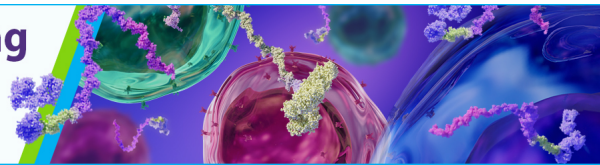


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High-Dose Leptin Activates Human Leukocytes Via Receptor Expression on Monocytes¹

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Leptin is capable of modulating the immune response. Proinflammatory cytokines induce leptin production, and we now demonstrate that leptin can directly activate the inflammatory response. RNA expression for the leptin receptor (Ob-R) was detectable in human PBMCs. Ob-R expression was examined at the protein level by whole blood flow cytometry using an anti-human Ob-R mAb 9F8. The percentage of cells expressing leptin receptor was $25 \pm 5\%$ for monocytes, $12 \pm 4\%$ for neutrophils, and $5 \pm 1\%$ for lymphocytes (only B lymphocytes). Incubation of resting PBMCs with leptin induced rapid expression of TNF- α and IL-6 mRNA and a dose-dependent production of TNF- α and IL-6 by monocytes. Incubation of resting PBMCs with high-dose leptin (250 ng/ml, 3–5 days) induced proliferation of resting cultured PBMCs and their secretion of TNF- α (5-fold), IL-6 (19-fold), and IFN- γ (2.5-fold), but had no effect on IL-4 secretion. The effect of leptin was distinct from, and additive to, that seen after exposure to endotoxin or activation by the mixed lymphocyte reaction. In conclusion, Ob-R is expressed on human circulating leukocytes, predominantly on monocytes. At high doses, leptin induces proinflammatory cytokine production by resting human PBMCs and augments the release of these cytokines from activated PBMCs in a pattern compatible with the induction of Th1 cytokines. These results demonstrate that leptin has a direct effect on the generation of an inflammatory response. This is of relevance when considering leptin therapy and may partly explain the relationship among leptin, proinflammatory cytokines, insulin resistance, and obesity. *The Journal of Immunology*, 2001, 167: 4593–4599.

Leptin, the product of the ob gene (1), is a peptide hormone derived mainly from adipocytes and genetic deficiency results in obesity (2). Leptin signals through the leptin receptor, which is a member of the class I cytokine receptor family (3). The Ob-R is widely distributed and expressed in both human and murine hemopoietic stem cell populations (4). Leptin has multiple biological actions on feeding, metabolism, and the neuroendocrine axis, and more recently leptin has been demonstrated to modulate the immune system toward the Th1 immune response (5, 6). Leptin-deficient children have an impaired cell-mediated immune response, an increased incidence of infectious diseases, and an associated increase in mortality (7).

Expression of the leptin receptor by PBMCs and CD4⁺ T lymphocytes has been reported at the mRNA level (5) and direct effects of leptin have been demonstrated in a number of studies. Human PBMCs and murine splenocytes show marked cellular clumping when incubated with leptin (7), and leptin increases Th1 (IFN- γ and IL-2) and suppresses Th2 (IL-4) cytokine production from activated human lymphocytes (5). Circulating concentrations of TNF- α and IL-6 strongly correlate with leptin levels in humans (8), and leptin levels rise after surgical stress (9). Leptin receptors

are expressed on human umbilical cord leukocytes (10), and leptin activates umbilical cord monocytes, polymorphonuclear cells, and lymphocytes (11). Rodents with genetic abnormalities in leptin and leptin receptor expression have deficits in macrophage phagocytosis and endotoxin-mediated induction of TNF- α and IL-6 (12).

High-dose leptin is now being used as a potential therapy for obesity (13), and high levels of leptin are found in extreme obesity due to leptin resistance and also in renal failure (14, 15). Injection of leptin elicits a mild localized inflammation (16), but the physiological basis for this reaction has not been established. To date, studies have focused on the effects of physiological levels (as determined by circulating plasma levels) of leptin on activated lymphocytes. In this study, we confirm the specificity of a mAb for the leptin receptor and demonstrate that monocytes are the predominant cells expressing Ob-R in the peripheral blood. Our results demonstrate that high-dose leptin induces the production of proinflammatory cytokines in resting PBMCs, which is compatible with the observed expression of Ob-R on peripheral blood monocytes.

Materials and Methods

All the materials were purchased from Sigma-Aldrich (Poole, U.K.) unless otherwise stated.

Blood samples

Peripheral blood (50 ml) was collected into heparinized tubes from healthy adult volunteers (aged 20–45 years).

Measurement of endotoxin activity in recombinant human leptin and blocking of endotoxin activity

Endotoxin activity in recombinant human leptin was measured using a QCL-1000 pyrogen testing kit (BioWhittaker, Walkersville, MD) according to the manufacturer's instructions. The potential influence of endotoxin contamination on observed effects was excluded by the addition of polymyxin-B in control experiments (17). This was added at the same time as endotoxin and leptin.

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Leptin functional assay

Biological activity of recombinant leptin was tested in transcription assays performed in human epithelial kidney (HEK293) cells transiently transfected with the leptin receptor long form and a reporter construct containing a Stat3-binding element fused to a minimal thymidine kinase promoter and luciferase. Luciferase activity was measured as previously reported (18).

