Whitney *U* test. The Spearman rank order correlation was used to analyse the relationship between CCR2 gene dosage and lesion area. Instat 2.01 software for the Macintosh was used for all calculations.

Received 11 May; accepted 3 July 1998.

1. Cocchi, F. *et al.* Identification of RANTES, MIP-1α, and MIP-1β as the major HIV-suppressive factors produced by $CD8^+$ T cells. *Science* **270,** 1811–1815 (1995).
2. Choe, H. *et al.* The β-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* **85,** 1135–1148 (1996).
3. Nelken, N. A., Coughlin, S. R., Gordon, D. & Wilcox, J. N. Monocyte chemoattractant protein-1 in human atheromatous plaques. *J. Clin. Invest.* **88,** 1121–1127 (1991).
4. Yiä-Herttuala, S. *et al.* Expression of monocyte chemoattractant protein 1 in macrophage-rich areas of human and rabbit atherosclerotic lesions. *Proc. Natl Acad. Sci. USA* **88,** 5252–5256 (1991).
5. Yu, X. *et al.* Elevated expression of monocyte chemoattractant protein 1 by vascular smooth muscle cells in hypercholesterolemic primates. *Proc. Natl Acad. Sci. USA* **89,** 6953–6957 (1992).
6. Cushing, S. D. *et al.* Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. *Proc. Natl Acad. Sci. USA* **87,** 5134–5138 (1990).
7. Boring, L. *et al.* Impaired monocyte migration and reduced type 1 (Th1) cytokine responses in C-C chemokine receptor 2 knockout mice. *J. Clin. Invest.* **100,** 2552–2561 (1997).
8. Plump, A. S. *et al.* Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombinant in ES cells. *Cell* **71,** 343–353 (1992).
9. Zhang, S. H., Reddick, R. L., Piedrahita, J. A. & Maeda, N. Spontaneous hypercholsterolemia and arterial lesions in mice lacking apolipoprotein E. *Science* **258,** 468–471 (1992).
10. Plump, A. S., Scott, C. J. & Breslow, J. L. Human apolipoprotein A-I gene expression increases high density lipoprotein and suppresses atherosclerosis in the apolipoprotein E-deficient mouse. *Proc. Natl Acad. Sci. USA* **91,** 9607–9611 (1994).
11. Nakashima, Y., Plump, A. S., Raines, E. W., Breslow, J. L. & Ross, R. ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree. *Arterioscler. Thromb.* **14,** 133–140 (1994).
12. Kraal, G., Rep, M. & Janse, M. Macrophages in T and B cell compartments and other tissue macrophages recognized by monoclonal antibody MOMA-2. An immunohistochemical study. *Scand. J. Immunol.* **26,** 653–661 (1987).
13. Gupta, S. *et al.* IFN-γ potentiates atherosclerosis in apoE knock-out mice. *J. Clin. Invest.* **99,** 2752–2761 (1997).
14. Duverger, N. *et al.* Protection against atherogenesis in mice mediated by human apolipoprotein A-IV. *Science* **273,** 966–968 (1996).
15. Suzuki, H. *et al.* A role for macrophage scavenger receptors in atherosclerosis and susceptibility to infection. *Nature* **386,** 292–296 (1997).
16. Purcell-Huynh, D. A. *et al.* Transgenic mice expressing high levels of human apolipoprotein B develop severe atherosclerotic lesions in response to a high-fat diet. *J. Clin. Invest.* **95,** 2246–2257 (1995).
17. Véniant, M. M. *et al.* Susceptibility to atherosclerosis in mice expressing exclusively apolipoprotein B48 or apolipoprotein B100. *J. Clin. Invest.* **100,** 180–188 (1997).

Acknowledgements. We thank D. Sanan, D. Newland and L. Jensen for help with tissue preparation and staining; D. Dichek for advice on statistical analysis; J. C. W. Carroll, N. C. Shea and S. Gonzales for figure preparation; J. Ernst for assistance with fluorescent microscopy; G. Howard and S. Ordway for editorial expertise; and A. Chen for manuscript preparation. We also thank R. Pitas, R. Farese and K. Weisgraber for careful readings of the manuscript. This work was supported in part by a grant from the NIH to I.F.C.

