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*J Immunol* 2006; 176:7745-7752; doi: 10.4049/jimmunol.176.12.7745

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Leptin Receptor Expression and Signaling in Lymphocytes: Kinetics During Lymphocyte Activation, Role in Lymphocyte Survival, and Response to High Fat Diet in Mice

Elizabeth Papanathanassoglou,* Karim El-Haschimi,* Xian Chang Li, † Giuseppe Matarese, ‡ Terry Strom, † and Christos Mantzoros*2*

Leptin has direct effects not only on neuroendocrine function and metabolism, but also on T cell-mediated immunity. We report in this study that leptin receptor (ObR) is expressed on resting normal mouse CD4+*, CD8*, B cells, and monocyte/macrophages. ObR expression is up-regulated following cell activation, but with different kinetics, in different lymphocyte subsets. Leptin binding to ObR results in increased STAT-3 activation in T cells, with a different activation pattern in resting vs anti-CD3 Ab-stimulated T cells. Leptin also promotes lymphocyte survival in vitro by suppressing Fas-mediated apoptosis. B lymphocytes appear to be more susceptible to the antiapoptotic effects of leptin, and they show higher surface expression of ObR, compared with T cells. Moreover, CD4+ T cells isolated from ObR-deficient mice displayed a reduced proliferative response, compared with normal controls. Furthermore, ObR/STAT-3-mediated signaling in T lymphocytes is decreased in the diet-induced obese mouse model of obesity and leptin resistance. In summary, our findings show that the ObR is expressed on normal mouse lymphocyte subsets, that leptin plays a role in lymphocyte survival, and that leptin alters the ObR/STAT-3-mediated signaling in T cells. Taken together, our data further support the notion that nutritional status acting via leptin-dependent mechanisms may alter the nature and vigor of the immune response. *The Journal of Immunology, 2006, 176: 7745–7752.

Nutritional status, especially starvation, has long been linked to immune dysfunction, including increased susceptibility to infection and/or diminished numbers or reactivity of lymphocytes (1–8). More limited evidence also suggests an association between obesity and immunoincompetence (3, 4). Circulating levels of the adipocyte-secreted hormone leptin are decreased or increased in malnutrition and obesity, respectively (9, 10), and have thus been proposed to be a link between nutritional status and immune function. Recent experimental evidence (11) supports a direct role for leptin in the regulation of immunity. Leptin-deficient mice (ob/ob),3 ObR-deficient mouse (db/db) mice, as well as mice with starvation-induced leptin deficiency, display an array of immune abnormalities (12, 13). Administration of leptin reverses the starvation-induced immunosuppression in vivo, and leptin enhances T cell proliferation and Th 1 proinflammatory cytokine production in vitro, conditional on the presence of a functional ObR (14). Moreover, leptin also may increase monocyte phagocytosis and cytokine production in animal models (15) and promotes differentiation and survival of human dendritic cells (16). Although leptin has been shown to reduce cytokine withdrawal-induced apoptosis in myeloid leukemia cells (17), to rescue pancreatic β cells from apoptotic elimination through a Bel-2-dependent manner (18), and to reduce thymocyte apoptosis when peripherally administered in ob/ob mice (19), leptin’s effect on lymphocyte apoptosis has not been studied in detail.

ObR isoforms include a long signaling isoform (ObRb), which activates the JAK/STAT pathway, and several short isoform variants with partial or no signaling capabilities (20, 21). The presence, kinetics, and signaling capacity of the ObR on normal mouse lymphocyte subpopulations have not yet been investigated. We have thus studied the following: 1) whether ObR is expressed on the surface of resting mouse lymphocytes; 2) whether the distribution of cell surface ObR expression differs among lymphocyte subpopulations (CD4, CD8, B cells, and macrophages); 3) whether ObR is up-regulated during lymphocyte activation, and whether it has a role in lymphocyte apoptosis and/or survival; 4) whether the proliferative capacity of CD4+ T cells from db/db mice is impaired; and 5) whether the ObR/STAT-3-mediated signaling in T cells is different in normal and leptin-resistant states in mice.

