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Leptin Modulates the Survival of Autoreactive CD4+ T Cells through the Nutrient/Energy-Sensing Mammalian Target of Rapamycin Signaling Pathway

Mario Galgani,*‡, Claudio Procaccini,*‡, Veronica De Rosa,*‡, Fortunata Carbone,*‡, Paolo Chieffi,§ Antonio La Cava,∥ and Giuseppe Matarese*‡

Chronic inflammation can associate with autoreactive immune responses, including CD4+ T cell responses to self-Ags. In this paper, we show that the adipocyte-derived proinflammatory hormone leptin can affect the survival and proliferation of autoreactive CD4+ T cells in experimental autoimmune encephalomyelitis, an animal model of human multiple sclerosis. We found that myelin oligodendrocyte glycoprotein peptide 35–55 (MOG35–55)-specific CD4+ T cells from C57BL/6J wild-type mice could not transfer experimental autoimmune encephalomyelitis into leptin-deficient ob/ob mice. Such a finding was associated with a reduced proliferation of the transferred MOG35–55-reactive CD4+ T cells, which had a reduced degradation of the cyclin-dependent kinase inhibitor p27kip1 and ERK1/2 phosphorylation. The transferred cells displayed reduced Th1/Th17 responses and reduced delayed-type hypersensitivity. Moreover, MOG35–55-reactive CD4+ T cells in ob/ob mice underwent apoptosis that associated with a down-modulation of Bcl-2. Similar results were observed in transgenic AND-TCR- mice carrying a TCR specific for the pigeon cytochrome c 88–104 peptide. These molecular events reveal a reduced activity of the nutrient/energy-sensing AKT-mammalian target of rapamycin (mTOR) pathway.

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everal studies have shown that calorie restriction (CR) can modulate metabolic and physiologic changes that can be beneficial in cancer, metabolic syndrome, inflammation, and autoimmunity (1–3). Leptin, an adipocyte-derived hormone of the long-chain helical cytokine family (4, 5), has been proposed to link nutritional status and certain immune responses (6, 7). Leptin concentration is proportional to fat mass and is positively associated with overweight, obesity, and increased susceptibility to chronic inflammation and autoimmunity (6, 7). At the immune level, leptin promotes Th1 proinflammatory responses in autoimmunity (8, 9), although it can also induce the expression of IL-1 receptor antagonist in human monocytes (10) or IL-4 (11) and

IL-10 (12) and suppress IFN-γ (12) or IL-6 production caused by endotoxin (13).

Leptin-deficient (ob/ob) mice and leptin receptor-deficient mice (db/db) mice display numerous immune abnormalities (14, 15), including CD4+ T cell lymphopenia, increased numbers of natural regulatory T cells, and a resistance to autoimmune disorders including experimental autoimmune encephalomyelitis (EAE), experimental colitis, Ag-induced arthritis, and type 1 diabetes (16).

The cytokines IL-6, IL-7, IL-15, and IL-21 have all been implicated in the homeostatic control of lymphocyte numbers (17–19), and homeostatic proliferation of regulatory T cells has been observed during T cell lymphopenia. An uncontrolled expansion of autoreactive T cells could favor autoimmunity (20–24), but in ob/ob mice, CD4+ lymphopenia does not associate with homeostatic proliferation of T cells and autoimmunity (14–16).

In this paper, we show that leptin can affect the survival and proliferation of autoreactive T cells by modulating the activity of the survival protein Bcl-2, Th1/Th17 cytokines, and the nutrient/energy-sensing AKT-mammalian target of rapamycin (mTOR) pathway. Findings that link body fat mass and autoactivity also suggest the possibility to regard obesity as a disease with autoimmune components (25–29).

Materials and Methods

Mice

Eight- to 10-wk-old female C57BL/6J (B6), wild-type (WT), and C57BL/6J-ob/ob-leptin–deficient mice were purchased from Charles River Laboratories (Calco, Italy) and from Harlan (Correzzana, Italy). The B10.Cg.Tg (TcrAND)53Hed/J (AND-TCR transgenic [Tg]) pigeon cytochrome c (PCC)34–104-specific transgenic mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6-WT and ob/ob mice were age-matched for individual experiments and were group-housed in the same cages according to different experimental condition, with a 12-hour light-dark cycle. All experiments were performed under approved protocol in accordance with animal use guidelines of the Istituto Superiore di Sanità (Rome, Italy).

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CD4+ T lymphocytes.

