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Effects of Intranasal Administration of a Leptin-Secreting *Lactococcus lactis* Recombinant on Food Intake, Body Weight, and Immune Response of Mice▽

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Leptin is an adipocyte-derived pleiotropic hormone that modulates a large number of physiological functions, including control of body weight and regulation of the immune system. In this work, we show that a recombinant strain of the food-grade lactic acid bacterium *Lactococcus lactis* (LL-lep) can produce and efficiently secrete human leptin. The secreted leptin is a fully biologically active hormone, as demonstrated by its capacity to stimulate a STAT3 reporter gene in HEK293 cells transfected with the Ob-Rb leptin receptor. The immunomodulatory activity of leptin-secreting *L. lactis* was evaluated in vivo by coexpression with the human papillomavirus type 16 E7 protein. In C57BL/6 mice immunized intranasally with a recombinant *L. lactis* strain coproducing leptin and E7 antigen, the adaptive immune response was significantly higher than in mice immunized with recombinant *L. lactis* producing only E7 antigen, demonstrating adjuvanticity of leptin. We then analyzed the effects of intranasally administered LL-lep in obese *ob*/*ob* mice. We observed that daily administration of LL-lep to these mice significantly reduced body weight gain and food intake. These results demonstrate that leptin can be produced and secreted in an active form by *L. lactis* and that leptin-producing *L. lactis* regulates in vivo antigen-specific immune responses, as well as body weight and food consumption.

Human leptin, the product of the *obese* (*ob*) gene, is a 167-amino-acid protein (~16 kDa) with a 21-amino-acid signal sequence and one disulfide bond (70). Leptin is an adipocyte-secreted hormone that bears structural similarity to the helical cytokine family (43, 69). Treatment with recombinant leptin has been shown to reduce food intake and body weight and to correct metabolic and hormonal perturbations in leptin-deficient *ob*/*ob* mice (10, 27, 53). In humans, leptin also plays a crucial role in the regulation of body weight, as demonstrated by morbid obesity in patients with congenital mutations in either leptin or the leptin receptor gene (15, 47, 64, 65). Although leptin treatment induced remarkable weight loss in patients with rare congenital leptin deficiency (19, 20, 25, 42), it showed poor efficiency in most obese patients. Indeed, clinical trials involving the subcutaneous administration of recombinant leptin to obese subjects indicated that a significant reduction of body weight was only observed if serum leptin concentrations were 20- to 30-fold higher than normal physiological levels (29). This poor response was attributed in part to insufficient transport of leptin across the blood-brain barrier in obese patients (11). Since intranasal delivery is an efficient route for the administration of drugs directly to the brain (8, 28, 37), intranasal leptin administration is considered an interesting strategy to bypass the blood-brain barrier in leptin-resistant humans. This has motivated several recent studies demonstrating rapid and effective intranasal leptin administration and transport to the brain (23, 60, 61). These observations suggest that leptin may still be an effective therapeutic agent for the treatment of obesity (33).

In addition to its effects on body weight control and energy metabolism, leptin is now known to be a pleiotropic hormone also involved in the regulation of immunity, sexual maturation and fertility, bone formation, angiogenesis, tumorigenesis, and wound healing (1). Leptin has been shown to be beneficial for the treatment of lipodystrophy (32, 38, 49) and hypothalamic amenorrhea (68). However, it could also have adverse effects on the development of autoimmune diseases (44), atherosclerosis (35, 59), and cancer (13, 14). Therefore, not only leptin, but also leptin antagonists, produced by site-directed mutagenesis of leptin, have the potential for multiple therapeutic applications, depending on the patients and the target tissues considered (50, 52).

Both leptin and leptin antagonists are generally produced in *Escherichia coli*, after extraction from insoluble inclusion bodies, followed by renaturation and purification of the renatured protein (21). The development of a novel bacterial expression system, allowing the secretion of a soluble, biologically active leptin, may considerably facilitate (i) large-scale production of leptin and (ii) rapid production and evaluation of the activities of leptin mutants. *Lactoccocus lactis* has been demonstrated to be an efficient vector for the production and secretion of heterologous proteins (39, 40, 46, 62). In addition, *L. lactis* is a food-grade, gram-positive bacterium that could be used to

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deliver therapeutic proteins at the mucosal level, including the intranasal mucosa (4, 6, 62, 63).

In this work, we demonstrate that human leptin can be produced and efficiently secreted by *L. lactis* in a soluble and biologically active form. In addition, we demonstrate that the intranasal administration of a leptin-producing *L. lactis* strain can modulate the adaptive immune response in C57BL/6 mice and also reduces food intake and body weight gain in leptin-deficient *ob*/*ob* mice.

