Defective Leptin/Leptin Receptor Signaling Improves Regulatory T Cell Immune Response and Protects Mice From Atherosclerosis

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Objective—Obesity is a major risk factor for atherosclerosis and is associated with increased cardiovascular morbidity and mortality. However, the precise molecular pathways responsible for this close association remain poorly understood.

Methods and Results—In this study, we report that leptin-deficiency (ob/ob) in low-density lipoprotein receptor knockout (ldlr−/−) mice induces an unexpected 2.2- to 6-fold reduction in atherosclerotic lesion development, compared with ldlr−/− mice having similar total cholesterol levels. Ldlr−/−/ob/ob mice show reduced T cell helper type 1 (Th1) response, enhanced expression of Foxp3, the specification transcription factor of regulatory T (Treg) cells, and improved Treg cell function. Leptin receptor-deficient (db/db) mice display marked increase in the number and suppressive function of Treg cells. Supplementation of Treg-deficient lymphocytes with Treg cells from db/db mice in an experimental model of atherosclerosis induces a significant reduction of lesion size and a marked inhibition of interferon (INF)-γ production, compared with supplementation by Treg cells from wild-type mice.

Conclusions—These results identify a critical role for leptin/leptin receptor pathway in the modulation of the regulatory immune response in atherosclerosis, and suggest that alteration in regulatory immunity may predispose obese individuals to atherosclerosis. (Arterioscler Thromb Vasc Biol. 2007;27:2691-2698.)

Key Words: leptin ■ obesity ■ metabolic syndrome ■ atherosclerosis ■ immunity

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Obesity, characterized by an excess of adipose tissue mass, is closely associated with an increase in cardiovascular morbidity and mortality attributable to atherosclerosis.1 Obesity is a major underlying risk factor for atherosclerosis through its association/promotion of other major risk factors for the disease, namely dyslipidemia, hypertension, and hyperglycemia. Given the increasing prevalence of obesity and the metabolic syndrome worldwide, there is an urgent need for a better understanding of the molecular mechanisms linking these conditions to atherosclerotic disease.

Our current understanding of the pathophysiology of atherosclerosis suggests a prominent role for the immunoinflammatory response in disease development, progression, and complications.2-4 Recent studies have provided both at the cellular and molecular levels, a substantial evidence that obesity is a chronic, low-grade inflammatory disease,5 suggesting that inflammation may affects the relationship between obesity/metabolic syndrome and atherosclerotic disease. However, the precise molecular mechanisms responsible for this low-grade inflammation and their roles in the development of atherosclerosis remain poorly understood.

Leptin, a cytokine-like hormone encoded by ob gene, is expressed most abundantly in adipocytes and its circulating concentrations rise with increasing adiposity.6 Leptin is primarily involved in the regulation of food intake and energy expenditure.7,8 Besides, several studies have described direct leptin effects on immune cells, including the promotion of T lymphocyte type 1 helper (Th1) response,9,10 of potential importance to the process of atherosclerosis.2,3 More recently, leptin has been involved in the susceptibility to autoimmune diseases, which are characterized by subtle alterations of the regulatory T cell (Treg) response.11-13 However, whether defective leptin/leptin receptor signaling in Treg cells may affect their suppressive response and alter the development of immunoinflammatory diseases is still unknown. In the present study, we focused on the role of leptin in the modulation of the Treg cell response in atherosclerosis.

