Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression


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Nutritional deprivation suppresses immune function1–3. The cloning of the obese gene and identification of its protein product leptin4 has provided fundamental insight into the hypothalamic regulation of body weight5,6. Circulating levels of this adipocyte-derived hormone are proportional to fat mass7,8 but may be lowered rapidly by fasting8,9 or increased by inflammatory mediators10,11. The impaired T-cell immunity of mice12,13 now known to be defective in leptin (ob/ob)4 or its receptor (db/db)14,15, has never been explained. Impaired cell-mediated immunity1–3 and reduced levels of leptin are both features of low body weight in humans. Indeed, malnutrition predisposes to death from infectious diseases16. We report here that leptin has a specific effect on T-lymphocyte responses, differentially regulating the proliferation of naive and memory T cells. Leptin increased Th1 and suppressed Th2 cytokine production. Administration of leptin to mice reversed the immunosuppressive effects of acute starvation. Our findings suggest a new role for leptin in linking nutritional status to cognate cellular immune function, and provide a molecular mechanism to account for the immune dysfunction observed in starvation.

Most immune responses are orchestrated by CD4+ helper T cells (Th). We first determined the effect of leptin on Th responses in the context of the mixed-lymphocyte reaction (MLR) resulting from the culture of T cells with major histocompatibility complex (MHC)-incompatible (allogeneic) stimulator cells. The doses of leptin used in these experiments were chosen to incorporate the range of serum levels measured in humans17. Leptin induced a


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marked dose-dependent increase in the proliferative response of peripheral blood lymphocytes (PBLs) in response to allogeneic peripheral blood mononuclear cells (PBMCs) (Fig. 1a). The allogenerative response was augmented in a similar way when the experiment was repeated using highly purified CD4\(^+\) cells as responders, suggesting that T cells, rather than accessory cells in the responder population, were the target of leptin action (Fig. 1b). To determine whether it was the responder or the stimulator cells that were being affected by leptin, either responder or stimulator populations were preincubated for 12 hours in the presence or absence of leptin and then washed extensively before co-culture in the absence of leptin. Preincubation of the responder CD4\(^+\) T cells with leptin produced a greater enhancement of proliferation in the subsequent MLR than did preincubation of the stimulator cells (Fig. 1c). The augmentation of proliferation was still seen, despite the fact that the leptin was only present for 12 hours during preincubation, rather than for the duration of the MLR (5 days) as in the previous experiments. Incubation of CD4\(^+\) T cells with leptin, in the absence of allogeneic stimulator cells, produced no increase in proliferation (data not shown), suggesting that cognate recognition by the T-cell antigen receptor (TCR) was required before leptin could have its effect.

To confirm the specificity of these effects, we compared the responses of T cells from leptin receptor-defective db/db mice with those of their littermate controls (db/+). Several splice variants of the leptin receptor (ObR) are expressed in vivo. Although all of the receptor isoforms bind leptin, the long isoform of the receptor (ObRb) appears to be of prime importance in signal transduction\(^1,4,15\). A point mutation within the ObR gene of the diabetic (db) mouse generates a new splice donor site that dramatically reduces the expression of the long isoform in homozygous db/db mice\(^1,15,16\) and renders them resistant to the weight-lowering effects of endogenous and exogenous leptin\(^5\). The allogenerative response of splenocytes from homozygous db/db mice was similar to that of heterozygous db/+ mice in the absence of leptin, consistent with reports that these cells can function normally ex vivo\(^1,17\). However, the leptin-mediated augmentation of proliferation in response to allogeneic splenocytes seen with responder cells from db/+ mice was not seen with db/db responder cells, despite the higher doses of leptin used (Fig. 1d). Similar results were obtained when MHC class II-transfected murine fibroblasts were used as allogeneic stimulators. All the MLR responses were inhibited by addition of an appropriate anti-MHC class II antibody (data not shown). These results indicate that the observed enhancement of proliferation by leptin was due to a specific effect of leptin receptor signalling, rather than to a nonspecific mitogenic stimulus.

Using long-isoform-specific primers, we were able to detect the expression of ObRb messenger RNA by the reverse transcriptase-polymerase chain reaction (RT-PCR) in human hypothalamus and PBMC, but not in kidney, in agreement with previous reports\(^15,18\). Consistent with our functional data, we detected ObRb mRNA in highly purified resting human CD4\(^+\) T cells but not in freshly isolated monocytes (Fig. 2). Taken together, these results indicate that leptin enhances T-cell responses primarily by binding to its receptor on the T cells, rather than through a direct effect on the stimulator cell.

