Leptin resistant Zucker rats with trinitrobenzene sulfonic acid colitis present a reduced inflammatory response but enhanced epithelial damage

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Short title: TNBS colitis in Zucker rats

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ABSTRACT

The role of leptin in the development of intestinal inflammation remains controversial, since proinflammatory and anti-inflammatory effects have been described. This study describes the effect of the absence of leptin signaling in intestinal inflammation. Experimental colitis was induced by intrarectal administration of trinitrobenzene sulfonic acid (TNBS) to lean and obese Zucker rats (n=10). Effects on inflammation and mucosal barrier were studied. Bacterial translocation and LPS concentration were evaluated together with colonic permeability to 4 kDa FITC-dextran. Obese Zucker rats showed a lower intestinal myeloperoxidase and alkaline phosphatase activity, reduced alkaline phosphatase sensitivity to levamisole, and diminished colonic expression of *Nos2*, *Tnf* and *Il6*, indicating attenuated intestinal inflammation, associated with attenuated STAT3, AKT and ERK signaling in the colonic tissue. *S100a8* and *Cxcl1* mRNA levels were maintained, suggesting that in the absence of leptin signaling neutrophil activation rather than infiltration is hampered. In spite of the lower inflammatory response, leptin resistance enhanced intestinal permeability, reflecting an increased epithelial damage. This was shown by augmented LPS presence in the portal vein of colitic obese Zucker rats, associated with induction of tissue non-specific alkaline phosphatase, LPS-binding protein and CD14 hepatic expression (involved in LPS handling). This was linked to decreased ZO-1 immunoreactivity in tight junctions and lower occludin expression. Our results indicate that obese Zucker rats present an attenuated inflammatory response to TNBS, but increased intestinal epithelial damage allowing the passage of bacterial antigens.

NEW & NOTEWORTHY

- Obese Zucker rats, which are resistant to leptin, exhibit a diminished inflammatory response in the trinitrobenzenesulfonic acid (TNBS) model of colitis, suggesting leptin role is proinflammatory
- At the same time, obese Zucker rats present a debilitated intestinal barrier function, with increased translocation of LPS
- Zuzker rats present a dual response in the TNBS model of rat colitis

INTRODUCTION

Intestinal barrier function may be defined as the ability of the intestine to provide adequate containment of luminal microorganisms and molecules while preserving the capacity to absorb nutrients (37). The epithelial layer, together with the secretion of mucus, IgA and antimicrobial peptides, the intestinal immune system and the microbiota, are all components of the intestinal barrier whose alterations have been related to a variety of systemic and intestinal conditions, including inflammatory bowel disease (IBD) and obesity (35, 37).

Leptin, the product of the *ob* gene, is a 16 kDa protein which has an important role in the regulation of satiety, food intake, energy expenditure and fertility, among other physiological functions (10, 29, 51). This protein is mainly secreted by adipose tissue and, to a lower degree, by muscle placenta and stomach, namely by the gastric mucosa. Leptin has been classically known for its effects on metabolism, i.e. increased thermogenesis in adipose tissue and the induction of satiety in the central nervous system. However several reports have identified leptin as a modulator of both the innate and adaptive immune system, and its receptor, LepR, is expressed by several immune cells, including macrophages, natural killers, T cells and polymorphonuclear cells (19). It has been reported that leptin is able to increase the activation and proliferation of monocytes, neutrophils and T cells, and it is required for Th17 polarization (13, 19, 25, 32). Plasma and adipose tissue leptin levels increase in response to inflammatory stimuli, including tumor necrosis factor (TNF) or lipopolysaccharide (LPS) (7, 14). It has been shown that leptin elevates T helper 1 (Th1) cytokine production, such as IL-2 and interferon gamma (IFNγ), while it decreases Th2 cytokines (24).

In addition, leptin receptors have been detected at the basolateral and apical sides of intestinal epithelial cells, suggesting a function in the homeostasis of intestinal tract (49). In fact, recent studies have shown that leptin has a role in reinforcing intestinal barrier function (21), stimulating gut mucosal cell proliferation, and modulating infants' intestinal microbiome (5, 9, 22, 43). Accordingly, mice lacking leptin receptor are reportedly more susceptible to the effects of *Entamoeba histolytica* (15) and a mutation in LepR was associated with increased

susceptibility to intestinal infection by this parasite in children (11), suggesting a protective effect of leptin against epithelial injury.

Taking into account the different actions of leptin at the intestinal level (protection of the intestinal barrier function and stimulation of the inflammatory response), it is perhaps no surprise that its role in intestinal inflammation is controversial. Thus leptin-deficient ob/ob mice are less susceptible to trinitrobenzenesulfonic acid (TNBS)-induced colitis as well as to dextran sulfate sodium (DSS) colitis (39). Moreover, in the T cell transfer model of colitis inflammation is attenuated in mice receiving lymphocytes obtained from leptin-resistant db/db mice donors compared with WT cells (40). Inhibition of leptin release has been reported to be protective in rat TNBS colitis, an effect that was reversed by administration of exogenous leptin (6). These studies would indicate a proinflammatory role of leptin. On the other hand, it has been demonstrated that the administration of a PEGylated leptin antagonist receptor has protective effects in $IL10^{-/-}$ colitic mice (42). However, leptin is suggested not to play an essential role in $IL10^{-/-}$ colitis by the finding that mice lacking both IL10 and leptin ($IL10^{-/-}$ ob/ob mice) do not exhibit changes in the onset or severity of colitis (41). Similarly, in the colitis model induced by the administration of acetic acid leptin treatment had an anti-inflammatory effect (8). An additional noteworthy observation is that leptin signal transduction deficiency in T cells increases the susceptibility to *Clostridium rodentium* (32), suggesting that weakening of the immune defense in the gut (a component of barrier function) may be proinflammatory *in vivo*.

Thus leptin may play different and even opposing roles in intestinal inflammation, which may result in various outcomes depending on the context. To date these separate actions have not been evaluated globally *in vivo*. Hence we undertook the present study, using Zucker leptin resistant obese rats and their corresponding lean controls, to simultaneously determine their sensitivity to TNBS experimental colitis and changes in mucosal barrier function. Our results show that obese Zucker rats had an attenuated colitis but an enhanced deterioration of the mucosal barrier, allowing bacterial and LPS translocation to extra-intestinal organs.

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MATERIALS AND METHODS

Chemicals. Except where indicated, all reagents and primers were obtained from Sigma (Barcelona, Spain). RNeasy Lipid Tissue Mini Kit was 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, Madrid, Spain).

Animals. Twelve-week-old male Zucker rats obtained from Charles River (Barcelona, Spain) were housed in makrolon cages and maintained in air-conditioned animal quarters with a 12 h/12 h light/dark cycle. They were provided with free access to tap water and a standard chow diet (Harlan-Teklad 2014, Harlan Ibérica, Barcelona, Spain). The present study was carried out in accordance with the European Union's Directive for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (86/609/EEC), and was approved by the Ethical Committee of the University of Granada (reference 789).

Induction of colitis and experimental design. Lean and obese rats were fasted overnight and anaesthetized with isoflurane, and were then given 10 mg TNBS dissolved in 0.25 ml of 50 % ethanol (v/v) by means of a Teflon cannula, inserted 8 cm into the anus. They were kept in a head-down position for an additional 30 s, and returned to their cages. They were then randomly assigned to four different groups (n=10), namely lean control (LC), obese control (OC), lean TNBS (LT) and obese TNBS (OT). Colitic groups received the TNBS challenge, while the non-colitic groups were administered a saline enema, and were killed after 7 days. Food and water intake and body weight were measured daily.

Assessment of colonic damage. After the animals were killed, the status of the large intestine was assessed, as described previously (26). Briefly, the large intestine was opened longitudinally and scored for visible damage (hyperaemia, fibrosis, thickening and ulceration) on a 0–25 scale by an observer unaware of the treatment. Colonic myeloperoxidase (MPO) and alkaline phosphatase (AP) activities were measured spectrophotometrically, as described (26). MPO and AP are expressed as mU/mg protein and U/mg protein, respectively (1 U= 1 µmol/min of substrate converted). In addition, the sensitivity of AP to the specific inhibitor levamisole was assessed *in vitro*. A distal colon fragment was processed for histology and

146 scored according to crypt length (0-2), leukocyte infiltration (0-4), submucous enlargement (0-
147 2), epithelial erosion (0-2), loss of crypts structure (0-2) and muscle hyperplasia (1-7).
148 Immunohistochemistry analysis was also performed using anti-ZO1 (Life Technologies, New
149 York, USA) ZO-1 as described (3).

150 *Analysis of gene expression by RT-qPCR analysis.* Total RNA was obtained by the
151 TRIzol method (Thermo Fisher Scientific, Alcobendas, Madrid, Spain), 1 μg was
152 retrotranscribed and specific RNA sequences were amplified with a Bio-Rad CFX Connect real-
153 time PCR device using the following primers at 200 nM. Table 1 includes the primers used for
154 qPCR analysis.

155 *Western blot.* Tissue samples were processed for Western blot as described (34). The
156 bands were detected by enhanced chemiluminescence (PerkinElmer, Waltham, MA, USA). The
157 primary antibodies were from: Cell Signaling (Danvers, MA, USA) (phospho-STAT3, ref.
158 9145, 1:1000; STAT3 ref. 9139, 1:2000; MLC2 ref. 3672, 1:1000); Thermo Fisher Scientific
159 (Alcobendas, Madrid, Spain) (occludin,ref. (331500), 1:1000; ZO-1, ref. 617300, 1:1000) and
160 Abcam (Cambridge, UK) (phospho-MLC2 ref. ab2040, 1:1000 and β actin, ref. ab3280)
161 1:2000). The bands were quantified with the National Institute of Health software Image J. The
162 Western blots shown in Figure 4a were obtained from a single membrane, which was cut
163 horizontally and incubated with the different antibodies, in order to minimize loading errors and
164 to avoid stripping. In the Western blots shown in Figure 4b the membranes were sequentially
165 incubated with pSTAT3 and STAT3; in this case, the lanes corresponding to samples unrelated
166 to this study have been excised from the blot.

167 *Bacterial translocation and LPS measurement.* In order to determine the bacterial
168 translocation (colony forming units, CFU) to liver and the presence of circulating LPS, liver
169 was removed at necropsy and mechanically homogenized in sterile PBS (GIBCO®, Waltham,
170 MA, USA) and portal plasma was sterily collected. The presence of CFU in liver was
171 determined via serial dilutions on MacConkey agar (BD, Madrid, Spain). Colonies were
172 counted after 24 h of culture at 37°C and results are expressed as log (CFU)/g of liver. Plasma
173 LPS was measured 1:5 (v/v) dilution in sterile PBS with Pyrosperse (Lonza, Porriño, Spain)

174 1:200 and making use of an Endpoint Chromogenic Limulus Amebocyte Lysate Assay (Lonza)
175 following the manufacturer's protocol.

176 In vitro *intestinal permeability assay.* Mucosa-submucosa preparations of the distal colon
177 were mounted in Ussing chambers as described (36) using Ringers solution, with the following
178 composition (in mM): 115 NaCl, 25 $NaHCO_3$, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 2.4 K_2HPO_4, 0.4 KH_2PO_4, and
179 10 glucose. Only one piece of tissue per colonic segment was used. Care was taken to avoid any
180 areas of necrosis in the distal colon. The preparations were allowed to equilibrate for 20-30 min
181 until stable basal readings of *Isc* (I_0) and conductance (G_0) could be obtained. FITC-dextran (MW
182 4000, 2.5 mg/ml) was added to the apical side and samples were taken after 45 minutes from the
183 basolateral side. FITC-dextran was measured with a fluorometer (FLUOstar-Control, Polarstar
184 Optima, BMG Labtech, Ortenberg, Germany). Results are expressed as a percentage of FITC-
185 dextran in basolateral side compared with the total amount in both apical and basolateral sides.

186 *Plasmatic measurements.* Plasma levels of glucose, triglycerides and cholesterol were
187 determined by colorimetric assays (Spinreact, Gerona, Spain), as well as free fatty acids
188 concentration (Wako Chemicals, Zaragoza, Spain). Zonulin, corticosterone and leptin were
189 measured using commercial ELISA kits from MyBioSource (San Diego, CA, US), Enzo®
190 LifeSciences Inc, (New York, US) and R&D Systems Inc., (Minneapolis, MN, US),
191 respectively, following manufacturer's instructions.