RT-PCR

For the analysis of leptin receptor and cytokine gene expression, total RNA was extracted from 4×10^7 PBMCs using TRIzol reagent (Life Technologies, Paisley, U.K.). Total RNA (1 μ g) was reverse-transcribed and PCR amplification was performed using previously published primers for Ob-Ra and Ob-Rb (4). For cytokine gene analysis, mRNA was extracted at the indicated time points, cDNA was prepared, and PCR amplifications were performed using previously characterized primers for IL-6, TNF- α , IFN- γ , and GAPDH (19).

Anti-leptin receptor mAb

A mAb (9F8) raised against the recombinant human leptin receptor extracellular domain (20) was used for the study. Specificity of mAb 9F8 was shown by the lack of cross-reactivity with extracellular domains from other closely related receptors of the same class I cytokine receptor superfamily (growth hormone and prolactin receptors).

Transfection of HEK293 cells with Ob-R

The leptin receptor expression plasmid was a kind gift from A. Welcher (Amgen, Thousand Oaks, CA). HEK293 cells were transfected with 9 μ g of leptin receptor cDNA using a calcium phosphate transfection kit (Life Technologies). Receptor expression was confirmed by assessing the binding of 125 I-labeled leptin (Biogenesis, Poole, U.K.).

Validation of 9F8 by flow cytometric analysis of transfected and nontransfected HEK293 cells

Transfected and nontransfected HEK293 cells were suspended in PBS/BSA (washing buffer) at a density of 1×10^7 cells/ml. Cells (10^6 ; 100 μ l) were transferred to 12×75 mm polycarbonate tubes (BD Biosciences, Oxford, U.K.) and incubated with 5 μ g of an anti-leptin receptor Ab (9F8) or isotype-matched negative control Ab (R&D Systems, Abingdon, U.K.) for 30 min on ice. Primary Ab binding was detected by incubation with biotinylated goat anti-mouse IgG polyclonal Ab (1 μ g; Calbiochem, Nottingham, U.K.), followed by incubation with streptavidin-R-PE (SAV-R-PE)³ conjugate (10 μ l; Serotec, Oxford, U.K.) for 30 min on ice. Cells were washed before flow cytometric analysis. The specificity of 9F8 was tested by preincubation with a recombinant form of the extracellular domain of the human leptin receptor rhOb-R (R&D Systems).

Detection of Ob-R on peripheral blood leukocytes by whole blood flow cytometry

Heparinized whole blood (150 μ l) and 10 μ l of goat serum (Serotec) were incubated for 15 min on ice, after which 1 μ g of 9F8 or isotype-matched negative control Ab were added. Cells were washed in PBS containing 7.5% v/v goat serum, incubated with 0.2 μ g of biotinylated goat anti-mouse IgG, and finally incubated with 10 μ l of the SAV-R-PE conjugate. Incubations were conducted for 30 min on ice and cells were washed twice with washing buffer. Erythrocytes were lysed with 2 ml of Erythrolyse (Serotec) before flow cytometry on a FACScan flow cytometer (BD Biosciences) using CellQuest data acquisition and analysis software. Polymorphonuclear leukocytes (neutrophils), lymphocytes, and monocytes exhibit characteristic light scatter properties that can be identified on a forward vs side light scatter plot. Leukocyte populations were located using these parameters and a live analysis gate was set around this population. Data were acquired from 10,000 cells (events) and the proportion of given cell populations expressing the leptin receptor was determined. For two-color flow cytometry, after staining with 9F8, cells were incubated with PBS containing 2% v/v normal mouse serum (Serotec) for 30 min on ice, washed, and incubated with 10 μ l of FITC-conjugated anti-human CD (CD14, CD15, CD3, CD4, CD8, and CD20) Abs (Serotec) for another 30 min on ice. Staining with mAbs specific for cell subsets revealed that the monocyte region contained $94.8 \pm 1.8\%$ CD14⁺ cells and the polymorphonuclear

cell region contained $92.2 \pm 1.7\%$ CD15⁺ cells. The lymphocytes region contained $70.4 \pm 1.7\%$ CD3⁺, $34.5 \pm 0.8\%$ CD4⁺, $42.4 \pm 1.06\%$ CD8⁺, and $4.4 \pm 0.3\%$ CD20⁺ cells.

Flow cytometry cytokine production assay

Heparinized whole blood (100 μ l) was treated with 10 μ l of either endotoxin, PBS, or leptin. Brefeldin A (BD Biosciences), a pharmacological inhibitor of secretion, was added, and cells were incubated for 6 h followed by surface staining of monocytes using FITC-conjugated mouse anti-human CD14 (Serotec). Cells were fixed and permeabilized, and intracellular staining of accumulated cytokines was performed using either PE-anti-human TNF- α mAb or PE-anti-human IL-6 mAb (BD Biosciences).

Isolation and culture of PBMCs

PBMCs were isolated by density gradient (Lymphoprep Nyegaard, Oslo, Norway), washed twice in normal saline and once in medium, and suspended at 1×10^6 cells/ml in medium RPMI 1640 growth medium supplemented with L-glutamine (2 mM), penicillin/streptomycin (100 U/ml penicillin, 100 μ g/ml streptomycin), and 2% v/v heat-inactivated normal human AB serum. The leptin concentration in this medium was below the sensitivity of the leptin ELISA kit (7.6 pg/ml; R&D Systems). The cell suspension (2 ml) was transferred to each well of a 24-well plate for the cytokine secretion studies. Cells were incubated with endotoxin (LPS from *Escherichia coli*, serotype 0111:B4) or leptin (recombinant human leptin, R&D Systems), at the concentrations indicated, for 72 h in 100% humidity, 5% CO₂, after which media were centrifuged at 400 g for 10 min. Supernatants were collected and stored at -70°C until assay. Control cultures were incubated with PBS or medium alone.

