Reduced inflammatory state promotes reinnervation of endometriotic-like lesions in TNFRp55 deficient mice

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Running title: Inflammation and reinnervation in endometriosis

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Abstract

Endometriosis is a chronic gynecological disease, characterized by growth of endometrial tissue in ectopic sites due to alteration of peritoneal homeostasis and deregulation of apoptosis. Here we have examined whether TNFRp55 deficiency modulates the pro-inflammatory state and the reinnervation of endometriotic-like lesions in mice. Two-month-old female C57BL/6 mice, eight wild type (WT) and eight TNFRp55$^{-/-}$ (KO), were used in the study. Endometriotic-like lesions were induced experimentally. The right uterine horn was removed from the animal, divided longitudinally, cut in three square pieces, and sutured to the intestine mesentery. After four weeks, the lesions and the peritoneal fluid were collected. The level of TNFα in the peritoneal fluid was evaluated by enzyme-linked immunosorbent assay (EIA). The expression of COX2, GRα, and GRβ were evaluated in the lesions by western blot and immunohistochemistry. β-III TUBULIN, BDNF, and NGF protein concentrations were evaluated in the lesions by western blot. Gene expression of *Pgp 9.5, SP* and *Th,* was analyzed by RT-PCR, whereas relative concentrations of TRKA, NTRp75, phosphorylated NFκB (pNFκB) and total NFκB in lesions were measured by EIA. Compared with the WT group, the KO mice showed lower TNFα levels in the peritoneal fluid and lower numbers of COX2 immunoreactive cells along with increased expression of GRα, β-III TUBULIN, *Pgp 9.5, SP, Th,* BDNF, NGF, NTRp75, and pNFκB in the lesions. Future histological studies will be necessary to confirm the sensory/sympathetic imbalance in the endometriotic-like lesions of the KO mice. Our results suggest that a reduced inflammatory state promotes reinnervation of endometriotic-like lesions in TNFRp55$^{-/-}$ mice. Chronic deregulation of TNF receptors can have serious consequences for women with advanced endometriosis.

Keywords: endometriosis, mice, tumor necrosis factor-alpha, tumor necrosis factor receptor p55, inflammation, innervation, glucocorticoid receptors, neurotrophins.

INTRODUCTION

Endometriosis is a chronic gynecologic disease associated with increased estrogen production and characterized by the presence of endometrial tissue outside the uterine cavity (Rizner, 2009; Greene *et al*., 2016). The presence of endometrial tissue in ectopic sites may be in part due to immunological evasion mechanisms (Burney and Giudice, 2012).

Tumor necrosis factor alpha (TNFα) is an important pleotropic cytokine produced mainly by classical M1 activated macrophages, which have been linked to endometriosis in several studies (Steff *et al*., 2004; Richter *et al.,* 2005; Cho *et al*., 2007). This cytokine has many biological effects, such as promotion of inflammation, mitogenesis, differentiation, immunological modulation, and antitumoral activity (Sriram and O'Callaghan, 2007). These activities are the result of TNFα binding to its receptors, TNFRp55 or TNFRp75. TNFRp55 is expressed in a constitutive manner in all cells and mainly activates pro-inflammatory pathways; TNFRp75 is highly regulated and expressed in immunological cells and mainly activates proliferative pathways (Grell *et al*., 1995). Since both receptors are expressed on the surface of several cell types, their participation in the remodeling and growth of tissues is possible.

Different studies show aberrant function of the TNF system in women with endometriosis. Lower levels of TNFRp55 expression are found in eutopic endometrium of women with endometriosis compared to healthy controls (Boric, 2013). Another study found that the levels of the soluble TNF receptor TNFRp55 in serum of patients were higher than in women without endometriosis, possibly antagonizing the effect of TNFα (Othman *et al*., 2016). We recently reported that TNFRp55 deficiency promotes endometriotic-like lesion growth and high levels of estradiol with positive correlation with metalloproteinase 2 (MMP2) activity in a murine model of induced endometriosis (Vallcaneras *et al*., 2017).