Materials and Methods

Animals

C57BL/6 ldlr−/−/ob/ob mice and ldlr−/− littermates were obtained as previously described.14 Ldlr−/−/ob/ob mice already display more than...
3-fold increase in plasma cholesterol levels at 6 weeks of age compared with ldlr–/– mice on chow diet, and show a rapid increase of cholesterol levels with age to reach a maximum from 12 weeks to 20 weeks of age. This spontaneous increase in cholesterol levels in ldlr–/–/lob/lob mice under chow diet closely reproduces the well-known increase in cholesterol levels when ldlr–/– mice are put under high-fat, high-cholesterol diet. Thus, we subjected 6-week-old ldlr–/– mice to a high-fat diet (1.25% cholesterol, 18% cacao butter, 0% cholate) for 12 weeks, to obtain the same range of total cholesterol as in ldlr–/–/lob/lob mice under chow diet. All mice were euthanized at 18 weeks of age. In a second experiment, 12-week-old female Apoe–/–/Rag2–/– mice were transferred with 8 × 10⁶ of Cd28-deficient (Treg cell deficient) splenocytes from C57BL/6CD28–/– female mice supplemented with 0.5 × 10⁶ CD4⁺CD25 Treg cells isolated from either wild-type mice (10-week-old C57BL/6 female mice) or 10-week-old C57BL/6 db/db mice (Charles River, Lyon, France). The mice were put on 18% fat, 1.25% cholesterol, and 0% cholate for 6 weeks.

**Size and Composition of Atherosclerotic Lesions**

These studies were performed as previously described. Briefly, mice were anesthetized with isoflurane before sacrifice. Fasting total cholesterol and triglycerides were measured with standard enzymatic assays (Boehringer Mannheim). After separation of VLDDL, LDL, and HDL fractions by gel filtration, free cholesterol and cholesterol ester levels in these fractions were determined by high-performance liquid chromatography (HPLC), as described before. The hearts were taken off, fixed in 4% paraformaldehyde for 2 hours; then they were placed in a PBS sucrose 30% solution overnight at 4°C, before being included in a cutting medium and frozen at −70°C. Successive 10-μm transversal sections of aortic sinus were obtained. Lipids and collagen were detected using Oil-red O and Sirius Red colorations, respectively. Plaque composition was determined by use of a monoclonal rat anti-mouse macrophage antibody (clone MOMA-2 MAB1852, Chemicon), a polyclonal goat anti-CD3ε antibody (DAKO) and an anti-Foxp3 antibody (clone FJK-16s, eBioscience).

Lesion size in aortic sinus represents the whole intimal surface.

**Flow Cytometry**

Splenocytes were labeled with fluorescein isothiocyanate (FITC)- conjugated anti-CD4 (GB1.5 clone miltenyi), biotin-conjugated anti-CD25 (PC61, Pharmingen), and then analyzed by flow cytometry on an Epics XL flow cytometer (Beckman Coulter). Intracellular Foxp3 staining was performed using PE-conjugated anti-mouse/rat Foxp3 (PFIK-16s, eBioscience) according to manufacturer’s instructions (eBioscience).

**Real-Time Polymerase Chain Reaction Analysis**

Total RNA from spleen was isolated using Trizol reagent (Invitrogen). Primer sequences for Foxp3, Ifn-γ and Tgf-β are respectively: Forward: 5'- TTGTGCGGTAGTGGCATTCTC-3'; Reverse: 5'- agcaacaacagg-gcggaaag -3'; Forward: 5'- cggctgtgctgtgtgtgta -3'; Reverse: 5'- GCAACATGTTAATCTACAGAA-3'; Reverse: 5'- AGCTGAAAAAGCCGACTCA-3'. The primers were purchased from Invitrogen. The real time PCR was performed on an ABI prism 7700 using Taqman Universal PCR master mix (Applied Biosystems) in triplicates. CT for GAPDH was used to normalize the gene expression of samples.

**Statistical Analysis**

Values are expressed as means±SEM. Differences between values were examined using nonparametric Mann–Whitney test, and were considered significant at P<0.05.