Correspondence and requests for materials should be sent to I.F.C. at the Gladstone Institute of Cardiovascular Disease (e-mail: icharo@gladstone.ucsf.edu).

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

Graham M. Lord*†, Giuseppe Matarese*†‡, Jane K. Howard†§, Richard J. Baker*, Stephen R. Bloom§ & Robert I. Lechler*

*Imperial College School of Medicine, Departments of *Immunology and §Endocrinology, The Hammersmith Hospital, Du Cane Road, London W12 0NN, UK*

† *These authors contributed equally to this work.*

Nutritional deprivation suppresses immune function[1–3]. The cloning of the *obese* gene and identification of its protein product leptin[4] has provided fundamental insight into the hypothalamic regulation of body weight[5,6]. Circulating levels of this adipocyte-derived hormone are proportional to fat mass[6,7] but may be lowered rapidly by fasting[8,9] or increased by inflammatory mediators[10,11]. The impaired T-cell immunity of mice[12,13] now known to be defective in leptin (*ob/ob*)[4] or its receptor (*db/db*)[14,15], has never been explained. Impaired cell-mediated immunity[1–3] and reduced levels of leptin[7] are both features of low body weight in humans. Indeed, malnutrition predisposes to death from infectious diseases[16]. 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 humans[17]. Leptin induced a

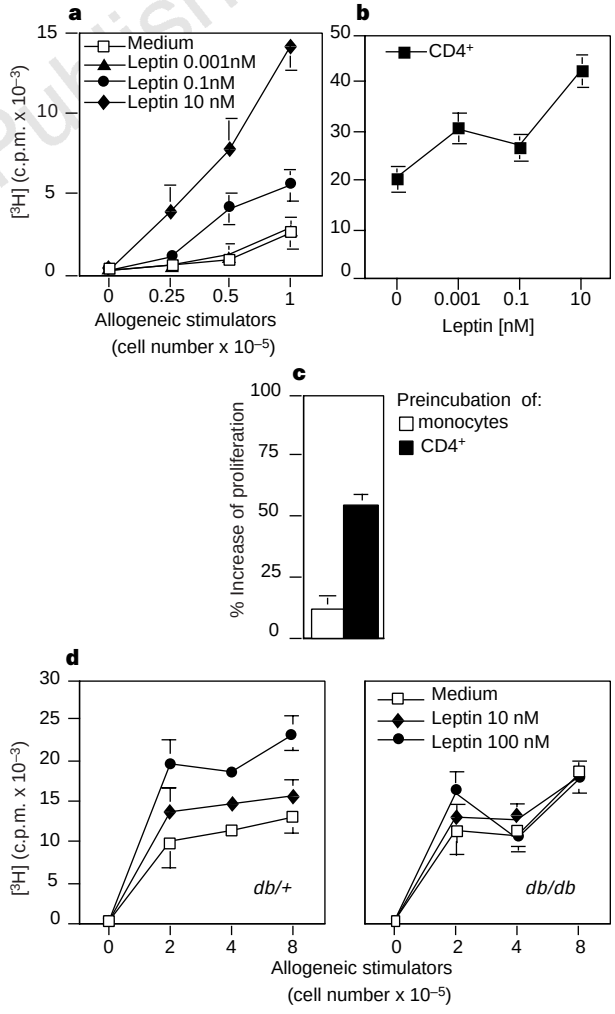

Figure 1 Leptin enhances the alloproliferative response. **a**, **b**, Thymidine incorporation in the presence of leptin in an MLR performed using human PBLs as responder and MHC-mismatched PBMCs as stimulator cells (**a**) or highly purified $CD4^+$ T cells as responders as a responder/stimulator ratio of 1:1 (**b**) (6 experiments). **c**, Preincubation of either responder $CD4^+$ T cells or irradiated stimulator allogeneic monocytes with 10 nM leptin before co-culture in the absence of leptin. **d**, MLR using murine heterozygous *db*/+ ($H\text{-}2^d$) and homozygous *db*/*db* ($H\text{-}2^d$) splenocytes as responders with irradiated C57BL/6 ($H\text{-}2^b$) allogeneic splenocytes (3 experiments). All data are expressed as mean c.p.m. of triplicate cultures ± s.e.m.

