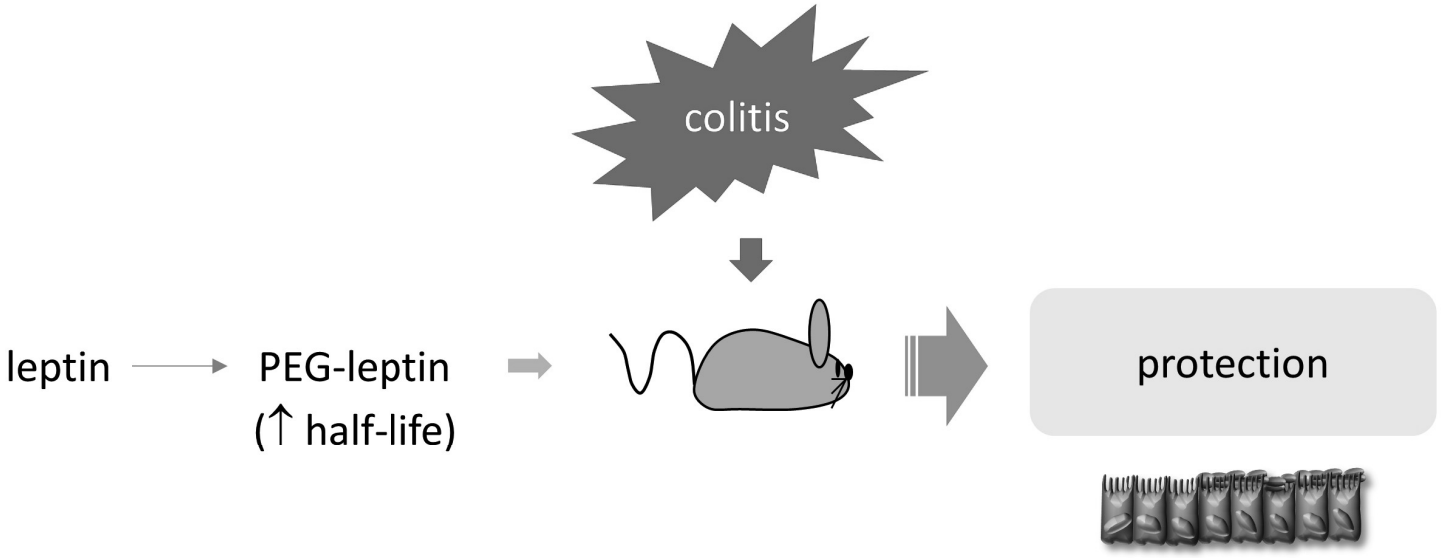
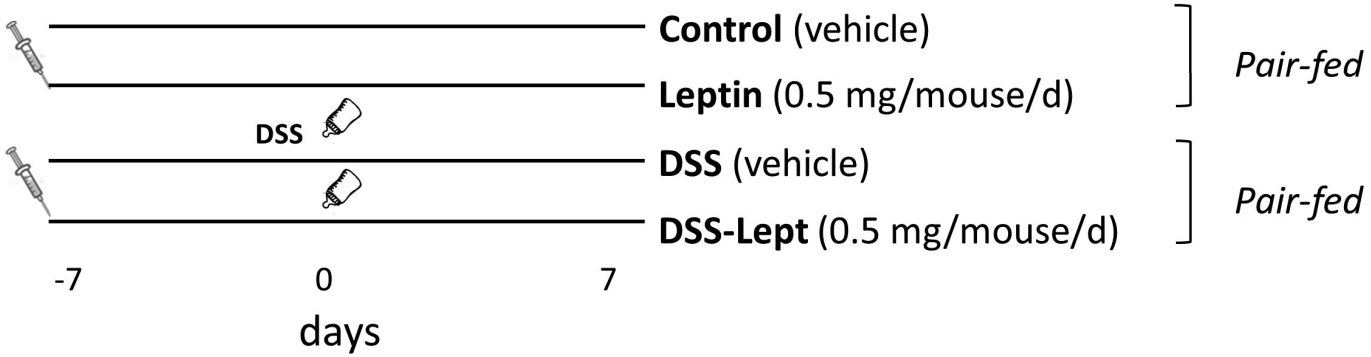

colitis
leptin
PEG-leptin
(↑ half-life)
protection
• enhanced barrier function
• augmented proliferation


Control (vehicle)
Leptin (0.5 mg/mouse/d)
Pair-fed
DSS
DSS (vehicle)
DSS-Lept (0.5 mg/mouse/d)
Pair-fed
-7
0
7
days

EXOGENOUS LEPTIN REINFORCES INTESTINAL BARRIER FUNCTION AND PROTECTS FROM COLITIS

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ABSTRACT

Besides its function controlling energy expenditure and food intake, leptin is an important modulator of inflammatory responses. The role of leptin in intestinal inflammation remains controversial, since both pro-inflammatory and anti-inflammatory effects have been reported. This study was carried out to further understand leptin contribution in the inflamed intestinal mucosa. Exogenous PEG-leptin or saline solution was given to C57BL/6 mice for two weeks. After 1 week, acute colitis was induced to C57BL/6 mice using dextran sulfate sodium (DSS) in drinking water. The severity of colitis, inflammatory parameters and mucosal barrier function were evaluated. Overall our results indicate that colitis was less severe in mice receiving leptin, as shown by a decrease in rectal bleeding, epithelial damage and colon inflammatory markers, and improved diarrhea. Leptin-treated mice displayed an increase in the expression of tight junction proteins and proliferative expression markers in colon, indicating a reinforcement in the mucosal barrier function induced by leptin administration. PEG-leptin treatment conferred protection to mice in the DSS model of colitis by reinforcing mucosal barrier function.

Keywords: leptin, barrier function, experimental colitis

1. Introduction

Leptin was discovered in 1994 as a metabolic hormone produced by adipose tissue, the first of the later called adipokines [1]. Circulating leptin levels are correlated with adipose mass [2] and are modulated by food intake [3,4]. Until the last decade, its main role was considered to be to the control of food intake and energy expenditure, since leptin is secreted in response to food stimuli and activates satiety in the CNS. Beside energy homeostasis, leptin exerts pleiotropic actions all over the organism, including neuroendocrine and immune functions, and glucose, lipid and bone metabolism [5]. Leptin affects both innate and adaptive immunity, where it can modulate the activity and function of neutrophils and macrophages, increase the cytotoxic ability of NK cells, modulate the maturation and survival of thymic T cells by reducing their apoptosis, or promote the switch towards Th1 and Th17 of memory T cells [6]. The leptin receptor, LEPRb, is expressed in numerous cell types, including neurons, intestinal epithelial cells (IECs), and immune cells, such as macrophages, T cells, natural killer cells and polymorphonuclear cells [7].

Although adipose tissue constitutes the main source of the hormone, leptin can be secreted by muscle, stomach and placenta [8]. It has been described that plasma leptin levels are augmented during intestinal colitis in individuals [9] irrespective of body mass index, and also in mice [8], where colonic epithelial cells were identified as a cellular source of leptin, suggesting the importance of leptin effects in the inflamed intestinal mucosa.

Different and opposite roles have been attributed to leptin in the gastrointestinal tract, where it has been linked to both harmful and protective effects depending on the biological context. Thus, Duggal *et al* found an increased susceptibility to intestinal infection by *Entamoeba histolytica* in children associated with an amino acid substitution of the LEPR [10]. Further, intestinal epithelium-specific deletion resulted in heightened susceptibility to infection and subsequent mucosal injury, indicating

the importance of leptin intestinal actions [11]. Similarly, leptin has a protective role in the intestinal mucosa in the *Clostridium difficile* model of colitis [12]. Of note, leptin can act from the luminal site, as mucus secretion can be induced by luminal perfusion in the rat [13,14]. Likewise, leptin could prevent gastric ulcer and promote its healing by increasing COX and NO activity, as well as mucus secretion and angiogenesis [15,16]. Thus several studies support the role of leptin as an intestinal anti-inflammatory agent.

Nevertheless, leptin is also considered a pro-inflammatory cytokine. Thus it has been shown that leptin deficient ob/ob mice are resistant to intestinal inflammation induced by DSS or trinitrobenzene sulfonic acid (TNBS) administration, associated with reduced cytokine secretion [17]. Mice receiving T cells from leptin resistant db/db mouse donor display a delay in the onset of T cell transfer model of colitis [18], as opposed to no difference with leptin deficient ob/ob mice donor cells [19], indicating that leptin signaling in T cells, but not its production, has a major role in colitis development. However, specific silencing of the leptin receptor *Lepr* in CD4+ T cells resulted in a selective defect in both autoimmune and protective Th17 responses. Reis et al. demonstrated a reduced capacity for differentiation towards Th17 phenotype by *Lepr*-deficient T cells associated with a lower activation of the signal transducer and activator of transcription 3 (STAT3) and its downstream targets [20].

Therefore, the evidence available about leptin function in the intestinal mucosa remains controversial. So far, most experiments have been performed either in animal models lacking leptin or its signaling or by blocking LEPRb. Therefore, we set out the present study with the aim of clarify this question and describe the effect of hyperleptinemia in C57BL/6 mice in steady state and under inflammation in the intestinal mucosa.

2. Methods

2.1. Reagents

Except where indicated, all reagents and primers were obtained from Sigma (Barcelona, Spain). MonoPEGylated leptin (PEG-leptin) was obtained from Protein Laboratories Rehovot (Rehovot, Israel). RNeasy mini kits were obtained from Qiagen (Hilden, Germany). Reverse transcription was achieved with the iScriptTM cDNA Synthesis Kit, and iQTM Sybr® Green Supermix was used for amplification (Biorad, Alcobendas, Spain).

2.2. Animals

Eight weeks old male C57BL/6 mice (20-25 g) were obtained from Envigo (Barcelona, Spain) (n=7-10). Animals were maintained at the University of Granada Animal Facility in air-conditioned animal quarters with a 12 h light-dark cycle. Control mice were pair-fed with leptin-treated mice, adjusting the quantity of food of control groups to the daily intake of chow diet of leptin-groups (one day of delay). All animals had free access to sterile water. Once the experiment finished, animals were sacrificed under ketamine-xylazine anesthesia, and blood and tissues were collected, processed and/or snap frozen and kept at −80°C. Whole blood samples were analyzed with a hematologic counter (Mythic® 22CT C2 diagnostics, Orphée, Geneva, Switzerland).

All animal procedures in this study were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health and were approved by the Animal Experimentation Ethics Committee of the University of Granada (ref. 2011-349).

2.3. Induction of acute DSS colitis and experimental design

Colitis was induced by adding 2.5% dextran sulfate sodium (DSS) to drinking water for 7 days. To induce hyperleptinemia, mice received a daily subcutaneous (sc) injection of PEG-leptin (0.5 mg/kg), whose half-life is ~20 hours. PEG-leptin treatment was initiated 7 days before the induction of colitis and kept until the mice were sacrificed after another week. Control groups received an equal amount of sterile saline solution sc.