Mean clinical score of adoptively induced EAE in B6-WT, Ob/ob and PBS–treated, and Ob/ob–leptin–treated mice groups develop clinical signs of disease, with a similar disease score. T cells were adoptively transferred with 5 × 10^6 encephalitogenic MOG35–55-specific CD4+ T cells, mice were injected i.v. with 5 × 10^6 CD4+ T cells in a final volume of 500 μL PBS. Mice received 200 ng pertussis toxin immediately after cell transfer and 1 d later. Individual mice were monitored daily for clinical signs of disease for up to 30 d after adoptive transfer, as described previously (30).

Treatment with leptin and rapamycin

Mouse r-leptin was purchased from R&D Systems (Oxon, U.K.); purity was >97%. The endotoxin level was <0.1 ng/μg leptin, as determined by the Limulus amebocyte lysate method. Mice comprised two groups (n = 6–11/group) of Ob/ob–leptin–deficient obese mice and one group (n = 6–10/ group) of B6-WT age- and sex-matched control mice. For adoptively-induced disease, mice were treated with r-leptin (0.5 μg/g initial body weight twice daily in 200 μL volume i.p.) starting 3 d before the transfer of MOG35–55-specific CD4+ T cells and continuing over a period of 30 d. Of the groups of leptin-deficient mice, one was injected with 200 μL PBS twice daily (at 10:00 AM and 6:00 pm); the second group was injected with murine r-leptin (0.5 μg/g initial body weight twice daily in 200 μL volume i.p.). For the group of B6-WT mice, PBS was injected twice daily accordingly to the same schedule. All mice were weighed, and food intake was recorded daily. Rapamycin was dissolved in DMSO and then brought to volume with PBS. For in vivo treatment with rapamycin (Sigma-Aldrich, St. Louis, MO), B6-WT mice adoptively-transferred with CFSE+ MOG35–55-specific CD4+ T cells were quantified using a time-dependent footpad swelling assay (32). Footpad thickness was measured at 7 d after transfer and at 12, 24, 48, and 72 h after challenge or at 1, 7, or 14 d by a “blinded” investigator, as described previously (30).

Proliferation assays

Spleen cells were obtained from mice at different time points after adoptive transfer and used for proliferation assays, as described previously (30). For experiments with AND-TCR-Tg mice, DCEK transfectants (murine fibroblasts cells transfected with the Eδ mouse MHC class II molecule) (31) were used as APCs to activate in vitro CD4+ AND-TCR-Tg mice T cells. T cells were incubated and harvested as described previously (30).

Delayed-type hypersensitivity

Delayed-type hypersensitivity (DTH) responses to MOG35–55 peptide by adoptively transferred MOG35–55-specific CD4+ T cells were quantified using a time-dependent footpad swelling assay (32). Footpad thickness was measured at 7 d after transfer and at 12, 24, 48, and 72 h after challenge or at 1, 7, or 14 d by a “blinded” investigator, as described previously (30).

Cytokine measurement

Leptin, IL-7, IL-15, and IL-21 were measured using ELISA kits from R&D Systems (Minneapolis, MN), BioScientific (Austin, TX), and BioLegend (San Diego, CA), respectively. Measurements were performed according to footpad-swelling responses; *p < 0.01 and **p < 0.05 of PBS versus B6-WT and ob/ob leptin treated. Dose-dependent MOG35–55 specific in vitro proliferation of splenocytes pulsed with MOG35–55 from the three groups of mice previously transferred with MOG35–55-specific CD4+ T cells. Data are representative of two independent experiments showing means ± SD of [3H]thymidine incorporation; *p < 0.01 of PBS versus B6-WT and ob/ob leptin treated. E. In vitro cytokine release in supernatants from in vitro proliferation of splenocytes to MOG35–55 from the three groups of mice previously transferred with B6-WT MOG35–55-specific CD4+ T cells; *p < 0.01 and **p < 0.05 of PBS versus B6-WT and ob/ob leptin treated.

Peptides

MOG35–55 peptide (MEGVYWRSPFSRVHLYRNGK) was used for the induction of EAE (30), and PCC88–104 peptide (KAERADLIAYKQ-ATAK) was used to stimulate AND-TCR-Tg cells (31).

