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**Supplementary Material**

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Cutting Edge: Leptin-Induced RORγt Expression in CD4+ T Cells Promotes Th17 Responses in Systemic Lupus Erythematosus

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Th17 CD4+ cells promote inflammation and autoimmunity. In this study, we report that Th17 cell frequency is reduced in ob/ob mice (that are genetically deficient in the adipokine leptin) and that the administration of leptin to ob/ob mice restored Th17 cell numbers to values comparable to those found in wild-type animals. Leptin promoted Th17 responses in normal human CD4+ T cells and in mice, both in vitro and in vivo, by inducing RORγt transcription. Leptin also increased Th17 responses in (NZB × NZW)F1 lupus-prone mice, whereas its neutralization in those autoimmune-prone mice inhibited Th17 responses. Because Th17 cells play an important role in the development and maintenance of inflammation and autoimmunity, these findings envision the possibility to modulate abnormal Th17 responses via leptin manipulation, and they reiterate the link between metabolism/nutrition and susceptibility to autoimmunity.  

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Leptin is an adipokine that has structural characteristics of the long-chain helical cytokine family (that includes IL-3, IL-6, and IL-12) (1) and binds to a receptor that shares homology with the IL-6 receptor (2). Although the most apparent characteristic of leptin is to control metabolism and energy expenditure, leptin has additional activities that include the ability to modulate immune responses (3). In innate immunity, leptin facilitates the activation of NK cells, chemotaxis of neutrophils, and the secretion of TNF-α, IL-6, and IL-12 from macrophages (4). In adaptive immunity, leptin stimulates the proliferation of naive T cells and inhibits CD4+CD25-Foxp3+ regulatory T cells (5). Importantly, leptin accelerates the development and progression of autoimmune diseases, including experimental autoimmune encephalomyelitis, Ag-induced arthritis, and experimentally induced colitis (6). Conversely, ob/ob mice, which have a genetic deficiency of leptin due to a mutation in the leptin gene (2), have a reduced susceptibility to develop autoimmunity and display elevated numbers of peripheral regulatory T cells (7).

In systemic lupus erythematosus (SLE), a chronic autoimmune disease that is characterized by the presence of multiple autoantibodies and elevated numbers of autoreactive CD4+ T cells (8), leptin is abnormally elevated (9). Moreover, an altered regulation of metabolism has been suggested to contribute to the pathogenesis of SLE (10, 11). Another proinflammatory cytokine, IL-17, is also significantly increased in SLE patients (12–14). IL-17 promotes inflammation and autoimmune responses in several animal models of autoimmune diseases (15–17) and is produced by the Th17 subset of CD4+ T cells (16).

In this study, we describe a link, both at the molecular and cellular levels, between leptin and IL-17. Leptin deficiency associated with a reduced frequency of Th17 cells that was restored to levels comparable to those found in wild-type (WT) animals after administration of leptin. We also found that leptin facilitated Th17 responses by inducing RORγt transcription in CD4+ T cells.

This capacity of leptin to promote Th17 cell differentiation identifies leptin as a new target for the modulation of Th17 immune reactivity in normal and pathologic conditions.

Materials and Methods

Mice

C57BL6/J (B6) wild-type (WT), leptin-deficient B6ob/ob (ob/ob), leptin receptor−deficient B6ob/ob (ob/ob), and (NZB × NZW)F1 (NZB/W) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). RORγt−/− (B6-Rorc−/−) mice were from Taconic (Hudson, NY). The mice...
were maintained at the University of California Los Angeles with a 12 h light/dark cycle and were 8–12 wk old and age-matched when used for the experiments, under approved protocols, in accordance with institutional and federal regulations.

**Human studies**

Blood was drawn from SLE patients that fulfilled the American College of Rheumatology criteria for the classification of SLE, as well as from healthy matched donors. All SLE patients had stable disease and a therapeutic steroid dose ≤ 10 mg/day. The study was conducted according to the principles of the Declaration of Helsinki and was approved by the Institutional Review Board of the University of California Los Angeles. SLE patients with comorbid conditions were excluded from the investigation.