The status of the animals was monitored daily by general examination and specifically by means of body weight evolution, stool consistency (0-3), and fecal occult blood, corresponding to the signs of the pathology in this model.

2.4. Evaluation of colitis

Animal blood was collected by heart puncture under ketamine-xylazine anesthesia. Then, mice were sacrificed by cervical dislocation. The entire colon was removed, gently flushed with saline and blotted on filter paper, placed on an ice-cold plate, cleaned of fat and mesentery and longitudinally opened so as to exhaustively eliminate fecal remains. Each specimen was weighed and its length measured under a constant load (2 g). A small segment was dissected from the intestine and used for RNA isolation. The colon was subsequently divided longitudinally in several pieces for biochemical determinations. The fragments were immediately frozen in liquid nitrogen and kept at −80°C until used. Alkaline phosphatase (AP) activity was measured spectrophotometrically, as described [21]. AP is expressed as mU/mg protein (1 U= 1 µmol/min of substrate converted).

Plasma leptin was measured by ELISA (R&D Systems Inc., Minneapolis MN, USA) following the manufacturer's instructions in mice untreated with PEG-leptin.

2.5. Histological assessment of colon damage

Distal colon tissue fragments were fixed in 4% paraformaldehyde (w/v). Five µm sections were obtained from paraffin-embedded colonic tissue and were placed on 3-amino propyl triethoxy silane coated slides. After being de-paraffinized, sections were rehydrated in serial dilutions of ethanol and water. Sections were then stained with hematoxylin & eosin (H&E) or alcian blue staining and mounted. H&E staining was conducted to study morphology and check for integrity of the colonic tissue attending to the following criteria. Crypt length (0-2), leukocyte infiltration (0-4), submucosal enlargement (0-2), epithelial erosion (0-2) and loss of crypts structure (0-2). Images were captured and digitalised using a Leica DMI3000B microscope equipped with a Leica DFC420 C Camera.

2.6. Fecal occult blood determination

Stool samples were freshly collected individually from each mouse and stored at −80◦C. The protocol used for the fecal occult blood (FOB) determination is an adaptation of the one first developed by the Dr. Donald S. Young [22]. Briefly, stool samples were homogenized in distilled ultrapure water (EIA Grade Ultra Pure Water, Spibio®, United Kingdom, 0.105 mg /µl). After that, 200 µl of that solution was taken into a 15 ml tube and incubated at 100°C for 10 minutes and mixed with 1.2 ml of acetic acid and distilled water (30/70). Next 1.8 ml of pure ethyl acetate was added on top and the whole mixture vortexed and centrifuged (350 g/3 minutes/room temperature). Finally, the upper phase was collected and exposed to 3,3',5,5'-tetramethylbenzidine in the presence of hydrogen peroxide and the reaction was monitored spectrometrically after 30 and 60 seconds at 660 nm. As a reference, a hemoglobin curve was generated in distilled water and subjected to the same protocol above described. Results are expressed as µg hemoglobin/mg of feces.

2.7. Colonic explants culture

After the mice sacrifice, the colon was removed and cleaned thoroughly. Here, in order to eliminate any bacterial remains, fragments were exposed to a PBS solution containing 500 U/mL penicillin, 0.5 mg/mL streptomycin, 12.5 µg/mL amphotericin B and 10 µg/mL gentamycin. Next, the colon was

divided into several equal portions (~0.5 cm^2), rinsed again in the antibiotic containing PBS and cultured with DMEM featuring heat-inactivated fetal bovine serum (10 % v/v), 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2.5 µg/mL amphotericin and 2 mM glutamine. After 24 h tissue fragments were collected and frozen. Culture medium was centrifuged for 5 minutes at 300 g and 4◦C and snap frozen at –80°C for subsequent ProcartaPlex® assay of the following analytes: TNFα, IFNγ, G-CSF, GM-CSF, IL-1β, IL-6, IL-12p70, IL-10, IL-17A and IL-22 (Affymetrix, eBioscience, Barcelona, Spain). Results are normalized to the protein amount of tissue fragments and results expressed as pg of cytokine/µg of protein (BCA assay) [23].

2.8. Western blot analysis

Tissue samples were homogenized in lysis buffer (0.1% w/v SDS, 0.1% w/v sodium deoxycholate, 1% v/v Triton X-100 in PBS) with protease inhibitor cocktail 1:200 (v/v) and phosphatase inhibitor cocktail (1:100 v/v, Santa Cruz®, Heidelberg, Germany). Homogenates were sonicated and centrifuged at 1100 g for 5 min at 4°C. Protein concentration was determined by the BCA assay. Samples were boiled for 5 min in Laemmli sample buffer (Bio-Rad®, Alcobendas, Spain) separated by SDS–PAGE, electroblotted to nitrocellulose membranes (Millipore, Madrid, Spain) and probed with the corresponding antibodies. The bands were detected by enhanced chemiluminescence (PerkinElmer®, Waltham, MA) and quantitated with the software Image J. The primary antibodies anti-pSTAT3 (1:2000, ref. 9145S lot 31) and anti-STAT3 (1:1000, ref. 9139S lot 8) were purchased from Cell Signaling (Denver, MA, US); anti-β-actin JLA (1:1000, ref. JLA20s lot 6/25/15) from DSHB (Iowa city, IA, US); anti-BCL2 (1:500, ref. 14-6992-81 lot E05256-1634) and anti-BAX (1:1000, ref. BMS163 lot 99486000) from eBiosciences (San Diego CA, USA). Ponceau staining was applied routinely to confirm equal loading [24].

2.9. RNA isolation and Quantitative Reverse-Transcription Polymerase Chain Reaction (RT-qPCR) Analysis

Total RNA was isolated by using RNeasy mini kit (Qiagen, Hilden, Germany) and checked for integrity by electrophoresis in 1% agarose gel (w/v). Quantification was determined by the 260/280 nm

absorbance ratio with a Nanodrop® (Thermo Scientific®). One µg RNA was subjected to reverse transcription, iQTM Sybr® Green Supermix was used for amplification and specific DNA sequences were amplified with a Biorad CFX connect real time PCR device (Alcobendas, Madrid, Spain). Primers used are shown in Table 1. Results are expressed as 2^{-ddCt} using hypoxanthine guanine phosphoribosyl-transferase (HPRT) as reference gene.

2.10. Intestinal permeability assay in Caco 2T cells

Caco 2T cells supplied by the European Collection of Authenticated Cell Cultures (ECACC, passes 54-56) were used as *in vitro* model to evaluate the effect of leptin on paracellular permeability to 4 kDa FITC-dextran in intestinal epithelial cells. Cells were seeded directly onto 3 µm pore size polycarbonate Transwell™ (Corning Life Science NY, USA) inserts in 12 well plates and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% v/v heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1% v/v penicillin [100U/ml], 0.1 mg/ml streptomycin and 2.5 µg/ml of amphotericin B. After two days, media was changed and leptin added at 0, 10 and 50 ng/ml into both the apical and basolateral compartments. Media and leptin were refreshed every other day and transepithelial electrical resistance (TEER) was measured to determine when the cells had reached full confluence and formed a tight and functional monolayer. Once TEER became stable, the experiment was performed. The cells were washed with sterile PBS and phenol red free medium added, with 4 kDa FITC-dextran in the apical side at 1 mg/ml (time 0). Aliquots of 60 µl were taken every hour from the basolateral compartment and media was replaced for a total of 7 hours. Finally, fluorescence was measured at 490/520 nm in a FLUOstarControl instrument (Polarstar OPTIMA, BMG Labtech). Paracellular flux was calculated as the relative change compared with control group. Three different experiments were carried out.

2.11. Statistical analysis

In all the experiments, samples were run in duplicate, and results are expressed as mean ± SEM. Differences among the means were tested for statistical significance by two-way ANOVA and a posteriori Fisher least significant difference tests. All the analyses were carried out with GraphPad Prism 6. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Leptin administration prevents intestinal damage in mice

We generated a hyperleptinemic condition by daily administering exogenous PEG-leptin to C57BL6/J mice for 14 days, 7 days before the induction of acute DSS colitis and another 7 days concurrently with the administration of DSS. To control for the anorexic effect of leptin, the intake of untreated healthy and colitic control animals was adjusted by pair-feeding. As expected, the administration of leptin prior to the induction of DSS colitis induced weight loss, that began to stabilize at day 4 of administration. Pair-feeding resulted in a similar behavior of the untreated groups with one day of lag (Figure 1A). The anorexic effect of leptin disappeared after 5 days of PEG-leptin administration, so that food intake was normal before the administration of DSS (data not shown).

DSS colitis had similar features to those described originally by Okayasu in 1990 [25] and was consistent with previous experiments in our laboratory. DSS is considered to elicit inflammation mainly by a direct toxic effect on intestinal epithelial cells, including stem cells at the base of the crypts, thereby affecting normal cell turnover and compromising epithelial barrier function. Colonic inflammation ensues, characterized by bloody diarrhea, ulcerations and an intense leukocyte infiltration. As expected, both DSS groups presented body weight loss, which was significant for the DSS control group from day 6 onwards, while the DSS-leptin group experienced a more pronounced weight loss, starting on day 2. In contrast, the body weight of the noncolitic control and leptin groups followed a parallel and stable evolution up to the end of the experiment (Figure 1B). After DSS administration the stool status was evaluated as a valuable marker of intestinal inflammation (Figure 1C). The stool consistency score increased in both the DSS and DSS leptin groups from day 2, and was significantly higher at 4-6 d in the DSS group compared with the DSS-leptin group, due to reduced diarrhea and blood content in the latter. Accordingly, fecal occult blood was measured in a sample

taken at the end of the experiment and was found to be increased in the DSS group vs. the DSS-leptin group, although statistical significance was not reached (P = 0.069) (Figure 1D).

To further reinforce these findings indicating an attenuated colonic damage in the DSS-leptin animals, bowel wall thickening (weight/length ratio) was diminished in the DSS-leptin group when compared to the DSS group (Figure 1E), and the histological study of colon cross sections using hematoxylin and eosin staining showed a better preservation of mucosal structure in leptin-treated animals as well as less leukocyte infiltration (Figures 2A and 2B). In accordance, AP activity and the expression of S100A8 (a calprotectin subunit) were reduced in colitic mice treated with leptin (p=0.07 for S100A8 expression) (Figures 2C and 2D). Leptin plasma levels were not measured in PEG-leptin treated mice due to interference with the ELISA assay, but a marked reduction was noted in the DSS group compared with the control (Figure 1F). Leptin administration resulted in upregulation of the leptin receptor in the colon of colitic but not control mice (Figure 1G).