In addition, high levels of TNFα in the peritoneal fluid of endometriosis patients have been found, principally, in early stages of the disease (Cheong *et al*., 2002; Pizzo *et al*., 2002), where the inflammatory process would appear to be higher than in the following stages. This cytokine is able to induce cyclooxygenase-2 (COX2) expression, an enzyme that regulates prostaglandin E2

(PGE2) synthesis (Wu *et al.*, 2010). In physiological conditions, this enzyme is undetectable, but it becomes overexpressed in response to infections or injuries. Wu *et al*. (2002) found an overexpression of COX2 in peritoneal macrophages of women with endometriosis, possibly due to inflammatory stimuli.

The study of anti-inflammatory factors is also crucial to understand this enigmatic disease. Glucocorticoids are the most important endogenous anti-inflammatory and immunosuppressive steroids of organisms, and mediate their actions principally through the glucocorticoid receptors alpha and beta (GRα and GRβ, respectively). Interestingly, previous reports showed enhanced expression of these receptors in endometriotic lesions (Rhen and Cidlowski, 2005; Grandi *et al*., 2016).

To this date, two distinct and opposed scientific positions exist regarding inflammation in endometriosis. Several studies show that this pathology takes place with increased levels of cytokines—such as IL-1, IL-6, and TNFα—in serum and peritoneal fluid of women with endometriosis (Harada *et al.*, 1997; Cheong *et al.*, 2002; Pizzo *et al*., 2002). However, other studies show an altered immune response through an increase of regulatory T cells in women with endometriosis (Basta *et al*., 2010; Chen *et al*., 2012; Podgaec, 2012; Olkowska-Truchanowicz *et al*., 2013). To shed new light on the pro-inflammatory state in endometriosis, it is essential to understand the survival of the lesions and the progress of the disease.

Another important mechanism for endometriotic lesion development is innervation. Berkley *et al*. (2004) demonstrated that endometriotic lesions develop a robust innervation in an induced endometriosis model in rats. In addition, women with endometriosis show an imbalance between sympathetic and sensorial reinnervation, which might directly be involved in the maintenance of inflammation and pain (Arnold *et al*., 2012). Increased levels of brain-derived neurotrophic factor (BDNF) and neural growth factor (NGF) have also been found in the peritoneal fluid (Barcena de Arellano *et al.*, 2013; Ding *et al*., 2018).

Interestingly, both estradiol (the main hormone involved in this pathology), and immune cells, can regulate the synthesis and release of neurotrophins and their receptors TRKA and NTRp75 in

endometriosis (Takei and Laskey 2008; Liang and Yao, 2016). In addition, macrophages stimulated with estradiol produce neurotrophins that participate in the sensory/sympathetic imbalance in ectopic endometrial tissue (Morotti *et al.*, 2014; Greaves *et al.*, 2015). Other studies indicate that BDNF and NGF promote nociceptor expression, contributing to nociceptive pain generation (Pezet and McMahon, 2006; Howard, 2009).

Considering all previous information, we investigated if the deficiency of TNFRp55 affects the pro-inflammatory state and reinnervation of the endometriotic-like lesions. This work was intended to shed light on some of the recommendations established in the 3rd International Consensus Workshop about endometriosis research priorities (Rogers *et al*., 2017).

MATERIALS AND METHODS

Animals

Two-month old female mice of the C57BL/6 strain, eight wild type (WT) and eight TNFRp55$^{-/-}$, weighing 19-21 g were used. The TNFRp55$^{-/-}$ mice were obtained from the Max von Pettenkofer-Institute (Munich, Germany). It is necessary to emphasize that cells from TNFRp55$^{-/-}$ mutant mice lack expression of TNFRp55 but display normal numbers of high-affinity TNFRp75 molecules (Pfeffer *et al.*, 1993). Breeding colonies were established at the Animal Facility of the National University of San Luis (San Luis, Argentina) under rigorous light conditions (12 h of light—07:00 to 19:00 h—and 12 h of darkness), controlled temperature (22 ± 2 °C), with water and sterile food *ad libitum*. The experiments were carried out according to the guidelines for the care and use of laboratory animals of the National Institutes of Health (NIH), and the Comité Institucional de Cuidado y Uso de Animales de Experimentación (CICUA) of Universidad Nacional de San Luis, Argentina (Protocols #B-201/15; B-225/16).

Rodent models of endometriosis are considered valid to study the development of this pathology (Vernon and Wilson, 1985; Grümmer, 2006; Vallcaneras *et al*., 2017). In fact, it has been demonstrated that the ectopic implants in rodents and women respond in a similar manner to

hormonal treatment, and show similar alterations in gene expression and protein production (Greaves *et al.*, 2014; Sharpe-Timms, 2002) .

