam cell formation. Thus, activated and lipid-laden macrophages were tested for cholesterol efflux activity (Figure 6C and 6D). IGF1R deficiency markedly reduced apolipoprotein AI–dependent cholesterol efflux in M1-activated cells (Figure 6C). We measured HDL-dependent cholesterol efflux in nonactivated, M1-activated, and M2-activated cells (Figure 6D) and found that IGF1R deficiency caused a small but significant reduction in cholesterol efflux to HDL (Figure 6D). To gain insights into potential mechanisms, we assessed expression levels of ABCA1, ABCG1, and SRB1, which are major lipid transporters responsible for cholesterol efflux. They were differentially regulated by IGF1R deficiency (Figure 7); ABCG1 expression levels were downregulated by \( \approx 50\% \) in IGF1R-deficient macrophages, regardless of activation status (Figure 7B and 7D), whereas ABCA1 and SRB1 were not regulated. To investigate ABCG1 expression in MΦ-IGF1R-KO cells, we exposed the cells to ligands of LXR, which is a major regulator of ABCG1
gene expression.40 LXR agonist GW3965 or T0901317 induced ABCG1 and ABCA1 expression in IGF1R-flox macrophages but not in MΦ-IGF1R-KO cells (Figure 7E). Thus, LXR-dependent regulation of ABCG1 expression is compromised by IGF1R deficiency, potentially accounting for downregulation of ABCG1 in MΦ-IGF1R-KO cells.

Monocyte Recruitment, Macrophage Proliferation, and Apoptosis in MΦ-IGF1R-KO Mice

Because MΦ-IGF1R-KO caused an increase in Mac3 positivity within plaques, we evaluated monocyte recruitment to lesions, macrophage proliferation,44 and macrophage apoptosis,3 major determinants of macrophage number in lesions, in plaques.42 To evaluate monocyte recruitment, we labeled circulating monocytes in vivo by intravenous administration of fluorescent microspheres.43 The microspheres are not capable of penetrating into tissue interstitial space; thus, red fluorescence–positive cells (identified by DAPI positivity) within plaque are considered recruited and infiltrated cells. We evaluated red microsphere labeling by flow cytometry. As described previously,16 intravenous injections of red microspheres labeled the Ly6C+ population among circulating monocytes (Figure VIA in the online-only Data Supplement), whereas clodronate administration 1 day before the injection of red microspheres introduced the label to Ly6C+ monocytes (Figure VIB in the online-only Data Supplement). As shown in Figure 8, we detected a significantly larger number of red fluorescence–positive cells in plaques from MΦ-IGF1R-KO mice both without (Figure 8C) and with (Figure 8D) clodronate administration. It is noteworthy that more red microsphere–positive cells are detected after clodronate administration, indicating that Ly6C+ monocytes are the dominant subpopulation to be recruited to lesions.35 To assess leukocyte adhesion and rolling on the luminal side of the endothelium in vivo, we performed intravital microscopy to detect CD11b+ positive cells in the mesenteric circulation. MΦ-IGF1R-KO significantly increased CD11b+ leukocyte adhesion (Figure 8E) and rolling (Figure 8F) on the luminal surface of the endothelium, consistent with increased recruitment of CD11b+ cells, that is, monocytes and neutrophils. Because IGF1R expression was undetectable in neutrophils (Figure 1 in the online-only Data Supplement), it is unlikely that IGF1R gene deletion in neutrophils contributed significantly to these findings.

To assess proliferation activity of macrophages in plaques, we assessed Ki67 and proliferating cell nuclear antigen gene expression levels44–46 (Figure VIC in the online-only Data Supplement). Macrophage Marker (SC-101447; a monoclonal antibody raised against isolated macrophages of mouse origin; Santa Cruz)77,78 –positive plaque area was laser dissected from aortic root for RNA isolation, followed by quantitative reverse transcription–polymerase chain reaction. Equal amplification of CD68 was confirmed between IGF1R-flox and MΦ-IGF1R-KO lesions. Macrophage-rich regions from MΦ-IGF1R-KO plaques expressed the same levels of Ki67 or proliferating cell nuclear antigen compared with IGF1R-flox (Figure VIC in the online-only Data Supplement), suggesting no difference in macrophage proliferation in plaques. Terminal deoxynucleotidyl transferase dUTP nick-end labeling/Mac3 double-positive cell numbers were not different (Figure VID in the online-only Data Supplement). Taken together, our data suggest that IGF1R deficiency enhances monocyte recruitment to lesions, thereby increasing the macrophage cell population.