The haematological analysis of the mice revealed that DSS colitis resulted in a significant decrease in hematocrit (HTC), in the concentration of hemoglobin (Hb) and in the number of circulating erythrocytes (RBC), consistent with the marked rectal bleeding inherent to the model. The administration of leptin normalized these parameters (Figure 3A). The presence of total leucocytes in blood (WBC) was increased by colitis, and the relative amount of basophils (BAS) and neutrophils (NEU) within them was significantly reduced in leptin-treated mice (Figure 3B-C). Finally, both colitic groups showed elevated levels of platelets in blood (platelet number, PLT, and thrombocrit, PCT), with no differences between them (Figure 3D).

Together, these results indicate lower inflammation in the colitic animals that received leptin.

3.2. Leptin stimulates the intestinal immune response in physiological conditions but inhibits inflammation in DSS induced colitis

To further study the effect of leptin on the intestinal inflammatory response, we cultured colonic explants and determined cytokine concentration in the culture medium (Figure 4). Leptin given to healthy, noncolitic animals showed a clear tendency to increase the secretion of most of the assayed cytokines, including TNF, IL-17A, IL-10, IL-6, GM-CSF, G-CSF and slightly that of IL-1β and IL-12, thereby showing an immunostimulant effect in these conditions. It should be noted that none of these changes reached statistical significance due to the distortion introduced by the data corresponding colitic groups. As expected, DSS inflammation induced the innate immune response, with augmented TNF or IL-1β, as well as the adaptive immune response, potentiating the release of Th1, Th17 and Treg cytokines IL-10, IL-17A, IL-22, IFN-γ, and also slightly that of IL-12p70 (Figures 4A-H). The colony stimulating factors GM-CSF and G-CSF were also significantly induced by DSS (Figure 4I-J). No significant differences were observed for the Th2 cytokines IL-13, IL-4 or for IL-27 (not shown). The DSS-leptin group showed levels of TNF, IFN-γ, IL-1β and IL17A intermediate between those of the control and DSS groups, while IL12p70 levels were equivalent to those of the control groups, indicating a regulatory anti-inflammatory effect of leptin in the colitis. In contrast, IL-10, IL-22, IL-6, GM-CSF and G-CSF were unaffected by leptin treatment.

3.3. Epithelial barrier function is reinforced by leptin

To evaluate the state of epithelial barrier function, we analyzed the expression of antimicrobial peptides and proteins involved in tight junctions by qRT-PCR. There was a marked increase in Reg3γ and haptoglogin (HP) expression in colitic animals compared with the controls, which was less pronounced in the DSS-leptin group (Figure 5A-B). Lysozime 2 (*Lyz-2*) was also induced in colitic mice (Figure 5C). Leptin induced the expression of ZO-1 and claudin 4, an effect that, notably, was observed in both control animals that received leptin (p=0.06) and in the DSS-leptin group, while claudin 5 was

significantly upregulated in the DSS-leptin group (Figure 5D-F). The glycocalix is another element of the intestinal barrier. Alcian blue and nuclear red staining of colonic tissue showed a substantial reduction of mucus in DSS colitis, which was partly counteracted in both the lumen and crypts in the DSS-leptin group compared with the DSS group (Figure 5H). Conversely, no differences were noted in noncolitic animals. Consequently, we measured the expression of mucin *Muc3* by qRT-PCR and found it to be significantly decreased in DSS colitis, an effect that was attenuated in DSS-leptin mice. Nevertheless, in the absence of inflammation, animals treated with leptin exhibited some decrease in *Muc3* expression in colonic tissue (Figure 5G). Finally, phosphorylation of myosin light chain 2 (MLC2), which has been related to an increased intestinal permeability, was reduced by leptin in the absence of inflammation (Figure 6A). This effect persisted in the colitic groups but was attenuated, so that it was no longer significant. An *in vitro* Caco-2T cell model was used to further study the effect of leptin on intestinal permeability. In agreement with the results obtained *in vivo*, culturing cells with different concentrations of leptin reduced the permeability to 4 kD FITC-dextran (Figure 6B).

The ability to restore the epithelial barrier is a determinant factor in the evolution and resolution of acute colitis induced by DSS, so we analyzed the expression of proliferative and apoptotic factors in colonic tissue. Western blot results for PCNA *(*Proliferating Cell Nuclear Antigen), STAT3 phosphorylation (pSTAT3/STAT3 ratio) and BCL2-associated x protein (BAX)/cell lymphoma 2 (BCL-2), shown in Figure 6. PCNA, a marker of cell proliferation, was increased by leptin administration (Figure 6D). STAT3 (in its phosphorylated active form pSTAT3) induces cell proliferation and is involved in the generation of antimicrobial peptides and mucins. In addition, it has been described as a key factor in the signaling cascade of leptin. The results obtained confirm enhanced phosphorylation of STAT3 in noncolitic leptin treated mice. The administration of DSS markedly increased the activation of STAT3, but this response was significantly lower in colitic mice treated with leptin (Figure 6C). BAX and BCL-2 are well known pro- and antiapoptotic factors, respectively. The BAX/BCL-2 ratio was increased in the

DSS group, consistent with a higher apoptosis in the colon of these animals, but not in the animals treated with leptin (Figure 6E).

4. Discussion

Various adipokines, including leptin, adiponectin, resistin, visfatin, ghrelin, and apellin, mostly derived from mesenteric adipose tissue, have been related to intestinal inflammation [26-30]. In particular, leptin, resistin, ghrelin and adiponectin are upregulated in the mesenteric adipose tissue of Crohn's disease patients [31,32]. The present study was carried out to assess the effect of exogenous leptin in the development of experimentally induced Th1 colitis in normal mice [33]. This direct experimental approach may harvest different results from those obtained in animals devoid of normal leptin signaling, such as Zucker rats or ob/ob mice, due to adaptive changes in the latter as mentioned above. In order to control for the expected anorexigenic effect of leptin pair feeding was applied. Of note, even in these conditions body weight remained somewhat lower in leptin treated mice, possibly reflecting augmented basal metabolism, which is a known effect of leptin [34].

Our results categorically indicate a diminished colonic inflammatory response in leptin treated mice, based on multiple parameters: attenuated bowel wall thickening, AP activity, fecal blood and anemia, neutrophil and basophil counts, S100a8 mRNA expression, Reg3γ and STAT3 phosphorylation, plus improved histology and stool consistency. In addition, inflammatory cytokine production by colonic explants (TNFα, IFN-γ, IL-1β, IL-17A, IL-12 p70) was also lower, albeit without reaching statistical significance in this case. Of note, cytokine levels were somewhat higher in explants from noncolitic leptin-treated mice, such as IL-6 and G-CSF. This was not significant, despite a relatively large increase, possibly owing to the statistical distortion introduced by the colitic groups. Thus this might represent a real effect of leptin, which may in turn be involved in tissue repair and intestinal homeostasis. In particular, IL-6 has been related to intestinal epithelial proliferative and repair response after injury [35], and to antimicrobial protection [36]. Similar effects have been described for G-CSF [37]. Related to these findings is increased STAT3 phoshorylation. STAT3 signaling has been

shown to be relevant to leptin intestinal effects [38]. Thus our results suggest that leptin may contribute to intestinal protection/repair in part by this mechanism.

These data globally point to an antiinflammatory effect of exogenous leptin. Because leptin has well documented effects on barrier function [13-15,39], we also measured barrier parameters in colonic samples. Leptin upregulated the expression of ZO-1 (*Tjp1*, p=0.06), *Cldn4* and *Cldn5* at the mRNA level. This effect was attenuated in colitic mice in the case of *Tjp1*. Leptin also increased the expression of the proliferation marker PCNA, augmented mucus production, and downregulated apoptosis (based on BAX/BCL2). Considered globally, these data suggest a mucosal barrier protective effect of leptin. Unfortunately we do not have direct evidence of enhanced barrier function *in vivo*, as DSS colitic animals did not exhibit measurable LPS or bacterial translocation (this is consistent with our experience with this model ([40] and data not shown). In Caco 2T cells leptin lowered paracellular permeability to 4 kD FITC-dextran. It should be noted that leptin failed to enhance epithelial wound healing *in vitro* (data not shown). Thus our results concur with previous reports showing a positive effect of leptin on mucosal barrier function, related at least in part with epithelial proliferation and regeneration, and, contrary to previous observations *in vivo* and *in vitro* [41], with a positive impact on tight junctions.

Protection from DSS colitis may result from such direct barrier enhancing actions. A possible alternative mechanism is activation of the mucosal immune system, inasmuch as it contributes to barrier function by controlling bacterial translocation [42-45]. Generalized [46] or T cell specific [20] blockade of leptin signaling enhances the susceptibility to infection, and leptin treatment protects against experimental sepsis [47]. Of note, mice with epithelial but not hematopoietic specific deletion of the leptin receptor are more sensitive to *Entamoeba histolytica* [10,11]. In addition to epithelial

mechanisms, leptin protection reportedly involves enhanced recruitment of neutrophils [48]. It is worth noting that leptin has been documented to have gastroprotective effects as well [49].

Our results are consistent with those reported by Cakir et al. using the acetic acid rat colitis model, using a 50-fold lower dose of unpegylated leptin and looking at short term effects (24 h) [50]. These authors implicated the glucocorticoid receptor, as the antiinflammatory effect was reversed by mifepristone. However, leptin has an inhibitory effect on the hypothalamus. Even though a substantial part of circulating corticosterone may proceed from peripheral sources in these conditions [51-53], plasma corticosterone levels were not increased in our study (data not shown), suggesting it is not involved in leptin effects. Conversely, colitis had a dramatic effect on leptin levels, which were reduced by 90%. This is in conflict with previous reports, which have documented increased circulating leptin [54-57], which may be secondary to augmented synthesis by the intestinal epithelium, mucosal T cells, or creeping fat/mesenteric adipose tissue [8,32,58]. It has been proposed that leptin expression may be induced by translocating bacteria [59]. The reason for this discrepancy is unknown, but we have also observed reduced plasma leptin in rat TNBS colitis (unpublished observations), and a similar result was observed in DSS colitis by Deboer et al. [60]. It should be noted that leptin has been also positively associated with body mass index and negatively with disease activity in IBD [61]. Thus, given that weight loss associated with colitis is substantial and results in reduction of adipose tissue mass, this may be the cause of hypoleptinemia. Our results suggest that it may aggravate inflammation. In turn, decreased circulating leptin arguably has little effect in the anorexia associated with colitis, as the latter, at least in experimental colitis, has been linked to increased IL-1β levels [62,63]. Due to cross-reactivity the levels of adipokine treated animals could not be assessed. Of note, the expression of the leptin receptor was upregulated in the colon of leptin treated colitic mice, possibly reflecting feed-forward regulation in an inflammatory context (since noncolitic mice showed normal expression levels). Such a mechanism has been previously documented in the liver of leptin treated mice [64].