Proliferation assay

The PBMCs suspension (200 μ l) was transferred to each well of a 96-well plate (Costar, Cambridge, U.K.), treated according to the protocol, and incubated for 5 days. During the last 24 h, 0.1 μ Ci [³H]thymidine was added and proliferation, on the basis of thymidine incorporation, was assessed by liquid scintillation counting. The influence of leptin on MLR-induced proliferation and cytokine production was also assessed. For MLR studies, cultures were established using equal numbers of responder and irradiated (30 Gy) allogeneic PBMCs as stimulator cells. Cells were incubated in the presence or absence of leptin for 3 (cytokine production) or 5 (proliferation) days.

Enzyme-linked immunosorbent assays

Cytokines (IL-6, TNF- α , IL-4, and IFN- γ) and leptin in media were measured using ELISA kits (R&D Systems). Intra-assay coefficient of variation values were 4.4, 8.7, 0.59, 3.7, and 3.3%, respectively.

MAPK inhibitors

To determine signaling pathways, PBMCs in whole blood were incubated with or without 0.01 nM of either extracellular-regulated protein kinase (ERK) inhibitor PD98059 (Calbiochem) or p38 kinase inhibitor SB2035580 (Calbiochem) 1 h before the addition of leptin or endotoxin. After an additional 6-h incubation, production of TNF- α on monocytes was assessed using flow cytometry.

Statistics

The paired *t* test was used to compare the effects of leptin on lymphocyte proliferation and cytokine production and ANOVA for the dose response. A value of *p* < 0.05 was considered to indicate statistically significant differences.

Results

Ob-R gene expression of PBMCs

PCR products for both short (Ob-Ra) and long (Ob-Rb) isoforms of leptin receptor were detectable in human PBMCs (Fig. 1).

Validation of 9F8 for detection of Ob-R expression by flow cytometry

HEK293 cells were transfected with Ob-R and expression of leptin receptor was confirmed by incubation with 125 I-labeled leptin in the presence or absence of unlabeled leptin. Ob-R-transfected HEK293 cells had high levels of leptin-specific binding ($8 \pm 1\%$),

³ Abbreviations used in this paper: SAV-R-PE, streptavidin-R-PE; ERK, extracellular-regulated protein kinase; rh, recombinant human; MAPK, mitogen-activated protein kinase; Ob-Ra, short isoform of Ob-R; Ob-Rb, long isoform of Ob-R.

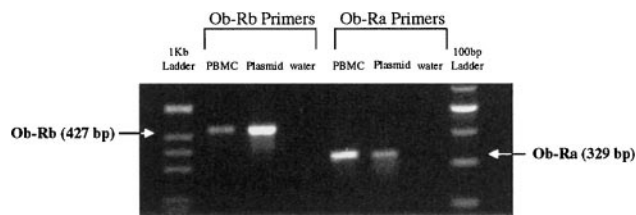


FIGURE 1. Ob-R gene expression in PBMCs. Results of RT-PCR mRNA expression for Ob-Ra and Ob-Rb in isolated human PBMCs. Positive controls are Ob-Ra and Ob-Rb plasmids and negative control is water. Expression of both Ob-Ra and Ob-Rb are detectable in human PBMCs.

but nontransfected cells also showed a low level of specific binding ($0.7 \pm 0.1\%$). Ob-R-transfected cells were used in flow cytometry to test the anti-leptin receptor Ab (9F8). Transfected and nontransfected HEK293 cells were incubated with either 9F8 or isotype-matched control Ab, and Ab binding was detected by flow cytometry. Binding of 9F8 to transfected cells was apparent, but 9F8 also bound to nontransfected cells, confirming that HEK293 cells express low levels of the leptin receptor (Fig. 2). The specificity of 9F8 for the leptin receptor was further tested by preincubation of Ab with recombinant human (rh)Ob-R, which com-