192 *Statistical analyses.* Results are expressed as mean ± standard error of the mean (SEM).
193 All measurements were performed at least in duplicate. Differences among means were tested
194 for statistical significance by two-way ANOVA and a posteriori Tukey tests or Kruskal-Wallis
195 followed by Dunn's tests when the normality requirement was not met, as indicated. PCR data
196 were log transformed prior to statistical analysis. All analyses were carried out with the
197 GraphPad Prism 5 (La Jolla, CA, USA). Differences were considered significant at $P < 0.05$.

198 **RESULTS**

199 *Effects of TNBS-induced colitis.* As expected, the rectal administration of TNBS
200 resulted in anorexia and weight loss (Figs. 1/2), with a severe inflammatory response in the
201 large intestine, characterized by mucosal erosions, epithelial necrosis, submucosal fibrosis and
202 edema, and crypt enlargement (Fig. 3 and Table 2), resulting in a marked increase in the
203 microscopic (Fig. 3B) and macroscopic colonic damage score (Table 3). Inflammation was also
204 associated with increased colonic MPO and AP activities, both of which are biochemical
205 parameters of inflammation (Fig. 3C/D). Furthermore, the sensitivity of AP activity to the
206 specific inhibitor levamisole *in vitro* was also heightened, consistent with a change in isoform
207 expressed in the inflamed intestine, as described in the literature (23). TNBS colitis resulted also
208 in the increased expression of a number of cytokine and inflammatory marker genes, including
209 *S100a8*, *Nos2*, *Tnf*, *Il1b*, *Il6* and *Foxp3* (Fig. 4). There was no significant change in the
210 expression of *Iap* (intestinal alkaline phosphatase), *Tlr4*, *Cox2*, *Ifng*, *Il10*, or *Il23.*

211 *Obese Zucker rats present a lower degree of colonic inflammation.* TNBS colitis
212 associated weight loss was generally comparable in both colitic groups when expressed in
213 grams (except at 3-4 days). When overall body weight gain was compared (Fig. 1B and C), lean
214 rats were found to lose more weight than obese rats, but colitis associated weight changes were
215 actually similar in both strains when compared with their respective noncolitic controls (i.e.
216 considering higher weight gain in noncolitic obese Zucker rats). Thus the main effect in obese
217 animals was failure to gain weight, while lean rats exhibited increased net loss. This happened
218 both with absolute and relative values. On the other hand, anorexia was comparable in colitic
219 obese and lean rats except at 1 d, when obese Zucker rats exhibited a higher food intake. In the
220 last day lean rats recovered their normal consumption, while obese rats remained below their
221 reference intake (which is 40-50% higher than normal, see Fig. 2A and 2B).

222 The colonic weight:length ratio was increased to a comparable extent in lean and obese
223 colitic rats (Table 3). The colonic damage score, necrotic area (Table 3) and histological
224 analysis (Fig. 3B) revealed a comparable degree of tissue injury in the LT and OT groups. At
225 the biochemical level, MPO activity was significantly lower in the OT group, failing to reach

significance vs. obese noncolitic rats (p=0.07, Fig. 3C). In addition, the sensitivity of colonic AP to the specific inhibitor levamisole *in vitro* was also significantly reduced in the OT group, although enzymatic activity was similarly increased in both colitic groups (Fig. 3D). RT-qPCR analysis showed that the gene expression level of inflammatory mediators in the OT group was generally similar (*S100a8*, *Il1b*, *Il6*, *IL-17a*, *Cxcl1*, *Tnf*, *Foxp3*) to that found in the LT group, although *Nos2* was downregulated (Fig. 4). *Alpl* –encoding the tissue nonspecific AP isoform– appeared to have a lower expression but failed to reach significance. Leptin is known to activate the MAPK and AKT pathways that regulate the immune system. As expected, in colitis our data showed increased phosphorylation for both proteins, indicating an induction in normal animals. This activation was impaired in the obese colitic rats (Fig. 5A/B).

Thus, obese Zucker rats display enhanced macroscopic colonic injury and protracted anorexia, while lean rats exhibited a greater severity of inflammation based on biochemical and molecular markers, plus a higher body weight loss.

Leptin resistance increases intestinal permeability and intestinal bacterial translocation in TNBS induced colitis. In order to explore the integrity of the intestinal epithelium, we decided to analyze the presence of luminal bacteria in the liver. Despite the lower severity of colitis, we found similar bacterial counts in the liver of obese colitic rats overall. However, only 1/9 rats did not display CFU in the OT group while 4/10 were negative in the LT group (Fig. 6A). Moreover, portal vein plasma LPS levels were higher in the OT group than in the controls (Fig. 6B). We also studied the hepatic expression of several proteins implicated in the management of LPS originating from the intestinal lumen (1). Lean rats with TNBS colitis exhibited an increased expression of *Lbp* and *Alpl* (TNAP) in the liver, compared with the lean control group (Fig. 6C), while augmented *Lbp*, *Cd14*, *Alpl* expression in colitic obese rats compared non colitic obese rats was noted. The barrier defect was further evaluated measuring the permeability to 4 kDa FITC-dextran *ex vivo*. Transepithelial passage of the tracer was significantly increased by TNBS colitis and obesity (two-way ANOVA), but not at group level (Fig. 6D).

Seeking a better understanding of the results described above, we studied other parameters of intestinal permeability. First, we analyzed the plasma concentration of zonulin by ELISA, since increased intestinal permeability has been associated with high circulating levels of this protein (48). Second, we investigated the expression of the tight junction proteins ZO-1 and occludin in the colon, performed by Western blot. As expected, and consistent with the above, zonulin concentration was significantly increased by colitis (single factor in two-way ANOVA), although not at group level (Fig. 6E). There was no change in ZO-1 protein expression, while occludin was upregulated in the LT group and less so in obese colitic rats group (Fig. 5D). Expression of the tight junction protein ZO-1 was further assessed by immunofluorescence (Fig. 7). ZO-1 immunoreactivity was found to be lower in obese rats, with no effect of colitis. Tight junctions are regulated in part via phosphorylation of myosin light chain 2, which is positively linked to permeability. Western blot analysis showed that phosphorylation was significantly increased by TNBS colitis in lean but not obese rats (Fig. 5D).

Haptoglobin is an acute phase α-sialoglycoprotein with protective properties against colitis. Colonic haptoglobin (*Hp*) mRNA expression was unaffected in lean animals, but increased in obese rats (Fig. 8B). The colonic expression of lysozyme 2 (*Lyz2* gene) was downregulated in obese control rats and upregulated after colitis, with no change in lean animals (Fig. 8D). Lysozyme like 1 (*Lyz1* gene) and cyclin D1 (*Ccnd1* gene) showed a similar profile. The expression of the antibacterial peptide REG3γ (*Reg3g* gene) and trefoil factor 3 (*Tff3*), as assessed by RT-qPCR, which are also involved in barrier function in the gut, was not affected (Fig. 8). Similarly, the status of STAT3 phosphorylation, which is relevant to epithelial dynamics, was comparable in LT and OT rats, although it was higher than in noncolitic animals (Fig. 5A). To further characterize epithelial dynamics, the expression of *Pcna*, *Erbb2*, *Wnt2* and *Egfr* was studied by RT-qPCR. No major differences were found in the expression of these markers. Interestingly, *Lgr5*, a marker of stem cells, was found to be downregulated in noncolitic obese rats, with a similar trend in inflammatory conditions, suggesting a negative impact on stem cell proliferation.

Leptin resistance alters the metabolic effect of TNBS colitis. As expected, obese Zucker rats presented with marked hyperleptinemia and hyperlipidemia (augmented triglycerides, free fatty acids and total cholesterol, Fig. 9A-C), not related to fasting or anorexia, since plasma blood glucose levels were unaltered by either obesity or colitis (Fig. 9D). TNBS colitis was associated with a slight (approximately 15-25%), nonsignificant decrease in all three lipid plasma parameters in lean rats. This became a much more prominent and significant effect in obese rats, where hyperlipidemia was fully normalized. Plasma leptin was increased in obese rats and was unchanged with colitis (Fig. 9E).

Because hyperlipidemia has been associated to increased corticoids such as in Cushing syndrome, plasma corticosterone was measured. Corticosterone was substantially increased in obese vs. lean rats in the absence of colitis (Fig. 9F). Remarkably, despite the expected stress and inflammation in the context of colitis, it was unchanged in lean rats and was actually decreased to control values in obese colitic rats.

DISCUSSION

Obesity is related to a basal inflammatory status and has been linked with several inflammatory diseases, including atherosclerosis, hypertension, cancer and autoimmune diseases. Thus a link between obesity and IBD has been long suspected; however, the evidence available in this regard is conflicting. For instance, in obese patients undergoing bariatric surgery IBD has been reported to improve (20), but also to be aggravated or to be elicited *de novo* (2). Several adipokines like leptin, adiponectin, resistin, visfatin, grelin or apelin have been reported to be related with intestinal inflammation (4, 18, 38, 45, 47). However, the exact role of leptin in IBD is equivocal thus far, and its effects remain controversial and poorly described in many ways. As explained above, leptin has been described to modulate the immune system, epithelial dynamics and permeability, and the microbiota, with conflicting reports in different experimental systems (12, 16, 21, 40, 41, 44). Other protective effects of leptin in inflammation have been described, including a model of puncture-induced sepsis and ileocecal ligation, in which survival of hyperleptinemic rodents was lengthened, while ob/ob mice had a higher mortality rate (46).

We used Zucker obese rats as an obesity model induced by leptin resistance to evaluate the influence of this phenotype on the experimental colitis induced by TNBS. Based on the above considerations, it was expected that obesity enhanced the inflammatory response, whereas leptin effects were difficult to predict. Our results globally indicate that colonic inflammation was attenuated in obese rats, based on MPO activity, AP sensitivity to levamisole, and the colonic expression of *Nos2*, *Tnf* and *Il6*. Comparable leukocyte infiltration was noted in lean and obese rats by histology. MPO, a neutrophil protein, was diminished in obese colitic rats while *Cxcl1*, one of the main neutrophil chemokines in rodents, and *S100a8*, encoding one of the main proteins expressed by neutrophils, were not significantly altered. Thus our data suggest that neutrophils, which typically dominate the infiltrate in the early stages of colitis, and specifically in the TNBS colitis model in rats (28), are similarly recruited, but possibly more weakly activated, in obese rats (19). This is consistent with the fact that leptin indirectly

326 activates neutrophils via TNF induction (50), a cytokine whose expression was upregulated in
327 lean but not obese rats in our study.

328 Despite the lower degree of colonic inflammation, colitic obese Zucker rats presented
329 increased colonic thickening and protracted anorexia, plus a trend to a larger intestinal area
330 affected by necrosis. Further, subjectively the appearance of obese rats was worse than that of
331 lean rats (shivering, spontaneous mobility, huddling behaviour). These data correlate with
332 measured parameters of barrier function. Thus obese colitic rats showed augmented
333 translocation of LPS and bacteria. Colonic expression of occludin and ZO-1 appeared to be
334 depressed in obese rats, but without reaching significance. Colonic permeability to 4 kDa FITC-
335 dextran *in vitro* was augmented by both obesity and colitis and was highest in obese colitic rats,
336 consistent with deterioration of barrier function, but statistical power was insufficient to pick up
337 further individual group differences, due to the known variability of this technique. Conversely,
338 MLC2 phosphorylation was actually reduced in obese colitis rats. In our model increased LPS
339 translocation is indicated additionally by the data obtained from the liver, which suggest
340 increased exposure to LPS via the portal vein in obese colitic rats, with induction of *Alpl*, *Lbp*
341 and *Cd14* expression.

