Leptin and adiponectin supplementation modifies mesenteric lymph node lymphocytes composition and functionality in suckling rats

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Abstract

At birth, when immune responses are insufficient, there begins the development of the defence capability against pathogens. Leptin and adiponectin, adipokines which are present in breast milk, have been shown to play a role in the regulation of immune responses. We report here, for the first time, the influence of *in vivo* adipokines supplementation on the intestinal immune system in early life. Suckling Wistar rats were daily supplemented with leptin (0.7 μg/kg/day, n = 36) or adiponectin (35 μg/kg/day, n = 36) during the suckling period. The lymphocyte composition, proliferation and cytokine secretion from mesenteric lymph node lymphocytes (on days 14 and 21), as well as intestinal IgA and IgM concentration (day 21), were evaluated. At day 14, leptin supplementation significantly increased the TCRαβ+ cell proportion in mesenteric lymph nodes, in particular due to an increase in the TCRαβ+ CD8+ cell population. Moreover, the leptin or adiponectin supplementation promoted the early development CD8+ cells, with adiponectin being the only adipokine capable of enhancing the lymphoproliferative ability at the end of the suckling period. Although leptin decreased intestinal IgA concentration, it had a trophic effect on the intestine in early life. Both adipokines supplementation modulated the cytokine profile during (day 14) and at the end (day 21) of the suckling period. These results suggest that leptin and adiponectin during suckling play a role in the development of mucosal immunity in early life.

1. Introduction

During human foetal development, the immune system is adapted to the intrauterine environment[1]. The transition from practically sterile environment in the uterus to the outside world is particularly critical because the neonate is suddenly newly exposed to a large number of microorganisms and food antigens. Therefore, the development of the human immune system undergoes the most radical and rapid changes. However, during the first months of life this system is still immature and, in consequence, there are high morbidity and mortality rates due to infectious diseases[2].

In the human and rat newborn, the immature immune system has significant quantitative and qualitative deficiencies, especially concerning acquired immunity. The underdeveloped acquired immunity is reflected by the existence of low immunoglobulin (Ig) levels, and the small number of naive T lymphocytes and antigen-presenting cells (APC) in the newborn of both species[3,4]. For this reason the survival of the human and rat neonate relies on the innate immune system[5,6]. However, this still has some deficiencies, such as low phagocytic activity, defects in neutrophil and monocyte numbers and function, reduced cytokine secretion and lower natural killer (NK) cytotoxic capacity compared to those present in adults[7]. On the other hand, physical barriers, such as the intestinal wall, are crucial for the human and rat neonate to avoid infections and control exposure to oral antigens, which in turn is a challenge for the development and maturation of the gut-associated lymphoid tissue (GALT)[4,8]. GALT, formed by the inductive sites (Peyer's patches, isolated lymph nodes, and mesenteric lymph nodes (MLN)) and the effector sites (intraepithelial and lamina propria lymphocytes), is not mature at birth in both species and develops in the postnatal period[9–11].

In humans, to compensate the immunological immaturity in intrauterine and early life, the mother provides important factors for protecting the newborn. Firstly, transferring antibodies through the placenta and, later, through the breast milk[12]. Human milk contains a wide variety of bioactive factors and hormones[13] including adipokines, such as leptin and adiponectin. The concentration of these adipokines in breast milk change throughout the lactation[14,15]. Leptin and adiponectin are involved in the control of endocrine and metabolic systems and they seem to play a role in the regulation of the immune system, in both the innate and adaptive immune responses[16,17]. In particular, leptin has been described as a pro-inflammatory cytokine that stimulates proliferation and NK cell and T lymphocyte responses, and the switch to a pro-inflammatory Th1

immunity, thus promoting the secretion of cytokines such as interferon (IFN)-γ, tumour necrosis factor (TNF)-α, interleukin (IL)-6 and IL-1[17]. However, the anti- or pro-inflammatory properties of adiponectin are still a matter of discussion[18]. It has been described that adiponectin has an anti-inflammatory function because of its capacity to suppress the synthesis of TNF-α and IFN-γ and to induce the production of several anti-inflammatory cytokines[19]. In contrast, other studies show an inflammatory response under certain circumstances, by activating NK cells and inducing inflammatory cytokines such as IL-1 and IL-6[20,21].

To date, there is little data available about the dietary effects of leptin and adiponectin on the GALT, and none about the effect of either adipokines in this compartment in early life, when the development of the immune system is taking place. We hypothesized that these breast milk adipokines, with immunomodulatory actions described in adults, may play also role in the neonatal immune system, and in particular in the maturation of the GALT. Therefore, the aim of the present study was to evaluate the impact of leptin or adiponectin supplementation on some aspects of the intestinal immune system development in neonatal rats. Specifically, we focused on the MLN lymphocytes, both in terms of their composition and functionality. Thus, immune responses of pups fed with leptin or adiponectin at the middle and at the end of the suckling period were established.

2. Materials & Methods

2.1. Animals

Sixteen pregnant Wistar rats (G15) were obtained from Janvier Labs (Le Genest Saint Isle, France) and individually housed in cages under controlled conditions of temperature and humidity in a 12 h light–12 h dark cycle in the Faculty of Pharmacy and Food Science animal's facilities. Rats were fed with commercial diet corresponding to the American Institute of Nutrition 93M formulation and water *ad libitum*.

The studies were performed in accordance with the criteria outlined by the Guide for the Care and Use of Laboratory Animals. Experimental procedures were reviewed and approved by the Ethical Committee for Animal Experimentation of the University of Barcelona (CEEA/UB ref. 220/15).

2.2. Experimental design and dietary supplementation

Pregnant rats were allowed to deliver naturally. The day after birth was registered as day 1 of life. Litters were unified to 9 pups per lactating dam, with free access to the nipples and rat diet. Handling was performed in the same time range to avoid the influence of biological rhythms.

According to the oral supplementation given, suckling rats were distributed into four groups (n = 36/group): reference, leptin, adiponectin and whey protein concentrate (WPC). Each group was composed of four litters of 9 pups each. Leptin group were administered with 0.7 μg/kg/day of leptin (i.e. 17.6, 26.6 and 43.8 ng/rat at days 10, 14 and 21, respectively) (PeproTech®, Rocky Hill, NJ, USA) in water, based on a reported study[22]. Due to the lack of similar studies, the adiponectin dose was calculated taking into account the concentration ratio of adiponectin vs. leptin in human milk, which was about 50 times higher[23,24]. Therefore, the adiponectin group was supplemented with a solution of 35 μg/kg/day of adiponectin (PeproTech®) in water (i.e. 0.8, 1.2 and 2.1 μg/rat at days 10, 14 and 21, respectively). In parallel, another group of animals was administered with a bovine WPC, containing high concentration of bioactive factors. This WPC was similar to that used in previous studies and it did show immunomodulatory effects on neonatal rats[25,26]. Thus, this intervention was used as a possible positive control of modulation by breast milk bioactive factors. Animals from the WPC group were supplemented with Lacprodan® MFGM-10 (Arla Foods Ingredients Group, Diby, Denmark) in a previously described physiological dose for suckling rats receiving infant formula[26] (0.3 g/kg/day) (i.e. 6.9, 10.8 and 17.9 mg/rat at days 10, 14 and 21, respectively). The reference group was administered with the same volume of vehicle (water) as the supplemented groups (10 mL/kg/day). To allow gastric emptying, litters were separated from their dam half an hour before oral supplementation. Meanwhile animals were weighed. Pups received the supplements daily by oral gavage throughout the suckling period (from day 1 to 21) using low-capacity syringes (Hamilton Bonaduz, Bonaduz, Switzerland) adapted to oral 25- or 23-gauge gavage tubes (ASICO, Westmont, IL, USA), as previously described[25].