Materials and Methods

Reagents

Mouse recombinant leptin was obtained from Eli Lilly. The following reagents also were used: serum-free UltraCulture medium, penicillin/streptomycin mixture, HBSS (BioWhittaker); Lymphocyte-M medium (Cedarlane); endotoxin (LPS from Escherichia coli 055: B5; Sigma-Aldrich); rabbit IgG control Ab (Zymed); purified anti-mouse CD16 and anti-CD3 mAb (clone 145–2C11; BD Pharmingen); PE-conjugated anti-CD4, anti-CD8, anti-CD19, and anti-CD11b (BD Pharmingen); FITC-conjugated anti-CD4 and anti-CD8 (BD Pharmingen); FITC-conjugated anti-rabbit...
anti-IgG Ab (R&D Systems); PE-conjugated annexin V, annexin V binding buffer, and 7-actinomycin (7-AAD; BD Pharmingen); and [3H]thymidine (Amersham Biosciences).

**Animals, diet, and leptin measurements**

Three- to 6-week-old C57BL/6j mice were purchased from The Jackson Laboratory. Animal procedures were in accordance with the guidelines of Beth Israel Deaconess Institutional Animal Care and Use Committees. For the high fat diet (HFD) vs low fat diet (LFD) experiments, 3-week-old mice were housed individually with free access to normal mouse chow diet (14 kcal/percent fat; PMI Feeds) for 7 days. Subsequently, animals were separated into two groups of 20 animals each with equal body weights. Animals had free access to LFD and HFD groups (10 or 45 kcal/percent fat; Research Diets) and water for 20–120 days. Food intake and body weight were recorded daily. Four-week-old C57BL/Ks-db/db and C57BL/Ks-db/+ control mice were purchased from Harlan Italy. Experiments were conducted in accordance with the guidelines of the Istituto Superiore di Sanità, Roma, Italy. Mice were age-matched for individual experiments and housed with a 12-h light/dark cycle in the animal facility at the Università di Napoli “Federico II” (Napoli, Italy). Leptin levels were quantified by radioimmunoassay (Lincor Research).

**Flow-cytometric detection of ObR expression**

Spleens were harvested from C57BL/6j mice, and single-cell suspensions were prepared in HBSS. RBC were lysed by hypotonic shock. Flow cytometric analysis was conducted with either fresh or anti-CD3-activated (incubation with 2 μg/ml purified (clone 145-2C11) anti-CD3 for 24 h) or endotoxin-activated (incubation with 12 μg/ml purified LPS from E. coli 055 for 24 h). A polyclonal rabbit anti-mouse ObR IgG Ab purified by our laboratory as described (20) was used to detect ObR expression on lymphocytes. Ab specificity and sensitivity was examined by labeling wild-type (WT) and ObR-transfected Chinese hamster ovary (CHO) cells (Fig. 1B). ObR-transfected CHO cells were generated as described previously (22). Incubation with control rabbit anti-mouse IgG Ab was used to exclude nonspecific binding. Briefly, lymphocytes were washed twice with PBS/1% BSA. One million cells were incubated (10 min, 4°C) with anti-CD16 mAb (1 μg/106 cells) to block non-Ag-specific binding to the mouse FcγRIII. The cells were then incubated with anti-ObR Ab (20 μg/106 cells; 20 min, 4°C) or rabbit IgG control and/or PE-conjugated anti-CD4, anti-CD8, anti-CD19, or anti-CD11b (BD Pharmingen), washed twice (PBS/1% BSA), and incubated with secondary FITC-conjugated anti-rabbit anti-IgG Ab (1 μg/106 cells; 20 min, 4°C) and subsequently washed twice. Ten thousand cells were analyzed per sample with an argon-laser FACS scanner (FACSort equipped with CellQuest software; BD Biosciences). Gates were set to exclude cell debris and nonspecific Ab binding, and results were analyzed by a FACS analysis software (CellQuest; BD Biosciences). Fluorescent surface labeling of ObR-transfected CHO cells with anti-ObR Ab was used as positive control to assure adequacy of ObR surface staining (Fig. 1B).

**Lymphoblast preparation for annexin V binding assays**

Resting primary splenocytes prepared as described above were activated with anti-CD3 (2 μg/ml) or endotoxin (12 μg/ml). Seventy-two hours later, cells were pelleted and washed twice in HBSS, resuspended in UltraCulture medium, and endotoxin-activated B lymphoblasts and anti-CD3-activated T lymphoblasts were separated by magnetic bead isolation (T and B cell isolation kits; Miltenyi Biotec).