**Cell preparation**

For the human studies, PBMCs were prepared by Ficoll gradient. After RBC lysis, PBMCs were washed prior to culture in AIM-V medium (Invitrogen, Carlsbad, CA) supplemented with penicillin and streptomycin. For the mouse studies, explanted spleens were teased into single-cell suspensions and filtered through a 70-μm cell strainer. After RBC lysis, splenocytes were either cultured in vitro or used for the purification of CD4+ T cells by negative selection using magnetic beads (Miltenyi Biotec, Auburn, CA), according to the manufacturer’s instructions. Mouse PBMCs were obtained from peripheral blood and cultured in HL-1 medium (Lonza, Walkersville, MD) supplemented with 2 mM glutamine, and 5% blood and cultured in HL-1 medium (Lonza, Walkersville, MD) supplemented with 2% mouse serum (Sigma-Aldrich, St. Louis, MO), penicillin and streptomycin, 2 mM glutamine, and 5 × 10^{-3} M 2-ME.

**Flow cytometry**

For intracellular staining, cells were incubated for 4–5 h with 25 ng/ml PMA (Sigma-Aldrich), 2 μg/ml ionomycin (Sigma-Aldrich), and 10 μg/ml brefeldin A (eBioscience, San Diego, CA) at 37°C/5% CO2. After surface staining with fluorescent-labeled anti-CD4, anti-ROTyt (eBioscience), and anti-leptin and leptin receptor (Ob-R) Ab (R&D Systems, Minneapolis, MN), cells were resuspended in fixation/permeabilization buffer (eBioscience) for intracellular staining with fluorescent-labeled anti–IL-17 (eBioscience) and anti-Stat3/anti-pStat3 Ab (BD Biosciences, San Jose, CA). Flow cytometry was performed on a FACSCalibur instrument (BD Biosciences) and analysis was done using FlowJo software (Tree Star, Ashland, OR).

**ELISA**

Plasma IL-17 or leptin in human and murine samples was measured using ELISA kits from R&D Systems. Measurements were done according to the manufacturer’s instructions.

**Leptin reagents**

Recombinant mouse leptin and human leptin were purchased from R&D Systems. Anti-leptin Ab was purchased from Cell Sciences (Canton, MA).

**Leptin treatment**

For in vivo experiments, ob/ob mice or NZB/W mice were injected i.p. twice daily with leptin dissolved in 200 μl saline at a dose of 1 μg/g body weight for 10 d. Age- and sex-matched mice received similar volumes of saline with the same schedule. A group of mice received 150 μg anti-leptin Ab or isotype-matched control Ab i.p. twice at 2-d intervals. On day 10, PBMCs and splenocytes were collected for flow cytometry analyses. For in vitro experiments with mouse cells, splenocytes were cultured in complete medium or under Th17 polarizing conditions (2 μg/ml anti-CD3 Ab, 2 μg/ml anti-CD28 Ab, 40 U/ml IL-2, 20 ng/ml IL-6, 5 ng/ml TGF-β, 10 μg/ml anti-IL-4 Ab, 10 μg/ml anti–INF-γ Ab) for 4 d in a 37°C/5% CO2 incubator. Leptin was added during the last 18 h before cells underwent intracellular cytokine staining and flow cytometry. For the in vitro experiments with human cells, PBMC were stimulated with Dynabeads CD3/CD28 T Cell Expander (Invitrogen) plus 20 ng/ml IL-6 and 5 ng/ml TGF-β for 4 d in a 37°C/5% CO2 for Th17 polarization. Human leptin was added during the last 18 h prior to flow cytometry analyses.

**Plasmin and retrovirus production**

The RORγt (RORγt-IRE6-GFP) and control pMIG (IRE5-GFP) plasmids (retrovirus-based vectors containing GFP under the regulation of an internal ribosome entry site) were provided by Dr. D.R. Littman (18). Phoenix cells were transfected with 4 μg plasmids using Lipofectamine 2000 (Invitrogen) on day 0. Viral supernatant was collected on days 2–3 and supplemented with 8 μg/ml polybrene (Sigma-Aldrich) before use.