MATERIALS AND METHODS

DNA manipulations. Plasmid constructions were established in *L. lactis* by electrotransformation (36). Isolation of plasmid DNA was performed by using a Mini-Scale purification system (QIAGEN S.A.). Lysozyme (10 mg/ml) and mutanolysin (100 ng/ml) were added prior to the lysis step and incubated for 30 min (37°C) to prepare the protoplasts. PCR (Cetus apparatus; Perkin Elmer, Norwalk, CT) was performed using Vent DNA polymerase (Promega), and DNA sequences were confirmed by sequencing (MWG-Biotech AG). Restriction and DNA-modifying enzymes were used according to the supplier's recommendations.

Cloning of the human leptin gene in *L. lactis*. A 462-bp DNA fragment encoding mature human leptin (i.e., without the signal peptide) was PCR amplified from the pcDNA3:leptin vector (64) by using primers NsiI-hlep (5′-CC A***ATGCAT***CAGTGCCCATCCAAAAAGTCCAAGATGAC-3′) and SpeI-hlep (5′-GG***ACTAGT***CCTCAGCACCCAGGGCTGAGGTCCAGCTGCCACAGC ATGTCCTGCAGAGACCCCTG-3′). The resulting fragment was directly digested with NsiI and SpeI enzymes (restriction sites on the primers are indicated in bold and italics) and cloned into purified backbone isolated from the NsiI-SpeI-cut pSEC-E7 vector (7), a derivative of the broad-host-range plasmid pWV01, which replicates in *E. coli* and several gram-positive bacteria (6), resulting in pSEC:lep (Fig. 1). This plasmid was introduced into *L. lactis* strain NZ9000 carrying the regulatory genes *nisR* and *nisK* (18) to obtain the strain LL-lep. As a negative control, NZ9000 was transformed with the pSEC empty vector to generate strain LL. Recombinant clones were selected by the addition of 10 μg of chloramphenicol per ml. Recombinant *L. lactis* strains were grown in M17 medium supplemented with 1% glucose (GM17) at 30°C without aeration.

Inducible expression of leptin. For the induction of leptin expression from the nisin promoter, strains were grown to an optical density at 600 nm of 0.6, followed by induction with 10 ng of nisin (Sigma) per ml for 1 h. *L. lactis* culture extraction and immunoblotting assays were performed as follows, using a polyclonal leptin antibody (Bio-Vendor). To quantify leptin production, protein samples were prepared from 2 ml of induced culture. After centrifugation (5 min, 10,000 × *g*), the cell pellet and supernatant were treated separately. The supernatants were treated with 1 mM phenylmethylsulfonyl fluoride and 10 mM dithiothreitol, followed by the addition of 100 μl of 100% trichloroacetic acid to precipitate proteins. Samples were incubated for 10 min on ice, and proteins were recovered from the pellets after centrifugation at 4°C for 10 min at 13,000 rpm. The cell fraction was obtained by cell lysis in lysis buffer (25% sucrose, 1 mM EDTA, 50 mM Tris-HCl [pH 8.0], and 10 mg/ml lysozyme) complemented with 1 mM phenylmethylsulfonyl fluoride and 10 mM dithiothreitol. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Western blotting, and immunodetection were performed as previously described (7). Human commercial leptin (PeproTech., Inc.) was used as a control in Western blotting. The concentrations of leptin secreted in the medium and retained in cell fractions were assessed by using an enzyme-linked immunosorbent assay (ELISA) kit (Bio-Vendor).

STAT3 reporter gene activation assay. *H*uman *e*mbryonic *k*idney (HEK293) cells maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Cergy Pontoise, France) supplemented with 4.5 g/liter glucose and 10% fetal calf serum were seeded at a density of 2.5×10^5 cells per 2-cm^2 well. Transient transfections were performed 1 day later using FuGENE 6 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's protocol. HEK293 cells were cotransfected in 2-cm^2 wells with 700 ng/well of Ob-Rb receptor, 300 ng/well of a *s*ignal *t*ransducer and *a*ctivator of *t*ranscription-3 (STAT3)-firefly luciferase reporter gene, and 0.02 ng/well of pcDNA3-*Renilla* luciferase (used as an internal control between samples) (17). Twenty-four hours after transfection, the culture medium was removed and cells were cultured for an additional 24 h in 0.5 ml of DMEM containing 1% fetal calf serum and either 10 nM human commercial leptin or 50 μl of strain *L. lactis* culture medium. The cells were then washed with phosphate-buffered saline (PBS) and lysed in passive lysis buffer (Promega) for