342 These data are consistent with our previous study showing enhanced barrier function in
343 experimental colitis by treatment with PEGylated leptin (33). Intestinal inflammation, including
344 IBD, is associated with significant barrier function defects. The colitis model used in this study,
345 one of the most widely applied in the field, is based partially on disruption of the barrier with a
346 subsequent immunological reaction to TNBS haptenated proteins, and is therefore associated
347 also to substantial barrier defects. Of note, our data are not generally consistent with changes in
348 epithelial dynamics, despite lower colonic AKT and ERK phosphorylation (consistent with
349 defective leptin signaling). In turn, resistance to leptin was associated to downregulated
350 expression of *Lgr5*, suggesting compromised stem cell proliferation. There was little or no
351 change in antibacterial defense genes (*Reg3γ*, *Lyz2*, *Lyz1*). In our study, increased permeability
352 may account for the overall worse appearance of obese colitic rats, as endotoxemia may lead to
353 fever and malaise. In addition, a less robust immune response may paradoxically tend to

354 increase inflammation in the intestine, because of defective containment of microbiota
355 components (37). In our case, this mechanism might explain the increase in colonic thickening
356 and the trend toward enhanced necrosis and histological injury. This may also account for the
357 protective effects of leptin in colitis, inasmuch as direct barrier defects may be offset by
358 activated innate immunity. It is possible that endotoxemia leads to anorexia as part of the
359 systemic response. The consequences are complex: weight loss may favor an improvement of
360 barrier function, consistent with the results obtained, but this is speculative as food intake was
361 not independently manipulated. Anorexia may debilitate barrier function (17).

362 During the time period where food intake was comparable (i.e. the first 4 days after
363 TNBS), the percentage of colitis induced weight loss was ~50% lower in obese rats when
364 compared to lean rats. However, colitis associated weight changes were comparable in lean and
365 obese rats when compared with the respective noncolitic controls. This suggests that lower
366 energy expenditure in Zucker obese rats is the most likely explanation for this finding, although
367 an effect of lower colitis severity cannot be ruled out. In addition, TNBS colitis had a very
368 marked effect in the metabolic profile of obese rats, with dramatic decreases in plasma levels of
369 free fatty acids, triglycerides and total cholesterol. These effects, only hinted in lean rats,
370 represent a normalization of these parameters, which were elevated in uninflamed obese
371 animals. Although decreased food intake associated to colitis may be suspected to account for
372 this, it has been previously described that the response of obese Zucker rats to fasting is the
373 opposite, i.e. an even greater increase in hyperlipidemia (30). Of note, noncolitic obese rats
374 presented increased corticosterone levels, which were virtually normalized with colitis, i.e. there
375 is a close correlation between lipidemia and plasma corticosterone levels. Since
376 hypertriglyceridemia and hypercholesterolemia are commonly seen in Cushing syndrome, it is
377 likely that excess corticosterone is causally involved in this phenotype in (noncolitic) obese
378 Zucker rats. In turn, this effect may be related to induction of the enzyme 11β-dehydrogenase
379 hydroxyesterol 1 in adipose tissue (31) and/or to lack of inhibition by leptin of the
380 hypothalamic-pituitary-adrenal axis. It is important to note that, although experimental colitis is

obviously a stressful situation, no changes are typically seen in plasma corticosterone levels in a semi-chronic setting (unpublished observations) (27).

Overall, our results indicate that obese Zucker rats present an attenuated inflammatory response to TNBS, but an enhanced defect in barrier function. The latter may be accounted for by lack of leptin signaling, by obesity, or both. In turn, decreased inflammation may be attributed to the absence of leptin signaling. Our data are consistent with the lower inflammatory response found in mice models of colitis in the absence of leptin (32, 39, 40) and with the higher susceptibility to sepsis (46).

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DISCLOSURES

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

OMA and FSM: Experimental design, analysis of results and manuscript writing. BR, RGB, MA: Carried out the experiments, analyzed data and contributed to manuscript writing.

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567
568

569 **FIGURE LEGENDS**

570 **Figure 1. Effects of TNBS-induced colitis on body weight in Zucker rats**. A. Body weight
571 evolution. B. Body weight change (g) at the end of the experiment. C. Body weight change as
572 percentage relative to initial weight. D. Body weight change (percent of initial weight). Data are
573 expressed as mean ± SEM. $^{+}P < 0.05$ vs. LC; $*P < 0.05$ vs. LT; $^{\#}P < 0.05$ vs OC. LC: lean
574 control; OC, obese control; LT, lean TNBS; OT, obese TNBS. Two way ANOVA followed by
575 Tukey tests was applied.

576 **Figure 2. Effects of TNBS-induced colitis on food intake in Zucker rats.** Food intake is
577 shown in absolute (A) and relative values (percent of basal consumption, B). Data are expressed
578 as mean ± SEM. $^{+}P < 0.05$ vs. LC; $*P < 0.05$ vs. LT; $^{\#}P < 0.05$ vs OC. LC: lean control; OC,
579 obese control; LT, lean TNBS; OT, obese TNBS. Two way ANOVA followed by Tukey tests
580 was applied.

581 **Figure 3. Effects of TNBS-induced colitis on morphological and biochemical parameters in**
582 **Zucker rats.** A. Representative photographs of colonic segments. Colitis is evidenced by
583 colonic shortening, the presence of distal necrotic areas in the process of progressive healing,
584 and thickening. B. Histological score. C. Representative haematoxylin & eosin micrographs. D.
585 Colonic MPO activity. E. Colonic AP activity and in vitro sensitivity to levamisole. Data are
586 expressed as mean ± SEM. $^{+}P < 0.05$ vs. LC; $*P < 0.05$ vs. LT; $^{\#}P < 0.05$ vs OC. LC: lean
587 control; OC, obese control; LT, lean TNBS; OT, obese TNBS. Two way ANOVA followed by
588 Tukey tests was applied.

589 **Figure 4. Evaluation of colonic inflammation by RT-qPCR**. The relative expression of
590 different genes using the 18S RNA subunit as a reference house-keeping gene is shown. Data
591 are expressed as mean ± SEM. $^{+}P < 0.05$ vs LC; $*P < 0.05$ vs LT; $^{\#}P < 0.05$ vs OC. LC: lean
592 control; OC, obese control; LT, lean TNBS; OT, obese TNBS. Two way ANOVA followed by
593 Tukey tests or Kruskal-Wallis followed by Dunn's tests was applied depending on normality of
594 the data. Log transformation was applied in all cases.

595 **Figure 5. Tight junction proteins and mucosal barrier function related genes in the**
596 **intestinal epithelium as assessed by Western blot.** (A. B, C) Colonic expression of
597 pSTAT3/STAT3, pAKT/AKT and pERK/ERK, respectively. (D) Colonic expression of zonulae
598 occludens-1 (ZO-1), occludin and phosphorylated myosin regulatory light chain-2 (pMLC-2).
599 Representative Western blots and quantification are shown. Data are expressed as mean ± SEM.
600 $^{+}P < 0.05$ vs LC; * $P < 0.05$ vs LT; $^{\#}$ $P < 0.05$ vs OC. LC: lean control; OC, obese control; LT,
601 lean TNBS; OT, obese TNBS. Two way ANOVA followed by Tukey tests was applied.

602 **Figure 6. Assessment of gut barrier integrity parameters and bacterial translocation.** A.
603 Bacterial translocation to liver, expressed as log CFU/g tissue. B. Plasma LPS concentration. C.
604 Relative mRNA expression of hepatic markers of endotoxemia. The relative expression of
605 different genes using the 18S RNA subunit as a reference house-keeping gene is shown. D.
606 Intestinal permeability of distal colon to FITC-dextran (4 KDa) assessed by Ussing Chambers.
607 E. Plasma zonulin concentration. Data are expressed as mean ± SEM. $^{+}P < 0.05$ vs LC; *$P <$
608 0.05 vs LT; $^{\#}P < 0.05$ vs OC.LC: lean control; OC, obese control; LT, lean TNBS; OT, obese
609 TNBS. Two way ANOVA followed by Tukey tests was applied. Log transformation was
610 applied to all PCR data.

611 **Figure 7. Immunohistochemical analysis of ZO-1 expression.** ZO-1 signal (red) was detected
612 by confocal microscopy. Nuclei are stained with DAPI (4', 6-diamidino-2-fenilindol). LC: lean
613 control; OC, obese control; LT, lean TNBS; OT, obese TNBS.

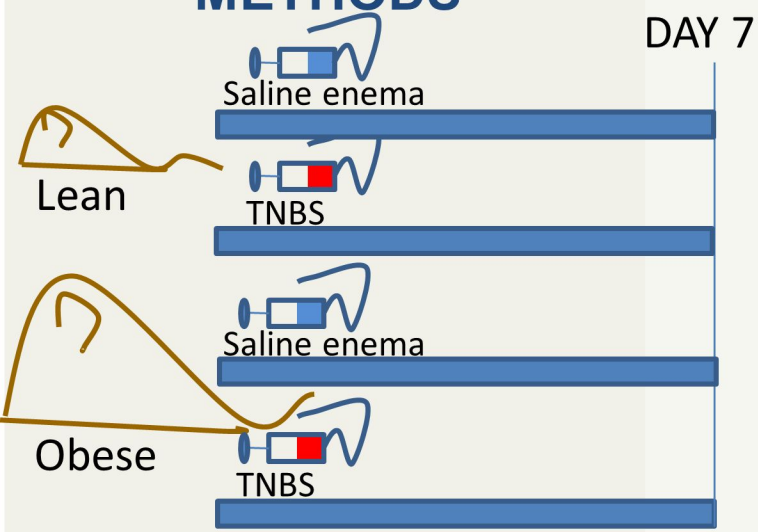
614 **Figure 8. Tight junction proteins and mucosal barrier function related genes in the**
615 **intestinal epithelium as assessed by RT-qPCR.** Colonic expression of: (A) claudin 4 (*Cldn4*);
616 (B) Haptoglobin (*Hp*); (C) EGF receptor (*Egfr*); (D) Lysozime 2 (*Lyz2*); (E) RegIIIγ (*Reg3g*);
617 (F) Lysozime-like 1 (*Lyzl1*); (G) Cyclin D1 (*Ccnd1*); (H) *Pcna*; (I) Trefoil factor 3 (*Tff3*); (J)
618 Erb-b2 receptor tyrosine kinase 2 (*Erbb2*); (K) *Lgr5*; (L) *Wnt2*. The relative expression of
619 different genes using the 18S RNA subunit as a reference house-keeping gene is shown. Data
620 are expressed as mean ± SEM. $^{+}$ $P < 0.05$ vs LC; * $P < 0.05$ vs LT; $^{\#}$ $P < 0.05$ vs OC.LC: lean

621 control; OC, obese control; LT, lean TNBS; OT, obese TNBS. Two way ANOVA followed by
622 Tukey tests was applied. Log transformation was applied to all PCR data.

623 **Figure 9. Metabolic parameters**. Plasma levels of triglycerides (A), free fatty acids (B),
624 cholesterol (C), glucose (D), leptin (E), corticosterone (F). Data are expressed as mean ± SEM.
625 $^{+}P < 0.05$ vs LC; $*P < 0.05$ vs LT; $^{\#}P < 0.05$ vs OC. LC: lean control; OC, obese control; LT,
626 lean TNBS; OT, obese TNBS. Two way ANOVA followed by Tukey tests was applied.