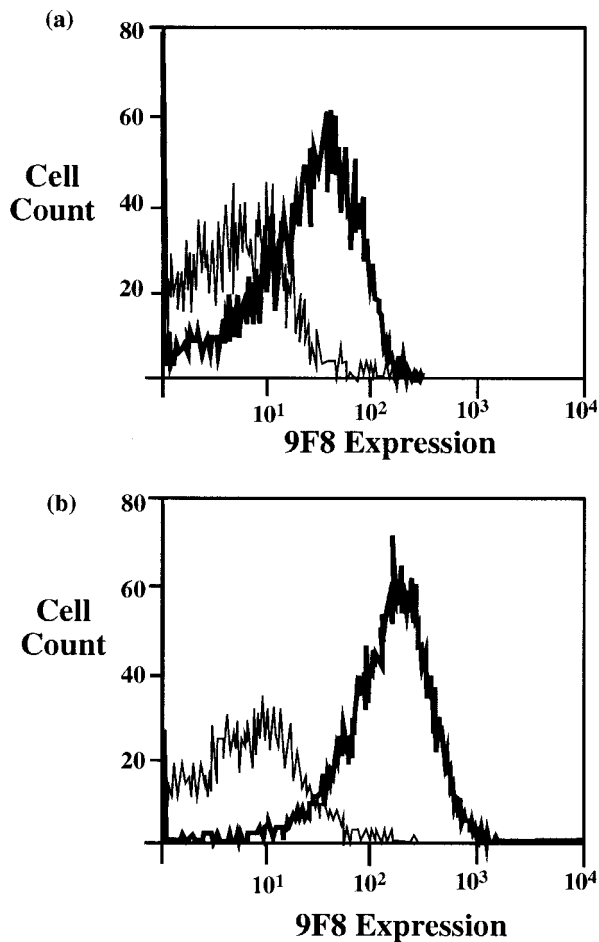


FIGURE 2. Detection of Ob-R on HEK293 cells by flow cytometry. HEK293 cells nontransfected (a) or transfected (b) with Ob-R plasmid were incubated with either 9F8 (thick lines) or control (thin lines) Ab and SAV-R-PE; fluorescence was detected by FACScan. Specific binding was detectable in the nontransfected and transfected cells with higher binding in transfected cells.

pletely abolished binding of 9F8 Ab to HEK293 cells (data not shown).

Leptin receptor expression on peripheral blood leukocytes

Whole blood flow cytometric analysis of samples from 10 subjects using 9F8 revealed that $25 \pm 5\%$ of monocytes, $12 \pm 4\%$ of polymorphonuclear cells, and $5 \pm 1\%$ of lymphocytes express the Ob-R (Fig. 3). Two-color flow cytometry revealed that lymphocyte expression was primarily related to CD20⁺ cells (B lymphocytes,

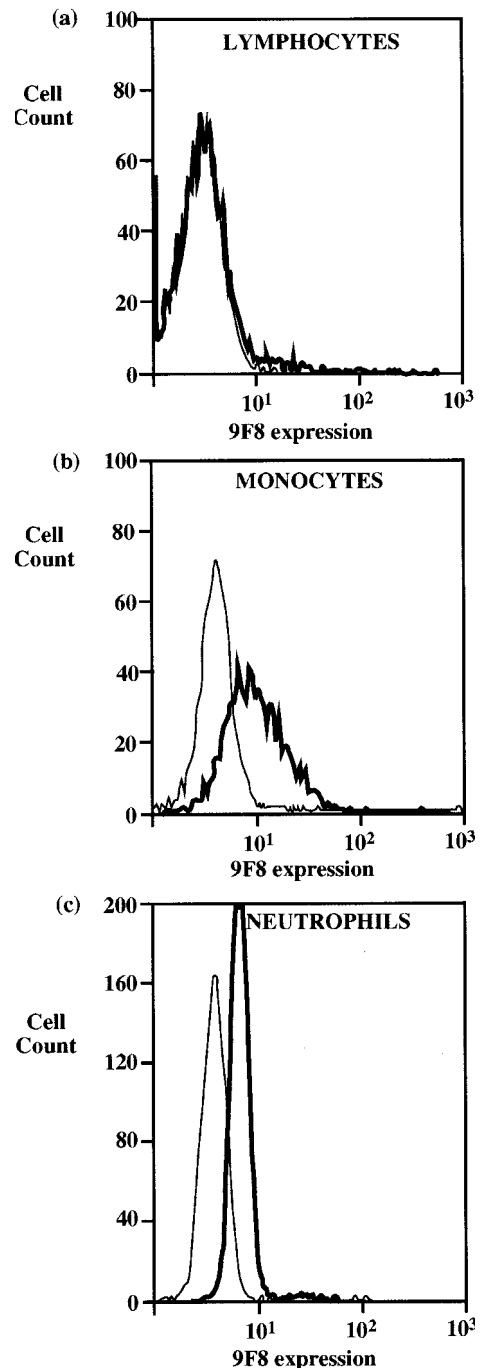


FIGURE 3. Ob-R expression on human blood leukocytes. A typical flow cytometry analysis of the cells obtained from the whole blood is represented. Whole blood was incubated with either isotype control (thin lines) or 9F8 (thick lines). After adding biotinylated secondary Ab and SAV-R-PE, fluorescence was detected by FACScan. Specific binding was mainly detectable in monocytes and to lesser extents in neutrophils.

50 ± 6%) with T lymphocytes showing no expression. Activation of PBMCs by incubation with endotoxin (10 ng/ml) for 2 h resulted in no change in Ob-R expression on monocytes, and incubation up to 3 days with endotoxin, PHA, or MLR did not alter Ob-R expression on lymphocyte populations.

Leptin bioassay

The biological activity of the recombinant leptin was tested in a function assay using a Stat3-luciferase reporter (Fig. 4a). Leptin showed a dose response between 1 and 100 ng/ml.