‡ Present address: Laboratorio di Immunologia, Dipartimento di Biologia e Patologia Cellulare e Molecolare, Università di Napoli "Federico II", via Pansini 5, 80131, Napoli, Italy.

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 alloproliferative 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[14,15]. 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[14,15] and renders them resistant to the weight-lowering effects of endogenous and exogenous leptin[5]. The alloproliferative 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*[12]. 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

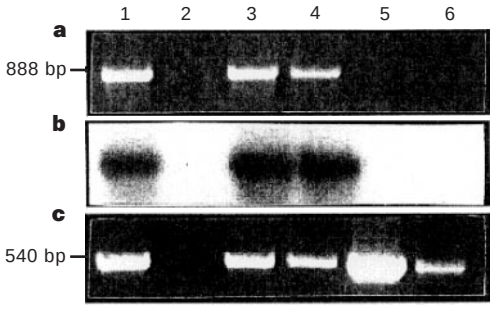

Figure 2 The long isoform of the leptin receptor is expressed in $CD4^+$ T cells. **a**, RT-PCR with human ObRb specific primers: lane 1, hypothalamus; lane 2, no RNA control; lane 3, PBMCs; lane 4, $CD4^+$ T cells; lane 5, monocytes; lane 6, renal mesangial cells. **b**, Southern blot analysis to confirm the specificity of the PCR product. **c**, RT-PCR with primers specific for β-actin.

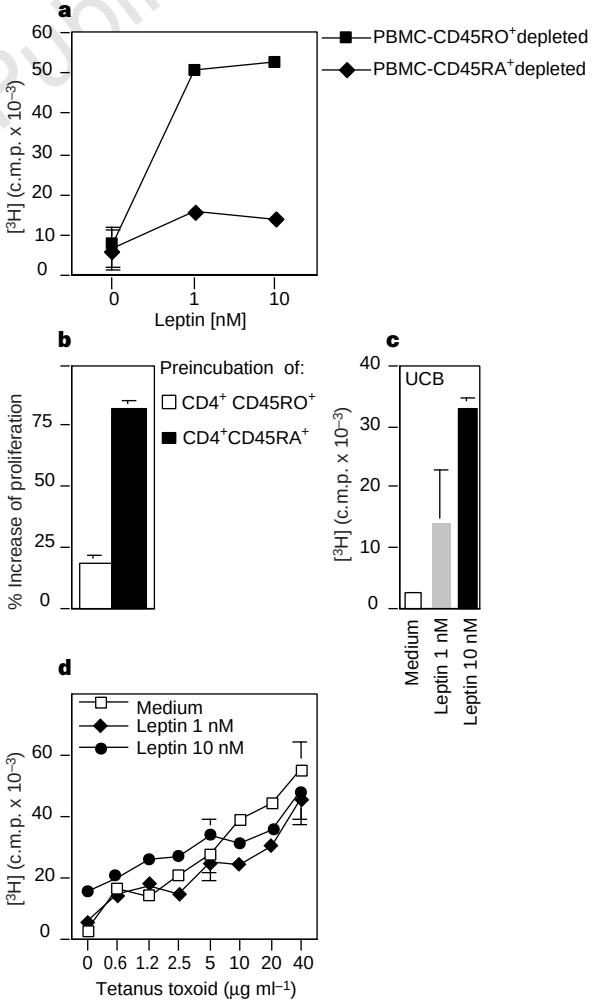

Figure 3 Leptin differentially affects the proliferative responses of naive and memory T cells. **a**, MLR with either human $CD45RO^+$ or $CD45RA^+$ depleted PBMCs as responders. **b**, Preincubation with leptin (10 nM) of either $CD4^+CD45RA^+$T cells or $CD4^+CD45RO^+$T cells before an MLR in the absence of leptin (3 experiments). **c**, MLR using human umbilical cord blood (UCB) responder cells (6 experiments). **d**, An adult T-cell recall antigen response to tetanus toxoid (3 experiments). All data are expressed as mean c.p.m. of triplicate cultures ± s.e.m.