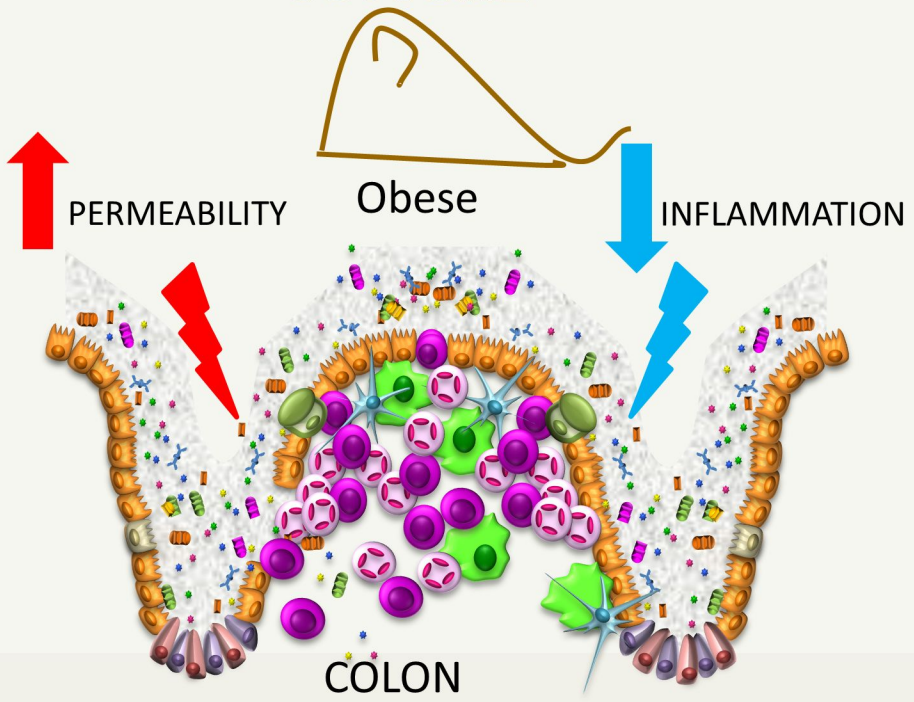
Leptin resistant Zucker rats with trinitrobenzene sulfonic acid colitis present a reduced inflammatory response but enhanced epithelial damage

METHODS



Permeability	Inflammation
COLON:	COLON:
FITC-Dextran	Myeloperoxidase
ZO-1	Alkaline phosphatase
Occludin	Nos2, Tnf, IL6, S100A8, Cxcl1
LIVER:	STAT3, AKT, ERK
LPS	
CD14	

OUTCOME



CONCLUSION Leptin resistant Zucker rats present increased intestinal permeability and decreased inflammation in response to TNBS.

Table 1. Sequence of primers used for PCR analysis.

Gene	Forward sequence	Reverse sequence
18S	CCATTGGAGGGCAAGTCTGGTG	CGCCGGTCCAAGAATTTCAAC
Cd14	AGAATCTACCGACCATGAAG	GATCTGAGAAGTTGCAGTAG
Cldn4	AAAAAGACTTTCTCAGCCC	AACTCAGGATGACTCCTAAC
Cxcl1	GCTCTGAGACAATGAACGCTACAC	TTCTTCCACATCTATGCCACTTGAG
Ccnd1	AAAAACAAACCACAAAGACG	AATTTTCCTCAGTTTGGATGG
Cox2	AGTCAAAGACACTCAGGTAGA	GAGTCTGCTGGTTTGGAATAG
Foxp3	CTGCTTGGCAGTGCTTGAGAA	CCCAGGAAAGACAGCAACCTT
Hp	AAAAACAAACCACAAAGACG	AATTTTCCTCAGTTTGGATGG
Iap	GACATTGATGTGATCCTTGG	CTCTCGATTCCAAACATACC
Ifng	GAAAGCCTAGAAAGTCTGAAG	AGTATTTTCGTGTTACCGTC
Il10	TCTCCCCTGTGAGAATAAAAG	TAGACACCTTTGTCTTGGAG
Il17a	TGGACTCTGAGCCGCAATGAGG	GACGCATGGCGGACAACAGAGG
Il1b	AATGACCGTTTCTTTGAGGCTG	CGAGATGCTGCTGTGAGATTT
Il6	GCTCTGGTCTTCTGGAGTTCCG	TTGGATGGTCTTGGTCCTTAGCC
Lbp	ATGTCAGTCCTGGGAATC T	CATTGAACATGCCGACTTTG
Lyz2	ATCAATAGCCGATACTGGTG	CCGATAGATCTCGGTTTTTAC
Lyzl1	CACAAGGGATGAACTATTGG	TGTGAGGAAAAGGGATACTC
Nos2	GGTCTTTGAAATCCCTCCTG	CAGAAGTCTCGAACTCCAATC
Reg3g	CTGTTCATATTTCAGGTACGAG	CTCCACTAAGAATAGACACAAG
S100a8	CTGGTATAAAAGGGAATCACC	TTATTCTGCACAAACTGAGG
Tff3	GTATGGCTCCAACAAATGTC	GTACATTCTGTCTCTTGCAG
Tlr4	ACCTAGATCTGAGCTTCAAC	TTGTCTCAATTTCACACCTG
Tnap	GCCAGAGAAAGAGAAAGACC	TCTTGGAGAGAGCCACAA
Tnf	GTCGTAGCAAACCACCAA	GCTGACTTTCTCCTGGTATG

Table 2. Microscopic parameters.

Group	Crypt length (μm)	Submucosal thickness (μm)
LC	252 ± 12	28 ± 12
OC	300 ± 12^{+}	48 ± 12
LT	392 ± 12^{+}	92 ± 11^{+}
OT	396 ± 16$^{+\#}$	132 ± 13$^{+\#}$*

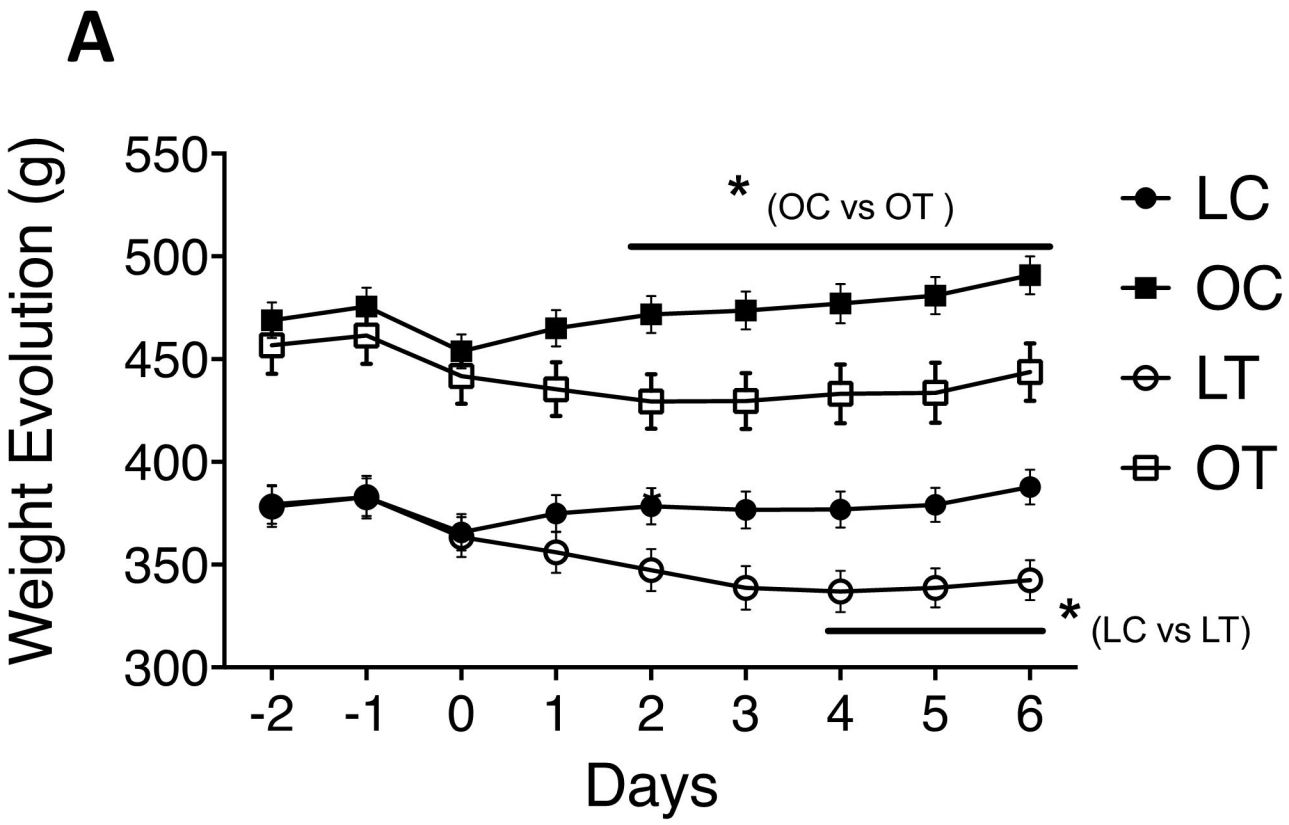
Data are expressed as mean ± SEM. $^{+}P < 0.05$ vs. LC; *$P < 0.05$ vs. LT; $^{\#}P < 0.05$ vs OC. LC: lean control; OC, obese control; LT, lean TNBS; OT, obese TNBS.

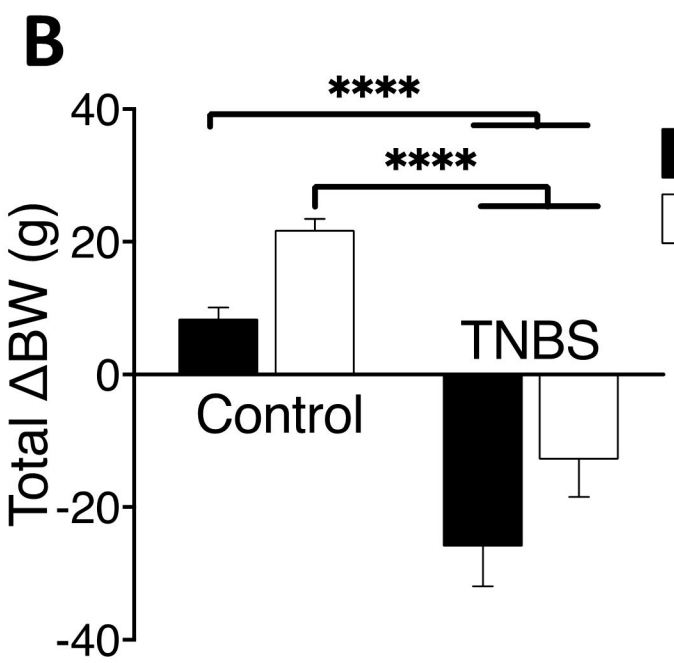
Table 3. Macroscopic parameters

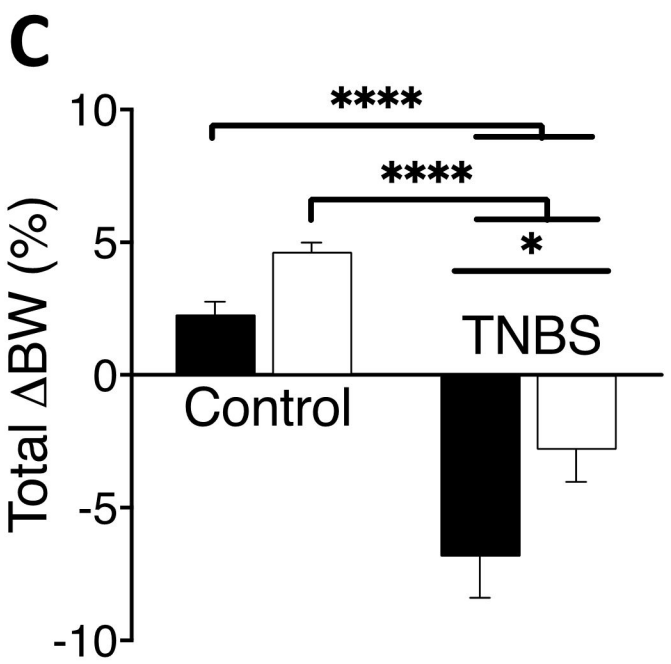
	LC	OC	LT	OT
Macroscopic Score (AU)	1.4 ± 0.5	0.5 ± 0.2	10.4 ± 0.2$^{+\#}$	11.9 ± 1.6$^{+\#}$
Necrotic area (cm^2)	-	-	8.4 ± 1.3	12.7 ± 2.8
Weight:Length ratio (mg/cm)	71.6 ± 0.0	83.5 ± 0.0^{+}	285.6 ± 0.0$^{+\#}$	431.4 ± 0.1$^{+\#}$
Spleen weight (g)	0.63 ± 0.03	0.63 ± 0.03	0.85 ± 0.05$^{+\#}$	1.10 ± 0.06$^{+\#*}$

Data are expressed as mean ± SEM. $^{+}P < 0.05$ vs. LC; $*P < 0.05$ vs. LT; $^{\#}P < 0.05$ vs OC. LC: lean control; OC, obese control; LT, lean TNBS; OT, obese TNBS.