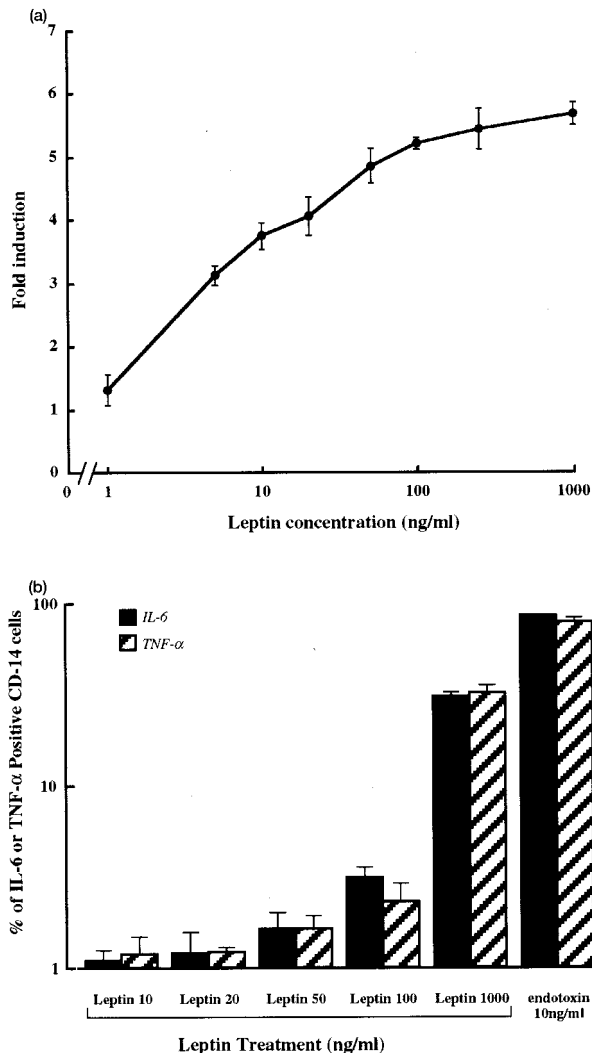


FIGURE 4. *a*, Dose response of luciferase activity to leptin stimulation in HEK293 cells. Cells were transiently transfected with Ob-R plasmid and a Stat3-luciferase reporter construct, and stimulated with varying doses of leptin. Luciferase activity was measured after 6-h incubation and was corrected for β -galactosidase levels, which were used as a transfection control. The results are represented as fold induction over unstimulated cells. *b*, Flow cytometry characterization of the dose response for the induction of IL-6 and TNF- α on CD14⁺-gated monocytes. Whole blood was incubated with or without increasing doses of leptin or endotoxin for 6 h and cytokines were detected by intracellular staining in gated CD14⁺ monocytes using two-color flow cytometry. The results are expressed as the percentage of CD14⁺ cells positive for IL-6 or TNF- α . Leptin induced TNF- α and IL-6 production in a dose-dependent manner. The response to leptin is compared with a maximal response to endotoxin. It should be noted that induction is presented in a logarithmic scale and the response to leptin is less than that seen after endotoxin.

Effect of leptin on intracellular cytokine production by peripheral blood monocytes

Whole blood was incubated with leptin in doses of 10–1000 ng/ml and production of IL-6 and TNF- α by CD14⁺ monocytes was determined using flow cytometry. Leptin at doses of 50–1000 ng/ml induced the production of both IL-6 and TNF- α ($p < 0.01$; Fig. 4b). The observed effects were not due to endotoxin contamination of the leptin preparation, as levels of endotoxin (<0.00036 ng/ml) were below those that we have previously shown to be the minimum dose (0.01 ng/ml) required to induce IL-6 (21). The possibility of endotoxin contamination was further excluded by the demonstration that polymyxin-B, an endotoxin inhibitor (17), completely inhibited the induction of TNF- α by 0.1 ng/ml endotoxin, yet it had no effect on leptin-induced TNF- α production (Fig. 5).

Effect of leptin on proinflammatory cytokine mRNA expression

PBMCs were incubated in the presence or absence of leptin for between 1 and 24 h and cytokine gene expression was determined by RT-PCR using cytokine-specific primers. Leptin induced IL-6 and TNF- α gene expression within 1 h (Fig. 6). IFN- γ was barely detectable in control samples and showed little induction.

Measurement of cytokine production from isolated PBMCs incubated with leptin

To confirm the activation of PBMCs by leptin, the effects of leptin (250 ng/ml) and endotoxin on cytokine secretion by PBMCs from six subjects were determined by ELISA. Medium from cells incubated without leptin showed very low levels of TNF- α , IL-6, IL-4, and IFN- γ . Leptin caused a 5-fold induction of TNF- α , a 19-fold increase in IL-6, and a 2.5-fold increase in IFN- γ (Fig. 7), whereas it had no effect on induction of IL-4. The IL-4 levels in samples from both resting and leptin-activated PBMCs were below the detection limit of the assay, although PHA-stimulated PBMCs produced high levels of IL-4 (122 ± 14 pg/ml). Leptin also significantly increased the proliferation of PBMCs (PBMCs only, 923 ± 178; PBMCs plus leptin, 1253 ± 254 cpm [³H]thymidine uptake, $p < 0.01$). When coincubated with endotoxin, leptin augmented