**Cell cultures and retroviral transduction**

CD4+ T cells (5 × 10^5/well) that had been negatively sorted from RORγt^−/− mouse splenocytes using magnetic beads were cultured in 96-well flat-bottom plates containing anti-CD3 Ab (2 μg/ml), anti-CD28 Ab (2 μg/ml), and mouse IL-2 (40 U/ml) on day 0. For viral transduction, on days 1 and 2, viral supernatant was added and cells were spun at 2500 rpm for 1.5 h at 30°C. After spin infection, the cells were put in culture media in the presence of anti-CD3 Ab/anti-CD28 Ab/IL-2 and harvested on day 5 for staining and flow cytometry. Cultures had scalar concentrations of leptin (or medium only) added during the last 18 h.

**Statistical analyses**

The t test was used for two-group analyses, and Kruskal–Wallis ANOVA was used for analyses of three or more groups using GraphPad Prism software.

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**Figure 1.** Leptin increases Th17 cell frequency in vitro and in vivo. Splenocytes from untreated ob/ob mice were stimulated under Th17 polarizing conditions and leptin was added at scalar doses during the last 18 h of culture. The figure shows representative (A) and cumulative data from total splenocytes (B) and gated CD4+ T cells (C) from eight mice in three independent experiments. (D and E) Representative (D) and cumulative data (E) from two experiments on splenocytes from ob/ob mice (n = 10) that were treated in vivo with leptin or saline (as control), according to the protocol described in Materials and Methods. (F) IL-17+ T cells in PBMCs from ob/ob mice treated with leptin (n = 10/group, two experiments). *p < 0.01, **p < 0.001.
Results and Discussion
Mice that are genetically deficient in leptin/leptin receptor have a reduced frequency of Th17 cells
Ob-R–deficient db/db mice had reduced numbers of peripheral Th17 cells among PBMCs and splenocytes in comparison with WT mice (Supplemental Fig. 1A–D). In line with this finding, ob/ob and db/db mice had lower plasma IL-17 levels than did WT mice (Supplemental Fig. 1E), suggesting that an impaired leptin/leptin receptor axis associated with reduced IL-17 responses in vivo. This result is consistent with the observation that in obesity, which is characterized by elevated leptinemia, there is an increase in plasma concentration of IL-17 (19).

Leptin increases Th17 cell frequency in vitro and in vivo
To investigate the above link between leptin and Th17 cells, we cultured ob/ob splenocytes with recombinant leptin. A dose-dependent increase in the number of IL-17+ T cells was observed in the presence of scalar doses of leptin (Fig. 1A–C). A concomitant upregulation of surface expression of Ob-R was also found by flow cytometry (not shown). To extend these findings to an in vivo system, we treated ob/ob mice with recombinant leptin or saline (as control). Leptin-treated mice had the expected weight loss (p < 0.01, not shown) associated with the inhibition of appetite by leptin (2). Interestingly, leptin treatment associated with an expansion of Th17 cells among splenocytes (Fig. 1D, 1E) and PBMCs (Fig. 1F), together with an upregulation of the surface expression of Ob-R in Th17 cells and an increase in the phosphorylation of Stat3 (which is activated by leptin) by flow cytometry (not shown).

Leptin induces RORγt transcription in CD4+ T cells
Because leptin increased Th17 cell frequency in vitro and in vivo (Fig. 1), we tested whether leptin could promote Th17 differentiation. RORγt (ROR nuclear hormone receptor family) is a transcription factor that acts as a master regulator in the differentiation of Th17 cells (20). To investigate the effects of leptin on the expression of RORγt, we evaluated RORγt expression in negatively sorted RORγt−/− CD4+ T cells infected with RORγt-expressing vector or control after culture in the presence of scalar doses of leptin. As shown