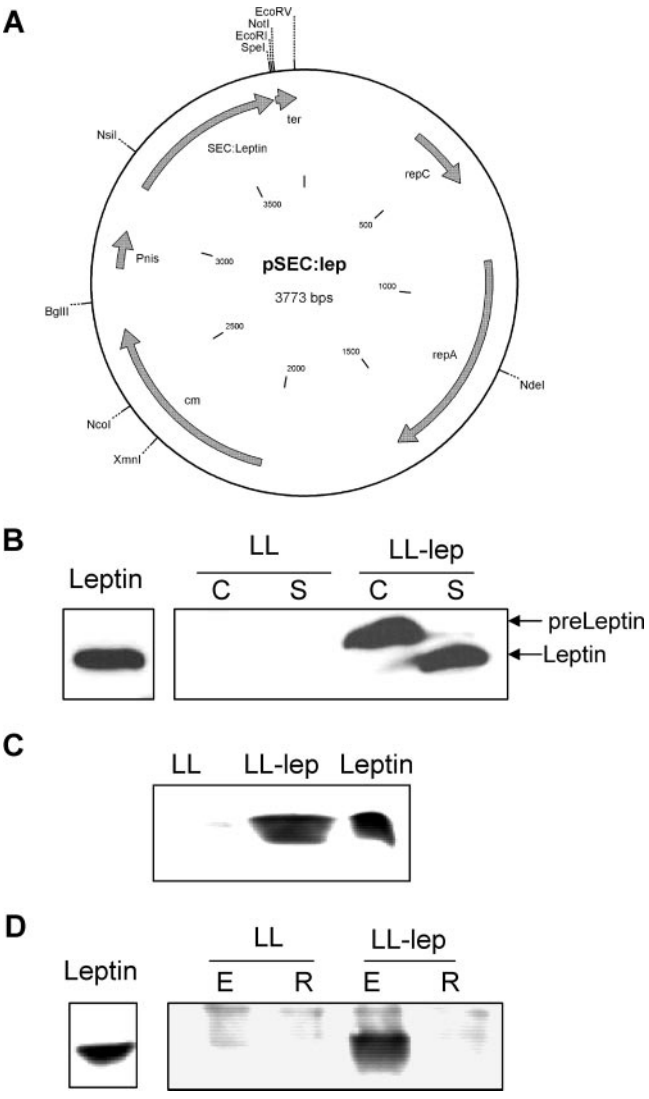

FIG. 1. Schematic representation of pSEC:lep vector and expression of leptin by *L. lactis*. (A) A 462-bp DNA fragment encoding mature human leptin was fused in frame with a DNA fragment containing the Usp45 signal peptide (SEC:Leptin), derived from the predominant *L. lactis*-secreted protein (67). In this plasmid, leptin expression is controlled by the nisin-inducible promoter (P_{nisA}) and harbors the Usp45 ribosome binding site and the *rho*-independent *trpA* transcription terminator (ter) (12) for clone stability. The pSEC:Lep carries the pC194 chloramphenicol resistance marker (cm) (34) (B) Strains LL and LL-lep were grown and induced with 10 ng/ml nisin for 1 h. After centrifugation, cell pellet and culture medium were treated as described in Materials and Methods. The antileptin antibody detected a protein in LL-lep culture supernatants with an apparent molecular mass identical to that of the commercial recombinant leptin. C, cell fraction; S, supernatant fraction. (C) Strains LL and LL-lep were grown as described in Materials and Methods. After centrifugation, leptin was immunoprecipitated from 1 ml of culture medium and immunodetected by Western blotting using antileptin antibody. (D) Strain LL or LL-lep culture supernatants were fractionated on 100-kDa centrifugal membranes as described previously (9). Leptin present in retentates (R) and eluates (E) was immunoprecipitated and detected by immunoblotting with antileptin antibody. Commercial leptin (Leptin) was used as a control in the assays.

15 min at room temperature. Total lysates were centrifuged for 2 min at 15,000 rpm, and the supernatants were used in a dual-luciferase assay system (Promega) using a Berthold Luminometer (Lumat LB 9507).

Administration of live *L. lactis* strain coproducing leptin and E7 antigen to C57BL/6 mice. Specific-pathogen-free C57BL/6 mice (females, 6 to 8 weeks of age; Charles River Breeding Laboratories) were housed in groups of four mice per cage in pathogen-free Texler-type isolators (La Calhène, Vélizy, France) under sterile conditions with water and were fed ad libitum in the animal facilities of the Unité d'Ecologie et de Physiologie du Système Digestif at the Institut National de la Recherche Agronomique (INRA, Jouy-en-Josas, France). Food intake and body weight were recorded every day. All experiments were performed according to protocols in accordance with INRA guidelines.