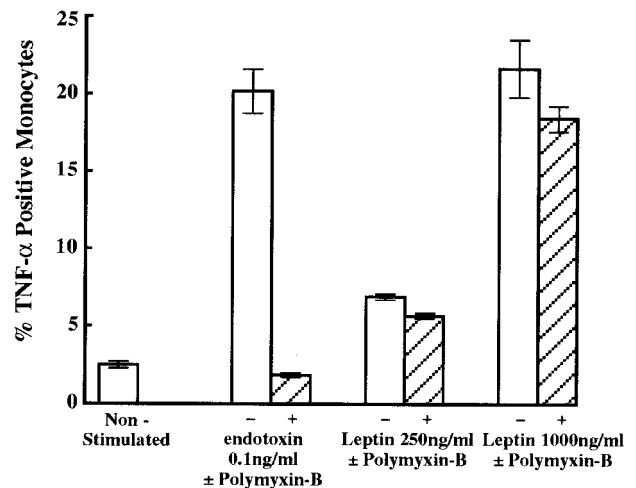


FIGURE 5. Effects of polymyxin-B on TNF- α production by endotoxin- and leptin-activated monocytes. Whole blood was treated with either endotoxin or leptin with and without polymyxin-B (1 μ g/ml) for 6 h and TNF- α was detected by intracellular staining in gated CD14⁺ monocytes using two-color flow cytometry. The results are expressed as the percentage of CD14⁺ cells positive for TNF- α . Polymyxin-B blocked endotoxin induction of TNF- α but had no effect on leptin activation.

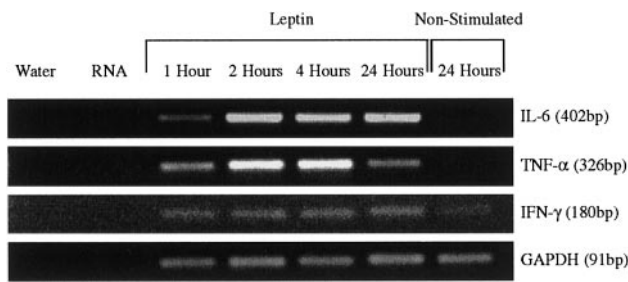


FIGURE 6. Results of RT-PCR for IL-6, TNF- α , and IFN- γ in PBMCs. PBMCs were isolated from whole blood and were incubated with leptin for periods of time up to 24 h and mRNA expression for IL-6, TNF- α , and IFN- γ were assessed using RT-PCR. Controls are lanes 1 and 2 (water and no RT step), and lane 7 incubated for 24 h in the absence of leptin. The lower panel shows RT-PCR in the same reactions with GAPDH. Leptin induced TNF- α and IL-6 mRNA expression.

the cytokine response to endotoxin with a pattern similar to the induction of cytokines by leptin alone. Endotoxin alone at the dose of 0.01 ng/ml did not induce IFN- γ (Fig. 7).

Effect of leptin on PBMCs activated by MLR

Leptin augmented the production of TNF- α , IL-6, and IFN- γ , but not IL-4 from MLR-activated PBMCs in a similar pattern to that seen in nonactivated PBMCs (Fig. 8). In addition, the MLR-induced proliferation was augmented by leptin (PBMCs only, 923 ± 178 ; MLR only, 3867 ± 372 ; MLR plus leptin, 6350 ± 584 cpm [3 H]thymidine uptake, $p < 0.001$).

MAPK inhibitors and leptin activation of PBMCs

PBMCs were preincubated with two mitogen-activated protein kinase (MAPK) inhibitors, PD98059 (inhibits ERK) and SB203580 (inhibits p38), and then exposed to either endotoxin or leptin. The p38 inhibitor, SB203580, consistently inhibited both the endotoxin and leptin induction of TNF- α by $\sim 30\%$, whereas PD98059 had no effect (Fig. 9). The biological activity of the ERK inhibitor PD98059 had previously been demonstrated in our functional bioassay.

Discussion

We have examined the possibility that high-dose leptin can induce proinflammatory cytokines in resting PBMCs and explored the mechanism by which this could occur. Our results confirm that

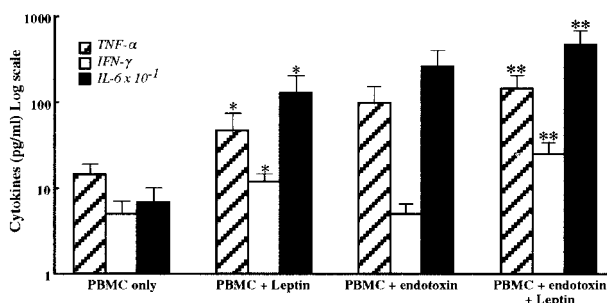


FIGURE 7. Cytokine production from cultured PBMCs incubated with leptin alone or leptin plus endotoxin. Mean (\pm SEM) levels of TNF- α , IFN- γ , and IL-6, in medium from PBMCs of six normal subjects. PBMCs were isolated from whole blood and were incubated with or without either leptin (250 ng/ml), endotoxin (0.01 ng/ml), or leptin plus endotoxin for 3 days. (*, $p < 0.03$ PBMCs plus leptin vs PBMCs incubated in the absence of hormone, **, $p < 0.01$ PBMCs plus endotoxin vs PBMCs with endotoxin and leptin). Leptin induced production of TNF- α , IL-6, and IFN- γ .