**Results**

**Leptin Deficiency Protects ldlr–/– Mice From Atherosclerosis**

Marked acceleration of atherosclerosis has been observed in ob/ob mice under ldlr–/– background. However, this was always associated with severe hypercholesterolemia, high triglyceride levels, and insulin resistance. Because cholesterol level is a major determinant of atherosclerosis, we first examined whether acceleration of atherosclerosis in leptin-deficient mice could be fully, or in part, explained by their high plasma cholesterol levels. We compared atherosclerotic lesion development between ldlr–/–/ob/ob mice put on chow diet and ldlr–/– mice put on high-fat diet to induce hypercholesterolemia. Mice were euthanized at 18 weeks of age. Ldlr–/–/ob/ob showed severe hypertriglyceridemia, hyperglycemia, and increased plasma insulin levels compared with ldlr–/– mice on high-fat diet (Figure 1). As expected, ldlr–/–/lob/ob mice also showed severe hypercholesterolemia (Figure 1). However, ldlr–/–/lob/ob mice on chow diet and ldlr–/– mice put on high-fat diet showed similar plasma total cholesterol levels (Figure 1). VLDL (13.2±1.8 versus 12.8±2.4 g/L, respectively, P=0.85) and LDL levels (7.3±0.9 versus 5.8±0.7 g/L, respectively, P=0.13). Interestingly, under these conditions, ldlr–/–/lob/ob mice (n=9) showed an unexpected 6-fold reduction in lesion development at the aortic sinus, compared with ldlr–/– mice (n=16; Figure 1), suggesting a profound protective effect of leptin deficiency in atherosclerosis, despite lower HDL-cholesterol levels, persistent hypertriglyceridemia, and insulin resistance (Figure 1). Reduction in atherosclerosis was associated with reduction in macrophage infiltration and lower collagen content, consistent with the phenotype of early atheromata (Amersham) for the last 18 hour of culture. Thymidine incorporation was assessed using a TopCount NXT scintillation counter (Perkin Elmer).
Thoracic aortas were available in some animals for analysis of the extent of atherosclerosis. We found that leptin deficiency also protected from lesion development at this atherosclerosis-prone site. Oil Red O–positive areas occupied 25.3 ± 2.4% of total aortic area in ldlr−/− mice (n = 6) compared with 11.4 ± 2.3% in ldlr−/−/ob/ob mice (n = 4; P < 0.01).

**Leptin Deficiency Improves Treg Cell Function in ldlr−/− Mice**

We next examined the potential antiatherogenic mechanisms induced by leptin deficiency. Cholesterol-independent reduction of atherosclerosis in leptin-deficient mice suggested a role for leptin in the modulation of the immune response. Consistent with this hypothesis, we found a marked increase in spleen mRNA levels of Foxp3 (Figure 2a), the transcription factor required for the commitment of T lymphocytes to the Treg cell lineage, along with an increase in Tgf-β mRNA levels (Figure 2b) but no change in Il-10 expression (data not shown). We next directly assessed Treg cell function in a well-validated coculture assay. Interestingly, we found a significant increase in the suppressive potential of Treg cells on the proliferation of effector T cells when the cells were recovered from ldlr−/−/ob/ob mice (Figure 2b). Similar results were obtained in ob/ob mice on ldlr−/− background (data not shown), suggesting that the effect of leptin on Treg function is independent of metabolic abnormalities. Of note, improved Treg cell response in ldlr−/−/ob/ob mice was associated with a marked reduction of Ifn-γ production by CD4+ T cells (Figure 2), suggesting inhibition of Th1-mediated pro-atherogenic immunity.