Body weight was recorded throughout the study, and on the last day body length (nose-anus) was measured. The body weight gain percentage at a specific day was calculated by subtracting the weight at the beginning of the study (day 1) from the weight at the subsequent days, divided by starting weight and multiplied by 100. These measures enabled the determination of the body mass index (BMI), calculated as body

143 weight/length^2 (g/cm^2) and the Lee index, calculated as $\sqrt[3]{}$ weight/length x 1000
144 ($\sqrt[3]{}$g/cm).

145 *2.3. Sample collection and processing*

146 At days 10, 14 and 21, animals were intramuscular anaesthetized with ketamine
147 (90 mg/kg) (Merial Laboratories S.A., Barcelona, Spain) and xylazine (10 mg/kg) (Bayer
148 A.G., Leverkusen, Germany), exsanguinated, and MLN and small intestine (SI) were
149 collected. The duodenum was removed and the distal 1/3 portion of the remaining
150 intestine was used to obtain the gut wash. For this, it was opened lengthwise, cut into 5
151 mm pieces, weighed, and incubated with 2 mL of phosphate buffer solution (PBS pH 7.2;
152 154 mM sodium chloride (NaCl), 3.99 mM sodium dihydrogen phosphate monohydrate
153 ($NaH_2PO_4 \cdot H_2O$), 16 mM disodium hydrogen phosphate dehydrate ($Na_2HPO_4 \cdot 2H_2O$)) for
154 15 min in a shaker (55 u/min) at 37 °C. After centrifugation (535 *g*, 5 min, 4° C),
155 supernatants were stored at -20 °C until Ig quantification.

156 *2.4. Immunoglobulin quantification*

157 At the end of the suckling period, intestinal IgA and IgM concentrations were quantified
158 in gut wash using a rat IgA or IgM enzyme-linked immunosorbent assay (ELISA)
159 quantification set (Bethyl Laboratories, Montgomery, TX, USA), as performed in
160 previous studies[27]. Data are expressed as µg of IgA or IgM per g of intestinal tissue used
161 for the gut wash.

162 *2.5. Lymphocyte isolation from mesenteric lymph nodes*

163 MLN cell suspensions were obtained by passing the tissue through a sterile 40 µm mesh
164 cell strainer (Thermo Fisher Scientific, Barcelona, Spain). The cell suspension was
165 centrifuged (538 *g,* 10 min, 4 °C) and resuspended in Roswell Park Memorial Institute
166 (RPMI) 1640 medium (Sigma-Aldrich, Madrid, Spain) enriched with 10% foetal bovine
167 serum (FBS; Sigma-Aldrich), 100 IU/mL streptomycin-penicillin (Sigma-Aldrich),
168 2 mM L-glutamine (Sigma-Aldrich) and 0.05 mM 2-β-mercaptoethanol (Merck
169 Millipore, Darmstadt, Germany). Cell counting and viability were assessed by
170 Countess™ Automated Cell Counter (Invitrogen™, Thermo Fisher Scientific).
171 Lymphocytes were immediately used to analyse their phenotype and their ability to
172 proliferate and secrete cytokines after mitogen stimulation.

173 *2.6. Lymphocyte immunofluorescence staining and flow cytometry analysis*

Lymphocytes (2–5 x 10^5) from MLN were stained with anti-rat monoclonal antibodies (mAb) conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridininchlorophylla protein (PercP), allophycocyanin (APC) or APC-cyanine (Cy)7, as in previous studies[28]. In this case, the mAb used were anti-CD4, anti-CD8α, anti-CD8β, anti-TCRαβ, anti-TCRγδ, anti-NKR-P1A, anti-CD25, anti-CD45RA (BD Biosciences, San Diego, USA), anti-CD62L and anti-CD103 (Biolegend, San Diego, CA, USA), anti-TLR-4 (Novus Biologicals, Littlon, CO, USA), anti-Foxp3 (eBioscience, Frankfurt, Germany). The cells were incubated with a mixture of optimal concentrations of mAb in PBS containing 2% FBS and 0.1% sodium azide (Merck Millipore), for 20 min at 4 °C in darkness. A negative control staining using an isotype-matched mAb was included in each cell sample. For intracellular staining, cells previously labelled with anti-CD4-PE and anti-CD25-FITC mAb were treated with Foxp3 fixation/permeabilization kit (eBioscience). Then, intracellular staining with anti-Foxp3-APC mAb was carried out under the same conditions as extracellular staining. All stained cells were fixed with 0.5% p-formaldehyde and stored at 4 °C in darkness until analysis by flow cytometry. Analyses were performed using a Gallios™ flow cytometer (Beckman Coulter Inc., Miami, FL, USA) in the Scientific and Technological Centres of the University of Barcelona (CCiT-UB). All samples were assessed by FlowJo version 10 software (TreeStar, Inc., Ashland, OR, USA). Results are expressed as percentages of positive cells in the lymphocyte population selected according to their forward-scatter characteristics (FSC) and side-scatter characteristics (SSC) or in a particular selected population.

2.7. Lymphocyte proliferative response

T lymphocyte~~s~~ activation was carried out in 96-well plates (TPP, Trasadingen, Switzerland) previously coated with anti-CD3/anti-CD28 mAb (10 µg/mL and 20 µg/mL, respectively, BD Biosciences). MLN lymphocytes (10^5/200 µL) from 14-day- and 21-day-old animals were incubated in quadruplicate with or without stimulus for 48 h. The proliferation was quantified by means of the BrdU Cell Proliferation Assay Kit (Merck Millipore) following the manufacturer's instructions. This assay is based on the measurement of 5-bromo-2'-deoxyuridine (BrdU) incorporated into proliferating cells during DNA synthesis. After stopping the enzymatic reaction, the absorbance (Ab) was measured at 450 nm on a microplate photometer (Labsystems Multiskan MS).

The proliferation rate (%) is expressed, as follows, considering the reference group as 100%:

Proliferation rate (%) = (A/B) x 100, where,

$A = [(Ab_{\text{stimulated cells}} - Ab_{\text{non-stimulated cells}}) / Ab_{\text{non-stimulated cells}}]_{\text{supplemented group}}$

$B = [(Ab_{\text{stimulated cells}} - Ab_{\text{non-stimulated cells}}) / Ab_{\text{non-stimulated cells}}]_{\text{reference group}}$

2.8. Quantification of cytokine production from MLN lymphocytes

Supernatants collected after the stimulation process described above were used to quantify cytokine production. Concentrations of IL-1α, IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-13, IL-17A, IFN-γ and TNF-α were quantified using ProcartaPlex® Multiplex Immunoassay (eBioscience). In brief, specific magnetic capture beads coded with distinct colours were bound to the analyte of interest. Then it was possible to reveal the specific concentration through different detection antibodies conjugated to PE by the Luminex MAGPIX analyser (Luminex®, Austin, TX, USA) at the CCiT-UB.