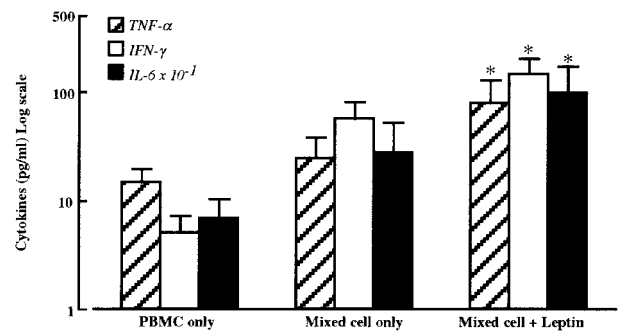


FIGURE 8. Leptin effects on cytokine production from PBMCs activated by MLR. Mean (\pm SEM) levels of TNF- α , IFN- γ , and IL-6 in medium from MLR-activated PBMCs of six normal subjects in the presence or absence of leptin (250 ng/ml) for 3 days. PBMCs were isolated from whole blood and were activated by MLR using PBMCs as responders and MHC-mismatched PBMCs as stimulator cells. (*, $p < 0.02$ MLR only vs MLR in the presence of leptin). Leptin augmented the cytokine response to MLR.

human circulating leukocytes express mRNA for both the long and short forms of the leptin receptor (5). We carefully validated the specificity of a mouse monoclonal anti-human Ob-R Ab and used this to examine Ob-R expression in human PBMCs. Ob-R expression was predominantly found on monocytes with low levels on neutrophils. Only a very small proportion of resting lymphocytes expressed Ob-R, and these were exclusively B cells. Compatible with monocyte expression of Ob-R was our demonstration that in vitro leptin rapidly induces the production of TNF- α and IL-6, this

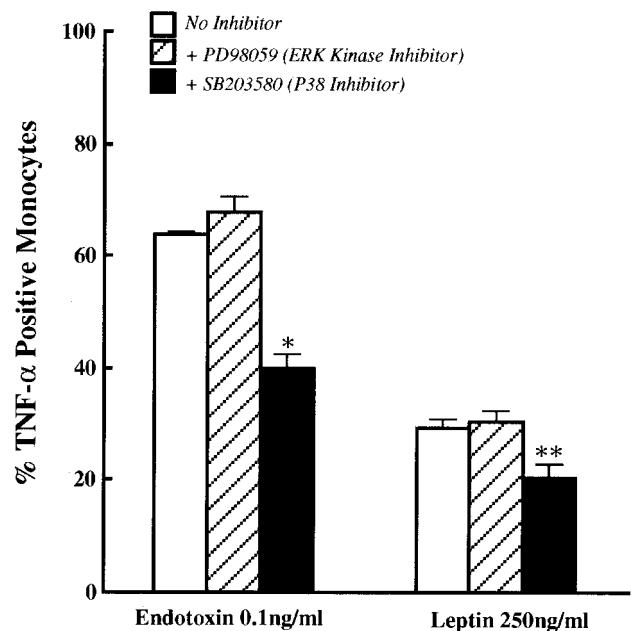


FIGURE 9. Effects of ERK inhibitor and p38 inhibitor on TNF- α production by either endotoxin- or leptin-activated monocytes. Whole blood was treated with and without ERK inhibitor (PD98059) and p38 inhibitor (SB203580) for 1 h before addition of either endotoxin (0.1 ng/ml) or leptin (250 ng/ml) for 6 h. TNF- α was detected by intracellular staining in gated CD14 $^+$ monocytes using two-color flow cytometry. The results are expressed as the percentage of CD14 $^+$ cells positive for TNF- α . The p38 inhibitor but not the ERK inhibitor partially inhibited the induction of TNF- α by both endotoxin- and leptin-activated monocytes. (*, $p < 0.01$ endotoxin only vs endotoxin plus p38 inhibitor, **, $p < 0.01$ leptin only vs leptin plus p38 inhibitor).

occurs in nonactivated as well as activated PBMCs, and the effect of leptin was seen to be additive to the actions of endotoxin.

The expression of Ob-R on human umbilical cord blood leukocytes has been reported using flow cytometry (10). In this study the proportion of monocytes expressing the Ob-R was comparable to that reported in this work ($23 \pm 4\%$); however, the proportion of lymphocytes expressing the receptor was greater ($16 \pm 4\%$) (10). The difference in results may be related to the different source of leukocytes. In our data, activation of monocytes and lymphocytes did not alter Ob-R expression.

Leptin treatment of activated PBMCs (MLR), in an experimental design similar to that reported in this study, has been shown to increase Th1 (IFN- γ) and suppress Th2 (IL-4) cytokine production (5). In the previous study, IL-6 and TNF- α were not measured and there was no effect of leptin on resting PBMCs. Our finding that leptin can activate resting PBMCs may relate to the higher dose of leptin used and confirms a study demonstrating that leptin can activate and stimulate the proliferation of resting human PBMCs (22). Leptin has been shown to have a direct effect on activated T lymphocytes (5), suggesting that leptin directly acts on T lymphocytes. We found no evidence of Ob-R on resting T lymphocytes by whole blood flow cytometry, and our data suggest that the predominant action on resting PBMCs is through activation of monocytes.

Our RT-PCR experiments demonstrate that there is an acute stimulation of cytokines by leptin, and the longer incubation studies confirm cytokine production by nonactivated PBMCs. Leptin treatment also enhanced the cytokine response to endotoxin and the MLR. Results from rodent studies confirm our observations in the human. In mice, thioglycollate-elicited peritoneal macrophages produce no detectable TNF- α or IL-6, whereas leptin pretreatment augments proinflammatory cytokine release from endotoxin-stimulated macrophages (12). In the RAW murine macrophage cell, leptin at a high dose of $1 \mu\text{g/ml}$ activates resting cells to produce significant levels of IL-1R antagonist after 24 h and also augmented the response to endotoxin in an additive manner (23). Intracerebroventricular and i.p. injection of leptin into rats induces the production of IL-1 β and increases body temperature (24).