Induction of adoptive EAE and clinical assessment

The induction of EAE has been described previously (30). Ten female donor B6-WT mice (6–8 wk old) were primed s.c. with 300 μg MOG35–55 peptide in CFA distributed over four sites. Recipient syngeneic naïve female ob/ob, PBS or recombinant leptin (r-leptin)–treated, and B6-WT control mice were injected i.v. with 5 × 10^6 CD4+ T cells in a final volume of 500 μL PBS. Mice received 200 ng pertussis toxin immediately after cell transfer and 1 d later. Individual mice were monitored daily for clinical signs of disease for up to 30 d after adoptive transfer, as described previously (30).
LEPTIN MODULATES CD4+ T CELL SURVIVAL VIA THE mTOR PATHWAY

Flow cytometry, cell sorting, and biochemical analyses

Untouched CD4+ T cells (isolated by negative selection using the Miltenyi Biotec T Cell Isolation kit and an AutoMACS cell separator) from donor mice were stained with the fluorescent dye CFSE from Molecular Probes (Eugene, OR) used at 1 μg/ml. A total of 5 × 10^6 CD4+CFSE^+ labeled T cells were injected into the tail vein of B6-WT, ob/ob, and leptin-treated ob/ob mice. For flow cytometric analyses of CFSE^+CD4^+ T cells 7 and 14 d after transfer, spleens of three mice groups were harvested, and 1 × 10^6 cells were analyzed using a FACS Calibur (BD Biosciences, San Jose, CA) and CellQuest software (BD Biosciences). For biochemical analyses, 0.5–1 × 10^6 CD4^+CFSE^+ cells were obtained from the spleen of each group of WT, ob/ob, and ob/ob-leptin–replaced mice using High-Speed Cells Sorting (MoFlo; DakoCytomation, Glostrup, Denmark); cells were >99% pure. For Western blotting, sorted cells were lysed as described previously (33). The Abs used were the following: anti-p27kip-1, anti-phospho (P)-AKT, anti-AKT and anti–Bcl-2, and anti-S6 ribosomal protein (S6) and anti-P-S6 (all from Cell Signaling Technology, Beverly, MA). All filters were quantified by densitometric analysis of the bands using the program ScionImage 1.63 for Macintosh (Scion, Frederick, MD). FACS analyses for intracellular signaling on ex vivo CFSE^+ cells fixed and permeabilized were performed by intracellular staining of P-S6 by PE-conjugated P-S6 Ab (Cell Signaling Technology).

Statistical analyses

Analyses were performed using Mann-Whitney U test (for unpaired two group analyses) and Kruskal-Wallis ANOVA test (for three or more group analyses). Results are expressed as mean ± SD; p < 0.05 was considered statistically significant.

Results

Autoreactive CD4+ T cells do not transfer EAE in ob/ob mice

We adoptively transferred 5 × 10^6 encephalitogenic MOG35–55-specific CD4^+ T lymphocytes into leptin-deficient ob/ob mice treated or not with mouse r-leptin. As shown in Fig. 1A, MOG35–55-specific CD4^+ T cells did not transfer EAE when injected into ob/ob recipients. In contrast, T cells did induce EAE when transferred into B6-WT and ob/ob mice treated with r-leptin with a similar frequency of disease and clinical score in the groups.

Transferred MOG35–55-specific CD4^+ T cells were studied in vivo and in vitro. Seven days after transfer of MOG35–55-reactive CD4^+ T cells, mice were challenged with 50 μg MOG35–55 peptide in the footpad, and DTH response was measured. Ob/ob mice had delayed DTH (12–72 h) as compared with B6-WT and r-leptin–treated ob/ob mice (Fig. 1B). The monitoring of DTH after adoptive transfer (1, 7, and 14 d) indicated that the DTH was lost when MOG35–55-specific CD4^+ T cells were transferred into ob/ob mice (Fig. 1C).

To also define whether leptin deficiency affected the proliferation of transferred MOG35–55-specific CD4^+ T cells, we performed dose-dependent in vitro stimulation with MOG35–55 in autologous serum (to preserve leptin deficiency). Reduced cell proliferation to MOG35–55 peptide of B6-WT cells from ob/ob mice was restored by r-leptin treatment (Fig. 1D). Also, secretion of proinflammatory cytokines IL-1α, IL-2, IL-6, IL-17A, GM-CSF, IFN-γ, and TNF-α from B6-WT MOG35–55-specific CD4^+ T cells from ob/ob mice was reduced and restored by r-leptin administration (Fig. 1E). Leptin deficiency did not alter the production of IL-4 and IL-10 from B6-WT T cells in response to MOG35–55, and IL-5 was reduced similarly to Th1 cytokines. Finally, in adoptively transferred CD4^+ B6-WT T cells stimulated in vitro with MOG35–55, the levels of IL-15 and IL-21 were significantly reduced (Fig. 1E), and IL-7 was undetectable (data not shown). To exclude that the observed

