

Leptin potentiates experimental autoimmune encephalomyelitis in SJL female mice and confers susceptibility to males

Giuseppe Matarese¹, Veronica Sanna², Antonio Di Giacomo³, Graham M. Lord⁴, Jane K. Howard⁵, Stephen R. Bloom⁵, Robert I. Lechler⁴, Silvia Fontana² and Serafino Zappacosta¹

¹ Cattedra di Immunologia, Dipartimento di Biologia e Patologia Cellulare e Molecolare, Università di Napoli "Federico II", Napoli, Italy

² Centro di Endocrinologia e Oncologia Sperimentale, CEOS-CNR, Napoli, Italy

³ Laboratorio di Immunologia Cellulare, Azienda Ospedaliera "V. Monaldi", Napoli, Italy

⁴ Department of Immunology, Imperial College School of Medicine, Hammersmith Hospital, London, GB

⁵ Endocrine Unit, Imperial College School of Medicine, Hammersmith Hospital, London, GB

SJL (H-2^s) female mice are more susceptible than males to experimental autoimmune encephalomyelitis (EAE) induced by immunization with myelin-derived peptides. The reasons for this sexual dimorphism are unclear, but may include such factors as sex-related differences in immune responsiveness, hormonal effects and sex-linked genetic factors. Recent evidence indicates that leptin modifies T cell immunity promoting T helper (Th) 1 pro-inflammatory immune responses. Circulating leptin levels show a marked sexual dimorphism, being higher in females than in males. In the present study, we investigated whether leptin treatment altered the course of relapsing-remitting EAE, induced by the proteolipid protein peptide (PLP_{139–151}), in SJL susceptible females and EAE-resistant males. Administration of leptin to female SJL mice before or after disease onset significantly worsened the disease, with a concomitant increase in the PLP_{139–151}-specific delayed-type hypersensitivity (DTH) reactivity and *in vitro* IFN- γ secretion. Leptin treatment at priming with antigen or before disease onset rendered male SJL mice susceptible to EAE, with the appearance of PLP_{139–151}-specific DTH reactivity and a switch from a Th2 to Th1 pattern of cytokine release. Our findings indicate that leptin administration to susceptible females resulted in a more severe disease, and that reduced leptin levels in male SJL mice may contribute to the gender-related differences in the induction phase of EAE.

Key words: EAE / Leptin / Autoimmunity / Sex hormones / Multiple sclerosis

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1 Introduction

It is well recognized that most autoimmune diseases are more frequently observed in females than males [1]. Such sexual dimorphism covers a broad range of autoimmune disorders, ranging from Graves' and Hashimoto's thyroiditis to rheumatoid arthritis (RA) and multiple sclerosis (MS) [1]. Female preponderance in animal models of autoimmunity, such as experimental autoimmune encephalomyelitis (EAE) in SJL mice [2] and type I diabetes in nonobese diabetic (NOD) mice [3], has also

been observed. The reasons for this sex difference in MS and other autoimmune disorders are still unclear. Emerging evidence is clarifying some of the mechanisms underlying the gender-related disease susceptibility. Factors such as sex-related immune responsiveness, response to infection, sex steroid and non-steroid hormonal effects, and sex-linked genetic factors have been implicated [1]. Specifically, the role of sex steroid hormones in controlling patterns of T cell lymphokines has been the focus of several recent studies on EAE [4, 5].

EAE is an animal model of autoimmune demyelinating disease of the central nervous system (CNS) resembling MS. It is induced by CD4⁺ T lymphocytes specific for myelin-derived proteins. Encephalitogenic T cells display the T helper (Th) 1 phenotype, whereas Th2 cells are protective [6]. Steroid sex hormones such as androgens and progesterone can favor the development of Th2 cells

[1 21599]

Abbreviations: EAE: Experimental autoimmune encephalomyelitis **leptin:** Recombinant leptin **PLP:** Proteolipid protein **CFA:** Complete Freund's adjuvant

[5, 7]. Estradiol has been observed to exhibit a dose-dependent biphasic effect on immune cells, whereby low concentrations promote and high doses inhibit cell-mediated Th1 immune functions [1, 5]. Other sexually dimorphic hormones, higher in females than males, such as prolactin and growth hormone have been shown to exert stimulatory effects on pro-inflammatory immune responses during autoimmunity [1, 8].