Contamination of any component of the cell incubation by endotoxin could activate cells and induce the production of inflammatory cytokines. This seems unlikely in our experiments because endotoxin levels in the leptin preparation were well below that which is required to activate PBMCs (21). In addition, polymyxin-B (an inhibitor of endotoxin) treatment inhibited the response to endotoxin but had no effect on leptin. The profile of cytokine induction by leptin was different from that of endotoxin, with leptin inducing significant levels of IFN- γ , an effect not seen with low levels of endotoxin.

We performed a dose response for the effect of leptin on TNF- α and IL-6 production. The biological activity of the recombinant leptin was tested in a functional bioassay measuring leptin signaling. This confirmed the biological activity of leptin at doses as low as 1 ng/ml . Higher doses of leptin were required to induce cytokine production ($50\text{--}1000 \text{ ng/ml}$) than are usually found in venous blood under physiological conditions ($1\text{--}100 \text{ ng/ml}$). Our repeated experiments on PBMCs were performed with a leptin dose of 250 ng/ml . This dose was chosen because it was clear from our dose response studies that this dose of leptin could activate PBMCs. Similar higher serum leptin levels are found in some nonphysiological conditions, such as in children with chronic renal failure (leptin levels up to 400 ng/ml are reported in Ref. 15) and following leptin treatment (25). Treatment of a patient with genetic leptin deficiency with low levels of leptin (0.028 mg/kg of lean body mass) increased the serum leptin up to 107 ng/ml (26). In obese but

otherwise healthy subjects treated with leptin (1 mg/kg/day), serum leptin levels up to 736 ng/ml have been reported (25). Thus the high leptin doses we used in our study were similar to those found in some nonphysiological conditions and may lead to activation of PBMCs.

The high dose response of leptin in our study may reflect the insensitivity of our method for measuring cytokine production from incubated PBMCs, although in activated PBMCs a lower dose of leptin was reported as effective (5). Another possibility is that incubation of PBMCs with leptin over time may result in degradation of leptin and/or loss of cytokines. The higher dose of leptin may be relevant, because leptin levels could vary between tissues and leptin levels in fat tissue may be greater than those found in serum. There are other possibilities for the high dose-dependent activation of PBMCs by leptin. It is recognized that short forms of the leptin receptor can activate the MAPK signaling pathway, and it may be that either the dose response of these receptors is different from that of the long form of the receptor (27) or that leptin is activating another receptor. Recombinant leptin is known to have a lower potency than native leptin, which may be due to a different pattern of glycosylation (28).

The class I cytokine receptors are known to act through the Janus kinase/STAT and MAPK pathways (29). Both long and short leptin receptor isoforms can activate the MAPK pathway (30). The major MAPK pathways are: ERK, c-Jun NH₂-terminal kinase, and p38 MAPK. A wide variety of inflammatory mediators, such as cytokines, activate p38 MAPK in several cell types. In neutrophils, TNF- α activates p38 MAPK specifically, whereas others, such as GM-CSF, fMLP, IL-8, and PMA preferentially phosphorylate ERK 1/2 (31). To determine which MAPK signaling pathway is recruited by leptin to produce TNF- α in monocytes, PD98059 and SB203580 were used to block ERK and p38 respectively. PD98059 did not show any effect on either endotoxin-stimulated or leptin-activated monocytes, despite its inhibitory effects on leptin induction of serum response element transfected cells (data not shown). SB203580 reduced the percentage of activated monocytes by 30% in leptin-activated cells, showing involvement of p38 MAPK signaling in leptin induction of TNF- α in monocytes. Currently there is no specific inhibitor of the Janus kinase/STAT signaling pathway, so we were unable to examine the potential role of this pathway in PBMCs.

The physiological significance of leptin-induced proinflammatory cytokine production requires investigation. Leptin-deficient or -resistant rodents are more susceptible to the toxic effects of TNF (32). Although one might therefore expect that leptin would reduce levels of TNF, the reverse appears to be true. It is possible that leptin induces a negative regulator of TNF, and that the rise in TNF levels occurs in response to partial TNF resistance and is therefore compensatory. It may be that the local induction of proinflammatory cytokines provides a pathway for some metabolic actions of leptin. The production of IL-6 in the hypothalamus is known to activate the hypothalamo-pituitary axis (33), and TNF alone can produce an anorectic action (34). Another possibility is that the high leptin levels seen in obesity could be responsible for some of the adverse effects of central obesity. The local activation of TNF could play a role in atherogenesis or insulin resistance (35). Our data suggest that leptin directly activates monocytes and that the activation of T lymphocytes may be in response to the release of inflammatory cytokines. To date, the presence of Ob-R on human B lymphocytes has not been demonstrated. It has been proposed that leptin treatment may augment the immune response in malnourished individuals (6).

The fact that leptin can increase the response to endotoxin and promote proinflammatory cytokine release from apparently non-activated PBMCs should be further investigated in humans. Particular care should be taken in human therapeutic trials that leptin therapy does not activate an unwanted inflammatory response, and leptin-treated patients may be more susceptible to an inflammatory response following infection.

In conclusion, human blood leukocytes express leptin receptor, and the predominant leptin receptor-expressing cell type is the monocyte. In vitro, leptin modulates the immune response, inducing the production of inflammatory cytokines, and has an additive action on cytokine production from PBMCs exposed to endotoxin.

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