The lower limits of detection were: 13 pg/mL for IL-1α; 2.10 pg/mL for IL-2; 0.85 pg/mL for IL-4; 1.65 pg/mL for IL-5; 14 pg/mL for IL-10; 4.93 pg/mL for IL-12p70; 3.17 pg/mL for IL-13; 2.61 pg/mL for IL-17A; 4.35 pg/mL for IFN-γ; and 3.08 pg/mL for TNF-α.

2.9. Statistical analysis

Analysis of the data was carried out by the software package IBM Statistical Package for the Social Sciences (SPSS, version 22.0, Chicago, IL, USA). To assess the homogeneity of variance and the distribution of the results, Levene's and Shapiro–Wilk tests were performed, respectively. Repeated measures analysis of variance using the Bonferroni correction was used to determine the time-related differences in the body weight in each supplementation. For other variables, when there was a normal distribution and equality of variance, conventional one-way ANOVA test followed by the post hoc Bonferroni was performed. Non-parametric Kruskal–Wallis test followed by the post hoc Mann–Whitney U test were used in order to assess significance. Significant differences were established at $P<0.05$.

3. Results

3.1. Growth and morphometry

Rats' body weight gain, calculated by monitoring from the day after birth and throughout the suckling period, showed no differences among groups (Suppl. Fig. 1). Morphometric variables such as BMI and Lee index were also evaluated during suckling (Table 1). The

age-increasing BMI pattern was not modified by any of the supplementations. However, while the Lee index was not affected by the diets in any of the groups at 10 and 14 days, a decrease was seen at day 21 ($P<0.01$) in all groups (Table 1).

Although relative weight of the small intestine, expressed as percentage with respect body weight, increased mainly the last week of the suckling period (Table 1), the relative length decreased all along this period. Moreover, leptin displayed an intestinal growth-enhancing effect at day 21 ($P<0.05$ vs. reference group).

3.2. Intestinal secretory IgA and IgM concentration

Intestinal secretory IgA and IgM production was established on day 21 (Fig. 1). In the reference group, secretory IgA concentration was five times higher than that of IgM (Fig. 1). Leptin dietary supplementation significantly lowered the secretory IgA content in the intestinal compartment ($P<0.01$ vs. reference group) (Fig. 1). With regard to IgM, none of the three experimental supplementations modified the levels of this Ig (Fig. 1).

3.3. Lymphocyte composition of mesenteric lymph nodes

A mean count of $2.0 \times 10^6 \pm 2.0 \times 10^5$ and $1.3 \times 10^7 \pm 7.6 \times 10^5$ of MLN cells were established during (day 14) and at the end (day 21) of the suckling period, respectively, from all groups, without differences among them. Their main lymphocyte subsets proportions are shown in (Table 2). Leptin and WPC supplementations significantly increased the T cell proportion compared with the reference group at day 14 ($P<0.01$ and $P<0.05$, respectively). These increases were due to a higher proportion of TCRαβ+ cells ($P<0.01$ and $P<0.05$, respectively), and in particular to an increase in the TCRαβ+ CD8+ cell population ($P<0.05$) (Table 2).

Although no significant effects were observed in TCRγδ+ cell proportions as a result of adipokine supplementation, an age-increasing proportion was found in the reference group that was particularly due to the increase in the TCRγδ+ CD8+ cell proportion ($P<0.01$). This was not the case for the rest of the groups in which the values observed at day 14 were already as high as those found on day 21 (Table 2). With regard to NK subset, a relative age-associated decrease was detected between 14- and 21-day-old rats due to a normal immune development in all groups. This decrease is also found in the proportion of NK CD8+ cells in MLN from leptin animals between day 14 and day 21, but not in the others. NK cell percentages at day 14 or at day 21 were not influenced by dietary

supplementation. On the other hand, the leptin group showed a decrease from day 14 to day 21 in NKT cell proportion in parallel to a decrease in NKT cells expressing CD8 coreceptor (Table 2).

In reference animals the whole proportion of CD8+ cells in MLN increased from day 14 to day 21 of age ($p<0.05$) (Fig. 2a). In contrast, both adipokines induced a higher proportion of CD8+ cells on day 14 ($p<0.05$ vs. reference) and on that day, they were able to promote the achievement of a percentage of CD8+ cells that was typical from day 21 (Fig. 2a). When looking at both forms of CD8 coreceptor (CD8αα and CD8αβ), a decrease in the CD8αα+/CD8αβ+ ratio in the reference and leptin groups was observed in comparison to their respective group at day 14 ($P<0.01$) (Fig. 2b).

The three dietary interventions did not modify the proportion of the other main subpopulations in the MLN compartment, such as Th, Treg, B and CD8+CD4+ cells, either at day 14 or day 21 of life (Table 2). The activation marker CD25 was also studied in the main subsets and in all cases its percentage was about 4%, without differences among groups or ages studied.

For the study of the intestinal homing, the expression of adhesion molecules CD62L selectin and αE integrin (CD103) was quantified. CD62L selectin was highly expressed in MLN cells at day 21, whereas αE integrin was poorly expressed (Suppl. Fig. 2). No significant differences were detected in the CD62L/αE integrin expression pattern due to the supplements in any studied group within each studied day (Suppl. Fig. 2). However, αE- CD62L+ cell percentages at day 21 from the reference, leptin and WPC groups (61.5 ± 1.7; 60.9 ± 1.6; 58.9 ± 1.3, respectively) were higher than their respective value at day 14 (52.9 ± 1.7; 54.5 ± 2.6; 50.9 ± 1.2, respectively) ($P<0.05$). In contrast, the adiponectin group showed no differences at day 14 and 21.

In addition, the expression of CD62L selectin/αE integrin molecules on T and B cells was also studied (Suppl. Table 1). No significant differences were detected between groups in Th (CD4+CD8-), Tc (CD8+CD4-), DP (CD4+CD8+) and B (CD45RA+) lymphocytes on the same day of analysis, with the exception of the changes found in the CD4+CD8+ subset at day 21, where leptin and WPC supplementation decreased the αE+CD62L+ percentage ($p<0.05$ vs. reference). Moreover, some statistical differences between day 14 and day 21 were found in Th and B subsets within their respective group. In this regard, the low αE-CD62L+ cell percentage in the adiponectin group found on day 21 (Suppl.

Fig. 2) could be explained by the relative increase in the percentage of CD4+ αE+CD62L- and CD4+ αE+CD62L+ (Suppl. Table 1). reference, adiponectin and WPC groups, but not the leptin group, showed some age-developmental CD62L/αE integrin changes in the B cell subset. In Tc cells no differences were found due to age or supplementations (Suppl. Table 1).

3.4. Mesenteric lymph node lymphocyte proliferation

To determine the functional capacity of MLN lymphocytes, we studied their lymphoproliferative response on days 14 and 21. At day 14, proliferative capacity was not modified by any supplementation (Fig. 3). At the end of the suckling period, adiponectin and WPC supplementation showed higher lymphoproliferative capacity than the reference group ($P<0.05$), whereas it was not modified by the leptin supplementation (Fig. 3).

3.5. Cytokine production by mesenteric lymph node lymphocytes

The cytokine pattern secreted by MLN cells differed between cells obtained at day 14 and 21 of age (Table 3). Cell culture supernatant from the reference group on day 21 showed lower IL-1α, IL-10, IL-13 and higher TNF-α concentrations than those quantified on day 14, while IL-10/TNF-α was particularly low on day 21 due to the decrease in IL-10.