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[20]. 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[20]. 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*[4]. 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

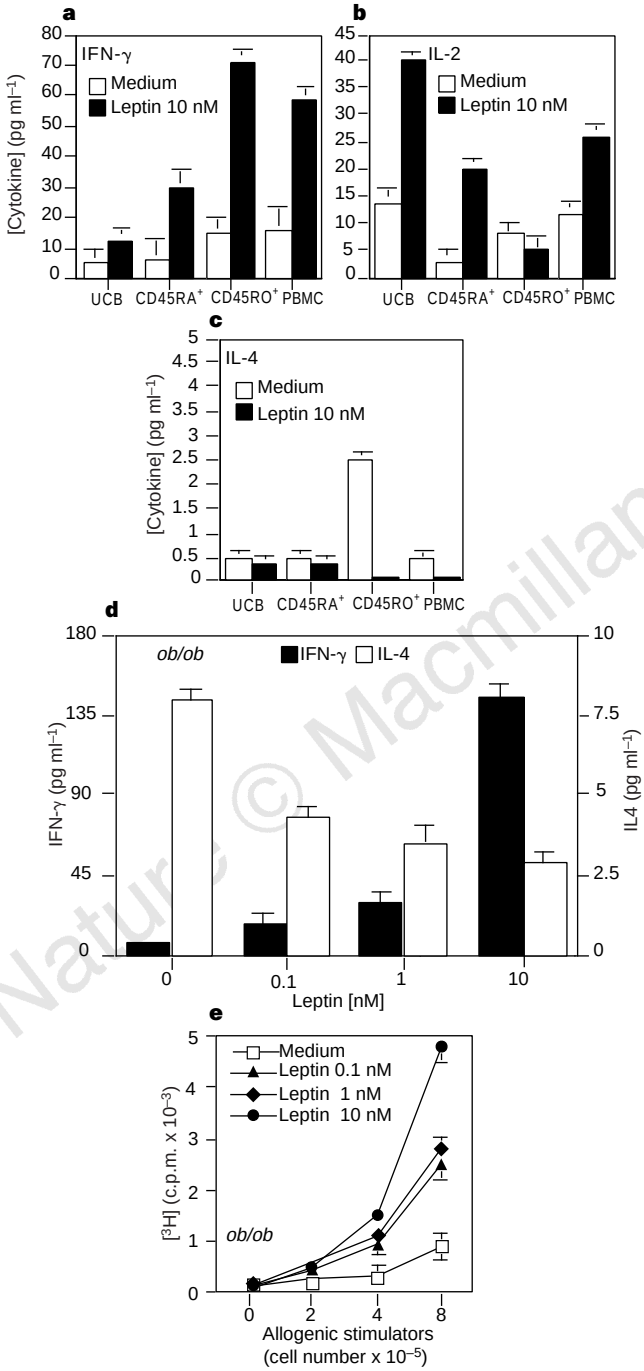

Figure 4 Leptin favours the production of proinflammatory cytokines. **a–c**, Cytokine production in the presence or absence of leptin in an MLR using human UCB cells, naive ($CD45RA^+$), memory ($CD45RO^+$) T cells or PBMCs as responders: **a**, Interferon-γ; **b**, interleukin-2; **c**, interleukin-4. **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.