Leptin, a 16-kDa adipocyte-derived hormone, also shows a marked gender-related dimorphism. It belongs to the long-chain helical cytokine family, such as interleukin (IL)-2, IL-12 and IL-15, and has been previously described as a central mediator of control of food intake, basal metabolism and reproductive function [9]. Circulating leptin levels are proportional to body fat mass and both rodent and human females display higher circulating leptin concentrations than males independent of the amount of body fat [10]. This difference in secretion is influenced by sex steroids such as estrogens and testosterone [11]. Leptin serum concentrations can also be rapidly increased by pro-inflammatory cytokines and reduced by starvation [12, 13]. Recent evidence suggests a role for leptin as a modulator of CD4⁺ T cell-mediated immune response [14, 15]. Specifically, leptin regulates the balance of Th1/Th2 cytokines and reduced circulating levels of this hormone, induced by acute starvation, cause immunodeficiency and lymphoid organ atrophy in rodents [14, 16]. Mechanisms underlying the reduced susceptibility of males to EAE involve a predominant Th2 response in those animals [4]. Since leptin has been shown to affect the Th1 response [14] and to be higher in females than males [10], its contribution to the gender-related susceptibility to EAE in SJL mice was explored.

2 Results

2.1 Leptin administration confers susceptibility to PLP_{139–151}-induced EAE in male SJL mice and potentiates disease in females

To determine the contribution of leptin to the gender-related differences in EAE susceptibility in SJL mice, we first tested the ability of proteolipid protein peptide (PLP_{139–151}) to induce EAE in age-matched male and female SJL mice with and without recombinant (r) leptin treatment. Specifically, SJL male mice immunized with PLP_{139–151} peptide were divided in three groups. The control group received PBS from day 0 to 10; the second was injected with rleptin one day before and 1 day after priming (from day -1 to 1 and from day 6 to 8) to increase circulating leptin levels only at time of priming with the antigen; the third group was treated with rleptin during

the whole immunization schedule from day 0 to 10. As previously observed [4, 17], immunized male SJL mice showed reduced susceptibility to active induction of EAE (Fig. 1a and Table 1) as testified by low incidence of disease, absence of mortality, very mild clinical symptoms and minimal cellular infiltration in both the brain and spinal cord (Table 1). Our results also confirmed the previously observed absence of relapse in males immunized with PLP_{139–151} peptide [18]. Conversely, rleptin replace-

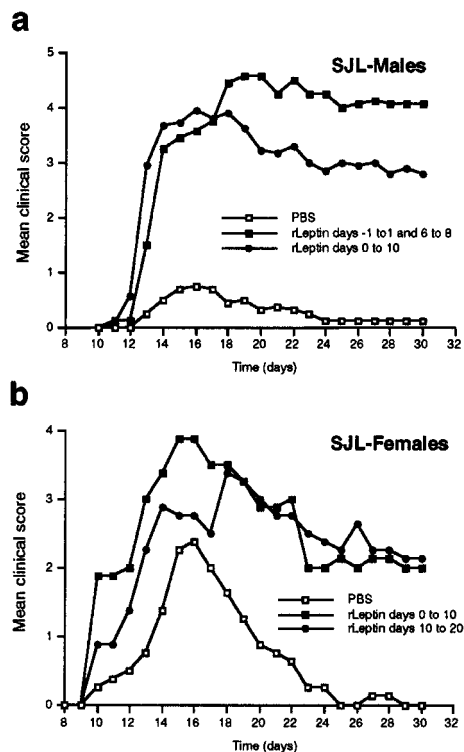


Fig. 1. Leptin administration reverses resistance to EAE in SJL male mice and worsens disease in females. (a) Mean clinical score and severity of EAE in male SJL mice treated with PBS, rleptin 24 h before and 24 h after priming with PLP_{139–151} (from day -1 to 1 and from day 6 to 8) and rleptin from day 0 to 10 of the observation, respectively. PBS-treated males showed only 25% of mice developing initial clinical symptoms. rLeptin treatment at priming determined the induction of a severe disease and very high mortality in treated males (see Table 1). Treatment with rleptin of male SJL mice from day 10 to 20, after priming with PLP_{139–151} peptide did not alter disease incidence and severity when compared with PBS treated males (data not shown). Data are representative of two independent experiments with similar results ($n=8-9$ mice/group). (b) Mean clinical score and severity of EAE in female SJL mice treated with PBS, rleptin from day 0 to 10 and rleptin from day 10 to 20 of the observation, respectively. rLeptin-treated females had a more severe clinical course than PBS-injected animals. Data are representative of two independent experiments with similar results ($n=8$ mice/group).