This research is the continuation of a study carried out by Vallcaneras *et al*. (2017) who, when using the same animal model of endometriosis surgically induced in WT and TNFRp55$^{-/-}$ mice, reported that in deficient animals all transplants progressed to endometriotic-like lesions [2.47 ± 0.21 (n = 12) *vs*. 3 (n = 11), $p<0.05$] and exhibited greater volume [11.77 ± 0.49 mm^3 (n=12) *vs*. 28.94 ± 3.30 mm^3 (n=11), $p<0.05$].

Surgical induction of endometriosis

The endometriotic-like lesions were induced experimentally, as reported previously (Bilotas *et al.*, 2010; Ricci *et al*., 2011). The induction of experimental endometriosis was carried out at random phases of the estrous cycle (Kiani *et al*., 2018). Eight animals per experimental group were anesthetized with 100 mg/kg of ketamine (Holliday Scott, Buenos Aires, Argentina) and 10 mg/kg of xylazine (Richmond, Buenos Aires, Argentina) by intraperitoneal injection. A mid-ventral incision was then made to expose the uterus and the intestine. The right uterine horn was removed from the animal, placed in DMEM-F12 (Gibco, Life Science, Great Island, NY, USA), opened longitudinally and cut into three square pieces of approximately 4 mm^2. Then, the three equal pieces of uterine horn were sutured onto the colonic mesentery with the endometrial layer facing the bowel serosa (autologous transplant) by means of a single stitch (supralong 6-0, Ethicon, NJ, USA). The abdomen was then closed with a 5-0 nylon suture. Mice were monitored daily in relation to body weight, food consumption, preening behaviour, and daily activity. No alteration in their behaviour was noted. After 4 weeks of surgery, animals were sacrificed by cervical dislocation. Then, a small medioventral hole was opened through which 1.5 ml of PBS (KH_2PO_4 0.015 M, NaH_2PO_4 0.017 M, KCl 0.076 M, NaCl 0.14 M, pH 7.4) was injected in the peritoneal cavity of each animal. The peritoneal lavage fluid was collected and centrifuged at 250 *g* for 10 min at 4 °C. The supernatant (peritoneal fluid) was separated from the precipitate (peritoneal lavage cells) and both were maintained at -80 °C until the corresponding determinations were made. Finally, the abdomen was

158 completely opened to have access to the endometriotic-like lesions, which were later randomly
159 selected to perform different analyses.

160 **Western blot**

161 Protein extracts were obtained from endometriotic-like lesions using TRIzol reagent, following the
162 manufacturer's indications (Invitrogen Life Technologies, Carlsbad, CA, USA). For each
163 experimental group, one lesion per animal was randomly selected. Protein concentration was
164 determined by the Lowry method (Lowry *et al*., 1951). Aliquots containing 40 µg of total protein
165 were subjected to electrophoresis in 10% (w/v) SDS-PAGE gels, and then electrotransferred to
166 PVDF membranes (Millipore Corporation, Burlington, MA, USA) at 100 V for 1 h in a transfer buffer
167 (25 mM Tris, 192 mM glycine, and 20% v/v methanol, pH 8.3). The membrane was immersed in 5%
168 (w/v) non-fat dry milk in PBS with 0.05% (v/v) Tween 20 for 1 h at room temperature, followed by
169 an overnight incubation at 4 °C with either rabbit polyclonal anti-COX2 antibody (1:1000; ab15191,
170 Abcam, Cambridge, UK), rabbit monoclonal anti-GR (D6HL2) XP antibody (1:1000; Cat# 12041,
171 Cell Signalling Technology, Danvers, MA, USA), mouse monoclonal anti β-III TUBULIN antibody
172 (1:8000; Cat# 2020-TUB, PhosphoSolutions, Aurora, CO, USA), rabbit polyclonal anti-BDNF
173 antibody (1:1000; sc-20981, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), mouse
174 monoclonal anti-NGF antibody (1:1000; sc-365944, Santa Cruz), or goat anti ACTIN antibody
175 (1:1000; sc-1615, Santa Cruz), all diluted in 1% (w/v) non-fat dry milk in PBS with 0.05% (v/v)
176 Tween 20. After incubation with the primary antibody, the membranes were washed in PBS-T and
177 incubated with goat anti-rabbit IgG peroxidase-linked antibody (sc-2004, Santa Cruz), goat anti-
178 mouse IgG peroxidase-linked antibody (sc-2005, Santa Cruz), or donkey anti-goat IgG peroxidase-
179 linked antibody (sc-2020, Santa Cruz), 1:5000 dilution in 1% milk for 3 h at room temperature,
180 respectively. Following washing in PBS-T, blots were developed using an enhanced
181 chemiluminescence Western blotting detection system, the Thermo Scientific SuperSignal West
182 Pico Chemiluminescent (Pierce Biotechnology, Rockford, IL, USA), and exposed to X-ray films. The
183 mean of intensity of each band was measured using the NIH ImageJ software (Image Processing