The effects of leptin in the inflamed intestine are complex, as the conflicting evidence available indicates. Thus ob/ob mice (which lack leptin) are resistant to both DSS and TNBS colitis [17], and become sensitive when exogenous leptin is administered or when WT MAT is transplanted [27], implicating mesenteric adipose tissue derived leptin as the colitogenic factor. Transfer colitis is also attenuated using KO leptin receptor T cells as donors [20]. Further, administration of a pegylated leptin receptor antagonist results in protection against IL10$^{-/-}$ colitis [65]. Underscoring the importance of adaptive changes in leptin signaling, double knockout IL10$^{-/-}$ ob/ob mice exhibit no changes in colitis severity compared with leptin expressing mice [66]. Thus no clear image emerges from existing evidence. It is likely that leptin effects depend on the biological context and experimental model. For instance, protection against colitis by the leptin antagonist has been assessed in the IL10$^{-/-}$ model, which is characterized by higher involvement of the adaptive immune system and lower barrier disruption than either DSS or acetic acid colitis. This suggests that the antiinflammatory effects observed in these latter models may be favored by the epithelial actions of leptin. At any rate, our results suggest that the usefulness of leptin receptor antagonists may be hampered by the loss of repair mechanisms at play. Further research is required to clarify the precise role of leptin in intestinal inflammation.

Conflict of interest disclosure

The authors have no conflict of interest.

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REFERENCES

[1] G. Fruhbeck, S.A. Jebb, A.M. Prentice, Leptin: physiology and pathophysiology, Clinical physiology (Oxford, England) 18 (1998) 399-419.

[2] T. Skurk, C. Alberti-Huber, C. Herder, H. Hauner, Relationship between adipocyte size and adipokine expression and secretion, J Clin Endocrinol Metab 92 (2007) 1023-1033.

[3] M. Pardo, A. Roca-Rivada, O. Al-Massadi, L.M. Seoane, J.P. Camina, F.F. Casanueva, Peripheral leptin and ghrelin receptors are regulated in a tissue-specific manner in activity-based anorexia, Peptides 31 (2010) 1912-1919.

[4] P.J. Havel, Peripheral signals conveying metabolic information to the brain: short-term and long-term regulation of food intake and energy homeostasis, Exp Biol Med (Maywood) 226 (2001) 963-977.

[5] H.K. Park, R.S. Ahima, Physiology of leptin: energy homeostasis, neuroendocrine function and metabolism, Metabolism 64 (2015) 24-34.

[6] A. La Cava, G. Matarese, The weight of leptin in immunity, Nat Rev Immunol 4 (2004) 371-379.

[7] E. Gorska, K. Popko, A. Stelmaszczyk-Emmel, O. Ciepiela, A. Kucharska, M. Wasik, Leptin receptors, European journal of medical research 15 Suppl 2 (2010) 50-54.

[8] S. Sitaraman, X. Liu, L. Charrier, L.H. Gu, T.R. Ziegler, A. Gewirtz, D. Merlin, Colonic leptin: source of a novel proinflammatory cytokine involved in IBD, FASEB J 18 (2004) 696-698.

[9] A. Tuzun, A. Uygun, Z. Yesilova, A.M. Ozel, A. Erdil, H. Yaman, S. Bagci, M. Gulsen, N. Karaeren, K. Dagalp, Leptin levels in the acute stage of ulcerative colitis, J Gastroenterol Hepatol 19 (2004) 429-432.

[10] P. Duggal, X. Guo, R. Haque, K.M. Peterson, S. Ricklefs, D. Mondal, F. Alam, Z. Noor, H.P. Verkerke, C. Marie, C.A. Leduc, S.C. Chua, Jr., M.G. Myers, Jr., R.L. Leibel, E. Houpt, C.A. Gilchrist, A. Sher, S.F. Porcella, W.A. Petri, Jr., A mutation in the leptin receptor is associated with Entamoeba histolytica infection in children, J Clin Invest 121 (2011) 1191-1198.

[11] X. Guo, M.R. Roberts, S.M. Becker, B. Podd, Y. Zhang, S.C. Chua, Jr., M.G. Myers, Jr., P. Duggal, E.R. Houpt, W.A. Petri, Jr., Leptin signaling in intestinal epithelium mediates resistance to enteric infection by Entamoeba histolytica, Mucosal Immunol 4 (2011) 294-303.

[12] R. Madan, X. Guo, C. Naylor, E.L. Buonomo, D. Mackay, Z. Noor, P. Concannon, K.W. Scully, P. Pramoonjago, G.L. Kolling, C.A. Warren, P. Duggal, W.A. Petri, Jr., Role of leptin-mediated colonic inflammation in defense against Clostridium difficile colitis, Infect Immun 82 (2014) 341-349.

[13] P. Plaisancie, R. Ducroc, M. El Homsi, A. Tsocas, S. Guilmeau, S. Zoghbi, O. Thibaudeau, A. Bado, Luminal leptin activates mucin-secreting goblet cells in the large bowel, Am J Physiol Gastrointest Liver Physiol 290 (2006) G805-812.

[14] M. El Homsi, R. Ducroc, J. Claustre, G. Jourdan, A. Gertler, M. Estienne, A. Bado, J.Y. Scoazec, P. Plaisancie, Leptin modulates the expression of secreted and membrane-associated mucins in

colonic epithelial cells by targeting PKC, PI3K, and MAPK pathways, Am J Physiol Gastrointest Liver Physiol 293 (2007) G365-373.

[15] T. Tanigawa, T. Watanabe, K. Otani, Y. Nadatani, H. Machida, H. Okazaki, H. Yamagami, K. Watanabe, K. Tominaga, Y. Fujiwara, N. Oshitani, K. Higuchi, T. Arakawa, Leptin promotes gastric ulcer healing via upregulation of vascular endothelial growth factor, Digestion 81 (2010) 86-95.

[16] E.O. Adeyemi, S.A. Bastaki, I.S. Chandranath, M.Y. Hasan, M. Fahim, A. Adem, Mechanisms of action of leptin in preventing gastric ulcer, World J Gastroenterol 11 (2005) 4154-4160.

[17] B. Siegmund, H.A. Lehr, G. Fantuzzi, Leptin: a pivotal mediator of intestinal inflammation in mice, Gastroenterology 122 (2002) 2011-2025.

[18] B. Siegmund, J.A. Sennello, J. Jones-Carson, F. Gamboni-Robertson, H.A. Lehr, A. Batra, I. Fedke, M. Zeitz, G. Fantuzzi, Leptin receptor expression on T lymphocytes modulates chronic intestinal inflammation in mice, Gut 53 (2004) 965-972.

[19] G. Fantuzzi, J.A. Sennello, A. Batra, I. Fedke, H.A. Lehr, M. Zeitz, B. Siegmund, Defining the role of T cell-derived leptin in the modulation of hepatic or intestinal inflammation in mice, Clin Exp Immunol 142 (2005) 31-38.

[20] B.S. Reis, K. Lee, M.H. Fanok, C. Mascaraque, M. Amoury, L.B. Cohn, A. Rogoz, O.S. Dallner, P.M. Moraes-Vieira, A.I. Domingos, D. Mucida, Leptin receptor signaling in T cells is required for Th17 differentiation, J Immunol 194 (2015) 5253-5260.

[21] C. Hernandez-Chirlaque, R. Gamez-Belmonte, B. Ocon, P. Martinez-Moya, S. Wirtz, F. Sanchez de Medina, O. Martinez-Augustin, Tissue Non-specific Alkaline Phosphatase Expression is Needed for the Full Stimulation of T Cells and T Cell-Dependent Colitis, Journal of Crohn's & colitis 11 (2017) 857-870.

[22] C.L. Welch, D.S. Young, Spectrophotometry of occult blood in feces, Clin Chem 29 (1983) 2022-2025.

[23] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Measurement of protein using bicinchoninic acid, Anal Biochem 150 (1985) 76-85.

[24] I. Romero-Calvo, B. Ocon, P. Martinez-Moya, M.D. Suarez, A. Zarzuelo, O. Martinez-Augustin, F.S. de Medina, Reversible Ponceau staining as a loading control alternative to actin in Western blots, Anal Biochem 401 (2010) 318-320.

[25] I. Okayasu, S. Hatakeyama, M. Yamada, T. Ohkusa, Y. Inagaki, R. Nakaya, A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice, Gastroenterology 98 (1990) 694-702.

[26] H. Tilg, A.R. Moschen, Adipocytokines: mediators linking adipose tissue, inflammation and immunity, Nat Rev Immunol 6 (2006) 772-783.

[27] J.A. Sennello, R. Fayad, M. Pini, M.E. Gove, G. Fantuzzi, Transplantation of wild-type white adipose tissue normalizes metabolic, immune and inflammatory alterations in leptin-deficient ob/ob mice, Cytokine 36 (2006) 261-266.

[28] Y. Ates, B. Degertekin, A. Erdil, H. Yaman, K. Dagalp, Serum ghrelin levels in inflammatory bowel disease with relation to disease activity and nutritional status, Dig Dis Sci 53 (2008) 2215-2221.

[29] A. Konrad, M. Lehrke, V. Schachinger, F. Seibold, R. Stark, T. Ochsenkuhn, K.G. Parhofer, B. Goke, U.C. Broedl, Resistin is an inflammatory marker of inflammatory bowel disease in humans, Eur J Gastroenterol Hepatol 19 (2007) 1070-1074.

[30] L. Valentini, E.K. Wirth, U. Schweizer, S. Hengstermann, L. Schaper, T. Koernicke, E. Dietz, K. Norman, C. Buning, B.M. Winklhofer-Roob, H. Lochs, J. Ockenga, Circulating adipokines and the protective effects of hyperinsulinemia in inflammatory bowel disease, Nutrition 25 (2009) 172-181.

[31] B. Bertin, P. Desreumaux, L. Dubuquoy, Obesity, visceral fat and Crohn's disease, Curr Opin Clin Nutr Metab Care 13 (2010) 574-580.

[32] K. Karmiris, I.E. Koutroubakis, E.A. Kouroumalis, Leptin, adiponectin, resistin, and ghrelin--implications for inflammatory bowel disease, Mol Nutr Food Res 52 (2008) 855-866.

[33] R. Perez-Navarro, I. Ballester, A. Zarzuelo, F. Sanchez de Medina, Disturbances in epithelial ionic secretion in different experimental models of colitis, Life Sci 76 (2005) 1489-1501.

[34] R. Pandit, S. Beerens, R.A.H. Adan, Role of leptin in energy expenditure: the hypothalamic perspective, Am J Physiol Regul Integr Comp Physiol 312 (2017) R938-r947.

[35] K.A. Kuhn, N.A. Manieri, T.C. Liu, T.S. Stappenbeck, IL-6 stimulates intestinal epithelial proliferation and repair after injury, PLoS One 9 (2014) e114195.

[36] S.M. Dann, M.E. Spehlmann, D.C. Hammond, M. Iimura, K. Hase, L.J. Choi, E. Hanson, L. Eckmann, IL-6-dependent mucosal protection prevents establishment of a microbial niche for attaching/effacing lesion-forming enteric bacterial pathogens, J Immunol 180 (2008) 6816-6826.