To construct a recombinant *L. lactis* strain coexpressing leptin and E7 antigen, we introduced a second plasmid encoding a cell wall-anchored form of human papillomavirus type 16 (HPV-16) E7 antigen (16) into the LL-lep strain, resulting in the LL-lep/E7 strain. Live bacterial inocula of the different *L. lactis* strains were prepared and induced as previously described (4). Briefly, cellular pellets were harvested by centrifugation at 3,000 × *g* at 4°C and washed three times with sterile PBS. The pellet was suspended in PBS to a final concentration of 1×10^9 CFU. Groups of C57BL/6 mice (five mice per group) were immunized intranasally with 1×10^9 CFU from each induced recombinant lactococcus strain suspended in 10 μl of PBS (5 μl was administered with a micropipette into each nostril) on days 0, 14, and 28. Control mice received identical quantities of *L. lactis* transformed with an empty vector (strain LL). The inocula were controlled for CFU and leptin and E7 production for all animal administrations. The antibodies used were human antileptin (Bio-Vendor) and anti-E7 (Santa Cruz Biotechnology).

Measurement of E7-specific $CD4^+$ and $CD8^+$ T cells. Splenocytes from immunized C57BL/6 mice were incubated with the $E7_{49–57}$ peptide (major histocompatibility complex [MHC] class I epitope [22]) or $E7_{30–67}$ peptide (MHC class II epitope [66]). The number of E7-specific gamma interferon (IFN-γ)-producing T cells was determined by enzyme-linked immunospot assay (mouse IFN-γ; R&D Systems) as previously described (4).

Administration of live bacterial inocula to *ob*/*ob* mice. Specific-pathogen-free *ob*/*ob* C57BL/6 mice (males, 6 to 8 weeks of age; Janvier) were housed as described above. After arriving, the mice were acclimatized for 1 week (including PBS pretreatment during the last 4 days). The room temperature was constant at 25°C, and a 12-h light, 12-h dark cycle was maintained. Mice (five animals per cage) were fed powdered ground rodent chow (food no. R03-40; SAFE, Augy, France) in powdered-food hoppers for mice (UAR, Epinay/Orge, France). Individual body weights and global food intake (food intake per cage) were monitored daily. For the preparation of the live bacterial inocula, LL-lep was grown and induced as described above. Cellular pellets were then harvested by centrifugation at 3,000 × *g* at 4°C and washed three times with sterile PBS. The pellet was suspended in PBS to a final concentration of 1×10^9 CFU. We previously demonstrated that after a 1-hour nisin pulse induction, nisin-induced protein production and secretion by the recombinant LL will continue for at least 10 h in the absence of nisin (5). Groups of *ob*/*ob* C57BL/6 mice were inoculated intranasally with 1×10^9 CFU of the nisin-induced lactococcus strain suspended in 10 μl of PBS (5 μl was administered with a micropipette into each nostril) daily for 19 days. Control mice received either PBS or identical quantities of LL. The quantities of CFU and leptin production were monitored as described above.

Statistical analyses. Student's *t* test was used to compare the differences between groups by using JMP statistical software. A P value of <0.05 was considered significant.

RESULTS

Characterization of leptin production by *Lactococcus lactis*. Lactococcus strains transformed with an empty vector (LL) or with the expression vector pSEC:lep (LL-lep) (Fig. 1A) were grown, and the leptin expression was induced with nisin as described in Materials and Methods. The ability of LL-lep to produce and secrete human leptin was then tested by Western blot using a polyclonal leptin antibody. As shown in Fig. 1B, bands of ~18 kDa and 16 kDa were detected in nisin-induced cultures of the LL-lep strain, corresponding, respectively, to SP_{Usp45}-Lep precursor (pre-Leptin), present in the cell fraction, and to mature leptin, present in the supernatant fraction. No signal was detected in the negative-control strain LL (Fig. 1B). In addition, the recombinant leptin secreted by *L. lactis* could be directly immunoprecipitated from the culture medium of the LL-lep strain, indicating that this protein is properly processed and secreted in an immunoreactive form (Fig. 1C).