Table 1. Neurological impairment in male and female SJL mice injected with PLP_{139–151} peptide and treated or not with recombinant leptin^{a)}

Group of mice	Figure	Indicence	Day of onset ^{b)} (range)	Mortality	Peak clinical score ^{c)}	Average CDI ^{d)}	Percentage body weight loss	CNS histopathology score ^{e)}
Males SJL-PBS	1 a	2/8 (25.0 %)	13.0 ± 0.0	0/8 (0.0 %)	0.7 ± 1.3	6.0 ± 10.0	3.6 ± 0.5 %	0.6 ± 1.1
Males SJL-rLeptin (days – 1 to 1 and 6 to 8)	1 a	8/8 (100.0 %)	12.8 ± 1.4 (11–16)	5/8 (62.5 %)	4.5 ± 2.1 ^{f)}	70.7 ± 38.0 ^{g)}	22.3 ± 1.3 % ^{f)}	4.0 ± 0.0 ^{g)}
Males SJL-rLeptin (days 0 to 10)	1 a	9/9 (100.0 %)	12.8 ± 1.1 (11–15)	4/9 (44.4 %)	3.9 ± 1.9 ^{f)}	59.0 ± 43.0 ^{g)}	16.9 ± 1.8 % ^{f)}	3.9 ± 0.3 ^{g)}
Females SJL-PBS	1 b	8/8 (100 %)	11.2 ± 1.6 (10–14)	0/8 (0.0 %)	2.3 ± 1.4 ^{h)}	15.8 ± 10.0 ^{h)}	16.6 ± 1.5 % ^{h)}	2.2 ± 0.4 ^{h)}
Females SJL-rleptin (days 0 to 10)	1 b	8/8 (100 %)	10.0 ± 0.0	2/8 (25.0 %)	3.9 ± 1.2 ⁱ⁾	53.8 ± 38.0 ⁱ⁾	24.2 ± 1.0 % ⁱ⁾	3.8 ± 0.3 ⁱ⁾
Females SJL-rLeptin (days 10 to 20)	1 b	8/8 (100 %)	10.2 ± 0.4 (10–11)	0.8 (0.0 %)	3.4 ± 1.6 ^{j)}	49.8 ± 29.2 ^{j)}	24.0 ± 1.1 % ^{j)}	3.7 ± 0.4 ^{j)}

a) Mice were immunized with 200 µg of PLP_{139–151} peptide emulsified in CFA and observed daily for 35–40 days. None of the control mice ($n = 3$ for each group) immunized with CFA alone and pertussis toxin developed disease (data not shown). Data are cumulated and averaged from two separate experiments and they are presented as mean ± SD.

b) Mean of day of disease onset of the group.

c) Data are presented as mean of the group clinical score of all the animals of that groups.

d) Cumulative disease index is the sum of daily scores determined for each mouse of that group and averaged.

e) Inflammation along the CNS was scored blindly and given a value of 0 to 4 for the degree of inflammation as described in Sect. 4.

f)–j) f) $p < 0.01$ compared with males SJL-PBS, g) $p < 0.001$ compared with males SJL-PBS, h) $p < 0.05$ compared with males SJL-PBS, i) $p < 0.05$ compared with females SJL-PBS, j) $p < 0.01$ compared with females SJL-PBS.

ment in male SJL mice in both groups of treatment (at priming or during the whole period of disease induction) caused a dramatic increase in incidence, severity and mortality of EAE (Fig. 1a and Table 1). Histological examination of the CNS of rleptin-treated males showed clear evidence of cellular infiltration in the brain and spinal cord, consistent with the severity of disease (Table 1). Notably, the mortality in SJL males treated with rleptin was very high also when compared with the females (Table 1). Furthermore, treatment of male SJL mice with rleptin after priming with the antigen (from day 10 to 20) did not significantly alter disease resistance (data not shown), supporting the hypothesis that in males increased leptin levels are needed at time of priming with antigen to induce EAE. In female SJL mice the PLP_{139–151} peptide immunization induced a classic relapsing-remitting disease in PBS treated control females (Fig. 1b) [6]. rLeptin administration from day 0 to day 10 to female SJL mice resulted in an increase in severity of disease when compared with PBS-treated animals, as indicated

by a more severe clinical score, percentage of body weight loss, CNS inflammatory infiltration and mortality (Fig. 1b and Table 1). Treatment of females after disease onset (from day 10 to 20) also caused an increase in disease severity, although to a lesser extent when compared with rleptin-treated mice during immunization (Fig. 1b and Table 1). Finally, the possibility that the adjuvants used for the immunization were pathogenic in these mice was ruled out by the fact that none of the female and male SJL mice ($n=3$ for each group) injected with complete Freund's adjuvant (CFA) alone and with pertussis toxin developed disease (data not shown).