[37] S.A. Eming, P. Martin, M. Tomic-Canic, Wound repair and regeneration: mechanisms, signaling, and translation, Science translational medicine 6 (2014) 265sr266.

[38] M.E. Gove, D.H. Rhodes, M. Pini, J.W. van Baal, J.A. Sennello, R. Fayad, R.J. Cabay, M.G. Myers, Jr., G. Fantuzzi, Role of leptin receptor-induced STAT3 signaling in modulation of intestinal and hepatic inflammation in mice, J Leukoc Biol 85 (2009) 491-496.

[39] C.S. Marie, H.P. Verkerke, S.N. Paul, A.J. Mackey, W.A. Petri, Jr., Leptin protects host cells from Entamoeba histolytica cytotoxicity by a STAT3-dependent mechanism, Infect Immun 80 (2012) 1934-1943.

[40] B. Ocon, C.J. Aranda, R. Gamez-Belmonte, M.D. Suarez, A. Zarzuelo, O. Martinez-Augustin, F. Sanchez de Medina, The glucocorticoid budesonide has protective and deleterious effects in experimental colitis in mice, Biochem Pharmacol 116 (2016) 73-88.

[41] G. Le Drean, V. Haure-Mirande, L. Ferrier, C. Bonnet, P. Hulin, P. de Coppet, J.P. Segain, Visceral adipose tissue and leptin increase colonic epithelial tight junction permeability via a RhoA-ROCK-dependent pathway, Faseb j 28 (2014) 1059-1070.

[42] F. Sanchez de Medina, I. Romero-Calvo, C. Mascaraque, O. Martinez-Augustin, Intestinal inflammation and mucosal barrier function, Inflamm Bowel Dis 20 (2014) 2394-2404.

[43] H. Wang, W. Zhang, L. Zuo, J. Dong, W. Zhu, Y. Li, L. Gu, J. Gong, Q. Li, N. Li, J. Li, Intestinal dysbacteriosis contributes to decreased intestinal mucosal barrier function and increased bacterial translocation, Letters in applied microbiology 58 (2014) 384-392.

[44] J. Meng, H. Yu, J. Ma, J. Wang, S. Banerjee, R. Charboneau, R.A. Barke, S. Roy, Morphine induces bacterial translocation in mice by compromising intestinal barrier function in a TLR-dependent manner, PLoS One 8 (2013) e54040.

[45] M. Zareie, K. Johnson-Henry, J. Jury, P.C. Yang, B.Y. Ngan, D.M. McKay, J.D. Soderholm, M.H. Perdue, P.M. Sherman, Probiotics prevent bacterial translocation and improve intestinal barrier function in rats following chronic psychological stress, Gut 55 (2006) 1553-1560.

[46] M. Ozata, I.C. Ozdemir, J. Licinio, Human leptin deficiency caused by a missense mutation: multiple endocrine defects, decreased sympathetic tone, and immune system dysfunction indicate new targets for leptin action, greater central than peripheral resistance to the effects of leptin, and spontaneous correction of leptin-mediated defects, J Clin Endocrinol Metab 84 (1999) 3686-3695.

[47] J. Tschop, R. Nogueiras, S. Haas-Lockie, K.R. Kasten, T.R. Castaneda, N. Huber, K. Guanciale, D. Perez-Tilve, K. Habegger, N. Ottaway, S.C. Woods, B. Oldfield, I. Clarke, S. Chua, Jr., I.S. Farooqi, S. O'Rahilly, C.C. Caldwell, M.H. Tschop, CNS leptin action modulates immune response and survival in sepsis, The Journal of neuroscience : the official journal of the Society for Neuroscience 30 (2010) 6036-6047.

[48] C. Naylor, S. Burgess, R. Madan, E. Buonomo, K. Razzaq, K. Ralston, W.A. Petri, Jr., Leptin receptor mutation results in defective neutrophil recruitment to the colon during Entamoeba histolytica infection, mBio 5 (2014)

[49] T. Brzozowski, P.C. Konturek, R. Pajdo, S. Kwiecien, A. Ptak, Z. Sliwowski, D. Drozdowicz, M. Pawlik, S.J. Konturek, E.G. Hahn, Brain-gut axis in gastroprotection by leptin and cholecystokinin against ischemia-reperfusion induced gastric lesions, J Physiol Pharmacol 52 (2001) 583-602.

[50] B. Cakir, A. Bozkurt, F. Ercan, B.C. Yegen, The anti-inflammatory effect of leptin on experimental colitis: involvement of endogenous glucocorticoids, Peptides 25 (2004) 95-104.

[51] R.C. Gaillard, E. Spinedi, T. Chautard, F.P. Pralong, Cytokines, leptin, and the hypothalamo-pituitary-adrenal axis, Ann N Y Acad Sci 917 (2000) 647-657.

[52] E.W. Roubos, M. Dahmen, T. Kozicz, L. Xu, Leptin and the hypothalamo-pituitary-adrenal stress axis, General and comparative endocrinology 177 (2012) 28-36.

[53] M.R. Stieg, C. Sievers, O. Farr, G.K. Stalla, C.S. Mantzoros, Leptin: A hormone linking activation of neuroendocrine axes with neuropathology, Psychoneuroendocrinology 51 (2015) 47-57.

[54] M. Waluga, M. Hartleb, G. Boryczka, M. Kukla, K. Zwirska-Korczala, Serum adipokines in inflammatory bowel disease, World J Gastroenterol 20 (2014) 6912-6917.

[55] R. Faggioni, K.R. Feingold, C. Grunfeld, Leptin regulation of the immune response and the immunodeficiency of malnutrition, FASEB J 15 (2001) 2565-2571.

[56] M. Barbier, C. Cherbut, A.C. Aube, H.M. Blottiere, J.P. Galmiche, Elevated plasma leptin concentrations in early stages of experimental intestinal inflammation in rats, Gut 43 (1998) 783-790.

[57] G. Biesiada, J. Czepiel, A. Ptak-Belowska, A. Targosz, G. Krzysiek-Maczka, M. Strzalka, S.J. Konturek, T. Brzozowski, T. Mach, Expression and release of leptin and proinflammatory cytokines in patients with ulcerative colitis and infectious diarrhea, J Physiol Pharmacol 63 (2012) 471-481.

[58] K. Karmiris, I.E. Koutroubakis, C. Xidakis, M. Polychronaki, T. Voudouri, E.A. Kouroumalis, Circulating levels of leptin, adiponectin, resistin, and ghrelin in inflammatory bowel disease, Inflamm Bowel Dis 12 (2006) 100-105.

[59] L. Peyrin-Biroulet, M. Chamaillard, F. Gonzalez, E. Beclin, C. Decourcelle, L. Antunes, J. Gay, C. Neut, J.F. Colombel, P. Desreumaux, Mesenteric fat in Crohn's disease: a pathogenetic hallmark or an innocent bystander?, Gut 56 (2007) 577-583.

[60] M.D. Deboer, Y. Li, Puberty is delayed in male mice with dextran sodium sulfate colitis out of proportion to changes in food intake, body weight, and serum levels of leptin, Pediatric research 69 (2011) 34-39.

[61] G. Chouliaras, I. Panayotou, D. Margoni, E. Mantzou, P. Pervanidou, Y. Manios, G.P. Chrousos, E. Roma, Circulating leptin and adiponectin and their relation to glucose metabolism in children with Crohn's disease and ulcerative colitis, Pediatr Res 74 (2013) 420-426.

[62] K.J. McHugh, S.M. Collins, H.P. Weingarten, Central interleukin-1 receptors contribute to suppression of feeding after acute colitis in the rat, Am J Physiol 266 (1994) R1659-1663.

[63] T. El-Haj, S. Poole, M.J. Farthing, A.B. Ballinger, Anorexia in a rat model of colitis: interaction of interleukin-1 and hypothalamic serotonin, Brain Res 927 (2002) 1-7.

[64] P. Cohen, G. Yang, X. Yu, A.A. Soukas, C.S. Wolfish, J.M. Friedman, C. Li, Induction of leptin receptor expression in the liver by leptin and food deprivation, J Biol Chem 280 (2005) 10034-10039.

[65] U.P. Singh, N.P. Singh, H. Guan, B. Busbee, R.L. Price, D.D. Taub, M.K. Mishra, R. Fayad, M. Nagarkatti, P.S. Nagarkatti, Leptin antagonist ameliorates chronic colitis in IL-10(-)/(-) mice, Immunobiology 218 (2013) 1439-1451.

[66] B. Siegmund, J.A. Sennello, H.A. Lehr, A. Batra, I. Fedke, M. Zeitz, G. Fantuzzi, Development of intestinal inflammation in double IL-10- and leptin-deficient mice, J Leukoc Biol 76 (2004) 782-786.

FIGURE LEGENDS

Figure 1. Leptin administration prevents intestinal damage in mice (I). Intestinal colitis was induced with 2.5% DSS in drinking water and PEG-leptin or vehicle were administered s.c. PEG-leptin was found to prevent intestinal damage according to fecal consistency and blood content and decreased bowel wall thickening. Body weight evolution before (A) and after (B) induction of DSS colitis. (C) Stool consistency. (D) Fecal occult blood as μg of hemoglobin (Hb) per mg of feces. (E) Colonic weight/length ratio. (F) Plasma leptin levels. (G) Colonic expression of *Lepr*. $^{+}P<0.05$ vs Control; $^{*}P<0.05$ vs DSS; $^{\#}P<0.05$ vs Leptin (n=7-10).

Figure 2. Leptin administration prevents intestinal damage in mice (II). Intestinal colitis was induced with 2.5% DSS in drinking water and PEG-leptin or vehicle were administered s.c. Histological staining significantly shows a better preservation of mucosal structure and less leukocyte infiltration, AP activity and *S100a8* expression in leptin-treated mice. (A) H&E staining in colon. (B) H&E quantification; (C) Alkaline phosphatase (AP) activity in colon.(D) Colonic expression of *S100a8* by qPCR. $^{+}P<0.05$ vs Control; $^{*}P<0.05$ vs DSS; $^{\#}P<0.05$ vs Leptin (n=7-10).

Figure 3. Hemogram results. Intestinal colitis was induced with 2.5% DSS in drinking water and PEG-leptin or vehicle were administered s.c. PEG-leptin reduced anemia and prevented neutrophilia and basophilia. Plasma levels of Hb, total red blood cells (RBC) and hematocrit (HTC) (A) as well as total white blood cells (WBC) (B). (C) Percentage of circulating lymphocytes (LYM), monocytes (MON), neutrophils (NEU), eosinophils (EOS) and basophils (BAO). (D) Total platelets (PLT) and thrombocrit (PCT). $^{+}P<0.05$ vs Control; $^{*}P<0.05$ vs DSS; $^{\#}P<0.05$ vs Leptin (n=7-10).