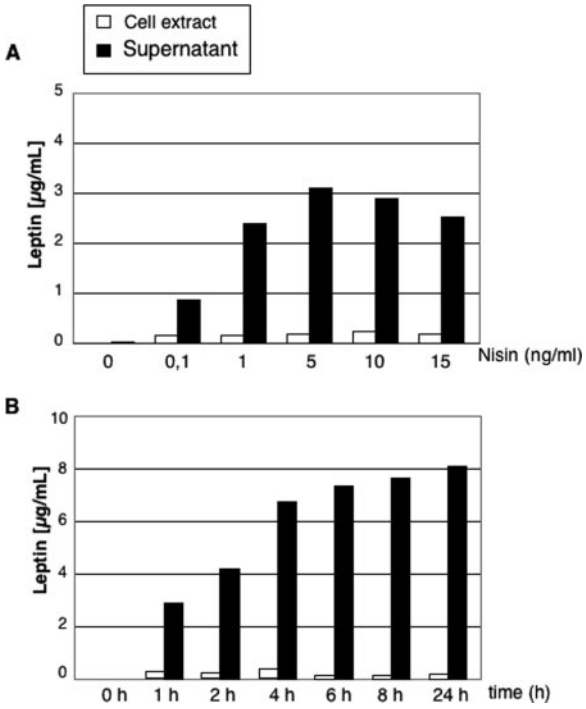

FIG. 2. Leptin expression by recombinant lactococci as a function of induction conditions. (A) Leptin quantification by ELISA of cell fractions or supernatant samples from LL-lep cultures induced (at an optical density at 600 nm of 0.6 U) with increasing concentrations of nisin (0 to 15 ng/ml). (B) Leptin quantification by ELISA of cell fractions or supernatant samples from LL-lep cultures induced (at an optical density at 600 nm of 0.6 U) with 10 ng/ml nisin at different times (0 to 24 h). The results of a representative experiment are shown.

Previous studies applying size-exclusion filtration to leptin mutants have shown that improperly folded leptin forms large molecular aggregates that cannot cross a filtration membrane with a cutoff of 100 kDa (9, 56). To ensure that leptin produced by *L. lactis* does not form such macromolecular aggregates, LL-lep culture medium was submitted to size exclusion membrane filtration. Leptin secreted by LL-lep was recovered in the eluate fraction, whereas no leptin was detected in the retentate fraction of the filtration unit (Fig. 1D). This result indicates that leptin secreted by LL-lep does not form large macromolecular aggregates.

Leptin secretion by the LL-lep strain was further characterized and quantified by ELISA. We first analyzed leptin production and secretion by the LL-lep strain at different nisin inducer concentrations (Fig. 2A). Incubation for 1 h with nisin resulted in a dose-dependent increase in leptin secretion, with a maximal effect obtained at a nisin concentration of 5 ng/ml. The very small amounts of leptin detected in the cell extracts indicated that most of the leptin produced by LL-lep was processed and secreted in the medium. We calculated that, under these experimental conditions, the secretion efficiency (i.e., the percentage of leptin found in the supernatant related

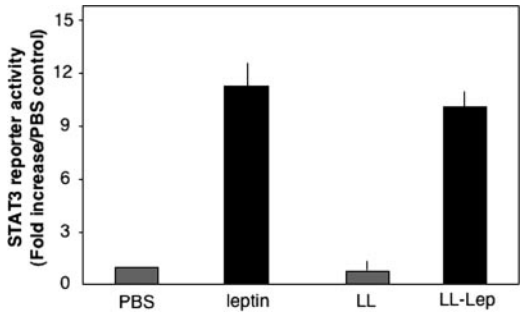

FIG. 3. In vitro biological activity of leptin produced by *L. lactis*. HEK293 cells were cotransfected with Ob-Rb receptor, STAT3-firefly luciferase reporter gene, and pcDNA3-*Renilla* luciferase. Twenty-four hours after transfection, cells were cultured for an additional 24 h in 0.5 ml of DMEM containing 1% serum and either 10 nM commercial leptin or 50 μl of *L. lactis* culture medium, from leptin-producing or -nonproducing strains. Results are expressed as ratios of activities for firefly luciferase over *Renilla* luciferase and represent the means of two independent experiments performed in triplicate.

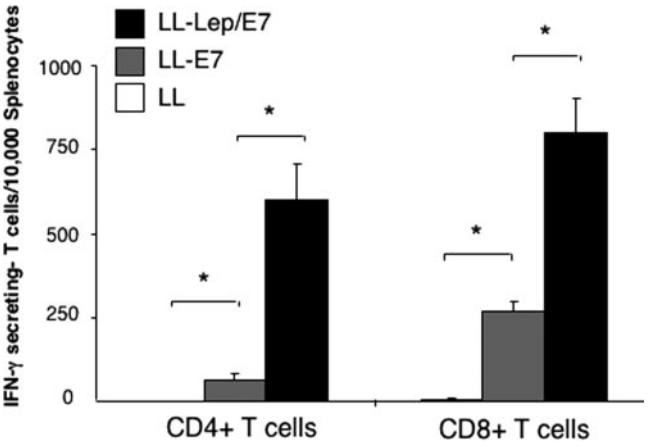

FIG. 4. Production levels of IFN-γ from spleen cells in mice immunized with live lactococci expressing human leptin and E7 antigen. Five C57BL/6 mice were intranasally immunized on days 0, 14, and 28 with strains LL, LL-E7, and LL-lep/E7. One week after the last immunization (day 35), splenocytes from immunized mice were pooled and stimulated in vitro with $E7_{30–67}$ peptide (MHC class II epitope) or $E7_{49–57}$ peptide (MHC class I epitope) for identification of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells, respectively, by enzyme-linked immunospot assay. *, differences are statistically significant ($P < 0.05$).

to the total amount of leptin found in both the supernatant and cell fraction) was at least 90%. Time-course experiments indicated that, at a concentration of 10 ng/ml of nisin, leptin accumulated in the culture medium for about 6 to 8 h, reaching a maximal concentration of about 7 to 8 μg/ml (Fig. 2B). These experiments demonstrate that LL-lep has a remarkably efficient capacity to secrete leptin in the culture medium.