**Increased Number and Suppressive Potential of Treg Cells in Leptin Receptor–Deficient Mice**

In order to further address the role of leptin-dependent pathway in Treg cell response, we examined Treg cell number and function in db/db mice, deficient in leptin receptor. Interestingly, we found a marked increase in the number of CD4+CD25+Foxp3+ Treg cells in the spleen of...
compared with wild-type mice (Figure 3a and 3b), suggesting enhanced survival or proliferation of Treg cells in vivo, in the absence of leptin receptor–dependent signaling. We next purified CD4$^{+}$/H11001 CD25$^{-}$/H11001 Treg cells and CD4$^{+}$/H11001 CD25$^{+}$/H11001 effector T cells and assessed their proliferative potential in vitro in response to CD3 stimulation, in the presence of dendritic cells. As expected, CD4$^{+}$/H11001 CD25$^{+}$/H11001 effector T cells proliferated vigorously in vitro, and CD4$^{+}$/H11001 CD25$^{-}$/H11001 Treg cells were anergic (supplemental Figure I, available online at http://atvb.ahajournals.org). Leptin receptor deficiency did not affect CD4$^{+}$/H11001 CD25$^{+}$/H11001 proliferation, nor it reversed the anergic state of CD4$^{+}$/H11001 CD25$^{-}$/H11001 Treg cells (supplemental Figure I). Interestingly, we found improved Treg suppressive function when the cells were recovered from db/db mice compared with wild-type mice (Figure 3c). Replacement of wild-type Treg cells by leptin receptor deficient Treg cells in the coculture experiment led to a better suppression of effector T cell proliferation, whereas replacement of db/db Treg cells by wild-type Treg resulted in reduced suppression (Figure 3d). Taken together, these results clearly show a critical role for leptin/leptin receptor pathway in Treg cell homeostasis and suppressive potential.

Defective Leptin Receptor Signaling in Treg Cells Reduces the Development of Atherosclerosis

Because db/db Treg cells showed increased suppressive potential, we examined their effect on lesion development in an experimental model of atherosclerosis. We hypothesized that replacement of wild-type Treg cells by Treg from db/db mice would inhibit lesion development. Thus, we supplemented Treg-deficient splenocytes recovered from Cd28$^{-}$/H11001 mice, by CD4$^{+}$/CD25$^{-}$/H11001 Treg cells purified from either wild-type or db/db mice, and examined their effect on lesion development in Apoe$^{-}$/H11001/Rag2$^{-}$/H11001 mice. We examined lesion development at the aortic sinus level, a site affected by immune deficiency. Remarkably, despite similar cholesterol levels, supplementation of Treg-deficient splenocytes with db/db Treg cells induced a significant reduction of lesion...
size compared with supplementation using wild-type Treg cells (Figure 4a and 4b). Atherosclerotic lesions were barely detectable in the thoracic aorta (data not shown). Reduction in lesion development was associated with a marked reduction of Ifn-γ expression in splenocytes in vivo (Figure 4c), suggesting enhanced suppression of pathogenic Th1 responses. Thus, leptin receptor signaling in Treg cells promotes lesion development in vivo.

Discussion

This study shows that defective leptin signaling increases the number and function of Treg cells, and is associated with reduced atherosclerotic lesion formation. Moreover, direct supplementation with Treg cells defective in leptin signaling reduces lesion development, unraveling a functional in vivo role for Treg cell-specific leptin signaling in the modulation of a major inflammatory disease.