Figure 1


A
Weight Evolution (g)
550
500
450
400
350
300
-2
-1
0
1
2
3
4
5
6
Days
* (OC vs OT)
* (LC vs LT)
LC
OC
LT
OT


B
Total ΔBW (g)
40
20
0
-20
-40
****
****
Control
TNBS
Lean
Obese


C
Total ΔBW (%)
10
5
0
-5
-10
****
****
*
Control
TNBS
Lean
Obese

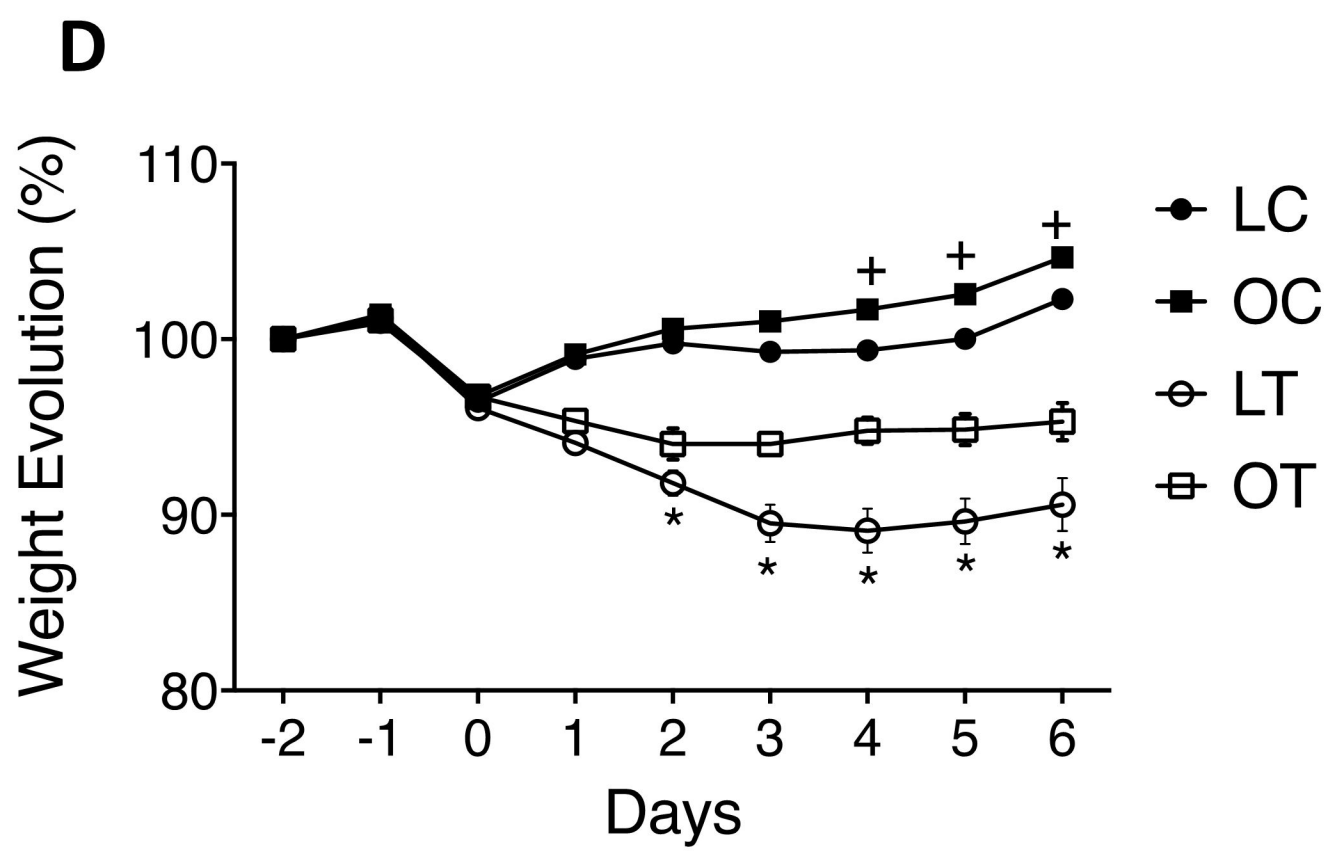

D
Weight Evolution (%)
110
100
90
80
-2
-1
0
1
2
3
4
5
6
Days
+
*
LC
OC
LT
OT

Figure 2

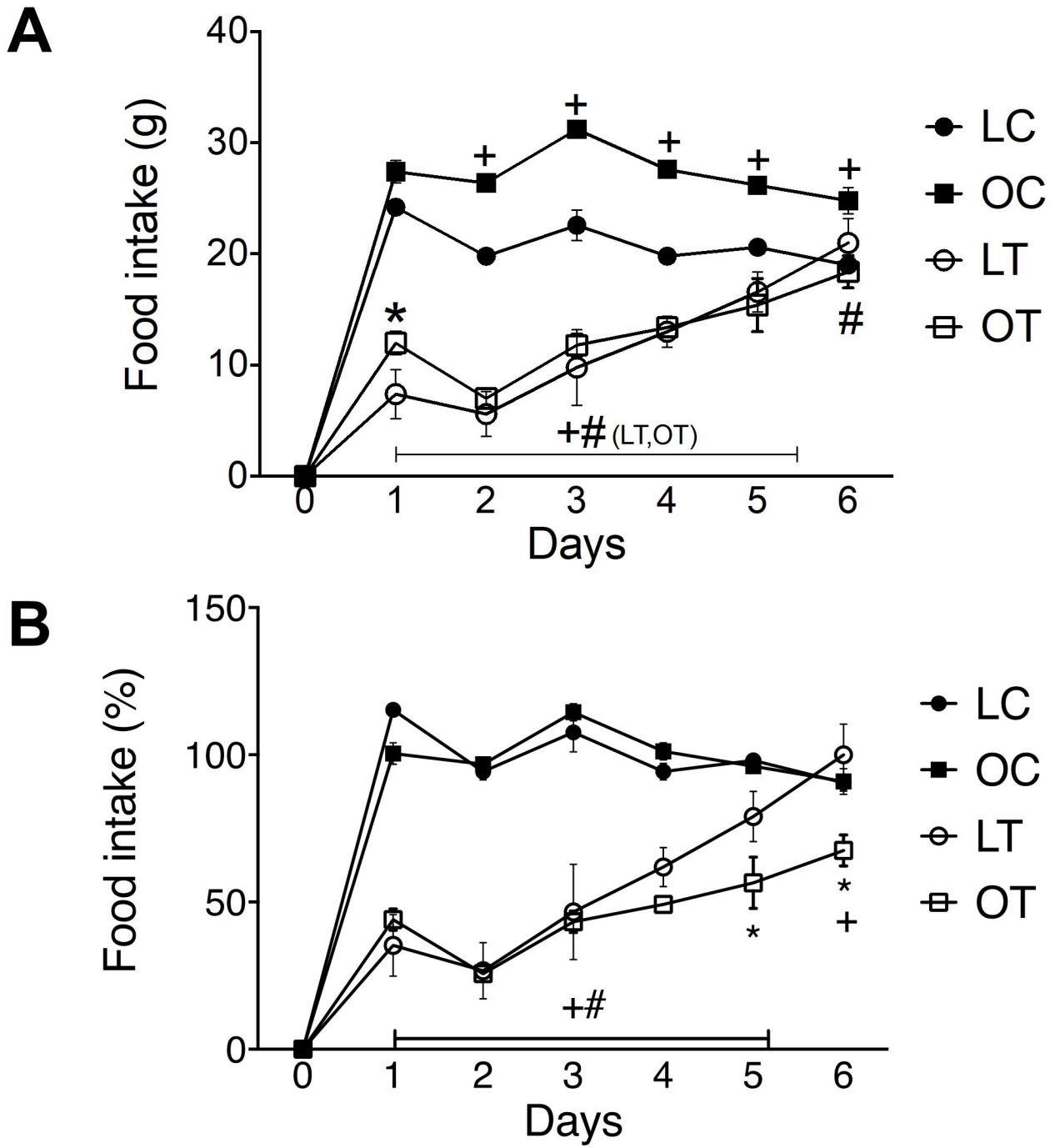
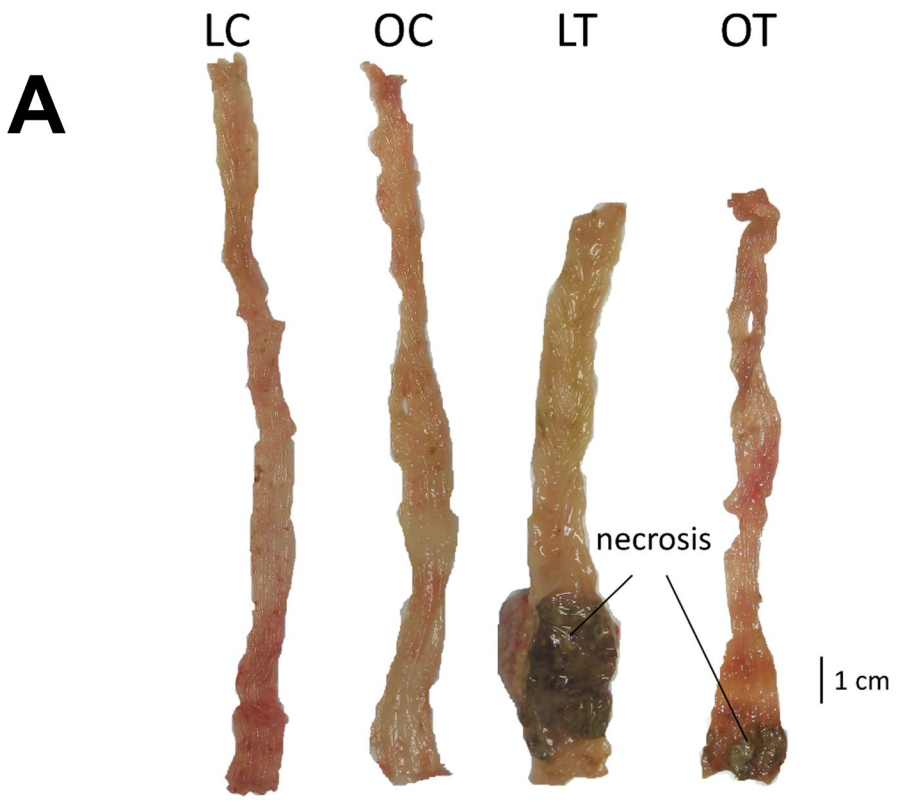
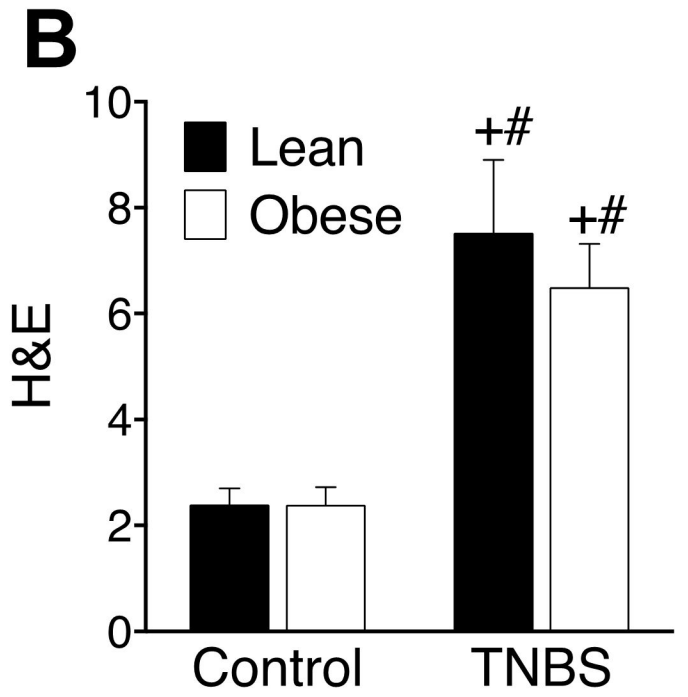
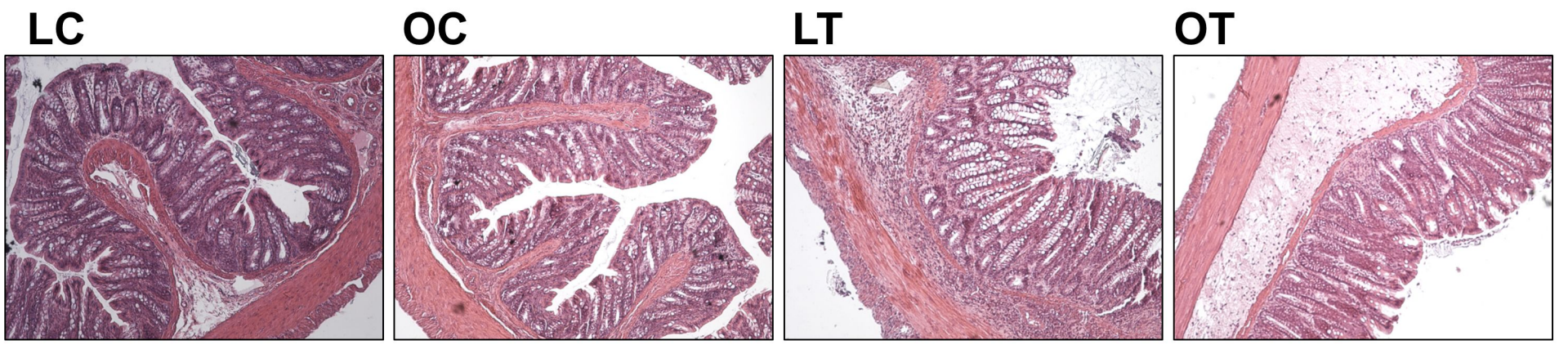
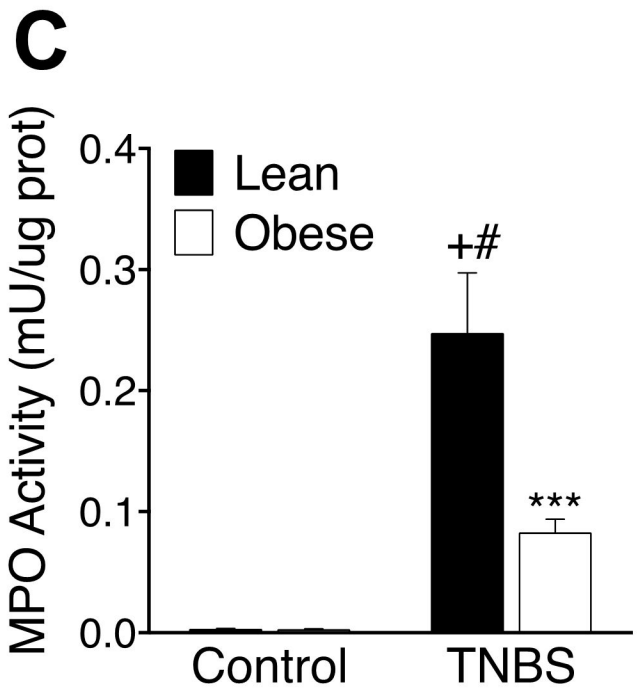