All three supplementations showed the same profile as that observed in the reference group for IL-1, IL-10 and IL-10/TNF-α. Leptin and adiponectin supplementation were able to lower the levels of IL-13 from day 21 to those observed at day 14. Moreover, apart from the reference group, only adiponectin was able to lower TNF-α to the values present at day 14. In addition, IL-4 and IL-5 concentrations at day 21 were lower than those at day 14 in the leptin and adiponectin groups, an effect not observed in the reference group.

At day 14, the leptin supplementation group showed higher IL-4 and IL-5 release, being both Th2-related cytokines, than the reference group ($P<0.05$) (Table 3). In contrast, lymphocytes from the adiponectin group produced significantly higher levels of IL-5, IL-13 and TNF-α at day 14 than the reference group at the same day ($P<0.01$) (Table 3).

At day 21, the leptin and adiponectin groups showed a notable decrease in IL-2 secretion in comparison to the reference group ($P<0.01$). The IL-12p70 secretion was also diminished by the three supplementations ($P<0.05$ in leptin group and $P<0.01$ in adiponectin and WPC vs. reference group) (Table 3). In addition, IL-13 concentration

331 from animals supplemented with adiponectin was lower than that produced by the
332 reference group (P<0.01).

333 Overall, the Th1/Th2 cytokine (INF-γ/IL-4) ratio was not influenced by age in the
334 reference , adiponectin or WPC groups, and an increase in that ratio at day 21 can only
335 be observed in the leptin group when comparing day 14 and day 21 (P<0.05) (Table 3).

336 **4. Discussion**

337 The neonatal benefits of the presence of leptin and adiponectin in breast milk are many;
338 leptin have an important role in regulation of appetite, growth and weight and adiponectin
339 was also associated inversely with obesity[15,29]. Moreover, both adipokines were
340 described to have immunomodulatory actions[18,30,31]. However, at present, there are no
341 studies on the influence of leptin and adiponectin on the immune system in early life,
342 particularly on the immature GALT.

343 The results of this study show that the adipokines supplementation did not modify the
344 body weight gain pattern, the body mass index or the Lee index, suggesting that the
345 administration of leptin and adiponectin did not modify the body mass of the animals.
346 These results are in accordance with other studies performed with rats that were orally
347 administered with leptin during the suckling period[22,32], and also in old male mice
348 receiving subcutaneous doses of leptin[33]. Taking into account the role of leptin in satiety
349 and suppression of appetite described in adults[34,35], a weight loss would have been
350 expected. However, studies in rats and mice suggest that leptin may have a different role
351 in neonates because the exogenous administration of leptin to newborn rats modifies the
352 expression of neuropeptides, known to affect appetite in adults, without altering appetite
353 in neonatal rats[35]. Furthermore, weight loss has been described as a potent inducer of
354 adiponectin synthesis[36]; however, there is almost no information regarding whether
355 adiponectin affects, directly or indirectly, body weight in adults, and none at all regarding
356 neonatal life.

357 When the adipokines are administered, they travel through the gastrointestinal system and
358 pass through the epithelial barrier into the blood[29]. A limitation of this study is that the
359 biodisponibility of leptin and adiponectin has not been measured in the suckling rats along
360 the intervention. In addition, although recent studies show that different multimers of
361 adiponectin can have different biological actions [38,39], we supplemented the rats with the

362 monomeric form, whereas the most abundant in human milk is the high molecular weight
363 (HMW) multimeric form [15,40].

364 Even though, as they reached the intestine there must be a direct interaction between these
365 compounds and the intestinal epithelial cells as well as with the immune cells scattered
366 throughout the intestine (effector sites) or grouped in aggregates (induction sites) of the
367 GALT. For this reason, considered it would be interesting to determine the effect of
368 adipokines on morphometric changes of the small intestine. Supplementation with leptin,
369 but not with adiponectin, for the three weeks of suckling was able to increase the relative
370 small intestine weight, therefore suggesting a certain local trophic effect of leptin on the
371 intestine. However, when we considered the intestinal production of secretory IgA and
372 IgM at the end of the suckling period, the levels were very low, far away from those
373 described in six-week-old Wistar rats[41], and in agreement with studies showing the
374 immaturity of B cells in the neonate[42]. The supplementation with leptin, but not
375 adiponectin, caused a reduction in the secretion of intestinal IgA without affecting IgM
376 levels. These antibodies are produced by plasma cells stimulated under Th2 conditions[43].
377 Some studies suggest the ability of leptin to stimulate Th1 responses and inhibit those of
378 Th2[30,44]. Thus, the reduction of intestinal secretory IgA associated with leptin
379 supplementation could be attributed to this inhibitory effect. This effect was not observed
380 in the case of adiponectin.

381 During suckling, rats undergo phenotypical changes in intestinal lymphoid tissues that
382 are a reflection of the immune system's maturation in this period[4]. Therefore, the study
383 of MLN lymphocyte composition and functionality in neonates is a useful strategy for
384 assessing the modulatory ability of leptin and adiponectin on the immune system. In this
385 study, none of the adipokines affected B, NK or NKT populations in MLN lymphocytes.
386 However, leptin significantly increased the proportion of TCRαβ+CD8+ lymphocytes in
387 a similar way to the WPC, suggesting an early maturation of this subset. The increase in
388 the TCRαβ+CD8+ cell proportion caused an increase in the overall T cell percentage.
389 These results are in concordance with a study carried out by Oral *et al*. in human patients,
390 in which an increase in the percentage of blood T cells and CD8+ subsets after leptin
391 intake was found, without changing NK cell percentage[45]. Moreover, another study in
392 mice splenocytes and thymocytes also described an increase in CD8+ population after
393 leptin administration[43]. This increase in T lymphocytes induced by leptin could be
394 explained by its suggested anti-apoptotic effect[47]. Leptin promotes T cell survival by

modulating the expression of anti-apoptotic proteins, such as Bcl-xL in stress-induced apoptosis[48]. The reason why this mechanism could be particularly affecting just the CD8+ subsets is yet to be established. On the other hand, little is known about the effect of adiponectin on lymphocyte subpopulations. We found here that adiponectin supplementation was also able to increase CD8+ cell percentage in MLN. This immune development-promoting effect found here is not in line with the negative regulation effect shown by Wilk *et al*.[49]. In particular, they investigated the *in vitro* and *in vivo* effects of adiponectin in antigen-specific T-cell responses and evidenced an increase in CD137+CD8+ and CD137+CD4+ proportions in adiponectin knockout mice. CD137 is a costimulatory molecule expressed by activated T cells that enhances T cell proliferation, IL-2 secretion, survival and cytolytic activity[50].

The proportion of cells bearing the adhesion molecules αE integrin and CD62L selectin related to the intestinal homing of lymphocytes has been also analysed. The surface αE integrin was practically non-existent in MLN lymphocytes, and the relative numbers of CD62L+ cells were high and age-related, being higher at the end of lactation. The presence of these molecules, involved in lymphocyte rolling and recruitment to peripheral lymphoid tissues, in early life rat MLN is similar to that in previous studies[28] and also in older animals[51]. The supplementation with adipokine did not exert profound changes in the expression of these homing molecules with the exception of an decrease in the αE+CD62L+CD4+CD8+ percentage in animals supplemented with leptin, the significance of which remains to be explored.