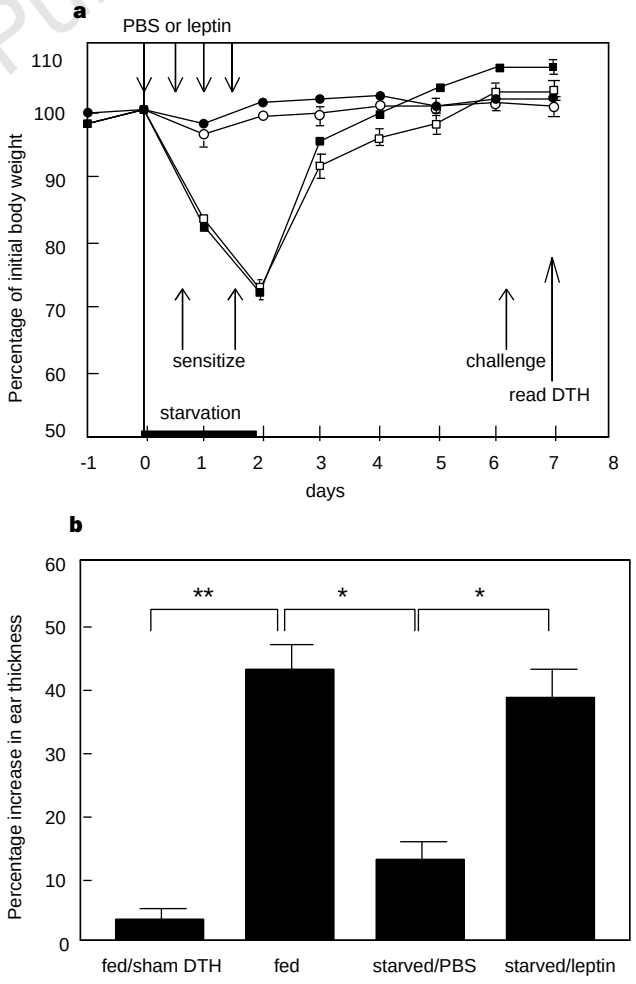

Figure 5 Leptin reverses starvation-induced immunosuppression. **a**, The effect of 48-h food deprivation on bodyweight of male C57BL/6 mice. ● = fed/shamDTH; ○ = fed; ■ = starved/PBS; □ = starved/leptin. **b**, The effect of leptin replacement during starvation on the DTH recall response to oxazolone in male C57BL/6 mice. All data are expressed as mean percentage increase in ear thickness ± s.e.m. ($n = 6$ per group). *$P < 0.0005$, **$P < 0.00001$, 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[21] and certain accessory molecules can bias a T-cell response towards the production of specific cytokines[22]. 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[8,9]. 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[9]. 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[9]. Immunoregulatory effects of these hormones are well recognized[23]. 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 fasting-induced changes in corticosterone and thyroid hormones and is without effect on the starvation-induced fall in plasma insulin and glucose levels[9]. Furthermore, our *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[16]: 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% pure, as judged by SDS–PAGE analysis and contained <0.1 ng mg^{-1} LPS as determined by the Limulus ameobocyte lysate method. Tetanus toxoid was from Evans Medical. The appropriate mAbs were used for purifying cells for the proliferation and cytokine assays, as described[24].

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 subpopulations were purified by immunomagnetic negative selection as described[24]. The purity of the separated cell subpopulations 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[25].

Mice. The leptin receptor mutant *db*/*db* mice (C57BL/Ks-db) (H–2^d), the heterozygous *db*/+ (C57BL/Ks) (H–2^d), the C57BL/6 *ob*/*ob* (H–2^b) and the C57BL/6 (H–2^b) 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 ($n = 6$ 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[26]. Briefly, mice were primed on two consecutive days by application of 50 μl of 3% oxazolone in acetone/olive oil (4:1 vol/vol) to the shaved flank. Five days later, mice were challenged with 10 μl of 1% oxazolone applied to the ear. Ear thickness was measured at 24 h using an electronic micrometer. 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 weights 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 (10^5 cells per well) and the same number of allogeneic irradiated (30 Gy) PBMCs or monocytes as stimulators unless otherwise indicated, as described[19]. 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 10^5 irradiated allogeneic PBMCs or monocytes for 5 days. The antigen-recall response was performed with PBMCs (10^5 cells per well) and tetanus toxoid and measured after 5 days. Mouse MLRs were performed as described[25]. 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[27]. 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[18]. The nature of the expected 888-bp PCR product was verified by Southern blot analysis[27] using an oligonucleotide probe 5′-TATCAGATCAGCATCCCAACAT-3′ (nucleotide positions 3,309–3,333 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.