2.2 Leptin restores DTH reaction to PLP_{139–151} peptide in male SJL mice and increases DTH in females

To determine the nature of the T cell response *in vivo* against PLP_{139–151} peptide in SJL mice, DTH responses [19] were measured in all groups of male and female SJL

mice with or without leptin supplementation. At 7 days after priming with the PLP_{139–151} peptide emulsified in CFA, mice were challenged with 25 µg PLP_{139–151} injected intradermally in the footpad. The degree of local footpad swelling was measured as a read out for the DTH reaction. Typical DTH kinetics were observed with footpad swelling peaking at 24 h (Fig. 2). DTH responses to the PLP_{139–151} priming epitope were deficient in male SJL PBS-treated mice, confirming previous observations [20, 21]. Male SJL mice treated with rleptin (both at time of priming only and during the immunization) exhibited a significant DTH response (Fig. 2, left graph), similar to that observed in PBS-treated female SJL (Fig. 2, right graph). These data show that leptin administration at the time of priming with the PLP_{139–151} antigen is able to restore DTH responsiveness in male SJL mice. In female SJL mice leptin administration led to a statistically significant increase in the DTH response when compared with the PBS-treated group (Fig. 2, right graph). This increase was comparable in animals treated at priming (days 0–10) and treated after priming (days 10–20). None of the female and male control mice injected with CFA

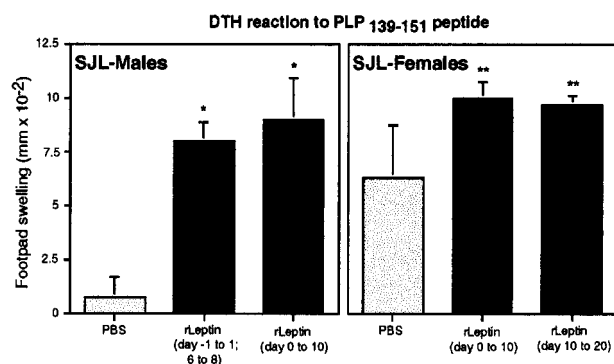


Fig. 2. Anti-PLP_{139–151} DTH reactivity is restored in male SJL mice and is increased in female SJL mice after leptin administration. Effect of leptin treatment on anti-PLP_{139–151} DTH reaction in male and female SJL mice was measured as footpad swelling. Seven days after sensitization with PLP_{139–151} in CFA, mice were challenged by injection of 25 µg PLP_{139–151} into the footpad and then assessed for footpad swelling assay 24 h later. Data are representative of two independent experiments with similar results, showing the means ± SD of footpad swelling responses. Male SJL mice controls injected with PBS did not show any significant reactivity against PLP_{139–151}. rLeptin administration to those mice restored the DTH reactivity to a level comparable to that of PBS-treated control females. rLeptin-treated females showed a significant increase in reactivity against the PLP_{139–151} antigen when compared with PBS treated control females. **p* < 0.01 compared with PBS-treated male SJL mice. ***p* < 0.05 compared with PBS-treated female SJL mice.

alone developed any significant anti-PLP_{139–151} DTH reaction (not shown).

2.3 Leptin administration reverses the pattern of helper T cell polarization in response to PLP_{139–151} peptide in male SJL mice and increases IFN-γ secretion in females