Leptin secreted by *L. lactis* is biologically active. The Ob-Rb leptin receptor is known to activate the JAK/STAT signaling pathway, leading to the stimulation of STAT3 transcriptional activity (24). To determine whether leptin secreted by *L. lactis* can bind to the leptin receptor and stimulate its downstream signaling pathways, HEK293 cells were cotransfected with the Ob-Rb leptin receptor and a STAT3-firefly luciferase reporter gene (17). After 24 h of transfection, cell cultures were followed for 24 h in the presence of commercial leptin (10 nM) or either strain LL or LL-lep culture medium (50 μl) derived from cells previously induced with 10 ng/ml of nisin for 2 h. Luciferase reporter activity was measured from HEK293 cell protein extracts. Both commercial leptin and culture medium from LL-lep markedly increased firefly luciferase activity, whereas culture medium from LL had no effect (Fig. 3). This result demonstrates that leptin secreted by recombinant lactococci binds to the human leptin receptor expressed in HEK293 cells and activates the JAK/STAT signaling pathway, leading to the stimulation of STAT3 transcriptional activity.

Intranasal administration of *L. lactis* coexpressing leptin and HPV-16 E7 protein in C57BL/6 mice enhances Th1 immune response. Previous work showed that the intranasal administration of an *L. lactis* strain expressing a cell wall-anchored form of HPV-16 E7 antigen induced an antigen-specific T-cell response in mice (3). Moreover, coadministration of an *L. lactis* strain secreting IL-12 had adjuvant effects, resulting in protection against HPV-16-induced tumors in mice (4). Since leptin is known to regulate immune function and to promote T helper 1 (Th1)-cell differentiation (44), we asked whether the coexpression of leptin with HPV-16 E7 antigen in the same strain could modulate the antigen-specific T-cell response after intranasal administration in mice. Three groups of four C57BL/6 mice were immunized on days 0, 14, and 28 with 1×10^9 CFU of the control *L. lactis* strain (LL), an *L. lactis* strain expressing E7 antigen (LL-E7), or an *L. lactis* strain coexpressing leptin and E7 antigen (LL-lep/E7). One week after the last immunization, splenocytes from immunized animals were used for the detection of IFN-γ, a cytokine characteristic of a Th1 type of immune response. Immunization with LL-lep/E7 resulted in a marked enhancement of IFN-γ secretion by $CD4^+$ and $CD8^+$ lymphocytes in response to E7-derived peptides (Fig. 4). These results further confirm that leptin produced by *L. lactis* is biologically active and demonstrate the in vivo immunomodulatory effects of intranasal administration of LL-lep in mice.

Intranasal administration of LL-lep reduces food intake and body weight gain in *ob*/*ob* mice. To further evaluate in vivo the biological activity of the leptin produced by *L. lactis*, we studied the effects of intranasal administration of LL-lep on food intake and body weight in leptin-deficient *ob*/*ob* mice. Three groups of five C57BL/6 *ob*/*ob* mice were subjected to intranasal administration of PBS or strain LL or LL-lep daily for 19 days. The total amount of food consumed by each group of mice (food intake per cage) was monitored every day during the duration of the treatment. Monitoring of the cumulative food intake at the end of the treatment period showed that, on average, a mouse from the LL-lep-treated group consumed 17 g and 13 g less than a mouse from the PBS-treated or the LL-treated group, respectively (Fig. 5A). Daily food intake was not significantly different between PBS- and LL-treated mice. In contrast, daily food intake was significantly lower in LL-lep-treated mice than in PBS- or LL-treated mice (Fig. 5B). The body weight of each mouse was monitored daily over the test period (Fig. 5C). Body weight gain from baseline values tended to be lower in mice receiving strain LL than in mice receiving PBS, but this effect reached significance at only a few time-points. In contrast, *ob*/*ob* mice that received LL-lep gained