A
Food intake (g)
40
30
20
10
0
0
1
2
3
4
5
6
Days
+
+
+
+
+
*
#
+# (LT,OT)
LC
OC
LT
OT
B
Food intake (%)
150
100
50
0
0
1
2
3
4
5
6
Days
+#
*
*
+
LC
OC
LT
OT

Figure 3


A
LC
OC
LT
OT
necrosis
1 cm


B
H&E
10
8
6
4
2
0
Lean
Obese
+#
+#
Control
TNBS


LC
OC
LT
OT


C
MPO Activity (mU/ug prot)
0.4
0.3
0.2
0.1
0.0
Lean
Obese
+#
***
Control
TNBS

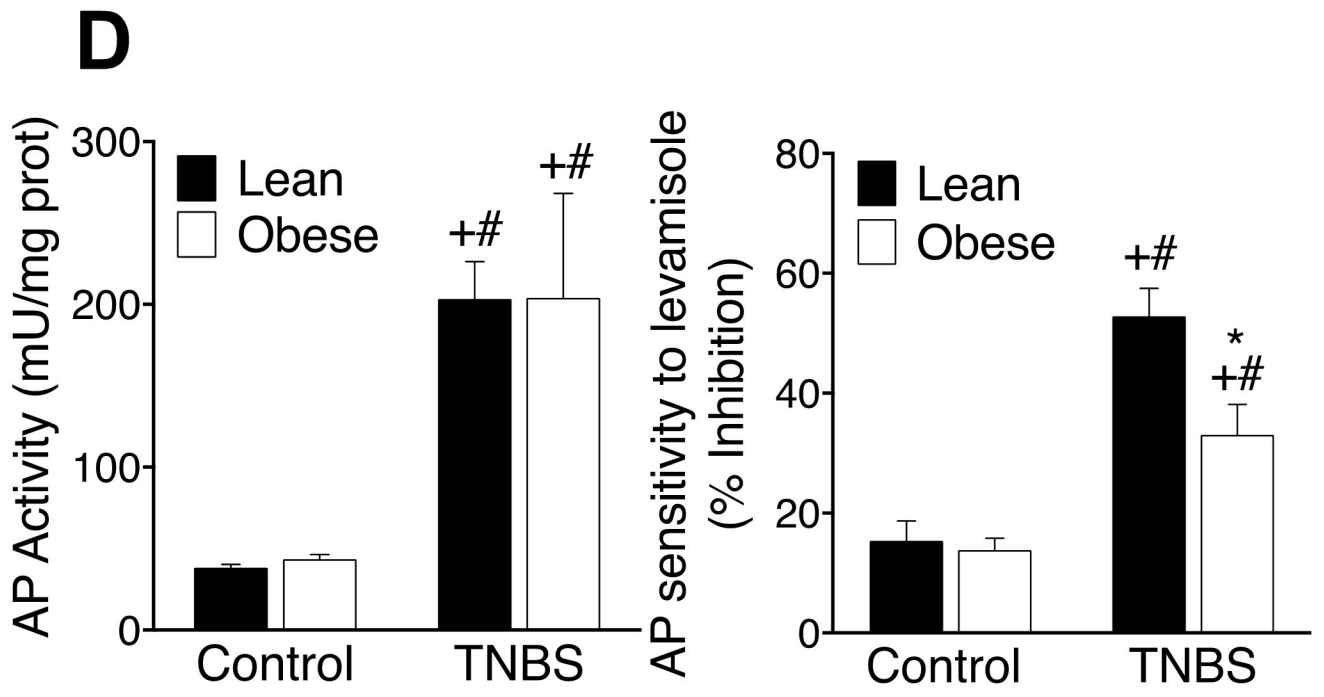

D
AP Activity (mU/mg prot)
300
200
100
0
Lean
Obese
+#
+#
Control
TNBS
AP sensitivity to levamisole (% Inhibition)
80
60
40
20
0
Lean
Obese
+#
*
+#
Control
TNBS

Figure 4

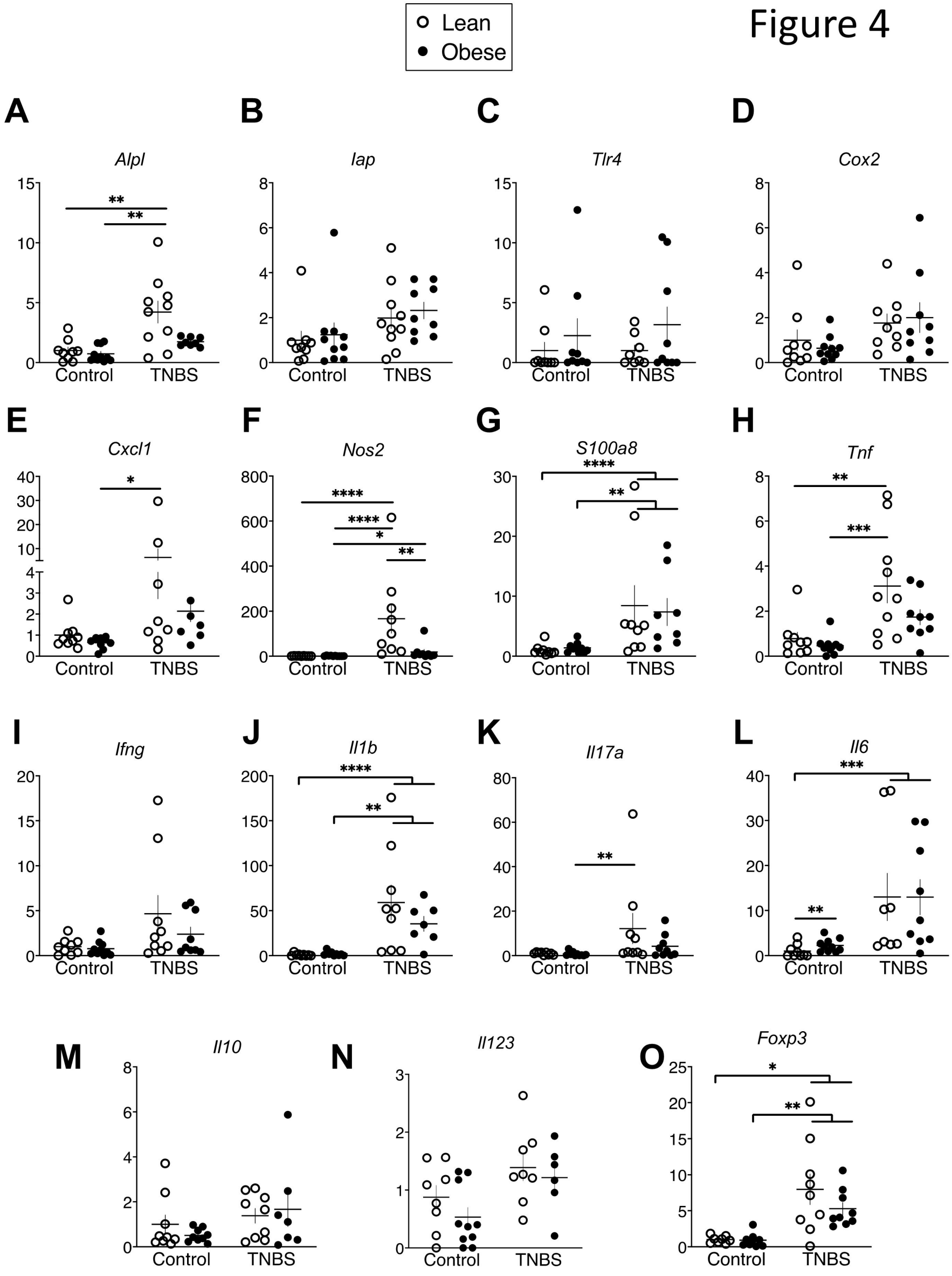

Lean
Obese
A
Alpl
B
Iap
C
Tlr4
D
Cox2
E
Cxcl1
F
Nos2
G
S100a8
H
Tnf
I
Ifng
J
Il1b
K
Il17a
L
Il6
M
Il10
N
Il123
O
Foxp3
Control
TNBS