The T cell response to PLP_{139–151} peptide was tested on draining lymph node (LN) and spleen (SP) cells, taken from all groups of mice after immunization and cultured in the presence or absence of different concentrations of antigen. As shown in Fig. 3a, LN cells derived from male SJL mice treated with leptin exposed to PLP_{139–151} peptide displayed little difference in proliferation when compared with LN from PBS-treated animals (Fig. 3a, left graph), whereas they showed an 8–30-fold higher IFN-γ production and subsequent suppression of IL-4 secretion compared with PBS-treated male mice (Fig. 3a, middle and right graphs, respectively). These results confirmed a prevalent Th2 response in male SJL mice, that was probably responsible for EAE resistance [4, 17]; leptin administration at time of priming or during the immunization schedule (days 0–10) determined a complete switch in the response from a Th2 to a Th1 phenotype. Viability and capacity of T cells to respond to polyclonal TCR-mediated stimulation was also assessed by culturing with anti-CD3-antibody (Fig. 3a, inset graphs). rLeptin treatment in this condition also significantly affected IFN-γ and IL-4 secretion, whereas it had little effect on proliferation. Similar results were also observed using SP cells as the responder population (not shown). *In vitro* T cell proliferative and cytokine profiles were also investigated in LN and SP cells from female SJL mice after exposure to different concentrations PLP_{139–151} peptide. As shown in Fig. 3b, LN cells derived from female SJL mice treated with leptin exposed to PLP_{139–151} peptide displayed both a three- to fivefold increase of proliferation (Fig. 3b, left graph) and a five- to eightfold higher IFN-γ production, but no difference in IL-4 production when compared to PBS-treated female mice (Fig. 3b, middle and right graphs). Viability and capacity of T cells to respond to polyclonal TCR-mediated stimulation was also assessed performing culture with anti-CD3-antibody (Fig. 3b, inset graphs). rLeptin treatment significantly affected IFN-γ secretion, whereas it had little effect on proliferation and IL-4 secretion.

3 Discussion

This report examines the contribution of leptin to the gender-related susceptibility to relapsing-remitting EAE in SJL mice induced by the immunodominant epitope