OxLDL Downregulates IGF1R in Macrophages

OxLDL plays a critical role in atherogenesis, and we have previously shown that oxLDL downregulates IGF1R in vascular smooth muscle cells.49 To determine potential regulation of macrophage IGF1R expression by oxLDL, we used the human mononuclear cell line THP-1, which was differentiated into macrophages and exposed to 100 µg/mL oxLDL (Figure VII in the online-only Data Supplement). OxLDL exposure for 24 hours downregulated IGF1R levels by 80%.

Discussion

IGF-1 production has been documented in macrophages8,9,50,51; however, precise effects of IGF-1 in macrophages in relation to the pathogenesis of atherosclerosis have not been elucidated. In this study, we generated macrophage/monocyte–specific IGF1R-deficient mice and discovered pivotal roles of IGF-1 in the regulation of inflammatory responses and lipid handling in macrophages, which are relevant to the antiatherogenic effects of IGF-1. Our data indicate that IGF1R deficiency in macrophages enhances proinflammatory activation (ie, M1 polarization), thereby promoting proinflammatory cytokine production (Figures 3 and 4), and enhances lipid accumulation as a result of reduced efflux (Figures 6 and 7). These phenotypic changes resulted in increased recruitment of macrophages to atherosclerotic plaques and increased atherosclerotic burden in MΦ-IGF1R-KO mice (Figures 1 and 2). Moreover, histological evaluation of brachiocephalic arteries indicated that the MΦ-IGF1R-KO induced features of unstable plaques (Table).

There is growing interest in the role of IGF-1 in cardiovascular disease. Low circulating IGF-1 levels have been associated with cardiovascular disease risk factors,32–36 and in particular, there is growing evidence for a role for IGF-1 deficiency in the pathogenesis of metabolic syndrome.57 Acromegaly (ie, excessive growth hormone and IGF-1) or otherwise growth hormone and IGF-1 deficiency have been linked to cardiovascular complications.58,59 However, epidemiological studies linking IGF-1 with cardiovascular disease report mixed results. Some cross-sectional and prospective studies60–65 suggest a positive association between IGF-1 and cardiovascular diseases, but others have found that low IGF-1 is a predictor of ischemic heart disease and mortality, consistent with the potential anti-apoptotic, antioxidant, and plaque stabilization effects of IGF-1.1–3,13,66–72 Methodological constraints could explain these contradictions because measurement of total IGF-1 levels represents only a crude estimate of the biologically active IGF-1. Thus, an IGF-1–specific kinase receptor activation assay may better reflect IGF-1 bioactivity.73 In fact, it has been reported that higher IGF-1 bioactivity is associated with significantly longer survival in subjects with a high inflammatory risk profile or history of cardiovascular disease.73 Additionally, polymorphisms in the IGF-1 gene promoter region that influence circulating IGF-1 levels have been
The alleles that indicate lower circulating IGF-1 levels have been associated with increased risk for type 2 diabetes mellitus, myocardial infarction, left ventricular hypertrophy, higher carotid intima-media thickness, higher aortic pulse-wave velocity, and lower endothelium-dependent vasodilation. Intriguingly, IGF-1 resistance in the endothelium was reported in an animal model of obesity, thereby blunting the vasodilatory response to IGF-1 via attenuated endothelial nitric oxide synthase phosphorylation and nitric oxide production. Our present finding that reduced macrophage IGF-1 signaling is highly proinflammatory and increases atherosclerotic burden is consistent with the growing evidence that decreased IGF-1 action may be a significant contributor to the pathogenesis of atherosclerosis. Of note, we found that oxLDL downregulates IGF1R levels in human-derived THP1 macrophage (Figure VII in the online-only Data Supplement), which is consistent with our previous reports that both IGF-1 and IGF1R expression were significantly lower in human atherosclerotic plaque intimal regions with macrophage infiltration, where oxLDL was highly detected.