Figure 5

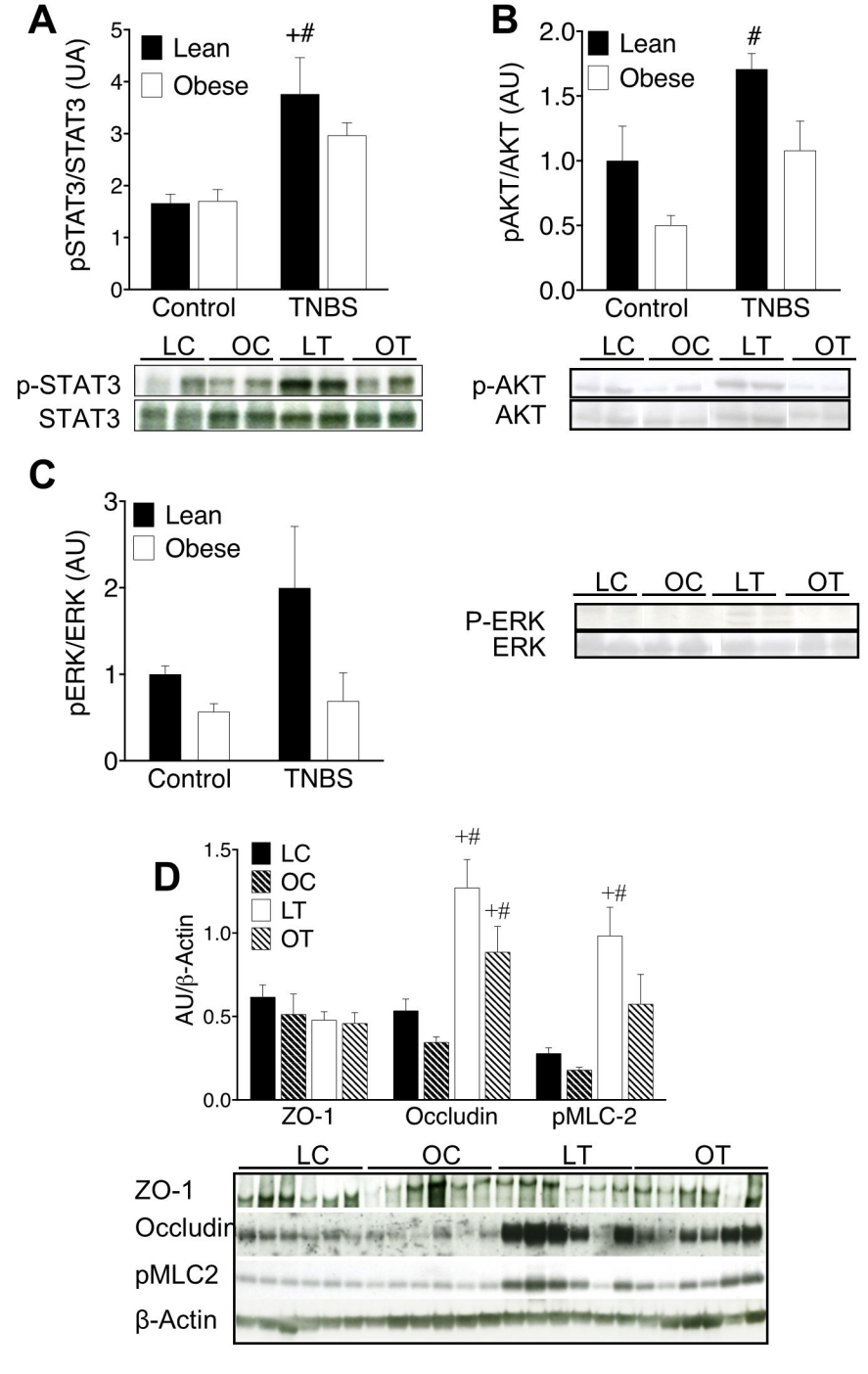
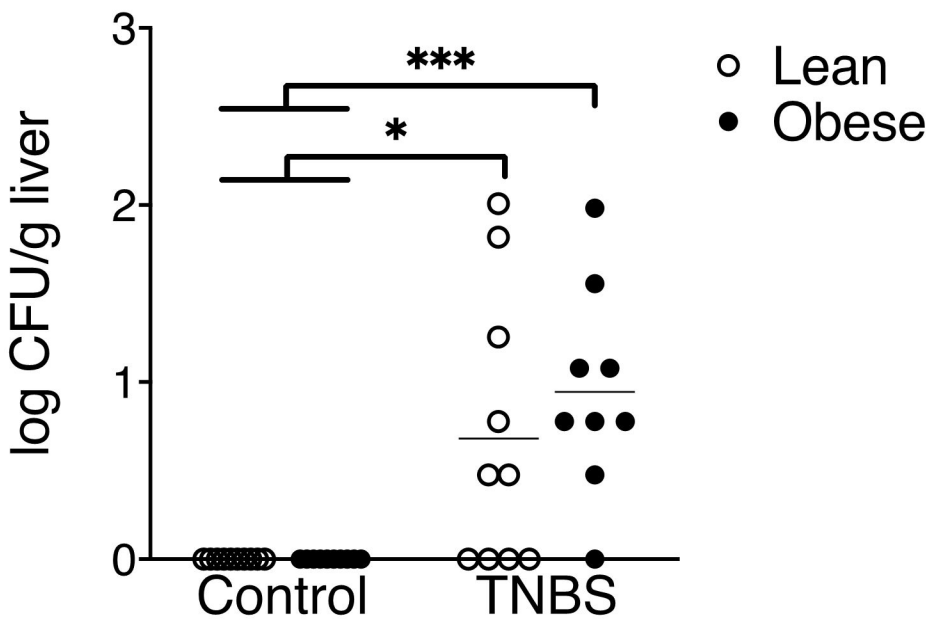
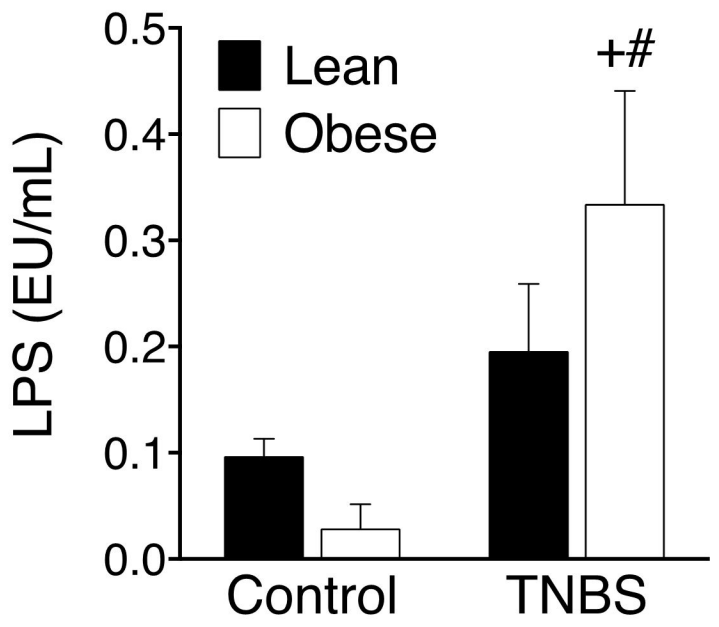
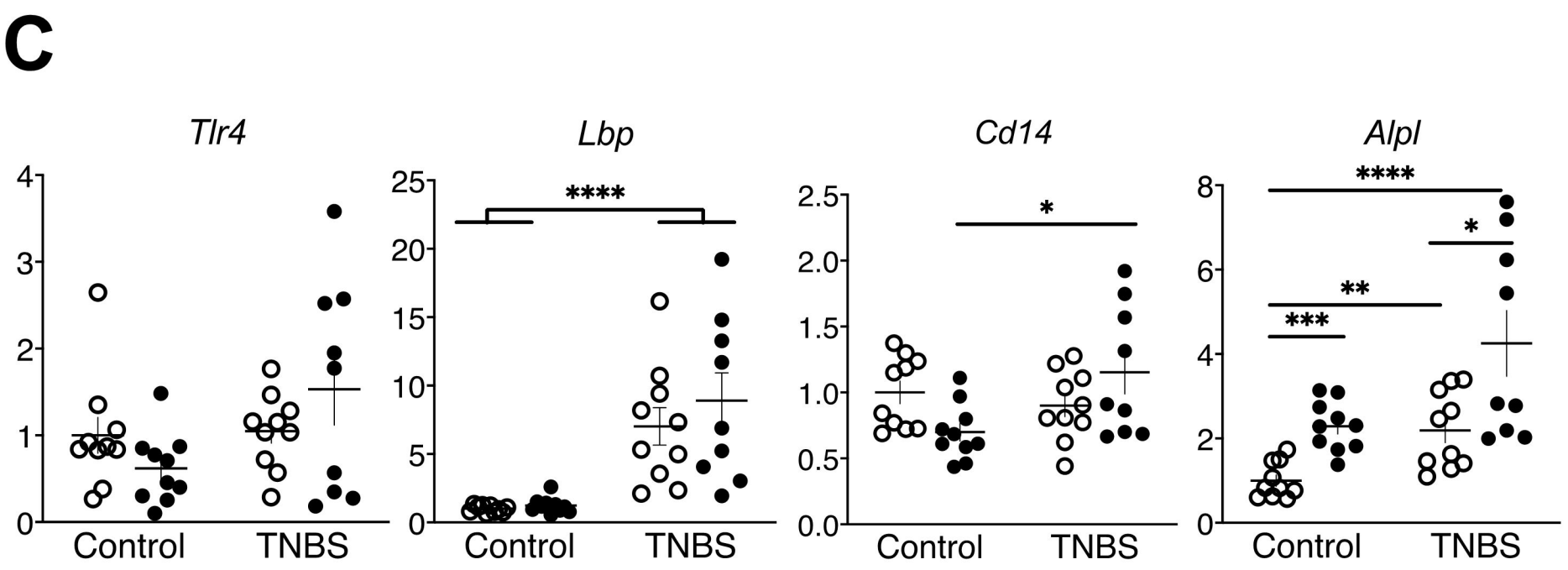
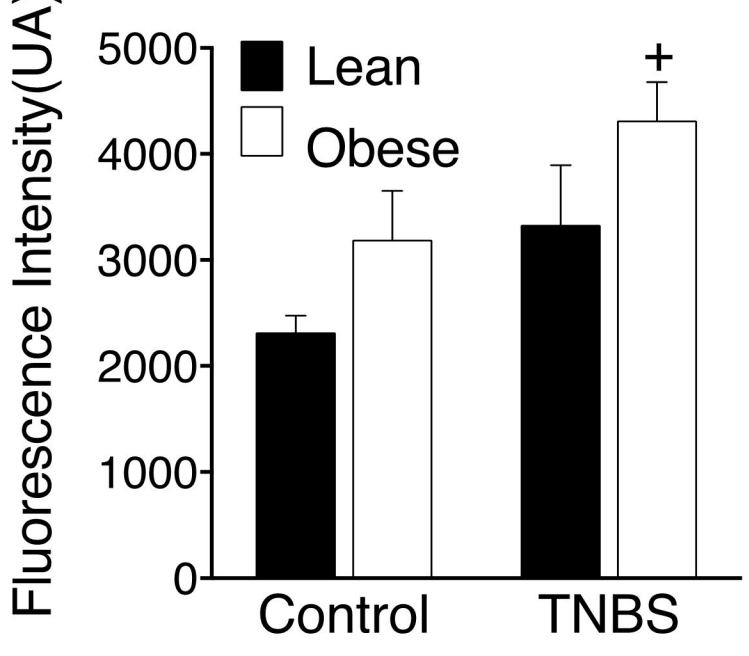

A
pSTAT3/STAT3 (UA)
Lean
Obese
+#
Control
TNBS
LC
OC
LT
OT
p-STAT3
STAT3
B
pAKT/AKT (AU)
Lean
Obese
#
Control
TNBS
LC
OC
LT
OT
p-AKT
AKT
C
pERK/ERK (AU)
Lean
Obese
Control
TNBS
LC
OC
LT
OT
P-ERK
ERK
D
AU/β-Actin
LC
OC
LT
OT
+#
+#
+#
ZO-1
Occludin
pMLC-2
LC
OC
LT
OT
ZO-1
Occludin
pMLC2
β-Actin

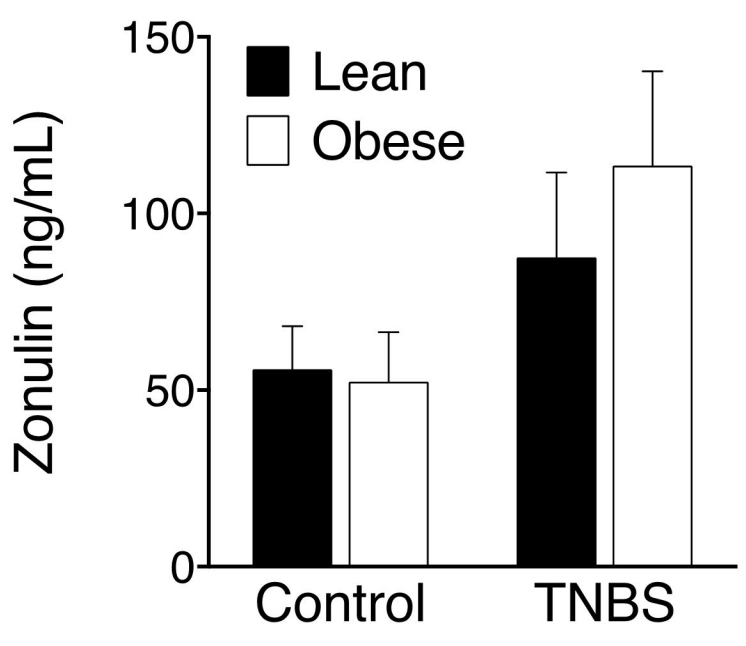
Figure 6


A
log CFU/g liver
3
2
1
0
***
*
Lean
Obese
Control
TNBS


B
LPS (EU/mL)
0.5
0.4
0.3
0.2
0.1
0.0
Lean
Obese
+#
Control
TNBS


C
Tlr4
Lbp
Cd14
Alpl
****
*
**
***
Control
TNBS


D
Fluorescence Intensity(UA)
5000
4000
3000
2000
1000
0
Lean
Obese
+
Control
TNBS