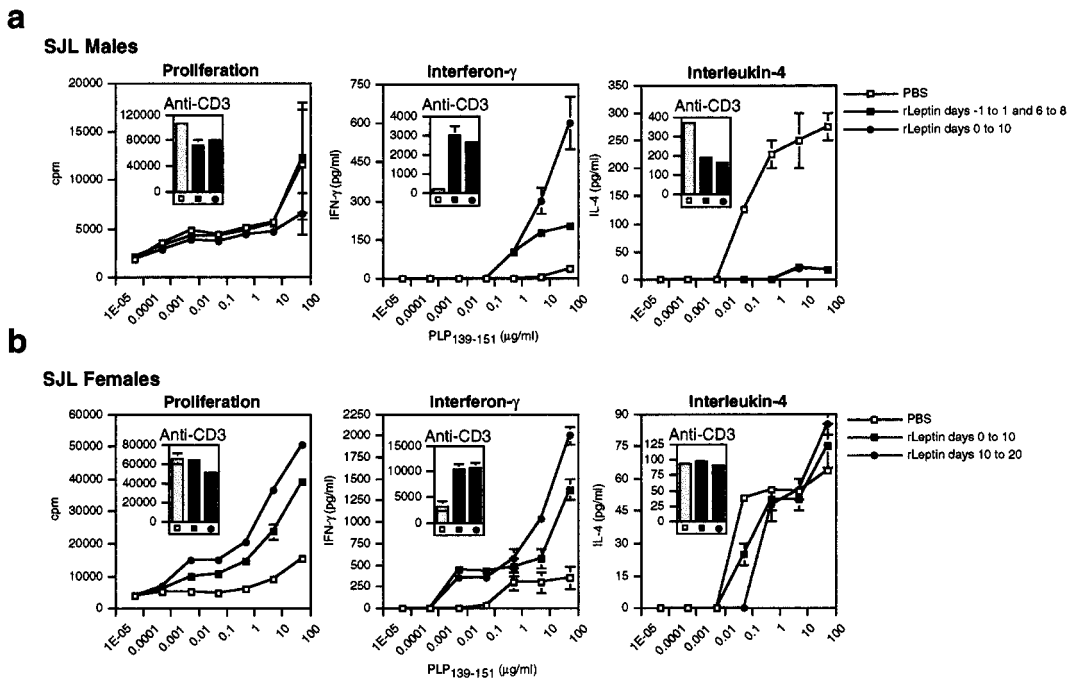


Fig. 3. Leptin administration switches the Th2 to a Th1 response in SJL male mice and increases T cell proliferation and IFN- γ production in females. (a) Antigen-specific proliferative, IFN- γ and IL-4 responses of LN cells from SJL male mice treated with PBS or rleptin, respectively. rLeptin replacement in males did not affect the proliferative response against PLP_{139–151}, whereas significantly increased IFN- γ and suppressed IL-4 secretion. Treatment of males 24 h before and 24 h after priming increased IFN- γ but to a lesser extent when compared with day 0 to day 10 treated mice; IL-4 was suppressed in both conditions of treatment with rleptin. (b) Antigen-specific proliferative, IFN- γ and IL-4 responses of LN cells from female SJL mice treated with PBS or rleptin, respectively. rLeptin treatment in females increased proliferative and IFN- γ responses when compared with PBS-treated controls; rleptin treatment did not significantly alter IL-4 secretion. Insets for each graph represent parallel polyclonal TCR-dependent anti-CD3-induced T cell proliferation, IFN- γ and IL-4 secretion of the same LN cells for all the single conditions tested. LN cells (5×10^5 /well) were taken after immunization and cultured in the absence or presence of varying concentrations of PLP_{139–151} peptide. Data are presented as means \pm SD of [3 H]thymidine incorporation (cpm) for proliferation and as means \pm SD of pg/ml cytokines secretion, all tested in duplicate. These data are representative of two independent experiments with similar results. In all cases, SD was less than 10% of the values. Similar results were also obtained with spleen cells as responders (not shown).

PLP_{139–151}. Our results show that leptin administration at priming with PLP_{139–151} peptide rendered male SJL mice susceptible to peptide-induced disease and increased sensitivity in females. In males, this was accompanied *in vivo* by the appearance of a significant anti-PLP_{139–151} DTH reaction together with an *in vitro* cytokine secretion switch from a Th2 to a Th1 pattern. In females, leptin administration determined an increase in the severity of clinical score, DTH reaction and IFN- γ secretion with little effect on *in vitro* IL-4 secretion. From the results obtained it is possible to hypothesize a model of leptin action together with sex hormones, all involved in the gender-related EAE susceptibility (Fig. 4).

Leptin is a cytokine that influences the immune system, endocrine function and nutritional status [9, 22]. Specifically, its circulating levels reflect the body mass index

(BMI) and can be rapidly reduced by starvation and increased during inflammation and food intake [12, 13]. These systemic effects are accompanied by secretion of gonadotropins and sex steroid hormones necessary for the reproductive function [23, 24]. The major aim of this study was to show that leptin as well as sex hormones may be part of the complex network responsible for the variations in susceptibility to autoimmune disease observed in rodents and humans. In females, leptin and estradiol may be required to sustain and promote pro-inflammatory CD4⁺ T cell responses. Indeed, androgen administration to females leads to reduction of leptin levels and estradiol, both associated with an increased capacity to secrete Th2 cytokines and reduced EAE susceptibility (Fig. 4) [17]. Conversely, estrogen administration to males or castration results in reduction of androgens and restored susceptibility to EAE (Fig. 4) [25, 26]. It

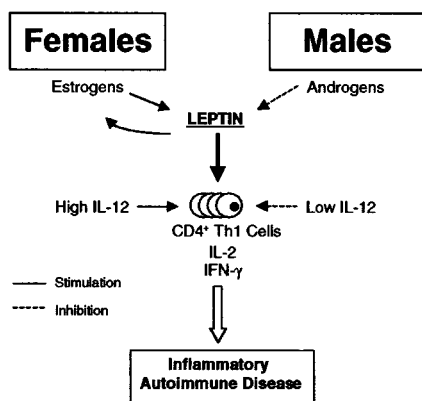


Fig. 4. Model summarizing the reciprocal influence of leptin and sex hormones on the immune T cell function in females and males. In females, high leptin levels are sustained by the reciprocal influence of estradiol on leptin secretion [9–11, 23, 24]. In addition, APC from females produce higher IL-12 than males [27]. These factors contribute to the increased capacity of females to produce a Th1 pro-inflammatory immune response [1]. Conversely, in males androgens, reducing the leptin levels [9–11], may prevent the induction of inflammatory autoimmune diseases [17].

has been shown that leptin levels rise after estrogen administration to males [11, 23], and this could be another mechanism contributing to the reversal of the resistance to susceptibility in male SJL mice. Furthermore, it has been recently demonstrated that the bias in the immune response is due to reduced ability of male SJL mice to produce IL-12, together with impaired capacity of APC to support Th1 pathogenic cell priming (Fig. 4) [27]. In agreement with this finding, relatively low leptin levels may also contribute to the reduced capacity to produce IL-12 by male SJL mice, thus impairing the Th1 response. This hypothesis will require further investigation.