We used Mφ-IGF1R-KO mice, which have 1 allele of Lys2 ablated; the IGF1R-flox mice (control) have both alleles intact. Previous studies in LysCre+ mice with regard to potential alterations in monocyte/macrophage biology caused by the hemizygous deficiency of Lys2 showed no evidence of a heterozygous phenotype. In addition, complete ablation of both alleles of Lys2 does not influence atherosclerosis in Apoe−/− mice. Lys2-cre-mediated gene excision occurs in monocytes and neutrophils. Confirmed deletion of exon 3 of the IGF1r gene in both macrophages and neutrophils isolated from the Mφ-IGF1R-KO mouse, but we were unable to detect IGF1R protein even in IGF1r-normal neutrophils (ie, IGF1R-flox neutrophils), indicating extremely low expression levels. Thus, although contribution of neutrophils to the phenotype of Mφ-IGF1R-KO mice cannot be excluded, it appears much more likely that macrophages, which express significant levels of IGF1R, play the predominant role.

IGF1R and InsR are structurally similar and form a heteromeric hybrid receptor consisting of α+/β+ subunits of IGF1R and α+/β+ subunits of InsR. The hybrid receptor binds IGF-1 with high affinity but does not bind insulin at physiological ranges. To the best of our knowledge, this is the first study evaluating the expression ratio between IGF1R and InsR in macrophages, showing predominant expression of InsR. Thus, IGF1R-flox macrophages (expressing both IGF1R and InsR) responded to a physiological dose of insulin (Figure I in the online-only Data Supplement). Intriguingly, IGF1R deficiency in macrophages, although it should free up InsR hemidimers to form holoreceptors, did not increase insulin signaling. This finding is relevant because macrophage InsR deficiency has been shown to modulate atherosclerosis development (although results have been contradictory). Because we found that Mφ-IGF1R-KO did not alter insulin signaling in macrophages, it is unlikely that changes in insulin action on macrophages plays a significant role in the phenotype of Mφ-IGF1R-KO mice.

Macrophages become activated as they infiltrate into a target tissue and are exposed to stimuli, expressing a highly proinflammatory phenotype or a less inflammatory but phagocytic and antigen-presenting phenotype. The former status was called classic activation or M1 activation; the latter was referred to as alternative activation or M2 activation. Recent investigations indicate, however, that macrophage activation status likely encompasses a broad spectrum in which M1 and M2 activation are likely 2 extremes. Recognizing that macrophage activation represents a continuum, we tested M1 and M2 activation, as well as Mox activation, which was described as a unique activation status found in plaque macrophages, to provide insights into the effects of IGF1R deficiency. Our results indicate that IGF1R deficiency resulted in macrophages acquiring a highly inflammatory (ie, M1) phenotype (Figure 3), whereas M2 marker gene expression levels were not altered (Figure 4F–4H). However, Mφ-IGF1R-KO macrophages are not simply skewed to a more inflammatory phenotype; these cells were also shifted to a phenotype induced by oxLDL, characterized by upregulation of the antioxidant genes Hmox1 and Tnnd1, described as Mox activation (Figure 5A and 5B). Reports of the effects of oxLDL and its specific lipid moieties on macrophage activation are variable and include enhanced of the inflammatory phenotype, induction of M2 activation, or a unique activation status. In summary, our results suggest that IGF1R deficiency skews macrophages to a unique activation state that can be characterized by enhanced proinflammatory response and elevated antioxidant system.