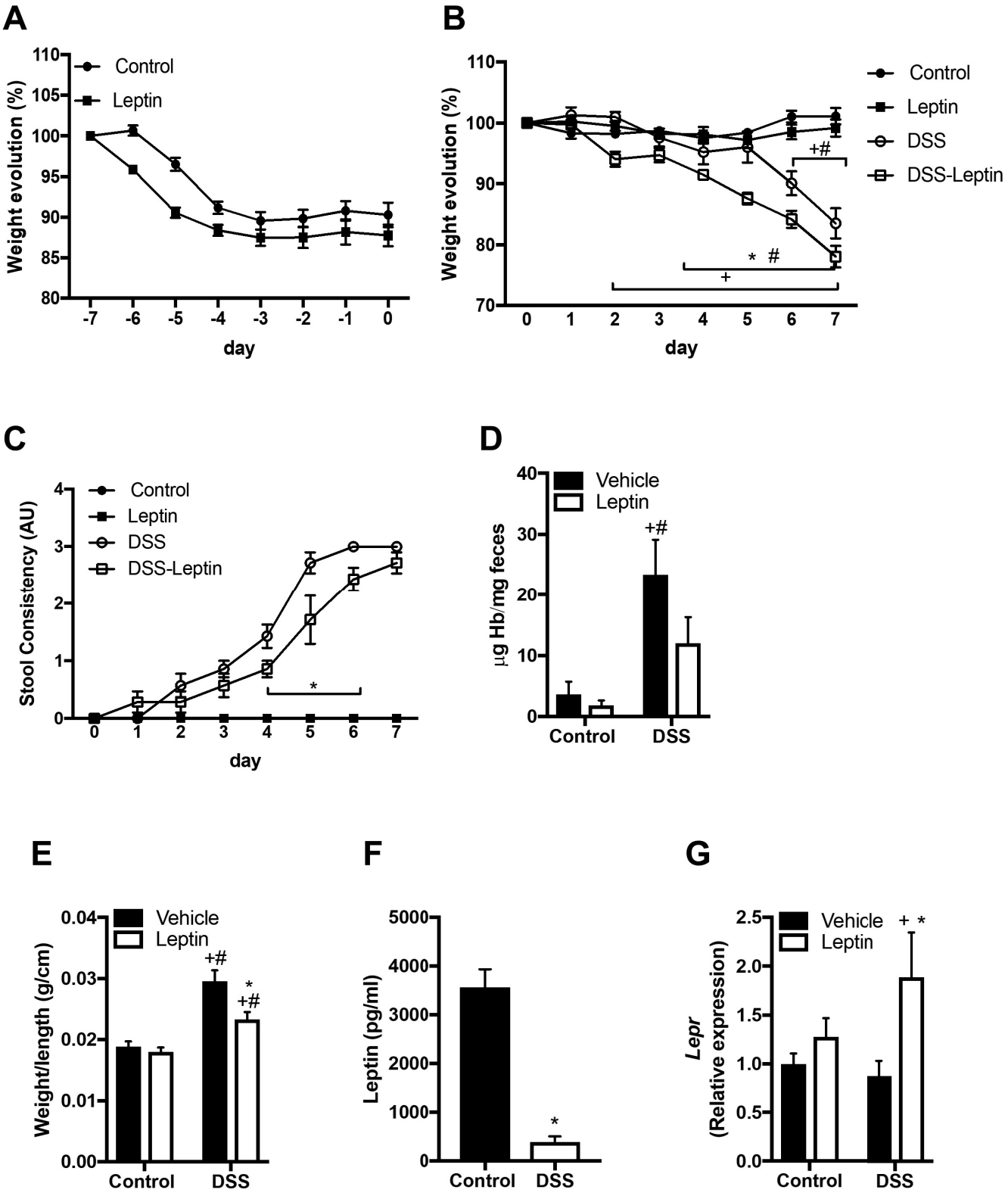
Figure 4. Cytokine and chemokine multiplex panel in colonic explants culture. Intestinal colitis was induced with 2.5% DSS in drinking water and PEG-leptin or vehicle were administered s.c. Cytokine/chemokine expression was generally upregulated in response to DSS, and PEG-leptin mice exhibited levels of TNF, IFN-γ, IL-1β and IL17A intermediate between those of the control and DSS groups, consistent with protection from colitis. A-J: TNF, IL-1β, IL-7A, IFNγ, IL-10, IL-22, IL-6, IL-12p70, GM-CSF and G-CSF. $^{+}P<0.05$ vs Control; $^{*}P<0.05$ vs DSS; $^{\#}P<0.05$ vs Leptin (n=7-10).

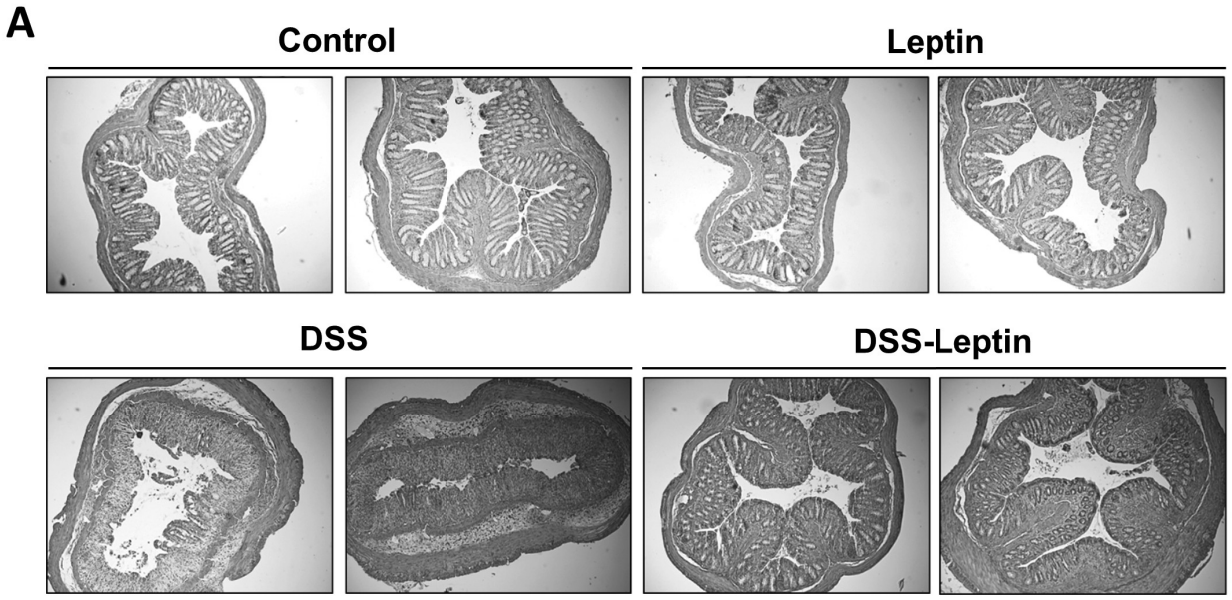
Figure 5. Epithelial barrier function is reinforced by leptin (I). Intestinal colitis was induced with 2.5% DSS in drinking water and PEG-leptin or vehicle were administered s.c. PEG-leptin reduced the increase of *Reg3γ* expression, augmented ZO-1 and claudin 4/5 (significant in some cases only, see text), and counteracted colitis-associated mucin depletion. Relative expression of *Reg3γ, Hp, Lyz-2, ZO-1, Cldn4, Cldn4* and *Muc3* in colon by RT-qPCR (A-G). Alcian blue and nuclear red staining in colon sections (H). $^{+}P<0.05$ vs Control; $^{*}P<0.05$ vs DSS; $^{\#}P<0.05$ vs Leptin (n=7-10).

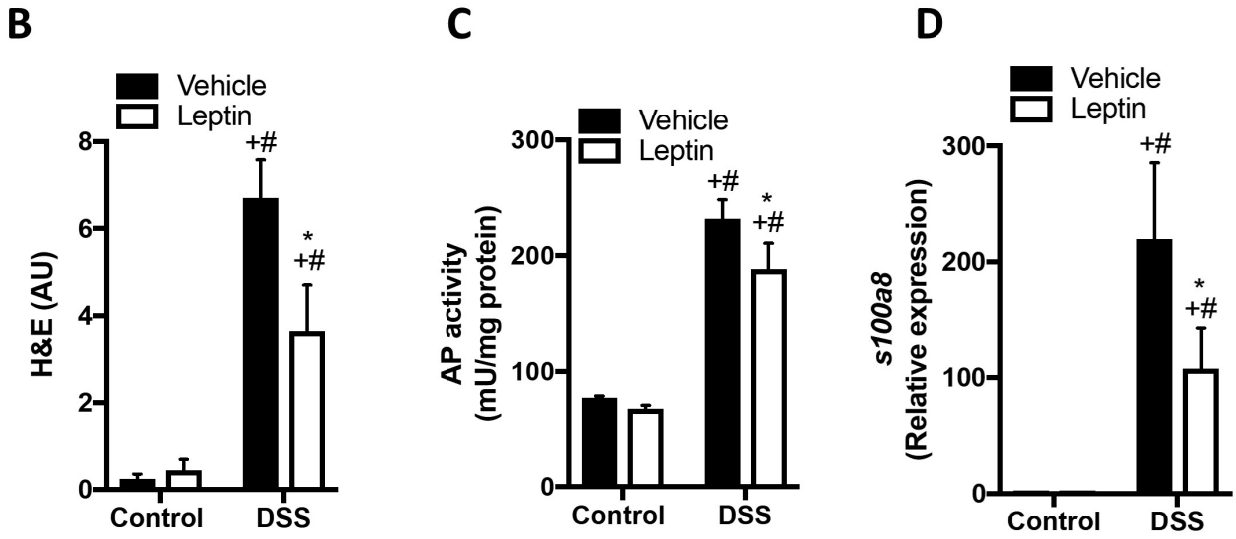
Figure 6. Epithelial barrier function is reinforced by leptin (II). Intestinal colitis was induced with 2.5% DSS in drinking water and PEG-leptin or vehicle were administered s.c. PEG-leptin reduced MLC2 phosphorylation, augmented cell proliferation and diminished apoptosis, while modulating STAT3 phosphorylation. In addition, leptin reduced epithelial permeability in Caco 2 cells. (A) Determination of pMLC2 by western blot in mouse colon. (B) Assessment of paracellular permeability of 4 kDa FITC-dextran in Caco-2 cells at 0, 10 and 50 ng/ml of leptin. Analysis of protein expression of (A) pSTAT3/STAT3, (B) PCNA and (C) BAX/BCL2 in colon homogenates by western blot. $^{+}P<0.05$ vs Control; $^{*}P<0.05$ vs DSS; $^{\#}P<0.05$ vs Leptin, except for (B), where $^{*}P<0.05$ versus Control (n=7-10).