FIGURE 2. Leptin deficiency impairs survival of autoreactive CD4^+ T cells in vivo. A, Representative flow cytometry of CFSE^-CD4^+ T cells recovered from B6-WT (upper black panel), ob/ob-PBS (middle blue panel), and ob/ob-leptin (lower red panel) treated mice 7 d after adoptive transfer of in vitro-activated MOG35–55-specific CFSE^-labeled CD4^+ T cells from B6-WT (see schematic diagram of experimental procedure). One experiment representative of three; *p < 0.05, ob/ob PBS versus B6-WT and ob/ob leptin treated. B, Histograms represent the percentage (upper panel) and absolute number (lower panel) of CFSE^- T cells detected at 7 and 14 d postadoptive transfer in spleen and B6-WT (black bars), ob/ob PBS-treated (blue bars), and ob/ob-leptin–treated (red bars) Data are representative of three independent experiments. Data are shown as mean ± SD; *p < 0.05 and **p < 0.01, respectively. C, Representative flow cytometry of annexin V staining in CFSE^-CD4^+ T cells recovered from B6-WT (upper panel), ob/ob-PBS (middle panel), and ob/ob-leptin (lower panel)-treated mice 7 d after adoptive transfer of in vitro-activated MOG35–55-specific CFSE^-labeled CD4^+ T cells from B6-WT. Data are representative of three independent experiments and indicate the percentage of Annexin V^+ cells. Data are shown as mean ± SD; *p < 0.01. D, Histograms showed average value of annexin V staining in CFSE^-CD4^+ T cells from three independent experiments. Data are shown as mean ± SD; *p < 0.01 and **p < 0.05, respectively.
differences were due to quantitative effects of cells recovery, we normalized the in vitro experiments for the same number of CFSE+CD4+ T cells in each well and confirmed that autoreactive CD4+ T cells exposed to a leptin-free microenvironment secrete less Th1/Th17 inflammatory and prosurvival cytokines.

Leptin controls the survival of autoreactive CD4+ T cells
To define whether leptin deficiency influences in vivo survival and proliferation of autoreactive MOG35-55-reactive T cells, CD4+ T cells purified from MOG35-55-immunized B6-WT mice 7 d after immunization were activated in vitro with MOG35-55 peptide, CFSE labeled, and adoptively transferred into B6-WT, ob/ob PBS, and ob/ob-leptin–treated mice 7 d postadoptive transfer. The graphs show quantity of each specific protein. Data are shown as averaged value ± SD (n = 3; *p < 0.05 and **p < 0.01). Immunoblot for P-AKT (D) and for P-S6 (E) on FACS-sorted B6-WT CFSE+ MOG35-55-specific CD4+ T cells isolated from B6-WT, ob/ob PBS, and ob/ob-leptin–treated mice 7 d postadoptive transfer. Graphs show quantity of each protein. Data are shown as averaged value ± SD (n = 3; *p < 0.05).

Leptin deficiency associates with a decrease of Bcl-2 expression, P-ERK1/2, and reduced degradation of the cell-cycle inhibitor p27kip1 in CD4+ T cells

The pathways for impaired survival of MOG35-55-specific CD4+ T cells in leptin-deficient ob/ob mice were studied. Western
blot analysis showed that FACS-sorted B6 WT MOG35-55-specific CD4+ T cells, isolated from ob/ob mice, had lower levels of Bcl-2 and P-ERK1/2; these data were associated with a reduced degradation of the cell cycle inhibitor p27kip1 as compared with B6 WT CD4+ T cells from B6 WT and leptin-treated ob/ob mice (Fig. 3A–C). These findings suggest an increased apoptosis, reduced proliferation, and cell cycle arrest in the absence of leptin.

Leptin modulates the survival of autoreactive CD4+ T cells through the nutrient-energy-sensing AKT-mTOR pathway

We tested whether leptin affects the expression of the protein kinase B (AKT) and its downstream energy-sensing mTOR pathway (34–36). We found that FACS-sorted MOG35-55-specific CD4+ T cells from ob/ob mice had lower levels of phosphorylation of both AKT and S6, which is downstream of the mTOR pathway (Fig. 3D, 3E). These results were confirmed by FACS for P-S6 in B6 WT CFSE+ MOG35-55-specific CD4+ T cells (Fig. 3F).