Received 20 May; accepted 26 June 1998.

1. Chandra, R. K. Nutrition and immunity: Lessons from the past and new insights into the future. *Am. J. Clin. Nutr.* **53,** 1087–1101 (1991).
2. Cason, J., Ainley, C. C., Wolstencroft, R. A., Norton, K. R. & Thompson, R. P. Cell-mediated immunity in anorexia nervosa. *Clin. Exp. Immunol.* **64,** 370–375 (1986).
3. Polack, E. *et al.* Low lymphocyte interferon-gamma production and variable proliferative response in anorexia nervosa patients. *J. Clin. Immunol.* **13,** 445–451 (1993).
4. Zhang, Y. *et al.* Positional cloning of the mouse *obese* gene and its human homologue. *Nature* **372** , 425–432 (1994).
5. Halaas, J. L. *et al.* Weight-reducing effects of the plasma protein encoded by the *obese* gene. *Science* **269,** 543–546 (1995).
6. Friedman, J. M. Leptin, leptin receptors and the control of body weight. *Eur. J. Med. Res.* **2,** 7–13 (1997).
7. Grinspoon, S. *et al.* Serum leptin levels in women with anorexia nervosa. *J. Clin. Endocrinol. Metab.* **81,** 3861–3863 (1996).
8. Boden, G., Chen, X., Mozzoli, M. & Ryan, I. Effect of fasting on serum leptin in normal human subjects. *J. Clin. Endocrinol. Metab.* **81,** 3419–3423 (1996).
9. Ahima, R. S. *et al.* Role of leptin in the neuroendocrine response to fasting. *Nature* **382,** 250–252 (1996).
10. Janik, J. e. *et al.* Interleukin 1 alpha increases serum leptin concentrations in humans. *J. Clin. Endocrinol. Metab.* **82,** 3084–3086 (1997).
11. Sarraf, P. *et al.* Multiple cytokines and acute inflammation raise mouse leptin levels: potential role in inflammatory anorexia. *J. Exp. Med.* **185,** 171–175 (1997).
12. Fernandes, G., Handwerger, B. S., Yunis, E. J. & Brown, D. M. Immune response in the mutant diabetic C57BL/Ks-*db*+ mouse. Discrepancies between *in vitro* and *in vivo* immunological assays. *J. Clin. Invest.* **61,** 243–250 (1978).

13. Chandra, R. K. Cell-mediated immunity in genetically obese (C57BL/6J *ob*/*ob*) mice. *Am. J. Clin. Nutr.* **33,** 13–16 (1980).
14. Ghilardi, N. *et al.* Defective STAT signalling by the leptin receptor in diabetic mice. *Proc. Natl Acad. Sci. USA* **93,** 6231–6235 (1996).
15. Lee, G. H. *et al.* Abnormal splicing of the leptin receptor in diabetic mice. *Nature* **379,** 632–635 (1996).
16. Shears, P. Epidemiology and infection in famine and disasters. *Epidemiol. Infect.* **107,** 241–251 (1991).
17. Considine, R. V. *et al.* Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *N. Engl. J. Med.* **334,** 292–295 (1996).
18. Gotoda, T. *et al.* Leptin receptor gene variation and obesity: lack of association in a white British male population. *Hum. Mol. Genet.* **6,** 869–876 (1997).
19. Lombardi, G. *et al.* Are primary alloresponses truly primary? *Int. Immunol.* **2,** 9–13 (1990).
20. Abbas, A. K., Murphy, K. M. & Sher, A. Functional diversity of helper T lymphocytes. *Nature* **383,** 787–793 (1996).
21. Boehm, U., Klamp, T., Groot, M. & Howard, J. C. Cellular responses to interferon-gamma. *Annu. Rev. Immunol.* **15,** 749–795 (1997).
22. Semnani, R. T., Nutman, T. B., Hochman, P., Shaw, S. & van Seventer, G. A. Costimulation by purified intercellular adhesion molecule 1 and lymphocyte function-associated antigen 3 induces distinct proliferation, cytokine and cell surface antigen profiles in human "naive" and "memory" CD4+ T cells. *J. Exp. Med.* **180,** 2125–2135 (1994).
23. Besedovsky, H. O. & del Rey, A. Immune-neuro-endocrine interactions: facts and hypotheses. *Endocr. Rev.* **17,** 64–102 (1996).
24. Young, J. L., Ramage, J. M., Hill-Gaston, J. S. & Beverley, P. C. L. *In vitro* responses of human CD45ROhright and CD45RAbright T cell subsets and their relationship to memory and naive T cells. *Eur. J. Immunol.* **27,** 2383–2389 (1997).
25. Laufer, T. M., DeKoning, J., Markowitz, J. S., Lo, D. & Glimcher, L. H. Unopposed positive selection and autoreactivity in mice expressing class II MHC only on thymic cortex. *Nature* **383,** 81–85 (1996).
26. Staite, N. D., Justen, J. M., Sly, L. M., Beaudet, A. L. & Bullard, D. C. Inhibition of delayed-type contact hypersensitivity in mice deficient in both E-selectin and P-selectin. *Blood* **88,** 2973–2979 (1996).
27. Beak, S. A. *et al.* Glucagon-like peptide-1 (GLP-1) releases thyrotropin (TSH): characterization of binding sites for GLP-1 on alpha-TSH cells. *Endocrinology* **137,** 4130–4138 (1996).