The effect of IGF1R deficiency on macrophage activation status prompted us to examine how IGF1R deficiency influences uptake of modified LDL, cholesterol efflux, and effrocytosis. It has been shown that M1-activated macrophages demonstrate reduced foam cell formation in response to oxLDL and that Mox-activated macrophages have attenuated phagocytosis and effrocytosis. It would thus have been reasonable to speculate that IGF1R deficiency, which enhances the proinflammatory phenotype and Mox marker expression levels, could lead to reduced foam cell formation. However, our results showed no evidence of altered acetylated LDL or oxLDL uptake by IGF1R-deficient macrophages (Figure 6A and 6B). We also assessed expression levels of genes that are functional in effrocytosis, but there was no apparent alteration caused by IGF1R deficiency (Figure V in the online-only Data Supplement). Thus, despite enhanced inflammatory responses and redox gene expression, IGF1R-deficient macrophages do not appear to have impairment in modified lipid uptake or effrocytosis. However, further assessment of lipid handling by IGF1R-deficient macrophages indicated that cholesterol efflux is impaired by IGF1R deficiency (Figure 6C and 6D), potentially promoting foam cell formation. Cholesterol efflux is mediated by the lipid transporters ABCA1, ABCG1, and SRB1. IGF1R deficiency caused lower expression levels of ABCG1 (Figure 7), which should, at least in part, account for lowered cholesterol efflux. Intriguingly, the LXR-dependent regulation of ABCG1 expression was compromised in Mφ-IGF1R-KO macrophages. LXR is a major regulator of ABCG1 expression. In fact, LXR drives ABCG1 expression on lipid loading by modified LDL (which causes accumulation of oxysterols). Thus, impaired LXR regulation of ABCG1 expression could be an important mechanism underlying lowered cholesterol efflux in Mφ-IGF1R-KO macrophages.
MΦ-IGF1R-KO increased atherosclerosis burden as assessed by Oil Red O staining of en face aortas and by histological analysis of aortic root cross sections (Figure 1). These mice had an increase in lesional macrophages and in recruitment of monocytes to atherosclerotic plaques (Figure 8). Consistent with the increased recruitment of monocytes, chemokine expression levels were upregulated in MΦ-IGF1R-KO macrophages (ie, MCP-1 and fractalkine; Figures 3–5). Moreover, MΦ-IGF1R-KO increased features of plaque vulnerability, as evidenced by histological features of intraplaque hemorrhage and medial elastin breaks in lesions from brachiocephalic artery (Figure IV in the online-only Data Supplement and Table). These findings were in accordance with the changes in plaque composition, notably the increased population of macrophages, the reduced population of smooth muscle cells particularly within the plaque cap, and decreased collagen (Figure 2). To obtain insights into the underlying mechanisms, we assessed MMP levels in peritoneal macrophages from MΦ-IGF1R-KO mice and showed significant upregulation of MMPs (Figure 2D), which have previously been shown to be relevant to atherogenesis and plaque vulnerability.96,97 In addition, we found significant upregulation of MMP-1, -2, -8, and -9 in lysates of ascending aorta from MΦ-IGF1R-KO animals, consistent with data obtained with peritoneal macrophages. A major source of collagen matrix synthesis and deposition in atherosclerotic plaques is smooth muscle cells, and thus, one can speculate that the enhanced inflammatory milieu induced by MΦ-IGF1R-KO altered smooth muscle cell homeostasis. In fact, it is noteworthy that MΦ-IGF1R-KO robustly enhanced macrophage production of proinflammatory cytokines such as IL-1α, TNFα, and IL-6 (Figure 3), and it has been reported that cytokines such as TNFα reduce IGF-1 and increase IGF binding protein-3 in vascular smooth muscle cells, leading to a reduction in bioactive IGF-1.98 IGF-1 positively regulates collagen synthesis by smooth muscle cells.99 Indeed, we have recently shown that increased IGF-1 signaling in vascular smooth muscle cells increases features of plaque stability, as determined by increased fibrous cap area, α-smooth muscle actin–positive smooth muscle cells, and collagen content, without affecting plaque burden,6 potentially mediated by IGF-1 induction of smooth muscle differentiation10 and collagen synthesis.100 Thus, it seems reasonable to speculate that increased production of inflammatory cytokines in MΦ-IGF1R-KO mice disrupts normal IGF-1 signaling in smooth muscle cells and reduces collagen deposition in plaques of MΦ-IGF1R-KO mice. Taken together with our previous results, the present study provides insights into potential interactions between macrophages and smooth muscle cells; that is, the enhanced inflammatory milieu by macrophages suppresses IGF-1 signaling in smooth muscle cells, leading to a reduction of plaque stability.

Our results are consistent with the growing body of experimental evidence that IGF1 has antiatherogenic effects. Infusion of IGF-1 in Apoe−/− mice reduces atherosclerotic burden,4 and overexpression of IGF-1 in vascular smooth muscle cells increases plaque collagen content and smooth muscle cell levels and reduces necrotic core size.6 IGF-1 has been shown to have antioxidant effects on the endothelium via upregulation of glutathione peroxidase levels.95 Our current findings demonstrate that IGF-1 signaling has a major effect on macrophage biology that is critical for atherogenesis. However, a limitation of experimental studies to date has been that they have been performed in murine models and that studies in larger animals phylogenetically closer to humans are lacking. Such studies will be important for the development of IGF-1–based therapeutic strategies.