Regarding the MLN cell functionality, although not in this particular tissue, there are previous studies attributing to leptin the capacity to induce lymphoproliferation *in vitro* and *in vivo*[17,51–55]. In contrast, with respect to the reference group we found no differences in lymphocytes from animals given leptin. Those animals supplemented with adiponectin had a higher proliferation rate than the reference group and similar values to those animals supplemented with WPC. Thus, it seems that adiponectin is able to accelerate the acquisition of this immune cell capacity in neonatal life. Conversely, Wilk *et al*. studied the effect of adiponectin on proliferation and apoptosis of antigen-specific T cells *in vitro* and showed an apoptosis-promoting effect on CD137+ subsets, which was not observed in CD137- subsets[49].

The release of cytokines after *in vitro* stimulation of lymphocytes from leptin- or adiponectin-supplemented animals was also studied. Reported studies with leptin show

its ability to stimulate the production of Th1-related cytokines such as IL-1, IL-6, IL-12 and TNF[30]. Nevertheless, we found an increase in IL-4 and IL-5, Th2-type cytokines, at day 14 and a decrease of IL-2 and IL-12p70 levels at day 21 due to leptin supplementation. These results agree with an *in vitro* study showing that low concentrations of leptin increase IL-4 production[56]. Overall, focusing on the Th1/Th2 cytokine ratio (calculated as IFN-γ/IL-4 ratio), leptin increased the age-related ratio (day 21 vs. day 14) thus reinforcing its suggested Th1-promoting and Th2-inhibiting role. In addition, adiponectin increased the release of IL-5, IL-13 and TNF-α at day 14 and decreased the secretion of IL-2, IL-12p70 and IL-13 at day 21. In consequence, it seems that adiponectin does not have a clear Th1/Th2-promoting effect. However, it has been described that adiponectin reduced the expression of the TNF-α in arterial and adipose tissues[57,58]. Furthermore, in human monocyte-derived macrophages, adiponectin inhibits the expression of IL-6 as well as TNF-α but increases the levels of IL-10[59]. Overall, further studies should be carried out to fully elucidate the contribution of these adipokines to the cytokine secretion in this period of life.

In summary, the results obtained here suggest that dietary supplementation with leptin and adiponectin during the suckling period is able to promote the maturation of the intestinal immune system. A daily supplementation with leptin induces a rise in the intestinal weight and in the proportion of T cells in MLN, suggesting an intestinal trophic and immunomodulatory effect. Moreover, adiponectin is also able to increase the CD8+ percentage and to promote a proliferative response in MLN cells. Both leptin and adiponectin modulate the production of Th1 and Th2 cytokines. This demonstrates the capacity of these adipokines to promote the development and maturation of cells involved in adaptive immune response, which is clearly immature at this stage of life.

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FIGURE LEGENDS

Figure 1. Supplementation effect on secretory IgA and IgM content in the intestinal compartment (gut wash) at the end of the suckling period (day 21) from the four 4 groups: reference, leptin, adiponectin and whey protein concentrate (WPC). Results are expressed as mean ± S.E.M. (n = 9–12 pups per group analyzed in duplicates). Statistical differences: * P<0.05 vs. reference group. Ψ P<0.05 vs. adiponectin group (Mann-Whitney U).

Figure 2. Percentage of CD8+ cell subset (a) and CD8αα/CD8αβ ratio (b) in mesenteric lymph node lymphocytes during (day 14) and at the end (day 21) of the suckling period from the 4 four groups: reference, leptin, adiponectin and whey protein concentrate (WPC). Results are expressed as mean ± S.E.M. (n = 9–12 pups per group analysed in uniplicates). Statistical differences: * P<0.05 vs. reference group. ‡ P<0.05 vs. same group at day 14 (ANOVA).

Figure 3. Supplementation effect on proliferation rate at day 14 and at day 21 in mitogen-stimulated MLN lymphocytes from the four 4 groups: reference, leptin, adiponectin, and whey protein concentrate group (WPC). Results are expressed as mean ± S.E.M. (n = 3–9 pups per group analysed in quadruplicates). Statistical differences: * P<0.05 vs. reference group. § P<0.05 vs. leptin group (Mann-Whitney U).

Table 1. Body mass index (BMI), Lee index, relative small intestinal (SI) weight and relative SI length in the four groups over the study (day 10, 14 and 21). The BMI was calculated as body weight/length^2 (g/cm^2) and the Lee index, calculated as $\sqrt[3]{}$ weight/length x 1000 ($\sqrt[3]{}$g/cm). Relative SI weight and relative SI length was calculated as weight/length of the intestine divided by the body weight x 100.

		BMI (g/cm^2)	**Lee Index ($\sqrt[3]{}$g/cm)*1000)**	**Relative SI weight (%)**	Relative SI length (%)
Day 10	**Reference**	0.3 ± 0.0	337.4 ± 4.2	3.3 ± 0.1	158.1 ± 5.9
	Leptin	0.3 ± 0.0	335.9± 3.6	3.2 ± 0.0	164.8 ± 8.4
	Adiponectin	0.3 ± 0.0	341.9 ± 3.2	3.0 ± 0.0	162.1 ± 5.4
	WPC	0.3 ± 0.0	337.2 ± 2.9	3.2 ± 0.1	171.0 ± 7.7
Day 14	**Reference**	0.4 ± 0.0 †	335.5 ± 2.6	3.3 ± 0.1	116.4 ± 2.4 †
	Leptin	0.4 ± 0.0 †	335.5 ± 3.1	3.4 ± 0.0 †	119.1 ± 7.6 †
	Adiponectin	0.4 ± 0.0 †	3354.0 ± 2.2	3.3 ± 0.0 †	122.3 ± 4.1 †
	WPC	0.4 ± 0.0 †	336.0 ± 3.6	3.4 ± 0.1	121.8 ± 5.3 †
Day 21	**Reference**	0.4 ± 0.0 †	314.5 ± 3.3 †‡	4.4 ± 0.1 †‡	86.9 ± 2.3 †‡
	Leptin	0.4 ± 0.0 †	316.3 ± 5.7 †‡	5.3 ± 0.1 *†‡	94.4 ± 4.0 †‡
	Adiponectin	0.4 ± 0.0 †	314.1 ± 4.4 †‡	4.6 ± 0.3 †‡§	92.8 ± 3.8 †‡
	WPC	0.4 ± 0.0 †	314.8 ± 6.2 †‡	4.4 ± 0.1 †‡§	96.6 ± 3.0 †‡

WPC, whey protein concentrate. Data are expressed as mean ± S.E.M. (n = 7-15). Statistical differences: * P<0.05 vs. reference group. † P<0.05 vs. same group at day 10. ‡ P<0.05 vs. same group at day 14. § P<0.05 vs. leptin group (ANOVA).

637 **Table 2.** Main lymphocyte subsets in mesenteric lymph nodes in the four groups during (day 14)
638 and at the end (day 21) of the suckling period.