Table 1. List of primer sequences used in qPCRs (*Mus musculus*)

Gene symbol	Forward sequence	Reverse sequence
s100a8	GATGGTGATAAAAGTGGGT	CTGTAGACATATCCAGGGAC
Reg3γ	CAGAGGTGGATGGGAGTGGAG	CACAGTGATTGCCTGAGGAAGAGA
Hp	ATGGACTTTGAAGATGACAG	GTAGTCTGTAGAACTGTCGG
Lyz-2	ATTTCCCCTCTAAGTCACAG	TGAAGAACTGACCTACAGAG
ZO-1	GGGGCCTACACTGATCAAGA	TGGAGATGAGGCTTCTGCTT
Hprt	AGGGATTTGAATCACGTTTG	TTTACTGGCAACATCAACAG
Cldn4	GACTGTGCAAAGTTACTAGC	ACCAGCAATTTGGATGTAAG
Cldn5	AACAGTTCCTACTGAGATCC	CTTTTTAACACGTCCCTCTG
Muc3	AAAGATTACCTCCCATCTCC	TAAAACTAAGCATGCCCTTG


A
Weight evolution (%)
Control
Leptin
day
B
Weight evolution (%)
Control
Leptin
DSS
DSS-Leptin
day
C
Stool Consistency (AU)
Control
Leptin
DSS
DSS-Leptin
day
D
µg Hb/mg feces
Vehicle
Leptin
Control
DSS
E
Weight/length (g/cm)
Vehicle
Leptin
Control
DSS
F
Leptin (pg/ml)
Control
DSS
G
Lepr
(Relative expression)
Vehicle
Leptin
Control
DSS


A
Control
Leptin
DSS
DSS-Leptin


B
Vehicle
Leptin
H&E (AU)
8
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0
+#
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Control
DSS
C
Vehicle
Leptin
AP activity
(mU/mg protein)
300
200
100
0
+#
*
+#
Control
DSS
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Vehicle
Leptin
s100a8
(Relative expression)
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200
100
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+#
*
+#
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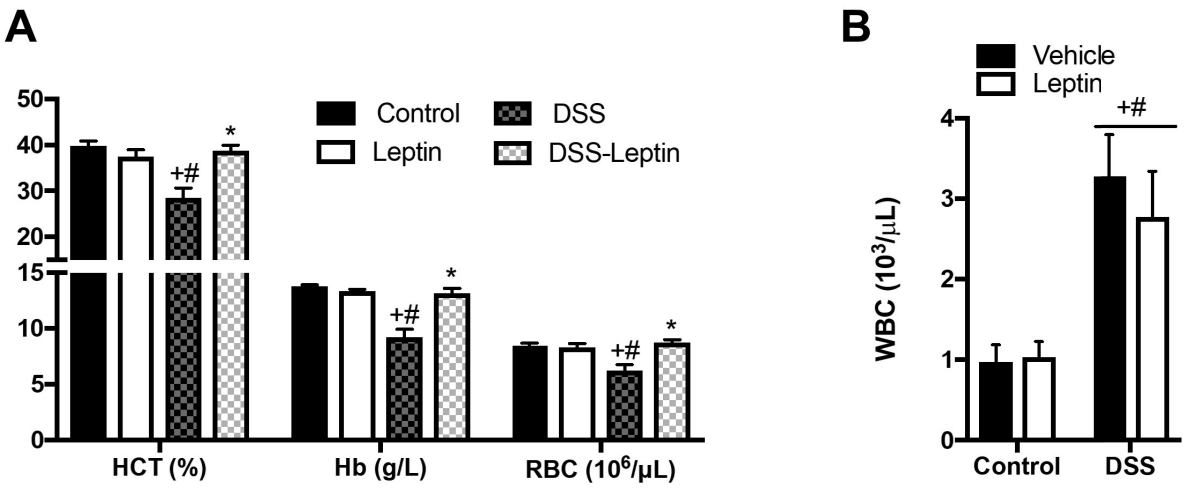
A
Control
Leptin
DSS
DSS-Leptin
50
40
30
20
15
10
5
0
+#
*
HCT (%)
Hb (g/L)
RBC (10^6/μL)
B
Vehicle
Leptin
WBC (10^3/μL)
4
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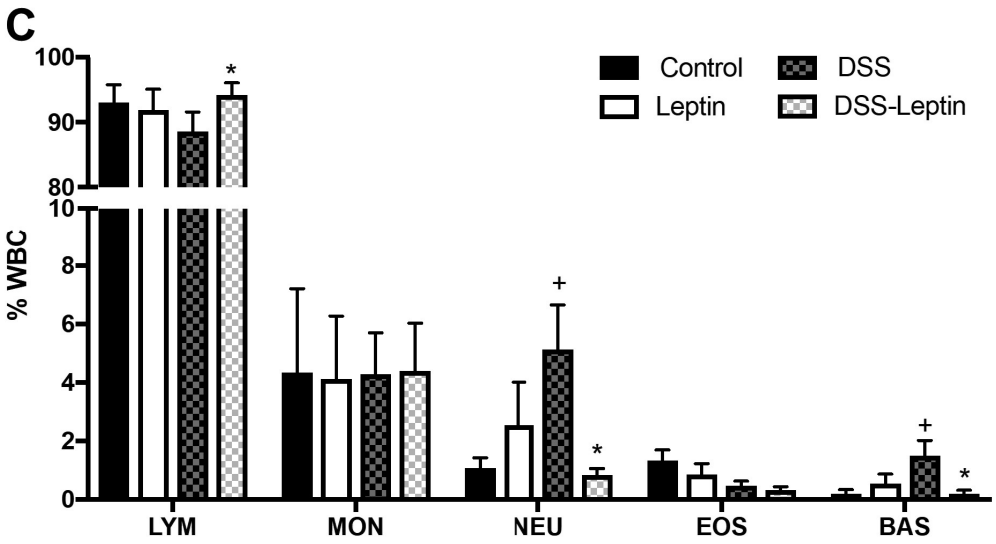
C
Control
Leptin
DSS
DSS-Leptin
% WBC
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90
80
10
8
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4
2
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*
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NEU
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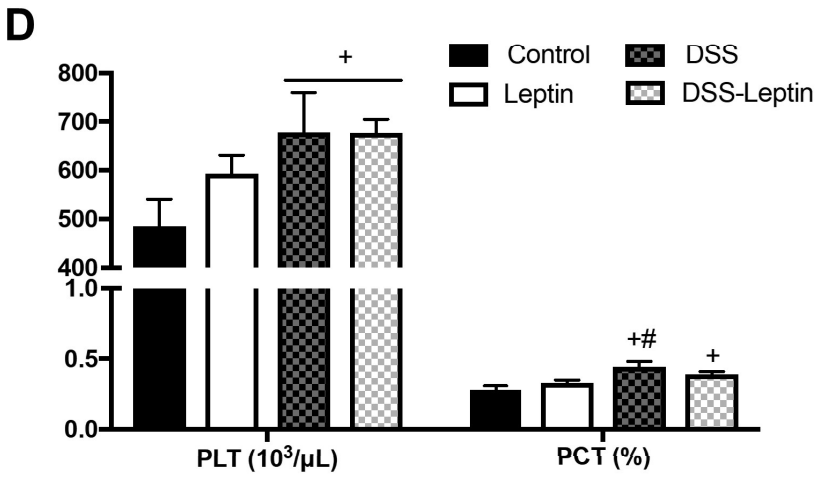
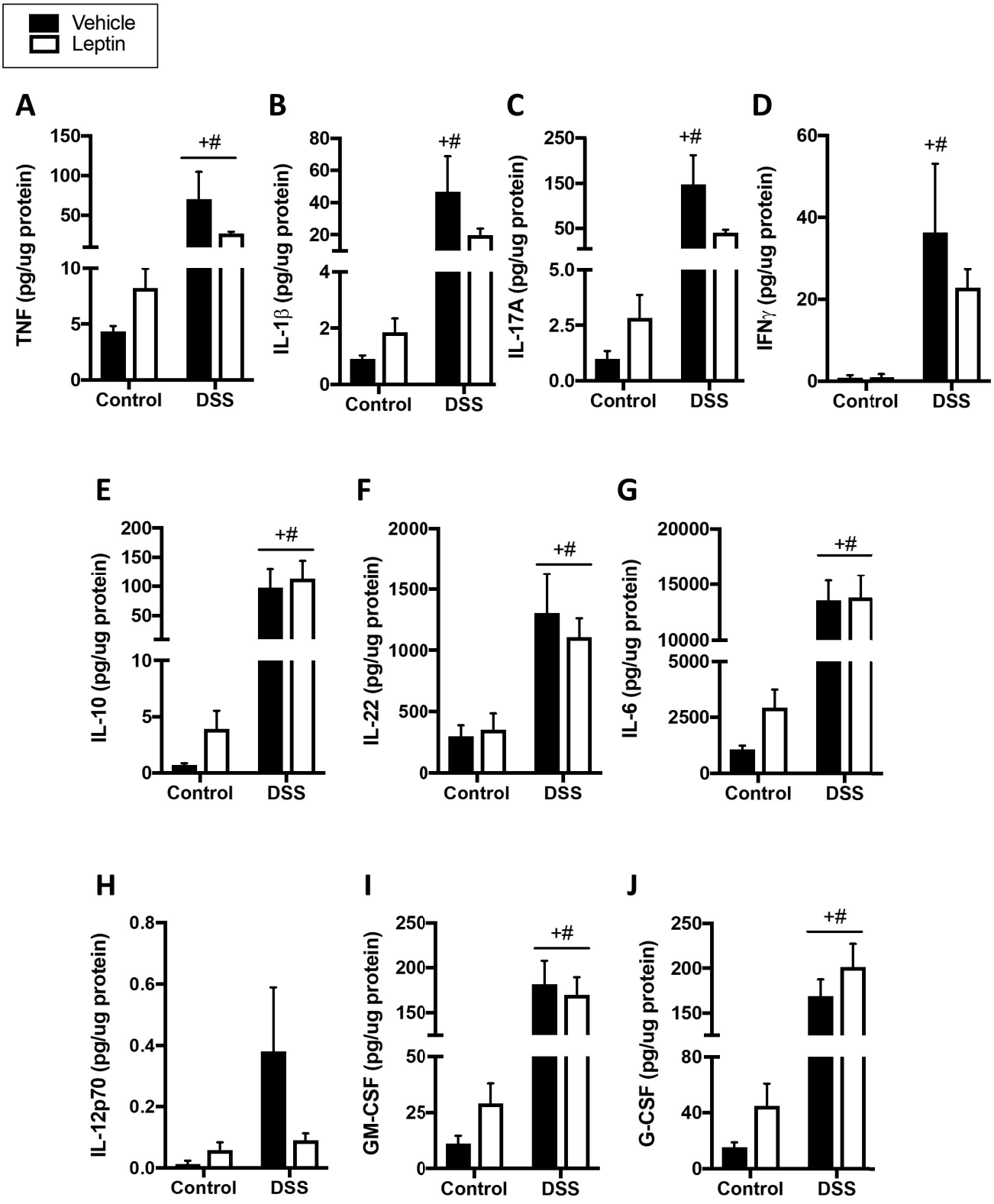
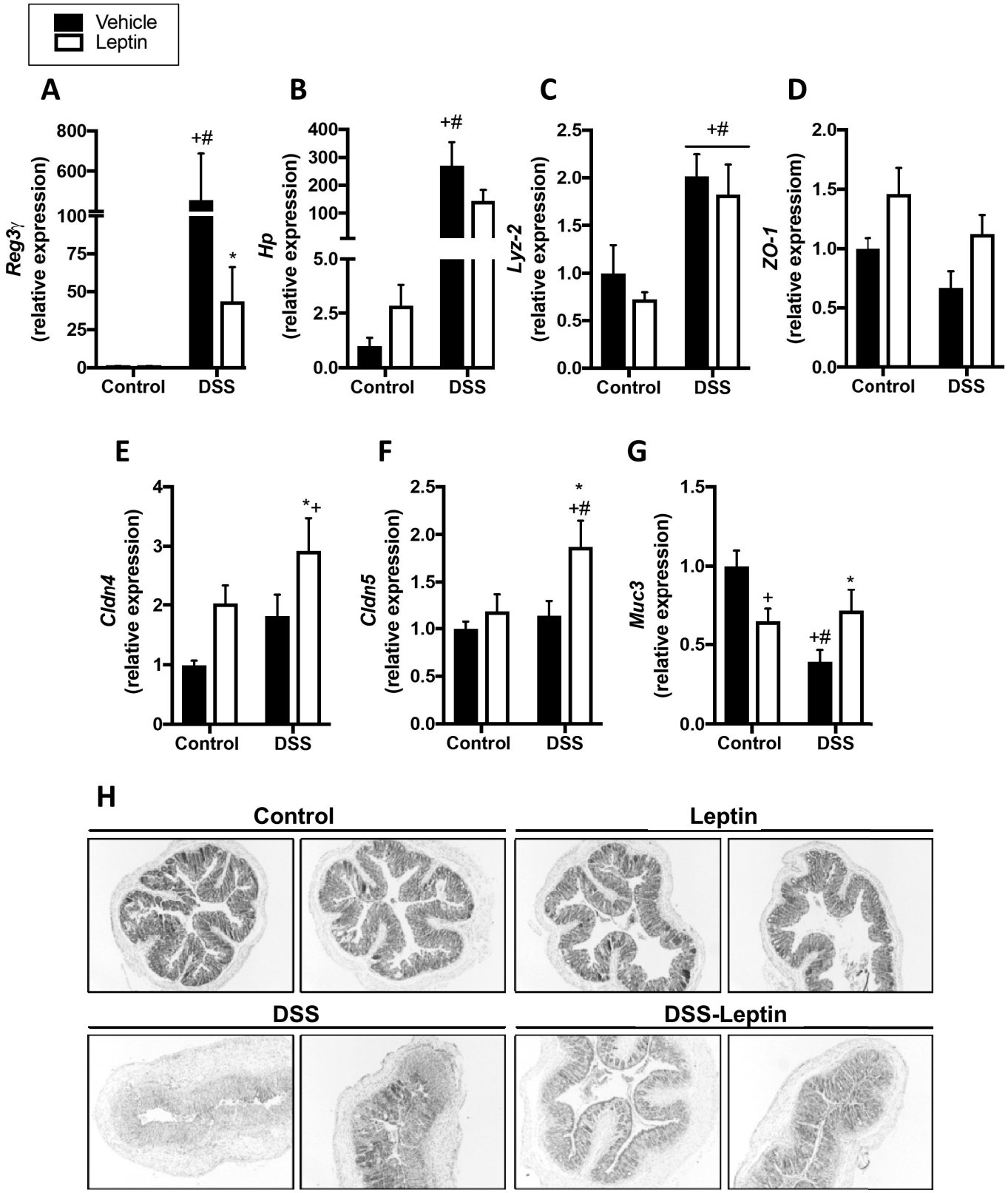
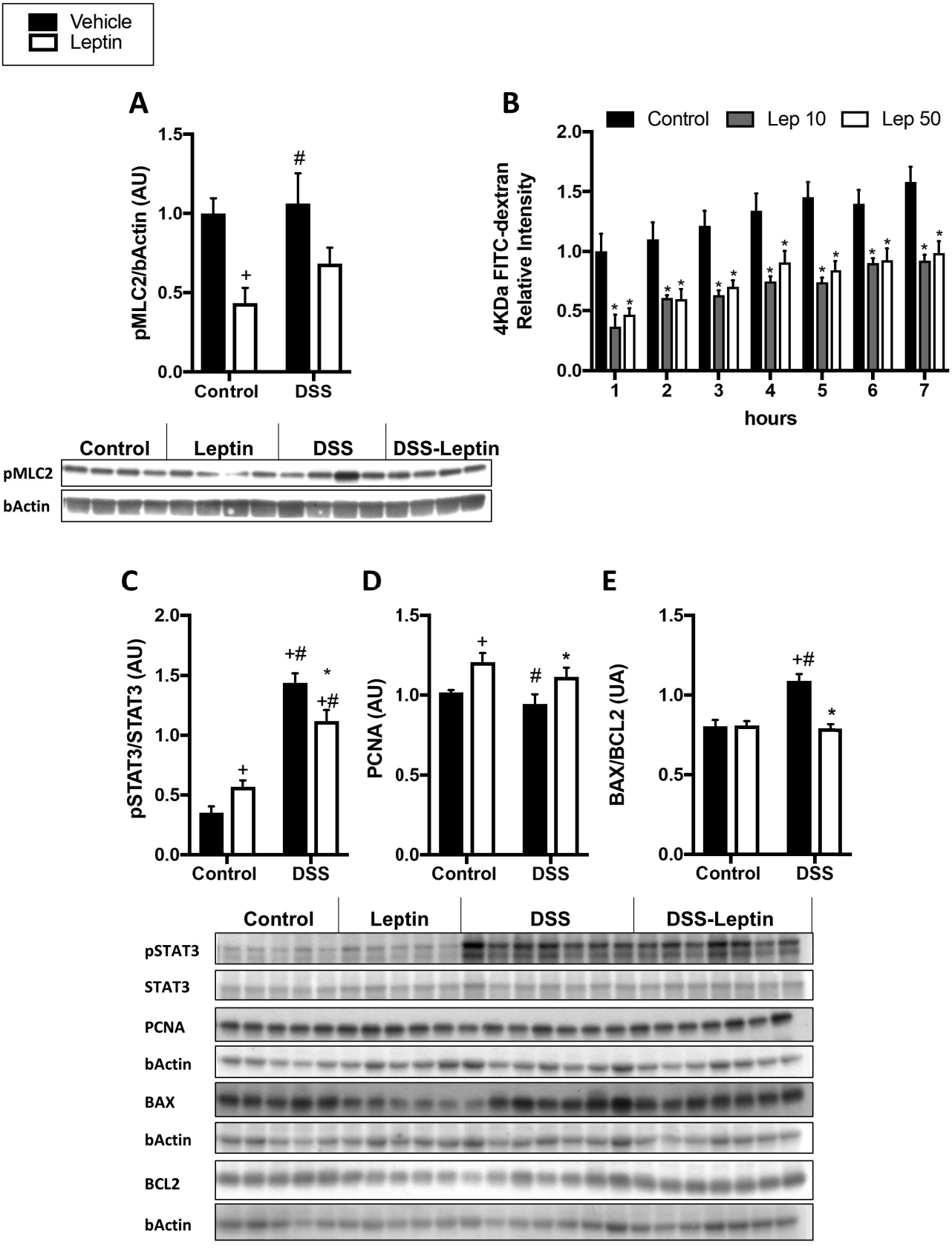