The MLR provokes a strong proliferative response by both naive and memory T cells\(^19\). In time-course studies, the effect of leptin in an MLR was maximal at day 7, a kinetic characteristic of a primary (naive) immune response (data not shown). We therefore investigated whether leptin had differential effects on naive (CD45RA\(^+\)) and memory (CD45RO\(^+\)) CD4\(^+\) T cells, which both express ObRb mRNA (not shown). Depletion of CD45RO\(^+\) rather than CD45RA\(^+\) cells from the responding population of PBMCs in an MLR enhanced the response to leptin (Fig. 3a). Proliferative responses were also determined in an MLR after preincubating highly purified naive or memory T cells for 12 hours either in the presence or absence of leptin, and then washing the cells extensively before culturing them with allogeneic stimulator cells. The effect of leptin was much more pronounced on the CD4\(^+\) CD45RA\(^+\) T cells than on CD4\(^+\) CD45RO\(^+\) T cells (Fig. 3b). Further evidence that leptin amplifies primary T-cell responses was provided by the use of responder T cells from umbilical cord blood (UCB), the purest preparation of naive

![Figure 2](image1.png) **Figure 2** The long isoform of the leptin receptor is expressed in CD4\(^+\) T cells. a, RT-PCR with human ObRb specific primers: lane 1, hypothalamus; lane 2, no RNA control; lane 3, PBMCs; lane 4, CD4\(^+\) T cells; lane 5, monocytes; lane 6, renal mesangial cells. b, Southern blot analysis to confirm the specificity of the PCR product. c, RT-PCR with primers specific for β-actin.
T cells. As shown in Fig. 3c, proliferation was increased tenfold in the presence of leptin. The reduced effect of leptin on proliferation by memory phenotype T cells in the MLR was paralleled by the finding that leptin had little effect on recall responses of previously immunized individuals to tetanus toxoid (Fig. 3d), or on proliferation of a panel of established human T-cell clones (data not shown).

CD4+ T cells (Th) determine the nature of immune effector function according to the pattern of cytokines that they secrete. Leptin favours the production of proinflammatory cytokines. This cell population can be divided into two subsets: Th1 cells, which secrete proinflammatory cytokines (such as interleukin(IL)-2 and interferon(IFN)-γ), and Th2 cells, which secrete cytokines with predominantly regulatory functions (such as IL-4). Several factors have been identified that polarize Th cells into these subsets. We tested the possibility that leptin influences Th cytokine production by measuring IL-2, IFN-γ and IL-4 in MLR experiments (Fig. 4). We found that leptin increased IL-2 production by all the T-cell populations studied apart from the CD45RO+ cells, which, despite a lack of proliferation and IL-2 production, showed a substantial increase in IFN-γ secretion (Fig. 4a, b). A similar increase in IFN-γ secretion was seen with the other T-cell preparations. Only the CD45RO+ population secreted measurable amounts of IL-4, and this was completely inhibited by the addition of leptin (Fig. 4c). These results indicate that leptin may bias T-cell responses towards a proinflammatory phenotype (Th1). We therefore analysed the cytokine and alloproliferative responses of responder cells from C57BL/6 ob/ob mice. This mouse strain is unable to produce functional leptin, and therefore its T cells have never been exposed to leptin in vivo. In the absence of leptin, ob/ob T cells produced minimal IFN-γ and a moderate amount of IL-4 (Fig. 4d). The addition of leptin induced the production of large amounts of IFN-γ and suppression of IL-4 production in a dose-dependent manner. Leptin increased both proliferation (Fig. 4e) and

**Figure 4** Leptin favours the production of proinflammatory cytokines. a–c. Cytokine production in the presence or absence of leptin in an MLR using human UCB cells, naive (CD45RA+), memory (CD45RO+) T cells or PBMCs as responders: a. Interferon-γ; b. interleukin-2; c. interleukin-4. d. Interferon-γ and interleukin-4 production by ob/ob T cells in an MLR in the presence of increasing doses of leptin. e. Proliferation of ob/ob responder T cells in an MLR in the presence of increasing doses of leptin, done in autologous ob/ob serum. All data are expressed as mean ± s.e.m.