	Day 14			
	Reference	**Leptin**	**Adiponectin**	**WPC**
T cells (%)	67.6 ± 2.0	76.4 ± 1.9 *	74.3 ± 2.2	75.6 ± 1.9 *
TCRαβ$^+$ (%)	65.4 ± 2.0	74.0 ± 2.0 *	71.6 ± 2.2	73.1 ± 1.8 *
TCRαβ$^+$ CD8$^+$ (%)	18.4 ± 0.8	21.9 ± 1.3 *	20.1 ± 0.9	21.0 ± 0.7 *
TCRγδ$^+$ (%)	2.2 ± 0.1	2.4 ± 0.1	2.7 ± 0.2	2.4 ± 0.2
TCRγδ$^+$ CD8$^+$ (%)	1.7 ± 0.1	1.9 ± 0.1	2.0 ± 0.2	1.9 ± 0.1
Th (%) (TCRαβ$^+$CD8$^-$CD4$^+$)	47.0 ± 1.5	51.0 ± 2.1	51.5 ± 2.1	52.1 ± 1.3
T reg (%)	-	-	-	-
NK (%)	1.2 ± 0.1	1.6 ± 0.3	1.2 ± 0.1	1.3 ± 0.1
NK CD8$^+$ (%)	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
NKT (%)	1.6 ± 0.2	2.2 ± 0.3	1.7 ± 0.2	1.8 ± 0.2
NKT CD8$^+$ (%)	1.0 0 ± 0.1	1.4 ± 0.2	1.0 ± 0.1	1.1-± 0.1
B (%)	18.7 ± 1.3	16.9 ± 1.6	16.3 ± 1.4	16.2 ± 1.1
CD8$^+$CD4$^+$ (%)	2.3 ± 0.4	2.8 ± 0.2	2.2 ± 0.1	2.4 ± 0.2
	Day 21			
T cells (%)	70.4 ± 2.1	74.3 ± 2.5	71.8 ± 2.6	71.0 ± 2.3
TCRαβ$^+$ (%)	67.5 ± 2.1	71.9 ± 2.7	69.0 ± 2.8	68.1 ± 2.4
TCRαβ$^+$ CD8$^+$ (%)	19.5 ± 0.7	21.4 ± 1.4	21.2 ± 1.3	21.5 ± 1.2
TCRγδ$^+$ (%)	3.0 ± 0.2 ‡	2.5 ± 0.3	2.8± 0.3	2.8 ± 0.2
TCRγδ$^+$ CD8$^+$ (%)	2.4 ± 0.2 ‡	1.9 ± 0.1	2.1 ± 0.2	2.1 ± 0.2
Th (%)(TCRαβ$^+$CD8$^-$CD4$^+$)	48.0 ± 1.5	50.5 ± 1.6	47.9 ± 1.6	46.7 ± 1.7 ‡
T reg (%)	4.2-± 0.2	4.4 ± 0.2	4.6 ± 0.2	4.1 ± 0.3
NK (%)	0.9 ± 0.1 ‡	0.7 ± 0.1 ‡	0.8 ± 0.1 ‡	0.9 ± 0.1 ‡
NK CD8$^+$ (%)	0.3 ± 0.0	0.2-± 0.0 ‡	0.4 ± 0.1	0.3 ± 0.1
NKT (%)	1.7 ± 0.2	1.3 ± 0.2 ‡	1.8 ± 0.3	1.8 ± 0.2
NKT CD8$^+$ (%)	0.9 ± 0.1	0.7 ± 0.1 ‡	1.1 ± 0.2	1.1 ± 0.1
B (%)	17.3 ± 1.9	13.7 ± 1.6	17.0 ± 1.9	16.5± 1.7
CD8$^+$CD4$^+$ (%)	2.2 ± 0.1	2.4 ± 0.2	2.4 ± 0.1	2.2 ± 0.1

639

640 WPC, whey protein concentrate. Values are expressed as mean ± S.E.M. (n = 8-14 pups per group
641 analyzed in uniplicates). Statistical differences: * P<0.05 vs. reference group. ‡ P<0.05 vs. same
642 group at day 14. (ANOVA).
643

Table 3. Cytokine production from mesenteric lymph node lymphocytes in the four groups over the study (day 14 and 21).

Cytokines (pg/mL)	Day 14			
	Reference	**Leptin**	**Adiponectin**	**WPC**
IL-1α	24.1 ± 8.0	34.5 ± 10.7	50.7 ± 5.4	18.0 ± 5.8
IL-2	321.2 ± 244.9	389.9 ± 163.9	1540.3 ± 732.7	1101.8 ± 931.8
IL-4	108.5 ± 18.3	225.4 ± 40.4 *	156.6 ± 24.2	70.4 ± 5.1 §
IL-5	1.3 ± 0.3	5.1 ± 0.9 *	7.7 ± 2.2 *	1.9 ± 0.6
IL-10	7147.5 ± 1452.3	6951.3 ± 950.8	7916.7 ± 1563.8	5075.1 ± 1493.8
IL-12p70	6.8 ± 1.4	8.6 ± 3.1	12.0 ± 0.9	8.6 ± 3.1
IL-13	30.1 ± 9.5	54.9 ± 17.2	85.8 ± 21.9 *	21.0 ± 10.0
IL-17A	33.1 ± 17.1	30.4 ± 13.4	92.8 ± 42.9	29.5 ± 11.8
INF-γ	14314.0 ± 8273.3	9281.4 ± 1495.3	14181.8 ± 2665.5	6059.8 ± 1516.1
TNF-α	6.1 ± 1.6	6.7 ± 2.9	16.2 ± 2.0 *	6.9 ± 2.7
IL-10/TNF-α	1943.5 ± 710.1	1954.4 ± 1179.0	551.5 ± 173.3	1148.7 ± 546.3
INF-γ /IL-4	113.2 ± 45.9	46.0 ± 14.2	106.9 ± 35.7	83.8 ± 16.0
	Day 21			
IL-1α	6.5 ± 0.0 ‡	8.2 ± 1.3 ‡	9.0 ± 1.3 ‡	7.9 ± 0.7 ‡
IL-2	716.7 ± 210.2	54.6 ± 6.3 *‡	120.4 ± 53.9 *‡	636.7 ± 176.4
IL-4	86.2 ± 9.5	50.9 ± 9.4 ‡	63.1 ± 17.0 ‡	70.4 ± 12.2
IL-5	1.8 ± 0.3	1.4 ± 0.5 ‡	1.3 ± 0.4 ‡	2.3 ± 1.0
IL-10	2745.8 ± 356.8 ‡	3060.2 ± 613.7 ‡	1971.0 ± 475.7 ‡	2084.9 ± 255.2 ‡
IL-12p70	13.8 ± 3.7	4.3 ± 1.2 *	2.5 ± 0.0 *‡	2.5 ± 0.0 *‡
IL-13	14.0 ± 2.3 ‡	7.7 ± 2.5 ‡	6.0 ± 1.4 *‡	8.4 ± 1.4
IL-17A	58.7 ± 11.0	45.8 ± 8.7	37.7 ± 8.7	64.1 ± 10.8
INF-γ	7090.1 ± 581.2	7407.5 ± 853.2	7043.1 ± 744.0 ‡	5424.3 ± 375.5
TNF-α	11.5 ± 1.6 ‡	8.8 ± 0.9	8.6 ± 0.5 ‡	8.9 ± 0.5
IL10/TNF-α	271.7 ± 49.1 ‡	357.0 ± 72.2 ‡	232.6 ± 51.9 ‡	231.9 ± 20.0 ‡
INF-γ /IL-4	106.1 ± 21.1	167.1 ± 34.8 ‡	195.2 ± 77.9	108.6 ± 29.0

WPC, whey protein concentrate. Values are expressed as mean ± S.E.M. (n = 3-12 pups per group analyzed in duplicates). Statistical differences: * P<0.05 vs. reference group. ‡ P<0.05 vs. same group at day 14. § P<0.05 vs. leptin group (ANOVA).