Because P-S6 appeared reduced by leptin deficiency in B6 WT autoreactive T cells, we performed in vivo experiments using rapamycin, an mTOR inhibitor. Interestingly, acute/short-term rapamycin treatment in vivo resembled the effects of leptin deficiency in terms of recovery of MOG35-55-specific CD4+ T cells in B6 WT mice. B6 WT CFSE+ MOG35-55-specific pathogenic CD4+ T cells were analyzed 7 d after adoptive transfer into B6 WT mice treated with a single dose of PBS, rapamycin, r-leptin, or r-leptin plus rapamycin (Fig. 4, ▬) on day 0. Rapamycin treatment significantly reduced the number of B6 WT CFSE+ MOG35-55 T cells; these effects were prevented by coinjection of r-leptin with rapamycin (Fig. 4, ▬). In this last condition, r-leptin could partially sustain mTOR activation, as demonstrated by P-S6 levels in CD4+ T cells from rapamycin plus r-leptin–treated mice. These results also correlated with the capacity of rapamycin to reduce serum leptin levels in B6 WT mice (Fig. 4, ▫); these effects were not secondary to weight loss (data not shown).

The serum leptin levels were unchanged in leptin-treated mice as compared with controls, likely because they were analyzed after 7 d of a single dose of r-leptin whose half-life is ∼180 min (37).

Discussion

Several reports have suggested that leptin can affect survival of immune cells in vitro (38, 39). To our knowledge, this study is the first to report that leptin has a key role in the survival and proliferation of autoreactive CD4+ T cells in vivo. Leptin-deficient ob/ob mice were protected from adoptively transferred EAE, and the protection was associated with a progressive decline in the survival of adoptively transferred autoreactive MOG35-55-specific CD4+ T cells and reduced secretion of Th1/Th17 proinflammatory cytokines. Also, CD4+ autoreactive T cells had a significant downregulation of the survival protein Bcl-2, reduction in P-ERK1/2, and cell cycle arrest associated with reduced degradation of the cell cycle inhibitor p27kip1. Importantly, a significant impairment occurred at the level of the nutrient-energy-sensing AKT-mTOR/S6 signaling pathway. These phenomena were all reversed in vivo by r-leptin administration, suggesting that leptin can activate the mTOR pathway in autoreactive CD4+ T cells (Supplemental Fig. 2).

Studies published by Piccio et al. (3) and by our group (40) have shown that nutritional deprivation or CR can reduce the magnitude of immune cell proliferation and cytokine secretion by IL-1 in EAE (46).

In summary, this study shows a previously unappreciated role of leptin in the promotion of the survival of CD4+ autoreactive T cells. The implications of these findings in relation to nutritional deprivation or CR can suggest targeting of leptin-based pathways of intervention for the modulation of autoreactive T cell activity.

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Disclosures

The authors have no financial conflicts of interest.

References

Fig. S1. Leptin deficiency impairs the survival of TCR transgenic, antigen-specific CD4+ T cells in vivo. (A) Representative flow cytometry of CFSE+ CD4+ T cells recovered from B6-WT (upper black panel), ob/ob-PBS (middle blue panel) and ob/ob (lower red panel) leptin-treated mice 7 days after adoptive transfer of in vitro-activated PCC88-104-specific CFSE-labelled CD4+ T cells from TCR-transgenic AND-TCR mice. One experiment representative of three. *p < 0.05, ob/ob PBS vs B6-WT and ob/ob leptin treated. (B) Histograms represent the percentage (upper) and number (lower) of CFSE+ CD4+ TCR transgenic T cells detected at 7 and 14 days post adoptive transfer in B6-WT (dark bars), ob/ob PBS (blu bars) and ob/ob leptin-treated mice (red bars). Data are representative of three independent experiments. Data are shown as mean ± SD. *p < 0.05, **p < 0.01.
Fig. S2. Schematic model of the effects of leptin-deficiency in autoreactive CD4+ T cells. Autoreactive CD4+ T cells in a leptin-free environment show reduced proliferation secondary to ERK1/2 and p27kip1, downmodulation of the survival gene Bcl-2 and of the nutrient-energy sensing AKT-mTOR pathway. Leptin deficiency also determines a reduced secretion of Th1/Th17 and survival cytokines. These phenomena can also be induced by rapamycin.