Cell-mediated autoimmune diseases, such as MS and RA, improve during pregnancy, a state characterized by increased levels of progesterone, estrogens and leptin [28, 29]. In this context, very high concentrations of progesterone and estrogens promote the switch of the immune response towards the Th2 phenotype [1], and high placental production of leptin causes peripheral leptin receptor (ObRb) desensitization [30], inhibiting the Th1 cell-mediated immune response. Conversely, systemic lupus erythematosus (SLE), mainly sustained by Th2 cytokines, is worsened during pregnancy possibly due to Th2 environment present in this condition [1, 31].

We have also observed that leptin serum concentration rises rapidly after priming with the PLP_{139–151} antigen in females, but not in males (unpublished findings). These

observations may explain why leptin replacement at priming with the antigen confers susceptibility to male SJL mice, whereas in females starvation, with consequent rapid fall of leptin serum concentration, is able to reduce disease severity and progression (unpublished data).

In conclusion our findings provide *in vivo* evidence for the effect of leptin on the activation and priming of autoreactive T cells in both sexes. Continued studies may lead to a better understanding of how leptin contributes to increased susceptibility to EAE and ultimately to the development of antileptin therapies for human autoimmune diseases.

4 Materials and methods

4.1 Mice

Female and male SJL/J mice, 4–6 weeks old, were obtained from Charles River Italia (Calco, Italy). Experiments were performed under an approved protocol in accordance with the animal use guidelines of the Istituto Superiore di Sanità, Italy. All mice were age matched for individual experiments and were group-housed two to six mice per standard cage according to the different experimental protocol, with a 12-h light/dark cycle. Male mice were housed individually to prevent effects of stress from fighting on immune function [32]. Paralyzed mice were afforded easier access to food and water to prevent dehydration.

4.2 Antigens

The peptide used in this study is the immunodominant mouse PLP_{139–151} peptide (HSLGKWLGHDPDKF) [33]. It was synthesized by PRIMM s.r.l. (Milan, Italy); purity was assessed by HPLC (>97% pure) and amino acid composition was verified by mass spectrometry. PLP_{139–151} peptide batches for *in vivo* and *in vitro* assays were all from one preparation, initially solubilized in LPS-free saline solution at 4 mg/ml concentration and stored at –80°C.

4.3 Immunization and EAE induction

For EAE induction, female and male mice were immunized in parallel subcutaneously in the flank with 100 µl of CFA (Difco Laboratories, Detroit, MI) emulsified with 200 µg PLP_{139–151} peptide on days 0 and 7, and with 200 ng pertussis toxin (Sigma, St. Louis, MO) intraperitoneally (i.p.), on days 0, 1, 7 and 8. Control mice (*n*=3 mice/group) were injected with CFA emulsified with PBS plus pertussis toxin, according to the same schedule [33].

4.4 Leptin administration

Mouse rleptin was purchased from R&D Systems Europe Ltd.; purity was >97%, as determined by SDS-PAGE and visualized by silver staining analysis. The endotoxin level was <0.1 ng/μg leptin as determined by the Limulus amoebocyte lysate method. For rleptin treatment *in vivo*, all injected mice received 0.5 μg rleptin/g initial body weight twice daily (at 10.00 and 18.00 h) in 200 μl i.p. (total: 1 μg/g/day of rleptin as previously described [16]). A first group of female SJL mice was injected i.p. with rleptin before disease onset from day 0 to 10 during the immunization schedule (with days 0 and 7 being the days of immunization with PLP_{139–151} in CFA). The second group of females mice immunized in parallel, received rleptin after priming with the antigen and during the onset of symptoms from day 10 to 20. The third group of control females received twice daily 200 μl PBS i.p. during the whole period (from days 0 to 20). Males were divided in three groups: the first group was injected with rleptin starting 1 day before and 1 day after the time of priming with the PLP_{139–151} in CFA (from day –1 to day 1; and from day 6 to day 8); the second group was treated with rleptin before disease onset from days 0 to 10; and the third control group received twice daily 200 μl PBS i.p. during the entire period (from days 0 to 20). All mice were weighed, and their food intake was recorded daily.