Fig. 1

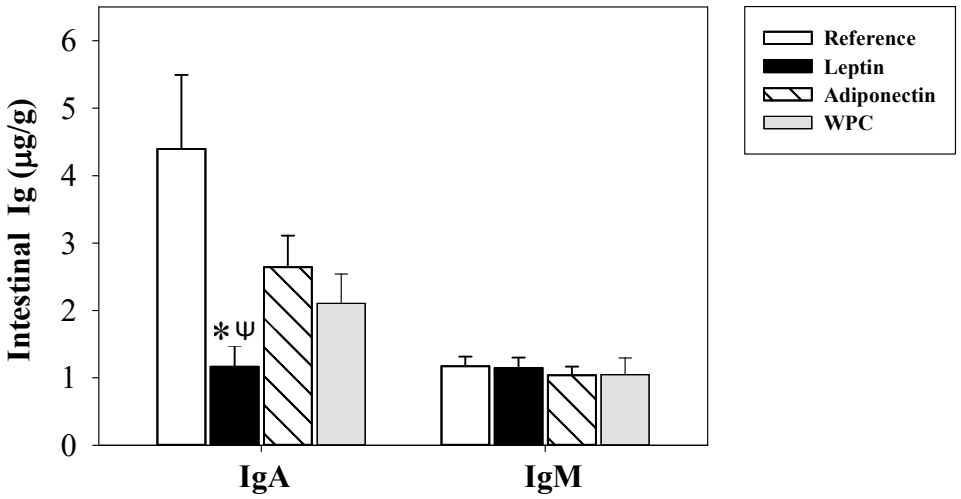
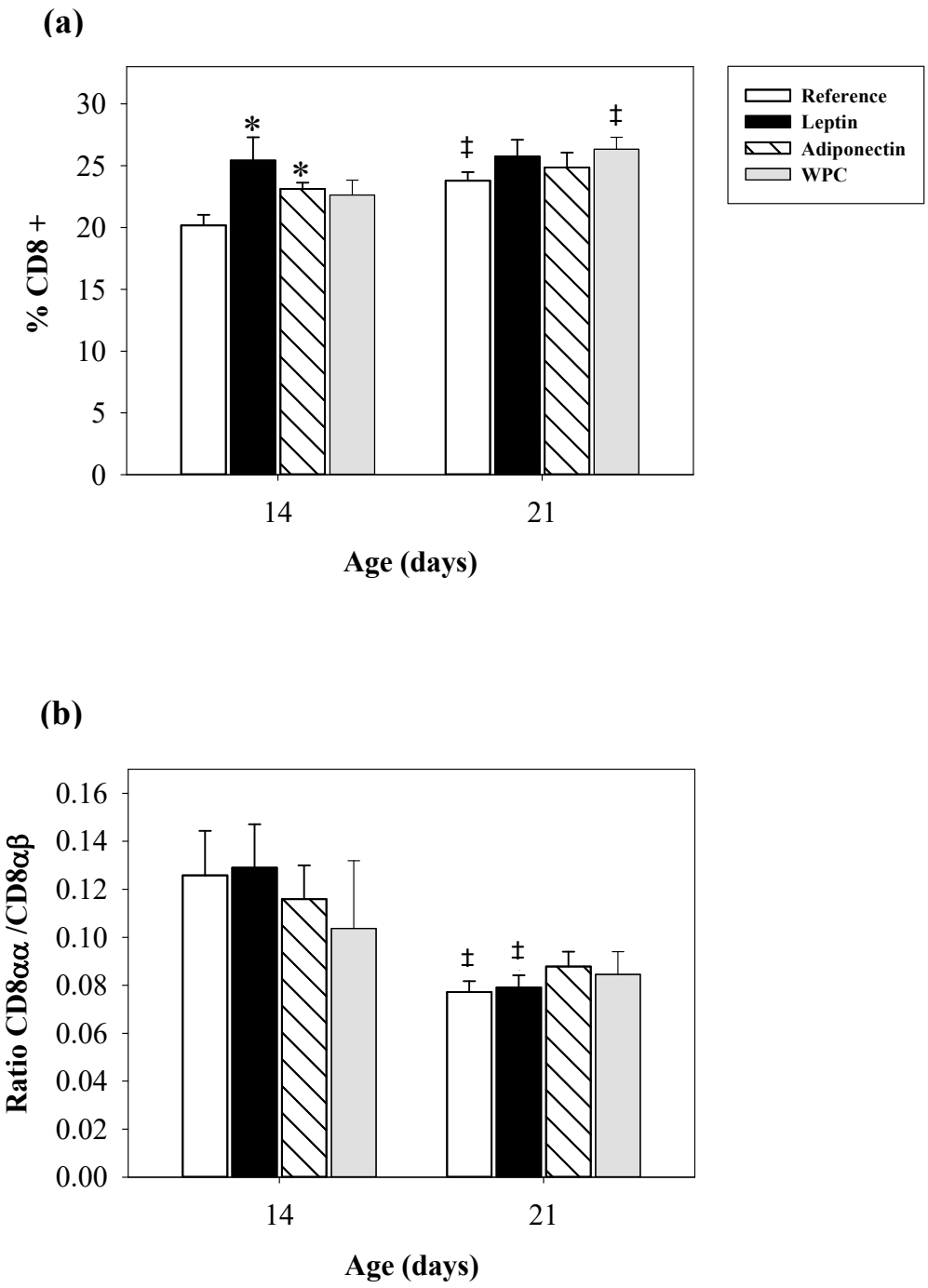

Intestinal Ig (μg/g)
0
1
2
3
4
5
6
*Ψ
IgA
IgM
Reference
Leptin
Adiponectin
WPC

Fig. 2


(a)
% CD8 +
30
25
20
15
10
5
0
14
21
Age (days)
Reference
Leptin
Adiponectin
WPC
(b)
Ratio CD8αα /CD8αβ
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
14
21
Age (days)

685 **Fig. 3**

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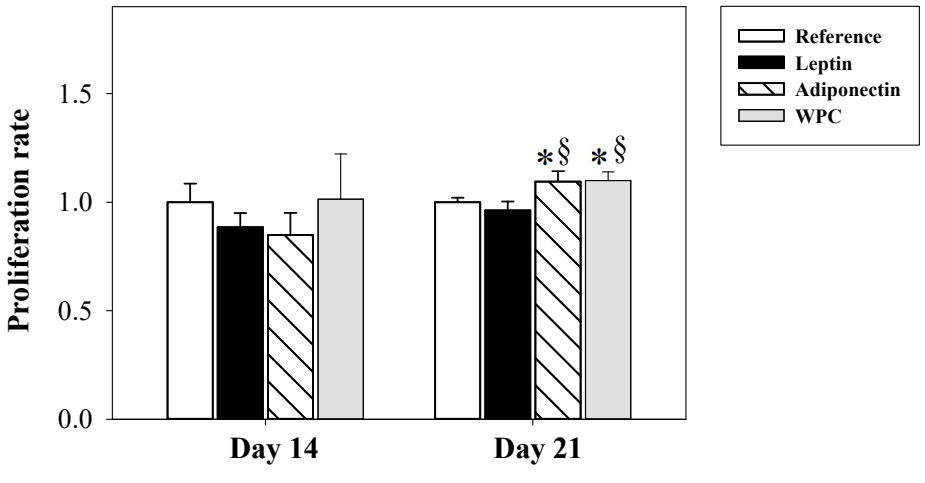