**Immune cell isolation and proliferation assays**

CD4+ T cells from db/db mice (n = 5) were isolated by magnetic bead isolation (CD4+ T Cell Isolation Kit, Miltenyi Biotec) (98% pure by FACS analysis) and stimulated with Dynabeads (Dynal Biotech) mouse anti-CD3/CD28 (0.5 bead per cell; 1 × 106 cells per well). T cells were cultured in round-bottom 96-well plates (BD Biosciences/Falcon) with RPMI 1640 medium supplemented with 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies), and 5% FCS (HyClone-Pierce). CD4+ T cells obtained from normal WT mice, triplicate samples of 2 × 106 cells, were cultured in 200 μl of serum-free UltraCulture medium in 96-well flat-bottom microtiter plates in the presence or absence of dose-response leptin (0.05–500 ng/ml) for 48 h. Cells were stimulated for 3 days, and on the last day, 0.5 μCi per well [3H]thymidine (Amersham Biosciences) was added to the cultures, and cells were harvested after 12 h. Radioactivity was measured with a beta plate scintillation counter (Wallac). These experiments were performed twice by two separate investigators independently.

**Nuclear extraction and EMSA**

Nuclear extractions were done as described earlier (22) with specific adjustments for nonadherent cells. Briefly, 2 × 106 fresh or anti-CD3-activated (anti-CD3, 2 μg/ml, 24 h) splenic leukocytes were placed in serum-free medium (10 × 106 cells in 5 ml of medium) and stimulated with OBR (10 ng/ml). Following incubation, cells were pelleted by centrifugation and washed in ice-cold Tris-buffered saline.

**Induction of apoptosis and annexin V binding assay**

Splenic leukocytes or lymphoblasts prepared as described above were cultured in serum-free UltraCulture medium on anti-Fas mAb-coated (1 μg/ml) 24-well plates in the presence or absence of leptin in concentrations ranging from 0.05 to 500 ng/ml for 3, 8, and 18 h. Cells were washed twice in PBS/1% BSA. One million cells were resuspended in 100 μl of annexin V binding buffer and incubated with PE-annexin V to detect apoptotic cells and/or 7-AAD to detect necrotic cells for 15 min at 4°C. Cells were subsequently washed twice in annexin V binding buffer and analyzed with a FACS scanner as described above.

**Statistical analysis**

Data are shown as means ± 95% confidence intervals (CIs), or means ± SD or SE as noted. Means were compared with Student's t test and

![FIGURE 1](http://www.jimmunol.org/)  
**FIGURE 1.** T cell-specific stimulation up-regulates the ObR expression over time. A, ObR expression in the mixed primary lymphocyte population after stimulation with anti-CD3 mAb. Following incubation with Fc blocker, lymphocytes were incubated with a polyclonal rabbit anti-mouse ObR Ab, washed twice, and incubated with a secondary FITC-conjugated Ab (anti-rabbit IgG). Lymphocyte subsets were determined through simultaneous labeling with PE-conjugated anti-CD4, -CD8, -CD19, and -mac-1 Abs (see also Table I). Gray line represents fluorescent labeling with rabbit IgG control Ab which was gated out of the analysis of ObR-positive cells. Ten thousand cells were analyzed per sample at an argon laser flow cytometry scanner. One of five experiments that yielded similar results is represented (results summarized in Table I; ObR). B, Fluorescent surface labeling of ObR-transfected CHO cells with anti-ObR Ab used as positive control. Gray line represents WT CHO cells, which do not express ObR.
Table I. Time course of ObR expression following lymphocyte stimulation with anti-CD3 (for T cells) or endotoxin (for B cells and macrophages) in vitro in the mixed lymphocyte population and specific lymphocyte subsets

<table>
<thead>
<tr>
<th>Time Post stimulation</th>
<th>Mixed Lymphocyte Population</th>
<th>CD4⁺</th>
<th>CD8⁺</th>
<th>B Cells</th>
<th>Macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percentage of ObR⁺ Cells</td>
<td>MFI</td>
<td>Percentage of ObR⁺ Cells</td>
<td>MFI</td>
<td>Percentage of ObR⁺ Cells</td>
</tr>
<tr>
<td>0 h</td>
<td>23.3 ± 3.6</td>
<td>~190</td>
<td>17.1 ± 2.1</td>
<td>~1430</td>
<td>13.7 ± 2.1</td>
</tr>
<tr>
<td>24 h</td>
<td>73 ± 7.8</td>
<td>~860</td>
<td>72.4 ± 6.5</td>
<td>~2640</td>
<td>52.7 ± 3.7</td>
</tr>
<tr>
<td>48 h</td>
<td>57.4 ± 2.9</td>
<td>~550</td>
<td>52.6 ± 5.2</td>
<td>~2300</td>
<td>35.6 ± 4.2</td>
</tr>
<tr>
<td>72 h</td>
<td>53.3 ± 3.9</td>
<td>~650</td>
<td>11.6 ± 1.6</td>
<td>~1800</td>
<td>18.4 ± 3.6</td>
</tr>
</tbody>
</table>

* Percentages represent means ± SD of n = 5 experiments.