Conclusions

We have shown that IGF1R deficiency in macrophages of Apoe−/− mice increases atherosclerotic burden and changes plaque composition to one of lowered smooth muscle cell and collagen content. Our data suggest that the loss of IGF-1 signaling skews macrophage activation to a proinflammatory status and promotes lipid accumulation in macrophages by lowering lipid efflux. There is increasing evidence linking low IGF-1 to multiple cardiovascular risk factors, including metabolic syndrome and aging.77,78 In fact, it has been reported that decreased IGF-1 bioavailability is an adverse prognostic factor for coronary heart disease (reviewed elsewhere89,90). Our findings herein suggesting that IGF-1 regulates macrophage inflammatory responses and lipid metabolism may provide the basis for a novel therapeutic approach for the treatment of atherosclerotic vascular disease development and progression.

Acknowledgments

We thank Chelsea Deroche, PhD (Biostatistics and Research Design Unit in the Office of Medical Research and Health Management and Informatics, University of Missouri School of Medicine), for her expert advice on statistical assessments.

Sources of Funding

This work was supported by National Institutes of Health grants R01-HL070241 (Dr Delafontaine), R01-HL080682 (Dr Delafontaine), R21-HL113705 (Dr Sukhanov), R01-HL059976 (Dr Korthuis), P01-HL095486 (Dr Korthuis), and R01-AA022108 (Dr Korthuis), as well as American Heart Association Grant-in-Aid 13GRNT17230069 (Dr Sukhanov).

Disclosures

None.

References

5. Sukhanov S, Higashi Y, Shai SY, Vaughn C, Mohler J, Li Y, Song YH, Titterington J, Delafontaine P. IGF-1 reduces inflammatory responses, suppresses oxidative stress, and decreases atherosclerosis progression...
2690. doi: 10.1161/ATVBAHA.111.304298.
1924. doi: 10.1161/ATVBAHA.110.210831.
8. Renier G, Clément I, Desfais AC, Lambert A. Direct stimulatory effect of insulin-like growth factor I on monocyte and macrophage tumor necro-
9. Hochberg Z, Hertz P, Maor G, Oktine J, Aviram M. Growth hormone and insulin-like growth factor I increase macrophage uptake and degrada-

## References

16. Carstairs KC. The identification of platelets and platelet antigens in histo-

## References

18. Fratallì AL, Pessin JE. Relationship between alpha subunit ligand occu-
24. Carstairs KC. The identification of platelets and platelet antigens in histo-

## References

26. Fratallì AL, Pessin JE. Relationship between alpha subunit ligand occu-
Atherosclerosis is an inflammatory disease, and acute coronary events result largely from erosion or rupture of unstable plaques with increased inflammatory cells and a relative reduction in vascular smooth muscle cells. Macrophages play a major role in atherogenesis by scavenging and accumulating lipids to become lipid-laden foam cells. Furthermore, proinflammatory macrophages induce smooth muscle cell death by secreting cytokines and degrade extracellular matrix by producing enzymes such as matrix metalloproteinases, weakening the tensile strength of plaques and predisposing them to rupture. In the past decade, insulin-like growth factor-1 (IGF-1) has demonstrated antiatherogenic effects in experimental and clinical models, but the mechanisms are poorly elucidated. In this study, we investigated macrophage–IGF-1 receptor deficiency in a murine model of atherosclerosis. We found that IGF-1 receptor deficiency increased monocyte/macrophage recruitment to the atherosclerotic lesion and promoted plaque instability.

**CLINICAL PERSPECTIVE**

Atherosclerosis is an inflammatory disease, and acute coronary events result largely from erosion or rupture of unstable plaques with increased inflammatory cells and a relative reduction in vascular smooth muscle cells. Macrophages play a major role in atherogenesis by scavenging and accumulating lipids to become lipid-laden foam cells. Furthermore, proinflammatory macrophages induce smooth muscle cell death by secreting cytokines and degrade extracellular matrix by producing enzymes such as matrix metalloproteinases, weakening the tensile strength of plaques and predisposing them to rupture. In the past decade, insulin-like growth factor-1 (IGF-1) has demonstrated antiatherogenic effects in experimental and clinical models, but the mechanisms are poorly elucidated. In this study, we investigated macrophage–IGF-1 receptor deficiency in a murine model of atherosclerosis. We found that IGF-1 receptor deficiency increased monocyte/macrophage recruitment to the atherosclerotic lesion and promoted plaque instability.

**REFERENCES**