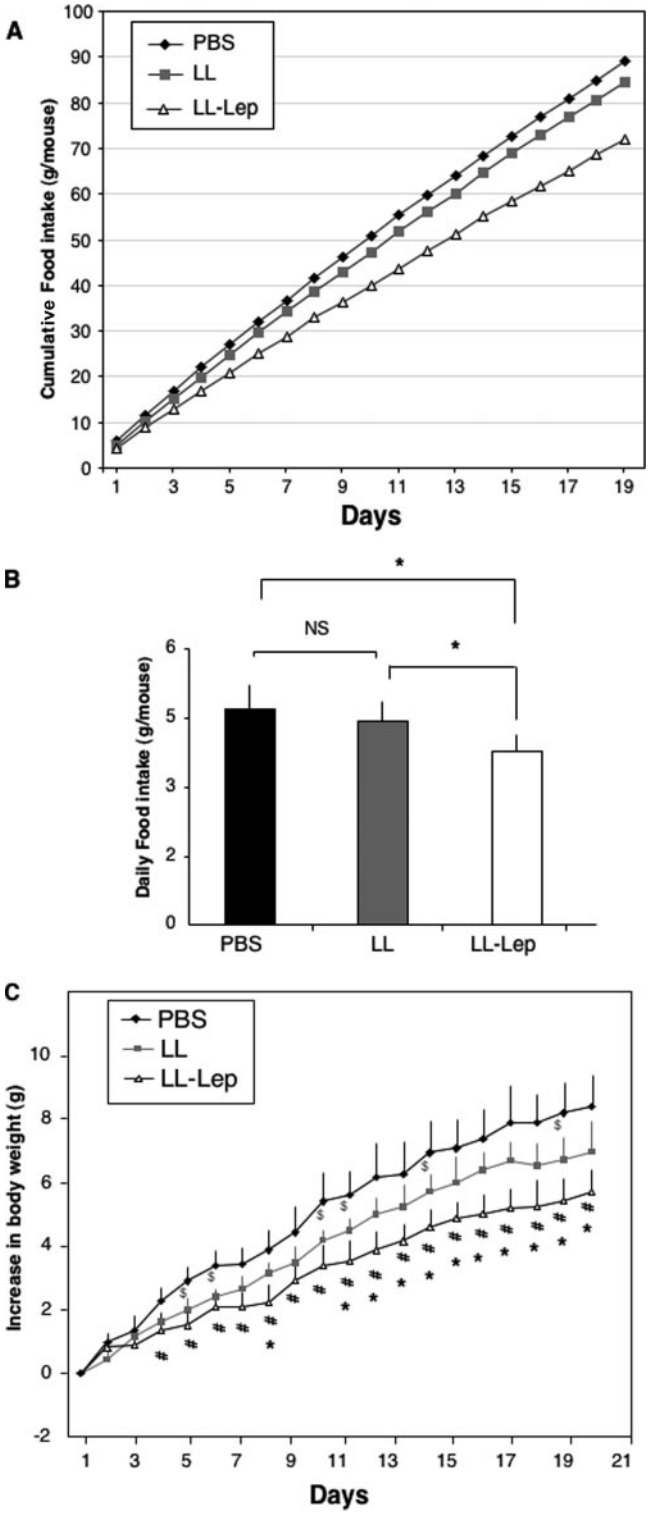

FIG. 5. Effects of daily intranasal administration of LL-Lep on food intake and body weight gain in *ob*/*ob* mice. *ob*/*ob* mice were inoculated daily with PBS or strain LL or LL-lep (1×10^9 CFU/inoculum). Food intake and body weight gain were measured every day for 19 days. (A) The total amount of food ingested by the mice present in the same cage and receiving the same treatment was measured every day. The figure represents the evolution of cumulative food intake per mouse during the period of treatment. (B) Mean food intake per animal and per day. *, differences are statistically significant ($P < 0.05$); NS, difference is not significant. (C) Evolution of body weight gain during treatment. Differences are statistically significant ($P < 0.05$) between LL-lep- and LL-treated mice (*), between LL-lep- and PBS-treated mice (#), and between LL- and PBS-treated mice ($).

significantly less weight than PBS- and LL-treated mice. Differences between LL-lep-treated mice and PBS- or LL-treated mice became significant from day 4 and 11, respectively, and remained significant up to the end of the treatment. We also evaluated the effect of daily intranasal administration of 10 μg of commercial leptin (i.e., the maximal amount of recombinant leptin produced in vitro by LL-Lep in 24 h, according to our in vitro experiments), and we did not observe any significant effect on body weight (data not shown). Altogether, our results demonstrate that leptin produced by LL-lep has biological activity in vivo on the regulation of food intake and body weight of *ob*/*ob* mice and confirm the interest in using lactococci for leptin delivery.

DISCUSSION

About 30% of the North American population and 20% of the European population is overweight. Given the world-wide prevalence of this pathology, peptides that regulate appetite and body weight represent major pharmaceutical targets for the next decades. In addition to its implication in the control of food intake and body weight, leptin may also have a high therapeutic potential in other biomedical fields, through its regulatory role on the immune system, for instance. To date, recombinant leptin has been produced essentially in *E. coli*, necessitating a multistep extraction process that generally includes disruption of the cells, centrifugation, extraction, and refolding of the denatured protein (21, 31, 57). Although in some cases leptin could be extracted in a soluble form from the *E. coli* periplasm, the recovery procedure involved osmotic shock and several centrifugation steps (26). In addition, all the methods described to date for recombinant leptin production require purification steps before the leptin's biological activity can be evaluated.