ANOVA. Statistical analysis was conducted using Statistical Package for Social Sciences software (version 8.0; SPSS). Statistical significance was set at the α = 0.05 level.

Results

Expression kinetics of the ObR on lymphocyte subpopulations

ObR expression was detected in ~20% of the total resting lymphocytes (C57BL/6j-derived fresh spleen cells) (Fig. 1 and Table I). Cells were subsequently activated in culture by either anti-CD3 mAb (2 μg/ml) or purified endotoxin (12 μg/ml), and cells were labeled and analyzed by flow cytometry at 0, 12, 24, 48, and 72 h following activation. ObR-positive cells increased gradually to a peak of ~73% of lymphocytes at 24 h and dropped to ~57% at 48 h and finally to 53% at 72 h (percentages represent means of five experiments) (Table I). Following lymphocyte activation, ObR fluorescence shifted to higher intensity, suggesting that stimulation induces heightened receptor density per cell. Although in resting cells, ObR-positive cells demonstrated low receptor density (mean fluorescence intensity (MFI) ~190), stimulation resulted in

FIGURE 2. Kinetics and dose response of STAT-3-mediated signaling in resting and anti-CD3-activated lymphocytes stimulated with leptin. A. One representative gel showing STAT-3 activation following time course of leptin stimulation (0, 5, 10, 15, and 30 min) in resting and activated lymphocytes. Activated splenocytes were stimulated with anti-CD3 (2 μg/ml) mAb in culture for 24 h. Following time course of leptin stimulation, nuclear extracts were prepared and analyzed by EMSA with a STAT-3 probe. Different kinetics of STAT-3 activation were observed in resting vs activated lymphocytes. One of six experiments that yielded similar results is presented in A. The control panel were ObR overexpressing CHO cells treated with leptin for 10 min that provided a solid response in the EMSA assay. B and C. Densitometric quantification of the STAT-3 activity over time (arbitrary units) in activated and resting lymphocytes. Following time course of leptin stimulation, nuclear extracts were prepared and analyzed by an EMSA with a STAT-3 probe. Different kinetics of STAT-3 activation were observed in activated (B), compared with resting (C) lymphocytes. Densitometric data (means ± 95% CI of the mean) averaging six experiments are shown. Data were analyzed by ANOVA (p < 0.05). Through posthoc comparisons, statistically significant differences were observed between the 5- and 30-min groups with all other groups in B and between the 5-min group with all other groups in C, D. One representative gel showing STAT-3 activation following dose response leptin (a, 0.05; b, 0.05; c, 0.5; d, 5; e, 50; f, 500 ng/ml) in activated and resting lymphocytes. Activated splenocytes were stimulated with anti-CD3 (2 μg/ml) mAb in culture for 24 h. Five minutes after leptin stimulation, nuclear extracts were prepared and analyzed by EMSA with a STAT-3 probe. E and F. Means and 95% CI of the mean STAT-3 activity (by densitometric analysis expressed as arbitrary units) following dose-response leptin stimulation (a, 0.05; b, 0.05; c, 0.5; d, 5; e, 50; f, 500 ng/ml) in activated (E) and resting (F) lymphocytes (n = 5 experiments). Densitometric data (means ± 95% CI of the mean) averaging five experiments are shown. Activated splenocytes were stimulated with anti-CD3 (2 μg/ml) mAb in culture for 24 h. Five minutes after leptin stimulation, nuclear extracts were prepared and analyzed by EMSA with a STAT-3 probe. Statistically significant differences in STAT-3 activity were revealed among lanes in activated (p < 0.0001) and in resting lymphocytes (p < 0.0001) by ANOVA analysis. Through posthoc comparisons, in both activated and resting lymphocytes, statistically significant differences (p < 0.05) were observed at the following pairs of lanes: a-b, a-c, a-d, a-e, a-f, b-d, b-e, b-f, c-d, c-e, c-f, and d-f.
the emergence of a lymphocyte population with higher receptor density (high expressers). Maximum receptor density appeared to be attained at 24 h (MFI ~860). At 48 and 72 h, all ObR-positive cells were high expressers (MFI ~650) (Fig. 1 and Table I).