D
Control
Leptin
DSS
DSS-Leptin
800
700
600
500
400
1.0
0.5
0.0
+
+#
PLT (10^3/μL)
PCT (%)


Vehicle
Leptin
A
TNF (pg/ug protein)
150
100
50
10
5
0
+#
Control
DSS
B
IL-1β (pg/ug protein)
80
60
40
20
4
2
0
+#
Control
DSS
C
IL-17A (pg/ug protein)
250
150
50
5.0
2.5
0.0
+#
Control
DSS
D
IFNγ (pg/ug protein)
60
40
20
0
+#
Control
DSS
E
IL-10 (pg/ug protein)
200
150
100
50
10
5
0
+#
Control
DSS
F
IL-22 (pg/ug protein)
2000
1500
1000
500
0
+#
Control
DSS
G
IL-6 (pg/ug protein)
20000
15000
10000
5000
2500
0
+#
Control
DSS
H
IL-12p70 (pg/ug protein)
0.8
0.6
0.4
0.2
0.0
Control
DSS
I
GM-CSF (pg/ug protein)
250
200
150
50
25
0
+#
Control
DSS
J
G-CSF (pg/ug protein)
250
200
150
80
40
0
+#
Control
DSS


Vehicle
Leptin
A
Reg3γ
(relative expression)
800
600
100
75
50
25
0
+#
*
Control
DSS
B
Hp
(relative expression)
400
300
200
100
5.0
2.5
0.0
+#
Control
DSS
C
Lyz-2
(relative expression)
2.5
2.0
1.5
1.0
0.5
0.0
+#
Control
DSS
D
ZO-1
(relative expression)
2.0
1.5
1.0
0.5
0.0
Control
DSS
E
Cldn4
(relative expression)
4
3
2
1
0
*+
Control
DSS
F
Cldn5
(relative expression)
2.5
2.0
1.5
1.0
0.5
0.0
*
+#
Control
DSS
G
Muc3
(relative expression)
1.5
1.0
0.5
0.0
+
+#
*
Control
DSS
H
Control
Leptin
DSS
DSS-Leptin


Vehicle
Leptin
A
pMLC2/bActin (AU)
1.5
1.0
0.5
0.0
+
#
Control
DSS
Control
Leptin
DSS
DSS-Leptin
pMLC2
bActin
B
Control
Lep 10
Lep 50
4KDa FITC-dextran
Relative Intensity
2.0
1.5
1.0
0.5
0.0
*
1
2
3
4
5
6
7
hours
C
pSTAT3/STAT3 (AU)
2.0
1.5
1.0
0.5
0.0
+
+#
*
+#
Control
DSS
D
PCNA (AU)
1.5
1.0
0.5
0.0
+
#
*
Control
DSS
E
BAX/BCL2 (UA)
1.5
1.0
0.5
0.0
+#
*
Control
DSS
Control
Leptin
DSS
DSS-Leptin
pSTAT3
STAT3
PCNA
bActin
BAX
bActin
BCL2
bActin