**Figure 5** Leptin reverses starvation-induced immunosuppression. a. The effect of 48-h food deprivation on bodyweight of male C57BL/6 mice. fed = fed/sham DTH; starved = starved/leptin. b. The effect of leptin replacement during starvation on the DTH recall response to oxazolone in male C57BL/6 mice. All data are expressed as mean percentage increase in ear thickness ± s.e.m. (n = 6 per group). P < 0.0005, **P < 0.0001, one-way analysis of variance with the Bonferroni adjustment for multiple group testing.
IL-2 production (not shown). Ob/ob responder T cells were more sensitive to exogenous leptin when compared with wild-type controls (21-fold as compared with a 5-fold increase in IFN-γ secretion; results not shown). This suggests that in the absence of leptin, Th2 responses are favoured, whereas addition of leptin seems to induce a switch to a Th1-type response in this system.

Proinflammatory cytokines such as IFN-γ can upregulate adhesion molecules and certain accessory molecules can bias a T-cell response towards the production of specific cytokines. Morphological examination of cultures of human PBMCs and murine splenocytes showed that leptin induced marked cellular clumping at 36 hours by both human and db/+ cells, but not by db/db cells (data not shown). In an MLR, leptin induced the expression of the adhesion molecules ICAM-1 (CD54) and VLA-2 (CD49b) on CD4+ T cells, but had no effect on the expression of other molecules that mediate adhesion (CD49a, c, d, f, CD50 or CD62L) (data not shown).

Nutritional deprivation affects immune function and also rapidly reduces the amount of circulating leptin in mice and humans. We investigated the capacity of exogenous leptin replacement to reverse the inhibitory effects of starvation on T-cell priming as measured by a delayed-type hypersensitivity (DTH) response. Starvation of mice for 48 hours led to a 69% reduction in the mean DTH response. This inhibition was completely reversed by injection of leptin during the period of starvation (Fig. 5).

Our observations further the hypothesis that a falling leptin concentration acts as a peripheral signal of starvation which serves to conserve energy in the face of limited reserves. In this situation, energy must be preserved for vital functions such as central nervous system metabolism. Less immediately essential systems, like the reproductive axis and a finely tuned cognate immune response, which requires large-scale clonal expansion, are inhibited. Starvation is accompanied by an adaptive neuroendocrine response which includes activation of the hypothalamic–pituitary–adrenal axis and suppression of the thyroid and gonadal axes. Leptin has been shown to blunt these adaptive changes. Immunoregulatory effects of these hormones are well recognized. The extent to which the immunomodulatory effects of leptin in vivo are mediated by a direct action on the CD4+ T cell, rather than by an indirect effect of leptin causing central suppression of the neuroendocrine response to fasting, remains to be determined. However, this regime of leptin repletion in mice only partially reverses the inhibition of the T-cell proliferation as measured by the immunological assays. Pre-in vitro findings indicate that leptin can modify T-cell responses directly. It is well recognized that starvation is associated with a higher frequency of infectious diseases; our results demonstrate that leptin modulates cognate T-cell immune function and may therefore be the key link between nutritional status and an optimal immune response.

Methods

Reagents. The human and murine recombinant leptin was purchased from R&D, Minneapolis, and from Peprotech, London. The recombinant leptin used was >97.5% pure, as judged by SDS–PAGE analysis and contained <0.1 ng mL−1 LPS as determined by the Limulus amoebocyte lysate method. Tetanus toxoid was from Evans Medical. The appropriate mAbs were used for purifying cells for the proliferation and cytokine assays, as described.

Preparation of cells. Blood was obtained from healthy adult volunteers or from the umbilical cords of neonates (Queen Charlotte’s Hospital, London). The various cell populations were purified by immunomagnetic negative selection as described. The purity of the separated cell populations was assessed by flow-cytometric analysis (results not shown) using a FACScan (Becton Dickinson) and was always >98%. All purified T cells were unresponsive to 72 h of culture with phytohaemagglutinin (PHA) (2 μg mL−1), confirming their functional purity. Mouse responder cells from the various mouse strains used were prepared as described.

Mice. The leptin receptor mutant db/db mice (C57BL/Ks-db) (H-2b), the heterozygous db/+ (C57BL/Ks) (H-2b), the C57BL/6 ob/ob (H-2b) and the C57BL/6 (H-2b) mice were from Harlan Labs, Oxford, UK.