Multicolor fluorescent staining was then used to investigate ObR expression on lymphocyte subsets. Approximately 17% of resting CD4+ T cells were ObR positive with moderate ObR density (Table I). Following anti-CD3 stimulation, the percentage of ObR-positive CD4+ cells increased markedly to ~72% (~20% high expressers) at 24 h, 52% at 48 h (~27% high expressers), and dropped to 11% at 72 h (all high expressers) (Table I). CD8+ T cells exhibited a similar time course of ObR expression with an

![Image of Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** Leptin inhibits apoptosis in B and T lymphocytes. **A,** Effect of dose-response leptin on the percentage of annexin V-positive B lymphocytes (n = 5 experiments) over time. B cells were separated by magnetic cell sorting (see Materials and Methods) of endotoxin-activated primary splenocytes (72 h) and were cultured in serum-free medium in 24-well plates precoated with anti-Fas mAb (1 μg/ml) and leptin in increasing doses or no leptin added. Apoptosis was quantified as the percentage of annexin V-positive cells by flow cytometric analysis. Necrotic cells were excluded from the analysis through the use of a vital dye (7-AAD). Means ± 95% CI of the mean are shown. Data were analyzed by ANOVA. Leptin decreased annexin V-positive cells in a dose-dependent manner at 6 h (p < 0.0001, between dose-group differences detected by ANOVA) and 18 h (p < 0.0001, between dose-group differences detected by ANOVA) but not at 3 h of culture (p = 0.647; between dose-group differences detected by ANOVA). The effect of all escalating leptin doses (0.05–500 ng/ml) differed statistically significantly from the “0” leptin concentration. Posthoc analyses revealed statistically significant differences at all pairs of doses, with the exception of the comparison of 0.5–5.0 ng/ml and 0.5–500 ng/ml at 6 h of culture, and the comparison of 0.5–500 ng/ml at 18 h of culture, which did not differ statistically significantly from each other. **B,** Effect of dose-response leptin on the percentage of annexin V-positive CD4+ T cells at 3, 6, and 18 h in culture (n = 6 experiments). T cells were separated by magnetic cell sorting (see Materials and Methods) of splenocytes cultured for 72 h with 2 μg/ml anti-CD3. T cells were cultured in serum-free medium in 24-well plates precoated with anti-Fas mAb (1 μg/ml) and leptin in increasing dose or no leptin. Apoptosis was quantified as the percentage of annexin V-positive cells by flow cytometric analysis. Necrotic cells were excluded from the analysis through the use of a vital dye (7-AAD). CD4-positive cells were detected through staining with FITC-conjugated anti-CD4. Means ± SE of the mean are represented. Data were analyzed by ANOVA. There were no statistically significant differences in the percentage of annexin V-positive cells at 3 and 6 h in culture (ANOVA, p > 0.10). At 18 h in culture, a significant decrease in the percentage of annexin V-positive cells with escalating doses of leptin was detected, but using posthoc analyses, no significant differences in-between groups of leptin concentrations were detected (p > 0.10). **C,** Effect of dose-response leptin on the percentage of annexin V-positive lymphocytes in culture (n = 6 experiments). Primary splenocytes were cultured in serum-free medium with anti-CD3 stimulation (2 μg/ml) and dose-response leptin or no leptin. Apoptosis was quantified as the percentage of annexin V-positive cells by flow cytometric analysis. Necrotic cells were excluded from the flow cytometric analysis through the use of a vital dye (7-AAD). Means ± 95% CI are presented. Data were analyzed by ANOVA (3 h, p < 0.05; 8 h, p < 0.01). By posthoc analyses, at 3 and 8 h of culture statistically significant differences were detected in between all pairs of doses with the exception of the comparison of 0.5 with 5.0 ng/ml and 50 with 500 ng/ml. **D,** Effect of dose-response leptin on the percentage of annexin V-positive CD8 T cells at 18 h in culture (n = 5 experiments), showing no statistically significant effect. Data at 3 and 6 h in culture were similar (data not shown).
early up-regulation at 24 h and a large decrease at 72 h (18% ObR-positive cells) (Table I). Interestingly, a larger proportion of resting B cells (57%) were ObR-positive, also containing a sub-population (20%) of B cells expressing high levels of ObR (Table I). Similar to T cells, the levels of ObR expression increased in endotoxin-activated B cells. Approximately 73% of B cells became positive at 24 h (~30% high expressers), 82% at 48 h (~50% high expressers), and ~87% at 72 h (all high expressers). Macrophages exhibited lower ObR-positive cells but with a comparable time course of ObR expression: ~28% of resting macrophages expressed ObR, which was increased to 76% at 48 h to remain at ~70% ObR-positive cells at 72 h. ObR density on macrophages was high (Table I). Hence, resting naive lymphocytes constitutively expressed low levels of ObR. Following lymphocyte stimulation, levels of ObR expression diverged among lymphocyte subsets: the percentage of T cells bearing the ObR increased early and subsequently decreased greatly, leaving a small percentage of cells with high receptor density. In B cells and macrophages, after an initial up-regulation the percentage of positive cells, the ObR remained high for the times analyzed.