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** Leptin increases RORγt transcription and IL-17 production in CD4+ T cells. Sorted RORγt−/− CD4+ T cells were stimulated with anti-CD3/CD28 Ab for 24 h, transduced with retrovirus encoding RORγt, and restimulated with anti-CD3/CD28 Ab and scalar doses of leptin for 18 h. The figure shows the flow cytometry results of RORγt (GFP) (A) and IL-17 expression (B) in the presence of scalar doses of leptin. (C) Flow cytometry for RORγt expression in ob/ob CD4+ T cells cultured under polarizing (●) or nonpolarizing (○) conditions in the presence of scalar doses of leptin during the last 18 h, stimulated (straight lines) or not (dashed lines) with PMA/ionomycin. *p < 0.05 in the comparison between polarizing and nonpolarizing conditions.
in Fig. 2A and 2B, leptin increased RORγt expression and IL-17 production in a dose-dependent fashion in CD4+ T cells. The surface expression of Ob-R was concomitantly upregulated on CD4+ T cells that differentiated into Th17 cells, together with an increased Stat3 phosphorylation by flow cytometry (not shown). Leptin also increased RORγt expression and IL-17 production in CD4+ T cells from ob/ob mice (Fig. 2C).

**Leptin promotes Th17 responses in human CD4+ T cells**

In the presence of increasing concentrations of leptin, Th17 cell numbers increased in a dose-dependent manner in cultures of healthy human PBMCs (Supplemental Fig. 2A, 2B). IL-17 was concomitantly higher in culture supernatants (Supplemental Fig. 2C). Moreover, a positive correlation between plasma leptin and IL-17 was found in SLE patients but not in healthy matched controls (Supplemental Fig. 2D).

**Leptin promotes Th17 responses in NZB/W lupus-prone mice**

Recent work has suggested that Th17 cells can contribute to the pathogenesis of SLE in humans (12, 13) and in lupus animal models (21, 22), where IL-17 blockade has been proven beneficial in reducing disease manifestations (23). When NZB/W splenocytes were cultured in the presence of scalar doses of leptin, a dose-dependent increase in Th17 cell frequency was observed (Fig. 3A, 3B). In vivo, the administration of leptin to NZB/W mice associated with an increased expression of Ob-R on Th17 cells by flow cytometry (not shown) and more peripheral Th17 cells, whereas treatment with anti-leptin Ab associated with a reduction in the number of Th17 cells (Fig. 3C–3E). Thus, leptin promoted (and leptin blockade inhibited) Th17 responses in NZB/W lupus mice.

In summary, we report a positive association between leptin and Th17 responses that associates with leptin-induced effects on the transcription of the Th17 master regulator RORγt. Together with the findings of a Th17-promoting effect of leptin on collagen-induced arthritis (24) and an inhibitory effect of anti-leptin Ab on Th17 responses in Hashimoto’s thyroiditis (25), our results suggest a potential for leptin-based manipulation of Th17 responsiveness in those conditions where Th17 cells have detrimental effects on the disease course.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**

Supplemental Figures

Supplemental Figure 1. Leptin-deficient ob/ob and leptin receptor-deficient db/db mice have reduced Th17 responses as compared to WT mice. Representative (a) and cumulative results (b) from PBMC in WT, ob/ob and db/db mice (n = 7 per group). *P<0.06, **P<0.04. Representative (c) and cumulative results (d) in splenocytes from 5 mice per group (WT, ob/ob and db/db mice). ***P<0.001, ****P<0.007. The results are from one of three independent experiments. Cells were stimulated in vitro with PMA/ionomycin for 5 hours before staining (a-b) or cultured under Th17 polarizing conditions (c-d) prior to stimulation with PMA/ionomycin during the last 5 hours of culture. (e) Plasma IL-17 detected by ELISA in age-matched, 12 weeks old untreated WT, ob/ob and db/db mice (n = 7 per group). *P<0.04, **P<0.01.

Supplemental Figure 2. Leptin promotes Th17 responses in normal human PBMC. Representative (a) and cumulative results (b) (n = 12) of IL-17 expression in CD4+ T cells from healthy donors’ PBMC cultured in the presence of scalar doses of leptin for the last 18 hours after culture under Th17 polarization conditions. (c) IL-17 detected by ELISA in the supernatant of the cultures shown in b. *P<0.006, **P<0.005, ***P<0.001. (d) ELISA for plasma leptin and IL-17 in SLE patients (n = 58) and healthy matched controls (n = 36) shows a positive correlation in SLE patients (P<0.008) but not in controls (P ns). Leptin concentration was higher in the SLE patients (32.72 ± 2.49 ng/ml) as compared to controls (13.02 ± 1.25 ng/ml) (P<0.0001).