Previous studies reported that leptin deficiency on atherosclerosis-susceptible ldlr−/− or Apoe−/− background resulted in the development of large atherosclerotic lesions, suggesting a protective role for leptin in atherosclerosis. We have recently argued against this hypothesis and suggested that lesion development in these mice should be compared with lesion development in ldlr−/− or Apoe−/− mice having equivalent cholesterol levels. In the present study, we provide direct evidence that leptin deficiency markedly reduces lesion development in mice, when comparison is made between animals with similar cholesterol levels. Our results are consistent with a very recent observational study showing that lesion size of leptin deficient hyperlipidemic mice (Apoe−/−/ob/ob mice) was significantly lower compared with Apoe−/− mice, when both groups were fed an atherogenic diet. In addition, leptin administration increases atherosclerotic lesion development in Apoe−/− mice. Our data are also consistent with clinical studies showing significant correlations between plasma leptin levels and cardiovascular disease, independently of body mass index and traditional risk factors.
In the present study, we also examined potential mechanisms that could account, at least in part, for the protective effect of leptin deficiency in atherosclerosis. We focused on leptin as a potential modulator of the immune response in atherosclerosis. Leptin directly affects the immune response and initial studies reported reversal of starvation-induced immunosuppression in vivo after leptin administration, associated with enhanced T cell proliferation and promotion of Th1 proinflammatory response. More recent studies clearly showed that lack or inhibition of leptin/leptin receptor pathway protects against the development of various immunoinflammatory diseases in experimental models, ranging from colitis to encephalomyelitis or diabetes. Protection was associated, at least in some experiments, with a shift of the cytokine profile toward increased Th2/Treg type, increased expression of Foxp3, and increased number of Treg cells in lymphoid organs of mice with defective leptin signaling. These studies were also consistent with clinical findings showing a switch from Th1 toward Th2/Treg cytokine profile in individuals with leptin or leptin receptor deficiency, and an inverse relationship between leptin secretion and the number of Treg cells in patients with multiple sclerosis. Thus, we hypothesized that improvement in Treg cell function in mice with leptin deficiency may account, at least in part, for the protective effect on atherosclerosis. Consistent with this hypothesis, we and others have recently identified an important role for natural Treg cells in the control of atherosclerosis. In the present study, we provide for the first time direct in vivo evidence that selective deficiency of leptin signaling in Treg cells inhibits the development of pathogenic Th1 response and reduces the development of atherosclerosis.

Additional studies are required to decipher the molecular pathways responsible for leptin-mediated alteration in Treg cell response. A very recent study suggested an important role for leptin signaling in the control of Treg cell proliferation. Treg cells proliferated after leptin neutralization and showed increased Foxp3 expression, which is consistent with the increased number of CD4⁺CD25⁺Foxp3⁺ Treg cells in lymphoid organs of ob/ob and db/db mice in vivo. However, the
authors failed to show any improvement in the suppressive function of Treg cells after inhibition of leptin/leptin receptor signaling. Proliferation of Treg cells in response to leptin neutralization even led to acute and transient reduction in their suppressive potential. In the present study, we confirm that alteration of leptin/leptin receptor signaling increases the number of Treg cells in vivo. In addition, our results clearly show that the mechanisms contributing to improved Treg cell response in the absence of leptin signaling go beyond the effect of leptin on Treg cell proliferation. In contrast with De Rosa et al, we consistently observed enhanced suppressive potential of Treg cells purified from \( \text{db/db} \) mice, compared with cells recovered from wild-type mice. We believe that the apparent discrepancy between these results could be attributed, at least in part, to differences in costimulatory pathways in vitro, ie, use of anti-CD28 antibody in their study, whereas in the present work, we stimulated Treg and T effector cells in the presence of purified CD1ic \( ^{+} \) dendritic cells. This explanation is also supported by the observation that, in their hands, stimulation of \( \text{db/db} \) CD4 \(^{+}\)CD25 \(^{+}\) effector T cells with anti-CD3/anti-CD28 was less effective in inducing proliferation than stimulation of CD4 \(^{+}\)CD25 \(^{+}\) effector T cells from \( \text{db/+} \) mice, whereas we found no difference in cell proliferation between \( \text{db/db} \) and wild-type effector T cells when stimulated with anti-CD3 in the presence of dendritic cells. Nevertheless, taken together, the results clearly show a major role of leptin signaling in the modulation of Treg cell response in vitro and in vivo, potentially affecting the development of diseases with immunoinflammatory component.

Finally, it should be noted that we did not detect Foxp3 \(^{+}\) cells within the atherosclerotic lesions (supplemental Figure II), suggesting that modulation of the immune response by Treg cells in atherosclerosis does not require their presence within the lesion, but is associated with increases in their number and function in secondary lymphoid organs.

In conclusion, we identify a critical role for leptin in the alteration of the regulatory immune response with a profound impact on atherosclerotic lesion development. A better understanding of the molecular pathways responsible for this alteration should lead to the development of novel and potent therapeutic strategies to limit disease development and complications.

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Disclosures

None.

References


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