**ObR/STAT-3-mediated signaling in T lymphocytes**

ObR can stimulate gene transcription through activation of the DNA binding activity of STAT proteins (signal transducers and activators of transcription). The activation of STAT-3 in response to leptin (10 ng/ml) stimulation was assessed over time in resting and activated spleen-derived lymphocytes after 24 h of anti-CD3 stimulation in vitro (serum-free medium, to reduce the risk of unspecific effects due to leptin present in culture serum) by EMSA. STAT-3 DNA binding activity was increased 5 min following leptin stimulation (10 ng/ml) in both resting and activated lymphocytes and subsequently gradually decreased at later time points (Fig. 2, A–C). This suggests that the long form of ObR is expressed on the surface of both resting and activated lymphocytes, and that different kinetics of STAT-3 activation were observed in resting compared with activated T lymphocytes (Fig. 2, B and C). Specifically, after initial activation, higher levels of STAT-3 activity were sustained in activated T lymphocytes, in contrast with STAT-3 activity in resting T lymphocytes, which returned to baseline 10 min after activation. In addition, STAT-3 activation in response to dose-response leptin (0.05–500 ng/ml) also was assessed. STAT-3 DNA-binding activity was stimulated with a dose of 0.5 ng/ml leptin and greater (Fig. 2, D–F).

**Protective effect of leptin on FAS-mediated T and B lymphocyte apoptosis**

To determine the role of ObR expression on lymphocyte survival, we investigated the effects of leptin on apoptosis of B and T lymphocytes (Fig. 3). Resting primary splenocytes were activated with anti-CD3 (2 μg/ml) or endotoxin (12 μg/ml) and B and T lymphoblasts were isolated (see Materials and Methods). T and B cells were plated on 24-well culture plates precoated with anti-Fas mAb (1 μg/ml) to cross-link the FasR. Apoptotic cell death was assessed after 3, 6, and 18 h in culture with either dose-response leptin (0.05–500 ng/ml) or no leptin by the annexin V binding assay and flow cytometric analysis. A vital dye, 7-AAD, was used to exclude necrotic cells. CD4+ and CD8+ cells were detected through staining with FITC-conjugated anti-CD4 or anti-CD8.

In B lymphocytes (Fig. 3A), leptin decreased annexin V-positive cells in a dose-dependent manner at 6 h (p < 0.001) and 18 h (p < 0.001) but not at 3 h of culture with FasR cross-linking (Fig. 3A). In CD4+ T lymphocytes, a significant decrease (p < 0.05) in the percentage of annexin V-positive cells was observed in CD4+ cells at 18 h but not at 3 and 6 h; however, there was no indication of dose response (p = 0.137; Fig. 3B). There was no decrease in the percentage of annexin V-positive cells in CD8+ lymphocytes (p = 0.562; Fig. 3D).

We further evaluated the effect of leptin on splenocyte apoptosis in culture (Fig. 3C). Leptin-treated cells exhibited a decrease in the amount of annexin V-positive cells in a dose-dependent manner at 3 h (p = 0.002) and 8 h (p < 0.001) of culture (Fig. 3C). At 36 h of culture, although a trend for decreased apoptosis with increasing doses of leptin was observed, the differences were no longer significant (p = 0.73).