4.5 Clinical and histological assessment

Individual mice were observed daily for clinical signs of disease up to 35 days after immunization. Mice were weighed and scored daily, by an observer blinded to group identity, according to the clinical severity of symptoms on a scale of 0–6 as described [33], with 0.5 points for intermediate clinical findings: 0, no abnormality; 0.5, partial loss of tail tonicity, assessed by inability to curl the distal end of the tail; 1, reduced tail tone or slightly clumsy gait; 2, tail atony, moderately clumsy gait, impaired righting ability or any combination of these signs; 3, hind limb weakness or partial paralysis; 4, complete hind limb paralysis or fore limb weakness; 5, tetraplegia or moribund state; 6, death. The data were plotted as daily mean clinical score for all animals in a particular treatment group. Scores for asymptomatic mice (score = 0) were included in the calculation of the daily mean clinical score for each group. The brains and spinal cords were dissected 20–30 days after immunization and fixed in 10% formalin. Paraffin-embedded sections, 5 μm thick, from brains and spinal cords were stained with hematoxylin-eosin and investigated for evidence of inflammation. Sections from 4–10 segments per mouse were examined blindly by one investigator using a published scoring system for inflammation [34].

4.6 Induction of DTH

DTH responses to PLP_{139–151} peptide during induction of disease were also quantitated using a time-dependent

(12–72 h) footpad swelling assay [19]. Briefly, mice previously sensitized with PLP_{139–151} in CFA were challenged by subcutaneous injection of 25 μg PLP_{139–151} (in 50 μl PBS) into the right hind footpad. PBS alone was injected into the left footpad to serve as control for measurements. As a negative control we used unimmunized mice (sensitized with CFA alone). Footpad thickness was measured 12, 24, 48 and 72 h after challenge by an experimenter (A.D.G.) 'blinded' to sample identity, using a caliper-type engineer's micrometer. The footpad swelling response was calculated as the thickness of the right footpad (receiving antigen) minus the baseline thickness of the left footpad (receiving PBS). Represented data in Fig. 2 are the 24-h time point corresponding to the peak of the DTH response.

4.7 Proliferation assays and cytokine analysis

SP and LN cells were obtained from mice between 20–30 days after PLP_{139–151} sensitization, dissociated into single-cell suspension and cultured for proliferation assays in flat-bottom 96-well microtiter plates (Falcon) at a density of 5×10^5 viable cells/well in a total volume of 200 μl RPMI 1640 medium (Gibco-BRL), supplemented with 2% FCS (Gibco-BRL), 2 mM L-glutamine (Gibco-BRL), 0.1 mM non-essential amino acids (Gibco-BRL), 1 mM sodium pyruvate (Gibco-BRL), 50 μM 2-mercaptoethanol (Sigma), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco-BRL). Cells were cultured at 37°C in 100% humidity and 5% CO₂ in the presence or absence of varying concentrations of PLP_{139–151} peptide (from 0 to 50 μg/ml). As control for proliferation, anti-CD3 antibody stimulation (2C11 hybridoma supernatant, diluted 1:100) was also performed. At 48–60 h after initiation of culture, cell supernatants (100 μl) were removed from single wells and frozen at –80°C for cytokine assay. IFN-γ and IL-4 were measured by an ELISA developed in our laboratory using cytokine-specific capture and detection Ab (PharMingen, San Diego, CA) according to the manufacturer's instructions (antibodies R4–6A2 and XMG1.2 for detection of IFN-γ; antibodies BVD4–1D11 and BVD6–24G2 for the detection of IL-4). Standard curves for each assay were generated using recombinant mouse cytokines (IFN-γ and IL-4, PharMingen) and the concentration of the cytokines in the cell supernatants was determined by extrapolation from the appropriate standard curve. The lower limits of detection for each assay were: <2 pg/ml for IFN-γ; <0.6 pg/ml for IL-4. The remaining cells were incubated for an additional 16 h, pulsed with 0.5 μCi/well of [³H]thymidine (Amersham-Pharmacia Biotech), harvested on glass-fiber filters using a Tomtec (Orange, CT) 96-well cell harvester and counted in a 1205 Betaplate liquid scintillation counter (Wallac, Gaithersburg, MD). Results are expressed as mean cpm ± SD from duplicates cultures.

4.8 Statistical analysis

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 \pm SD; *p* values less than 0.05 were considered to be statistically significant.

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Correspondence: Serafino Zappacosta, Cattedra di Immunologia, Dipartimento di Biologia e Patologia Cellulare e Molecolare, Università di Napoli “Federico II”, via S. Pansini, 5–80131, Napoli, Italy
Fax: +39-081-746 3252
e-mail: zappacos@unina.it.