Acknowledgements. We thank C. Campagnoli, J. G. Chai, A. Di Giacomo, J. Gardiner, G. Lombardi, A. Macrae and L.-Q. Gao for technical assistance and for helpful suggestions and D. Gray, H. Reiser and D. Smith for critically reviewing the manuscript. G.M.L., J.K.H. and R.J.B. are MRC Clinical Training Fellows.

Correspondence and requests for materials should be addressed to R.I.L. (e-mail: rlechler@rpms.ac.uk).

Csk controls antigen receptor-mediated development and selection of T-lineage cells

Christian Schmedt*§, Kaoru Saijo*§, Tetsuhiro Niidome†‖, Ralf Kühn‡, Shinichi Aizawa† & Alexander Tarakhovsky*

** Laboratory for Lymphocyte Signalling and ‡ Department of Immunology, Institute for Genetics, University of Cologne, Weyertal 121, 50931 Cologne, Germany*
† Department of Morphogenesis, Institute of Molecular Embryology and Genetics, Kumamoto University School of Medicine, Kumamoto-860, Japan
§ These authors contributed equally to this work

The development and function of αβT lymphocytes depend on signals derived from pre-T and αβT cell receptors (preTCR and αβTCR) (reviewed in refs 1, 2). The engagement of these receptors leads to the activation of Lck and Fyn[3,4], 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 αβTCR. Here we show that the inactivation of the negative regulator of Src-family PTKs, carboxy-terminal Src kinase (Csk)[5], in immature thymocytes abrogates the requirement for preTCR, αβTCR and major histocompatibility complex (MHC) class II for the development of $CD4^+8^+$ double-positive and $CD4^+$ single-positive thymocytes as well as peripheral CD4 αβT-lineage cells. These data show that Csk and its substrates are required to establish preTCR/αβTCR-mediated control over the development of αβT 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 αβTCR 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 (reviewed in ref. 6). The early block in thymocyte development in $Lck^{-/-}$; $Fyn^{-/-}$ mice[7,8], and the ability of constitutively active Lck to trigger the preTCR-independent generation of DP cells[9], shows that preTCR-mediated thymocyte development depends on Src-family PTKs. However, the failure of constitutively active Lck to induce αβTCR-independent progression past the DP stage of thymocyte development suggests that differentiation into SP thymocytes and peripheral T cells may require the concerted activation of Lck and Fyn, or may depend on additional factors. The activity of Src-family PTKs is negatively regulated by Csk and is increased in Csk-deficient cells[10,11]. We hypothesized that the inactivation of Csk and concomitant activation of Src-family PTKs in double-negative (DN) thymocytes may result in the preTCR/αβTCR-independent development of mature αβT-lineage cells. We inactivated the gene encoding Csk conditionally, because mice homozygous for a *csk* null mutation die before the onset of embryonic lymphopoiesis[10,11].