To decipher potential mechanisms accounting for the protective effect of leptin on apoptosis of B lymphocytes we quantified the expression of Bcl-2 (a mitochondrial protein inhibiting apoptosis) and of Fas (a prominent apoptotic receptor on lymphocytes). No difference in the expression of either Bcl-2 or Fas was observed with increasing doses of leptin (0–500 ng/ml; data not shown). These results suggest that leptin may prolong B cell survival following activation and FasR cross-linking; however, this effect appears to be independent of Bcl-2 and Fas expression.

**Effect of ObR and leptin on anti-CD3-induced proliferation**

Stimulation with anti-CD3/CD28 of CD4+ T cells from db/db mice was not as effective as CD4+ from db/+ control mice, indicating lesser responsiveness of CD4+ T cells from ObR-deficient mice (Fig. 4A) (p = 0.0001). These data suggested that absence of the ObR could impair expansion of CD4+ T cells.

In addition, to investigate the effect of leptin on lymphocyte proliferation, primary lymphocytes were activated by anti-CD3 (2 μg/ml) and cultured with increasing concentrations of leptin (0–500 ng/ml). No significant increase in proliferation was observed with doses of leptin in the physiologic leptin range (0.05–30 ng/ml) using ANOVA (data not shown).

**Effect of HFD vs LFD on ObR/STAT-3-mediated signaling in T lymphocytes**

To investigate the effect of diet on leptin signaling and ObR expression in lymphocytes, 4-wk-old C57BL/6d mice were given either HFD or LFD. Animals were studied at 20 and 120 days after...
the initiation of diet; body weight and serum leptin levels were significantly higher in the HFD group ($p < 0.003$) (Fig. 5). STAT-3 activity and ObR expression were determined in splenocytes. At 20 days after the initiation of diet, 5 min after leptin stimulation, the difference in STAT-3 activity in the HFD group was not significantly different from baseline, as opposed to the LFD where a significant activation of STAT-3 was observed in resting and in activated cells ($p < 0.05$) (Fig. 6, A and B). The same results were replicated at 120 days of HFD and LFD (Fig. 6, A and B). Flow-cytometric analysis of ObR expression revealed a small and not statistically significant decrease in percentages of ObR-positive lymphocytes at the HFD group, especially in the B and
Discussion

Our data suggest that macrophages and B and T lymphocytes constitutively express low levels of ObR, and that this expression is up-regulated, in terms of both percentage of positive cells and receptor density, in response to activation of B cells, T cells, and macrophages. Moreover, ObR expression appears to be higher in macrophages, compared with T and B cells at baseline, following lymphocyte stimulation. Lord et al. (14) have demonstrated that leptin may amplify CD4+ T cell responses, and ObR mRNA expression has been detected in human CD4+ T lymphocytes. In addition, human monocytes that have been shown to be activated by high-dose leptin also express ObR (24). ObR expression has been demonstrated not only on CD4+ but also on CD8+ human T cells (25), and more recently, expression of ObR on T cells mediating the regulation of immune responses has been reported in a mouse model of colitis (26). Finally, Zhao et al. (27) reported constitutive expression of both long and short ObR forms on human NK cells, which mediate regulation of NK cytotoxicity.

We now demonstrate through detection of activation of STAT-3 binding activity that a signaling form of ObR is expressed on both resting and activated T lymphocytes. This result is in agreement with the findings of Maccarrone et al. (28) who reported STAT-3, but not STAT-1 or STAT-5, activation in human T lymphocytes by leptin binding to its receptor. Although there are several different cell surface ObR isoforms with similar extracellular domains, only the long signaling isoform, ObRb, is able to activate STAT-3 (29, 30). Because the anti-ObR used for cytofluorimetric analysis recognizes only the common extracellular domain, no definitive inference can be made on the type of ObR implicated in our observations. The evidence that the STAT-3 activity is higher in activated splenocytes, however, suggests that the long signaling isoform, ObRb, is probably involved in these phenomena. Furthermore, these findings suggest that leptin may exert its actions on both naive and activated lymphocytes; however, B cells, macrophages, and activated lymphocytes may be more susceptible to leptin’s effects. Dose-response experiments of leptin signaling on lymphocytes revealed initiation of signaling at the expected leptin concentration based on the ObR dissociation constant ($K_d = 0.3$ nmol/l) (22).