E
Zonulin (ng/mL)
150
100
50
0
Lean
Obese
Control
TNBS

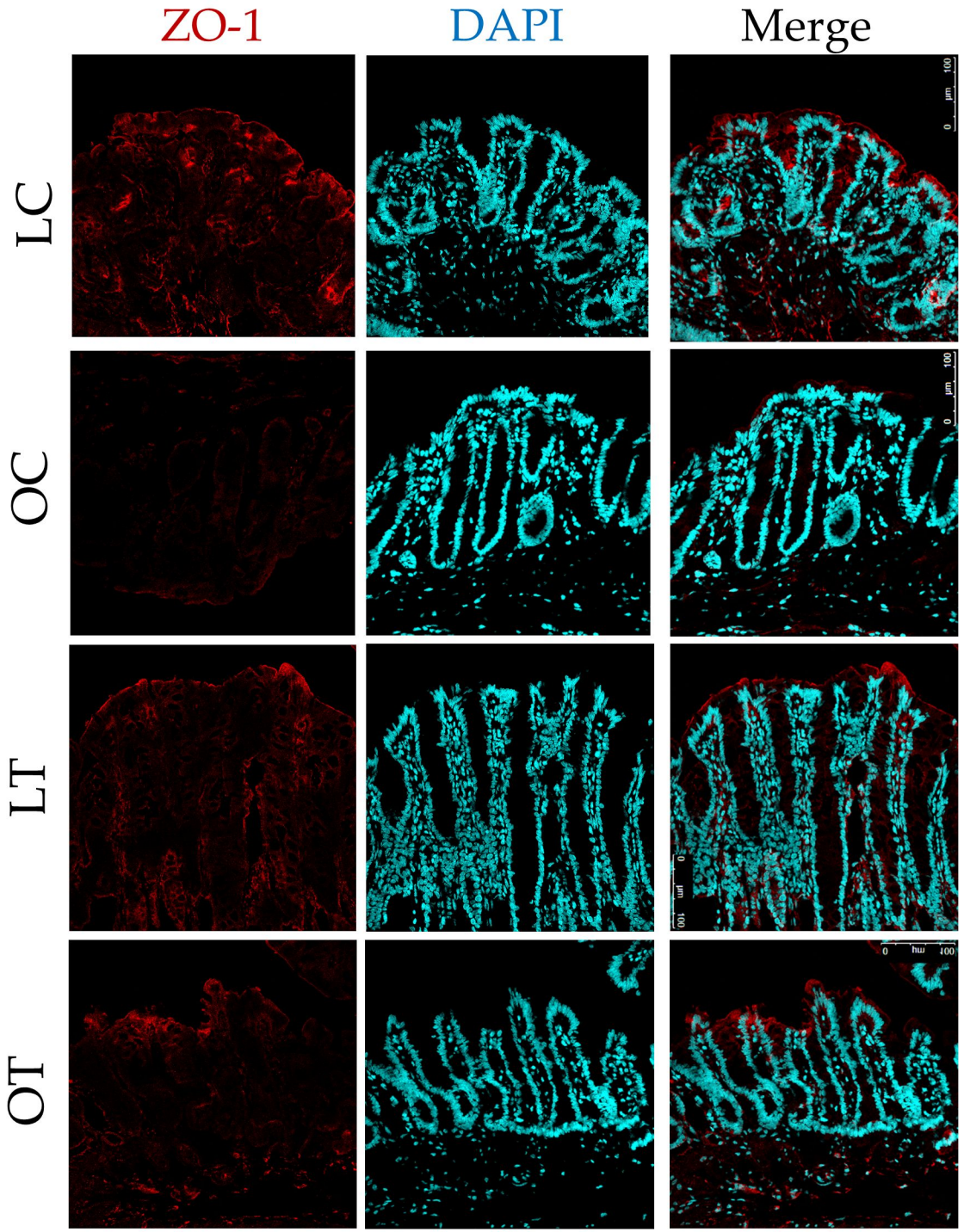


Figure 7

Figure 8

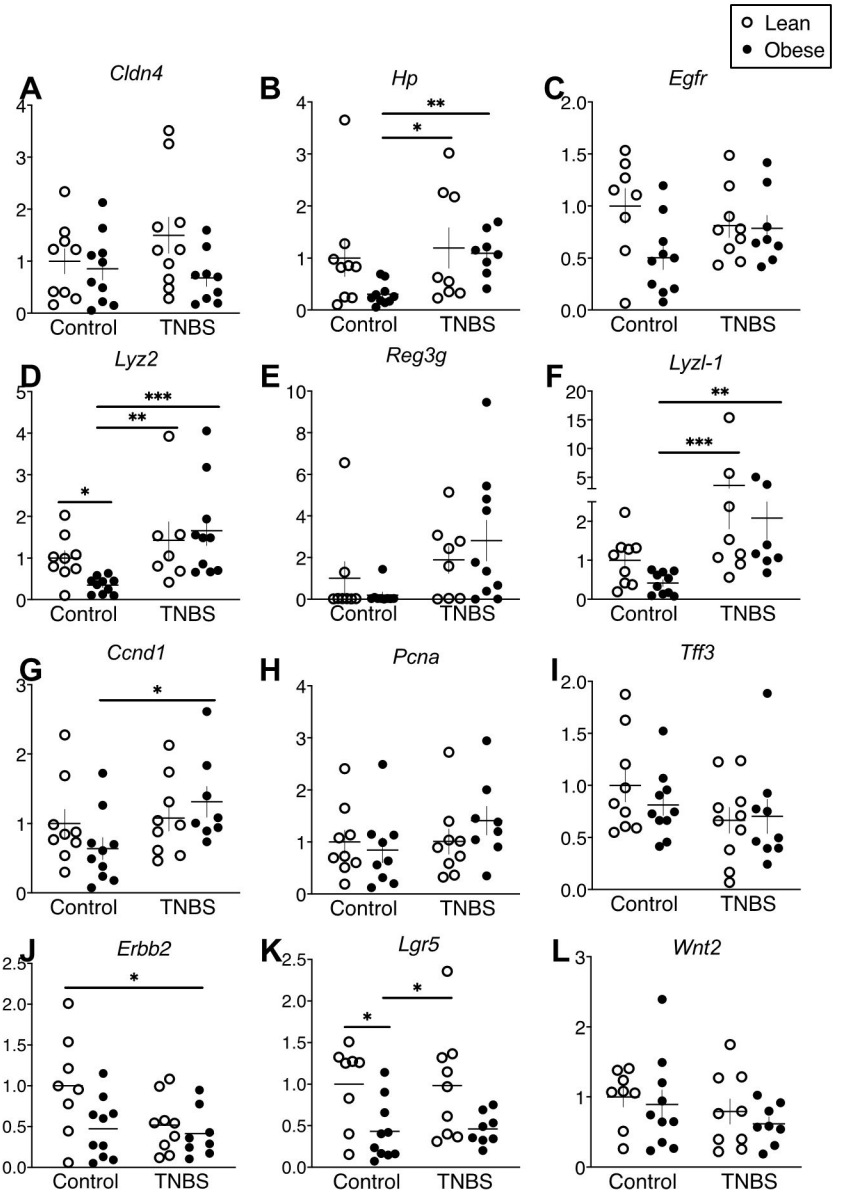
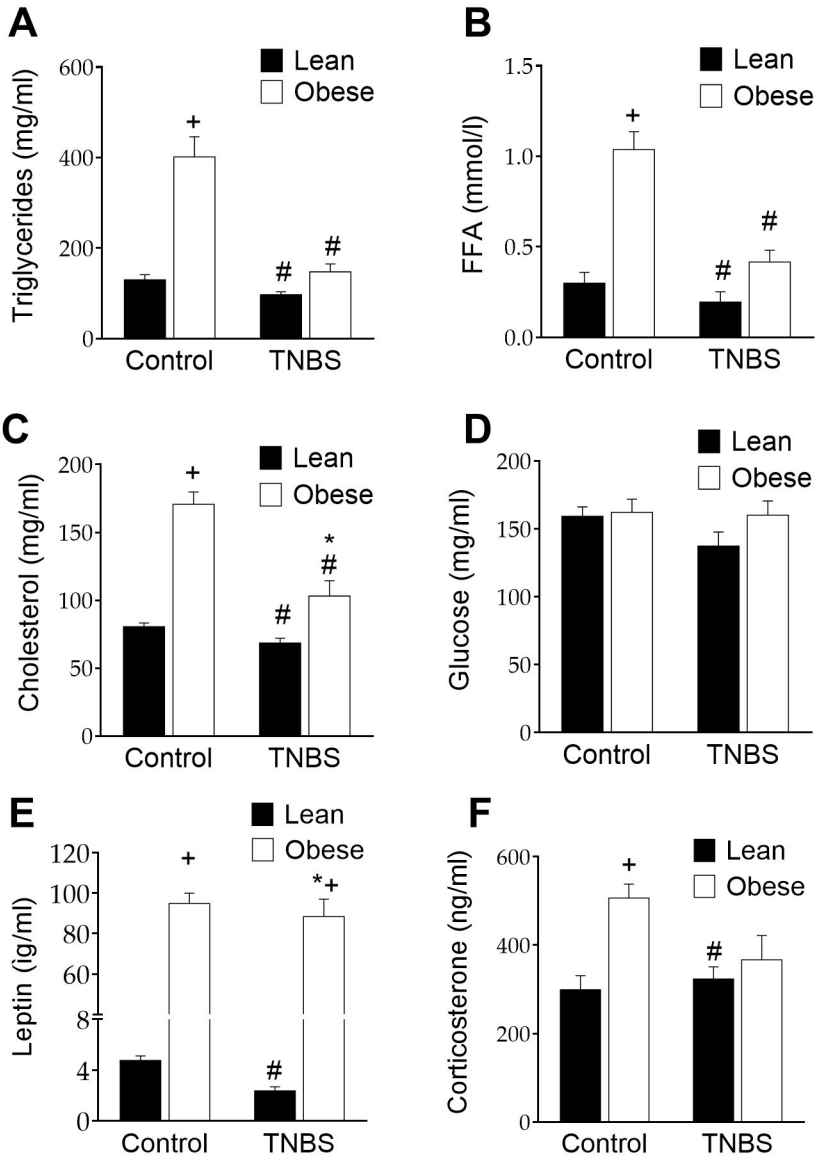

Lean
Obese
A
Cldn4
B
Hp
C
Egfr
D
Lyz2
E
Reg3g
F
Lyzl-1
G
Ccnd1
H
Pcna
I
Tff3
J
Erbb2
K
Lgr5
L
Wnt2
Control
TNBS

Figure 9


A
Triglycerides (mg/ml)
600
400
200
0
Lean
Obese
+
#
#
Control
TNBS
B
FFA (mmol/l)
1.5
1.0
0.5
0.0
Lean
Obese
+
#
#
Control
TNBS
C
Cholesterol (mg/ml)
200
150
100
50
0
Lean
Obese
+
*
#
#
Control
TNBS
D
Glucose (mg/ml)
200
150
100
50
0
Lean
Obese
Control
TNBS
E
Leptin (ig/ml)
120
100
80
60
8
4
0
Lean
Obese
+
*+
#
Control
TNBS
F
Corticosterone (ng/ml)
600
400
200
0
Lean
Obese
+
#
Control
TNBS