For the in vivo experiments, 6-week-old male C57BL/6 mice were housed in pairs under controlled temperature (21–23°C) and a 12-h light/dark cycle (lights on at 07:00 h). Animals were handled twice daily at 09:00 and 18:00 h and food intake and body weight were recorded daily. Animals comprised four groups (n = 8 per group) Two groups were allowed continuous access to laboratory chow and two groups were starved for 48 h, during which they received twice-daily intraperitoneal injections of either PBS or recombinant murine leptin (1 μg g−1 initial body weight). Before and after the period of starvation, mice were allowed to feed freely. Cellular immune function was assessed by delayed-type hypersensitivity (DTH) response, which was measured essentially as described. Briefly, mice were primed on two consecutive days by application of 50 μl of oxazolone acetate/olive oil (4:1 vol/vol) to the shaved flank. Five days after priming, mice were challenged with 10 μl of 1% oxazolone applied to the ear. Ear thickness was measured at 24 h using an electronic pachymeter. Mice undergoing ‘ sham DTH’ were primed with 50 μl of vehicle. Control challenge with 10 μl of vehicle was performed on the contralateral ear of all mice. There was no statistical difference in the mean body weight between groups at the start of the study or at the time of challenge or reading of the DTH response.

Proliferation assays. The human mixed-lymphocyte reactions (MLRs) were done using various purified cell populations as responders (105 cells per well) and the same number of allogeneic irradiated (30 Gy) PBMCs or monocytes as stimulators unless otherwise indicated, as described. The concentration of endogenous leptin in the human AB serum used was at or below the lower detection limit in a leptin radioimmunoassay (<0.5 ng mL−1; Linco, USA). Cells purified for preincubation were incubated for 12 h in the presence or absence of leptin (10 nM), washed four times, counted and plated in triplicate in 96-well plates with 104 irradiated allogeneic PBMCs or monocytes for 5 days. The antigen-recall response was performed with PBMCs (105 cells per well) and tetanus toxoid and measured after 5 days. Mouse MLRs were performed as described. The ob/ob MLRs were done in 2% autologous ob/ob serum.

Cytokine analysis. The production of IL-2, IL-4 and IFN-γ was measured by ELISA (Amersham Life Science) in supernatants collected after 72 h using irradiated allogeneic PBMCs or splenocytes as stimulators, in the presence or absence of leptin.

RT-PCR and Southern blotting. Total cellular RNA was extracted using RNazol reagent (AMS Biotechnology). 5 μg of total RNA was reverse-transcribed in a 20-μl reaction volume using oligo(deoxythymidine)12-18 primer, as described. Primer selection and PCR amplification to generate a product specific for the long form (ObRb) of the human leptin receptor (nucleotide positions, 2,831–3,719) were done as described. The nature of the expected 888-bp PCR product was verified by Southern blot analysis using an oligonucleotide probe 5′-TATCAGATCAGCATCCCAACAT-3′ (nucleotide positions 3,039–3,333 of human leptin receptor cDNA). cDNA quality was determined by the relative amplification of the human β-actin gene, which generated a 540-bp product. The T cells used in these experiments were extensively purified and were >99.5% pure on flow-cytometric staining and unresponsive to PHA. The remaining 0.5% were negative for B-cell, monocyte, NK and CD8 surface markers.

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The development and function of αβ T lymphocytes depend on signals derived from pre-T and αβ T cell receptors (preTCR and αβ TCR) (reviewed in refs 1, 2). The engagement of these receptors leads to the activation of Lck and Fyn
, which are protein tyrosine kinases (PTKs) of the Src family. It remains unclear to what extent the activation of Src-family PTKs can direct the differentiation steps triggered by preTCR and αβ TCR. Here we show that the inactivation of the negative regulator of Src-family PTKs, carboxy-terminal Src kinase (Csk), in immature thymocytes abrogates the requirement for preTCR, αβ TCR and major histocompatibility complex (MHC) class II for the development of CD4\(^+\) double-positive and CD4\(^+\) single-positive thymocytes as well as peripheral CD4 αβ T-lineage cells. These data show that Csk and its substrates are required to establish preTCR/αβ TCR-mediated control over the development of αβ T cells.

Signals transmitted through the preTCR are required for the generation of CD4\(^+\)CD8\(^+\) double-positive (DP) thymocytes\(^1\). The binding of MHC class I/II plus peptide to αβ TCR and CD4/CD8 co-receptors is essential for the differentiation of DP cells into CD4\(^+\) or CD8\(^+\) single-positive (SP) thymocytes and peripheral T cells.