Restimulation of activated T lymphocytes results in apoptotic elimination by activation-induced cell death. To mimic activation-induced cell death culture, lymphocytes were subjected to FasR cross-linking to trigger apoptotic cell death. Leptin treatment decreased the amount of apoptotic elimination in B lymphocyte cultures following anti-Fas cross-linking in a dose-dependent manner. B cells appear to be more susceptible to the rescuing effects of leptin. Whether this difference is linked to the higher ObR expression on B lymphoblasts remains unclear. Interestingly, survival of mixed lymphocytes of mice with inactivating mutations of the ObR is impaired in vitro. In agreement with these in vitro observations, continuous leptin administration has been shown to prevent lymphocyte apoptosis in vivo in a mouse model of stress (31). Leptin also may rescue myeloid leukemia cells, but not normal T cells, from growth factor-induced apoptosis (17). In addition, although maintenance of Bcl-2 expression has been proposed as a mechanism for the antiapoptotic effects of leptin on pancreatic $\beta$ cells (18), no differences in Bcl-2 expression were observed in our study, presumably suggesting that leptin may affect an antiapoptotic pathway distinct from Bcl-2 in lymphocytes.

The finding that physiologic leptin concentrations did not affect proliferation of mixed lymphocyte populations significantly may be understandable in light of the observations by Lord et al. (32), who reported that the anti-CD3 driven proliferation of naive T cells was enhanced, whereas, proliferation of memory T cells was inhibited by leptin; therefore leptin effects on unsorted leukocyte populations may be blunted. In addition, the finding that CD4+ T cells from ObR-deficient $db/db$ mice showed a reduced proliferative capacity when compared with normal heterozygote $db/+\$ controls suggests that the presence of the long signaling isoform of the ObR is important in CD4+ T lymphocyte proliferation and expansion during TCR-mediated stimulation. Consequently, ObR/STAT-3 signaling was significantly suppressed in leptin-resistant HFD animals, compared with their LFD-matched counterparts. The differences in ObR signaling in HFD- vs LFD-fed mice is an interesting finding with potential physiologic and therapeutic significance. Inasmuch as levels of ObR expression on total lymphocytes and specific subtypes were not significantly different between the two groups, we presume that the blunted ObR signaling following HFD could be due to postreceptor defects. Our data are the first to show that HFD-induced leptin resistance is associated with altered signaling capacity of the ObR, not only centrally (hypothalamus), but also in the periphery. Decreased responsiveness to leptin has been well documented in mice fed a HFD, potentially dependent on the gender and/or duration of exposure to diet and/or the strain of mice (33–35). The decreased STAT-3 activity in lymphocytes of mice receiving a HFD are consistent with prior reports showing decreased STAT-3 activity in the hypothalamus of HFD mice (36). This dietary effect has been found recently to be associated, at least in part, with increased level of the suppressor of cytokine signaling 3, an inhibitor of leptin signaling, that is specifically increased in the hypothalamus of HFD mice (36). Therefore, the elevated expression of suppressor of cytokine signaling 3 may account for the state of leptin resistance and the reduced STAT-3 activity, observed in these mice. Future studies are needed to elucidate differences in HFD-induced obesity and in ObR signaling defects on the basis of gender, strain of animals, as well as on the duration of high fat feeding. In this context, it is reasonable to hypothesize that similar to relative leptin deficiency, leptin-resistance could lead to alterations of immune responsiveness in immune cells of obese subjects. In addition to unequivocal evidence linking starvation, a leptin deficiency state, with immune dysfunction, clinical and epidemiological evidence suggests that obese individuals who have leptin resistance may also be more prone to infections. Obesity has been linked to an altered immune response in both humans and genetically obese rodents, but the underlying factors are not fully understood, and it remains unclear whether obesity itself or complications of obesity are responsible for immune abnormalities (3, 4, 37). It remains to be elucidated by more extensive investigation whether leptin resistance, often found in obesity, could be perceived by T cells as a state of functional leptin deficiency due to impaired ObR signaling and reduced STAT-3 activity, as seen in HFD-fed obese mice studied herein.

In summary, this is the first study to explore ObR distribution among different lymphocyte subpopulations in mice and to study kinetics after immune cell activation. In addition, we investigated the intracellular signaling capacity following leptin stimulation of T cells before and after TCR stimulation. Finally, we also examined the effect of HFD, and thus leptin resistance, on T cell-specific ObR-STAT-3-signaling. These results may elucidate the role of
leptin in immunity and in immune alterations observed in leptin-resistant subjects and may help to develop novel strategies for therapeutic interventions.

Acknowledgments
We thank Prof. Jeffrey Flier for helpful discussions and comments.

Disclosures
The authors have no financial conflict of interest.

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leptin receptor expression on lymphocytes.