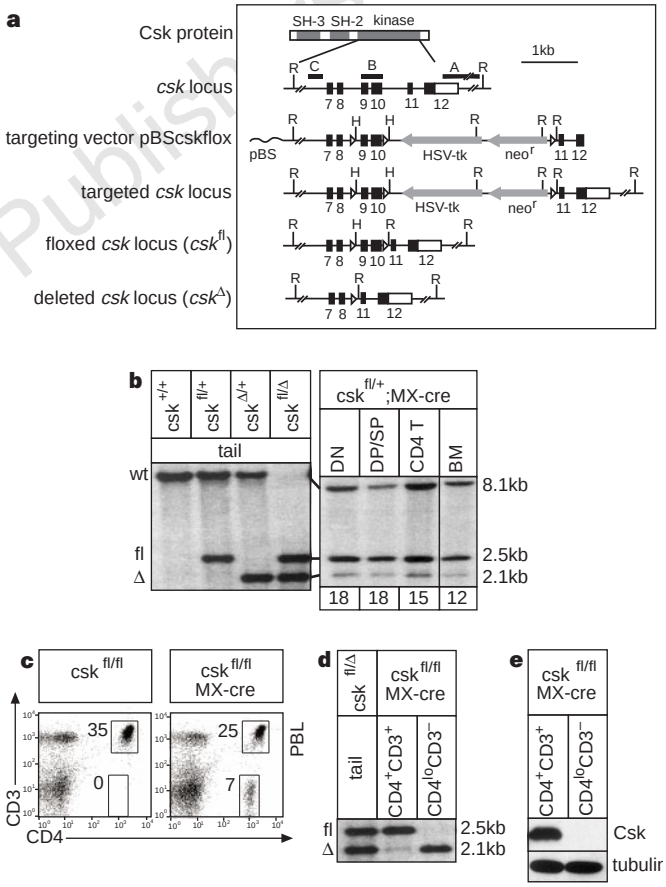

Figure 1 Conditional inactivation of *csk* results in the appearance of $CD3^-CD4^{lo}$ peripheral cells. **a**, Domain structure of Csk protein, partial structure of the *csk* locus, and maps of the targeting vector and the targeted *csk* locus before and after Cre-mediated recombinations. Numbered rectangles represent exons, coding regions are filled; open triangles represent *loxP* sites; grey arrows indicate the position and transcriptional orientation of selection marker genes. Restriction sites are: R, *Eco*RI; H, *Hin*dIII. Probes A, B and C used in Southern blots are shown as bars. **b**, Uninduced deletion of the floxed *csk* fragment in cells of $csk^{fl/+}$; MX-cre and control mice. The size of the DNA fragment corresponding to wild-type (wt), floxed (fl) and deleted (Δ) csk alleles and per cent of deletion are indicated. **c**, $CD3^-CD4^{lo}$ cells in the peripheral blood of $csk^{fl/fl}$; MX-cre mice. PBLs of $csk^{fl/fl}$ and $csk^{fl/fl}$; MX-cre mice were stained with anti-CD3 and anti-CD4 antibodies. Numbers in larger font show percentages of gate cells. **d**, Deletion of *csk* in splenic cells. Genomic DNA of purified $CD3^+CD4^+$ and $CD3^-CD4^{lo}$ cells or tail of $csk^{fl/fl}$; MX-cre or $csk^{fl/\Delta}$ mice, respectively, was analysed by Southern blotting. **e**, Csk protein expression in $CD3^+CD4^+$ and $CD3^-CD4^{lo}$ cells. Lysates of purified cells were analysed by western blotting with anti-Csk antibody, and reprobed with an anti-α-tubulin antibody.

‖ Present address: CNS Research Unit, Department of Drug Discovery, Tsukuba Research Laboratories, Eizai Co.Ltd, 1–3 Tokodai 5-chome, Tsukuba-shi, Ibaraki 300-26, Japan.