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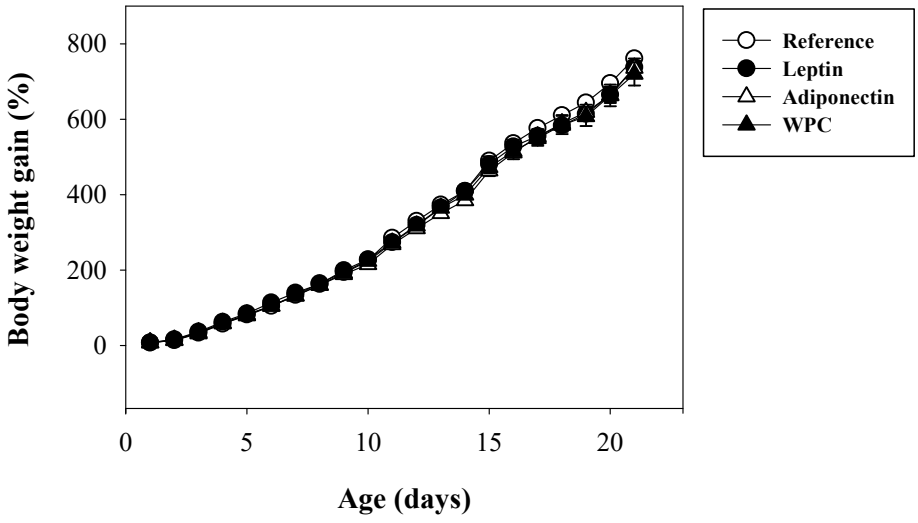
Suppl. Table 1. Expression of the αE integrin and the CD62L selectin in Th cells (CD4+CD8-), DP cells (CD4+CD8+), Tc (CD8+CD4-) and B (CD45RA+) lymphocytes in mesenteric lymph nodes from the four groups of the study.

			Th cells (CD4+CD8-)	DP cells (CD4+CD8+)	Tc cells (CD8+CD4-)	B cells (CD45RA+)
Day 14	αE+ CD62L- (%)	Reference	1.1 ± 0.2	3.0 ± 0.8	1.5 ± 0.2	3.4 ± 1.0
		Leptin	1.0 ± 0.5	2.6 ± 0.7	1.3 ± 0.4	1.1 ± 0.5
		Adiponectin	0.7 ± 0.1	2.1 ± 0.5	1.7 ± 0.2	2.3 ± 0.7
		WPC	0.8 ± 0.3	2.8 ± 0.6	1.5 ± 0.2	4.4 ± 2.1
	αE+ CD62L+ (%)	Reference	0.6 ± 0.2	6.0 ± 1.1	1.6 ± 0.4	0.5 ± 0.2
		Leptin	0.4 ± 0.1	3.8 ± 1.0	0.7 ± 0.4	1.2 ± 0.7
		Adiponectin	0.3 ± 0.1	3.9 ± 0.6	1.0 ± 0.2	1.1 ± 0.4
		WPC	0.4 ± 0.1	4.0 ± 1.2	0.8 ± 0.3	0.2 ± 0.1
	αE- CD62L+ (%)	Reference	55.1 ± 1.8	68.6 ± 2.1	62.0 ± 2.4	35.0 ± 4.6
		Leptin	54.6 ± 2.6	70.0 ± 2.2	58.7 ± 2.2	45.6 ± 6.4
		Adiponectin	52.0 ± 1.9	68.7 ± 1.2	60.3 ± 1.6	43.9 ± 3.8
		WPC	51.3 ± 1.2	67.7 ± 2.5	59.0 ± 2.3	33.3 ± 6.6
	αE- CD62L- (%)	Reference	43.3 ± 2.0	22.4 ± 2.1	34.9 ± 2.8	61.1 ± 4.8
		Leptin	44.1 ± 2.3	23.7 ± 1.6	39.4 ± 2.4	52.1 ± 6.3
		Adiponectin	47.0 ± 2.0	25.3 ± 1.2	37.0 ± 1.5	52.7 ± 4.1
		WPC	47.4 ± 1.3	25.5 ± 2.1	38.7 ± 2.5	62.2 ± 54.0
Day 21	αE+ CD62L- (%)	Reference	1.3 ± 0.2	3.9 ± 1.0	1.0 ± 0.1	3.1 ± 0.8
		Leptin	0.9 ± 0.1	2.5 ± 0.6	1.5 ± 0.8	2.0 ± 0.7
		Adiponectin	1.1 ± 0.1‡	2.9 ± 0.9	3.9 ± 1.8	2.6 ± 0.7
		WPC	0.9 ± 0.1	1.6 ± 0.4	2.6 ± 1.3	1.6 ± 0.4
	αE+ CD62L+ (%)	Reference	0.8 ± 0.2	5.2 ± 0.9	1.2 ± 0.3	0.3 ± 0.1
		Leptin	0.5 ± 0.1	2.3 ± 0.2*	0.7 ± 0.2	0.2 ± 0.1
		Adiponectin	0.6 ± 0.1‡	3.2 ± 0.5	1.1 ± 0.2	0.4 ± 0.1
		WPC	0.5 ± 0.1	2.3 ± 0.3*	1.0 ± 0.2	0.3 ± 0.1
	αE- CD62L+ (%)	Reference	61.6 ± 1.7‡	70.8 ± 2.4	65.9 ± 2.4	53.4 ± 3.2‡
		Leptin	60.4 ± 1.8	73.5 ± 1.6	63.6 ± 2.0	54.0 ± 4.9
		Adiponectin	57.4 ± 2.5	73.6 ± 3.2	60.4 ± 3.0	59.2 ± 2.8‡
		WPC	58.2 ± 1.5‡	73.9 ± 2.0	63.6 ± 1.7	60.2 ± 3.6‡
	αE- CD62L- (%)	Reference	36.2 ± 2.0‡	20.5 ± 2.0	31.9 ± 2.3	43.2 ± 2.8‡
		Leptin	38.3 ± 1.6	21.6 ± 1.4	34.3 ± 1.7	43.8 ± 4.9
		Adiponectin	40.9 ± 2.4	20.4 ± 2.4	34.6 ± 2.9	37.8 ± 2.5‡
		WPC	40.4 ± 1.5‡	22.2 ± 1.7	32.7 ± 2.4	38.0 ± 3.6‡

WPC, whey protein concentrate. Values are expressed as mean ± S.E.M. (n = 8-14 pups per group analyzed in uniplicates). Statistical differences: * P<0.05 vs. reference R group (ANOVA). ‡ P<0.05 vs. same group data at day 14 (ANOVA).

Suppl.Figure 1. Rat body weight gain during the suckling period from the four groups Reference, leptin, adiponectin and whey protein concentrate (WPC). Results are expressed as percentage of body weight gain from day 1 and represented as mean ± S.E.M. (n = 9–36 pups per group).



Suppl. Figure 2. Representative histograms of the CD62L selectin/αE integrin pattern in the total lymphocytes from the mesenteric lymph nodes of reference (a), leptin (b), adiponectin (c) and whey protein (WPC) (d) groups at day 21. In each quadrant the values expressed as mean ± S.E.M. (n = 9–12 pups per group analyzed in uniplicates) are included. Statistical differences: ‡ <0.05 vs. same group at day 14 (ANOVA).