In this work, we cloned the leptin gene in our usual expression vector, resulting in a vector allowing rapid and efficient secretion of soluble human leptin by *L. lactis*. We demonstrated that this recombinant leptin is directly secreted in a biologically active form, as shown by its capacity to efficiently stimulate the STAT3 promoter in HEK293 cells expressing the leptin receptor. This procedure could easily be adapted for the large-scale production of biologically active recombinant leptin. In addition, leptin production in secreted form allows for direct assessment of its biological activity in small volumes of culture medium, without any purification steps. This opens important perspectives for the production and rapid evaluation of the biological activity of leptin mutants, which may act as potent leptin receptor agonists or antagonists, with valuable therapeutic potential (51). Furthermore, in some cases, it may also be important to be capable of rapidly evaluating the biological activity of such mutants in vivo, without having to purify them. We showed that the activity of leptin produced by LL-lep can be studied in vivo in mice, by direct intranasal administration of live lactococci. Indeed, we first demonstrated that

leptin produced by LL-lep has immunomodulatory properties in C57BL/6 mice. Intranasal administration of food-grade bacteria expressing antigens has been proposed as an alternative vaccination approach for the stimulation of mucosal and systemic immune responses (45), and concomitant delivery of proinflammatory cytokines reportedly has adjuvant effects (4, 6, 63). Previous works using *L. lactis* as a live vaccine to immunize mice against E7 antigen have shown that coadministration of two strains, one expressing IL-12 and the other E7, resulted in about two- and sixfold increases, respectively, in $CD4^+$ and $CD8^+$ IFN-γ responses to E7 antigen (4, 6). In the present work, the administration of *L. lactis* coexpressing leptin and E7 antigen resulted in about 3- and 9.5-fold increases, respectively, in $CD4^+$ and $CD8^+$ IFN-γ responses to E7. This result suggests that leptin may have interesting immunostimulatory properties in the context of mucosal vaccination.

In vivo biological activity of leptin secreted by LL-lep was also demonstrated by the intranasal administration of *Lactococci* in leptin-deficient *ob*/*ob* mice. Several peptidic hormones need to be transported into the cerebrospinal fluid through the blood-brain barrier to exert their effects, and intranasal administration has been described as a means to bypass the blood-brain barrier and deliver active molecules directly into the brain. The nasal route for drug delivery to the brain via the olfactory region has been widely investigated. Various studies indicate that large molecules, such as peptides, can be directly transported from the nasal mucosa into the cerebrospinal fluid, the olfactory bulb, and the brain parenchyma (30). Several recent studies have shown that intranasal delivery of leptin resulted in the reduction of food intake and body weight (60, 61). We observed that intranasal administration of LL-lep significantly reduced food intake and body weight gain compared to administration of LL, suggesting that leptin secreted by LL is biologically active on the central nervous system. At the end of the treatment period, the animals were sacrificed and blood samples were taken for the measurement of peripheral leptin. Using a human leptin ELISA, we observed that virtually no leptin was detected in the plasma of LL-lep-treated *ob*/*ob* mice (data not shown). This suggests that the effect of intranasally administered LL-lep may be the result of direct transport of the secreted leptin from the nasal cavity to the brain. In agreement with this, a very recent study, using intranasally administered radioiodinated leptin, clearly supported circumvention of the blood-brain barrier and direct transport of leptin from nose to brain (23).

Interestingly, in mice treated with control strain LL, body weight gain tended to be lower than in PBS-treated mice, although this effect generally did not reach statistical significance, except for a few time-points. Since appetite and food preference are known to depend tightly on olfactory stimuli (2, 41, 55), the presence of LL in the intranasal cavity may somehow affect animal feeding behavior. However, food intake was not significantly decreased in LL-treated mice compared to the intake of PBS controls (Fig. 5B). On the other hand, the presence of a foreign microorganism in the intranasal cavity may induce local production of inflammatory cytokines that could impact on body weight gain through catabolic mechanisms, by directly acting on the central nervous system (48, 54, 58). Further work will be needed to firmly establish whether LL alone has any effect on the regulation of body weight and to elucidate the potential mechanisms involved.

In summary, we have designed an *L. lactis* strain for highly efficient secretion of biologically active leptin, which can be directly recovered from the culture medium. Biological activity of the leptin produced by *L. lactis* was also demonstrated in vivo in mice. Indeed, we showed that the intranasal administration of leptin-secreting lactococci can regulate immune responses in mice. Finally, we demonstrated, for the first time, the potential of *L. lactis* to produce peptides with therapeutic interest for the treatment of obesity.

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