and Analysis in Java from http://rsb.info.nih. gov/ij/). Protein levels were normalized against β-actin (ACTIN).

Enzyme-linked immunosorbent assay

The determination of TNFα in peritoneal fluid was done via ELISA (#560478, eBioscience, San Diego, USA) according to the manufacturer's instructions. The rest of enzyme-linked immunosorbent assays (EIAs) were done as follows: twenty µl of sample (20 µg of total proteins from endometriotic-like lesions) were added to 180 µl of 0.1 M bicarbonate buffer pH 9.6, in clear 96-well microplates (Corning Incorporated, Corning, NY, USA) and incubated for 1h at 37 °C. After washing with 0.05% (v/v) Tween 20 in PBS (500 ml/ 96-well plate), and blocking with 5% (w/v) non-fat dry milk in PBS with 0.05% (v/v) Tween 20 (10 ml/96-well plate) for 1 h at 37 °C, the microplates were incubated with 50 µl of rabbit polyclonal anti-TRKA antibody (1:1000; sc-118, Santa Cruz), rabbit polyclonal anti-NTRp75 antibody (1:1000; sc-8313, Santa Cruz), mouse monoclonal anti-NFκBp65 (1:1000, sc-8008, Santa Cruz) or rabbit monoclonal anti-phopho-NFκBp65 (1:1000, 3033S, Cell Signaling Technology) overnight at 4 ºC. After three washes, 50 µl of goat anti-rabbit IgG-HRP conjugate (1:10000, Jackson Immuno-Research Labs, West Grove, PA, USA) or goat anti-mouse IgG peroxidase-linked antibody (1:10000, sc-2005, Santa Cruz) was added to each well and incubated for 1 h at 37 °C. Finally, immunocomplexes were quantified using 3,3',5,5'-Tetramethylbenzidine (TMB). The oxidation reaction of the substrate was stopped with 2 M sulfuric acid, and the optical density (OD) at 450 nm was measured using a TECAN microplate reader (Infinite M200 PRO, Research Triangle Park, NC, USA).

Immunohistochemistry

Immunohistochemistry was done in Bouin-fixed, paraffin-embedded tissues of 4 µm; antigen retrieval was carried out by placing tissue sections in a solution of 0.034 mM citrate buffer (pH: 6.0) for 40 min at 96–98 °C, followed by cool-down at room temperature (RT) for 20 min. Permeabilization and reduction of the non-specific binding was achieved by incubating the sections with 2.5% (v/v) normal horse serum and 0.5% (v/v) Triton-100 for 20 min at RT. Slides were incubated with the specific primary antibodies in a moist incubation chamber at 4 ºC overnight.

Endogenous peroxidase activity was blocked with 3% H_2O_2 for 20 min at RT following incubation with the primary antibody. Antibodies and dilutions were as follows: COX2 (#ab15191; 1:200, Abcam) and GR D6HL2 XL (#12041; 1:200, Cell Signaling). Samples were incubated with secondary antibody [ImmPRESS HRP Anti-Rabbit (#MP-7401) Ig peroxidase; Vector Laboratories, Burlingame, CA, USA] for 30 min at RT. Specific peroxidase activity was developed with the following substrates: ImmPACT DAB Peroxidase (#SK-4105) or ImmPACT NovaRED Peroxidase (#SK-4805) (Vector Laboratories). In negative controls, one per slide, the primary antibody was replaced with 2.5% (v/v) normal horse serum (Vector Laboratories). Positive cell percentage was established with standard light microscopy at 200X. One hundred random epithelial and stromal cells per field were counted; 4-6 fields per section were analyzed. Samples from five different animals per experimental group were used. Total positive cell percentage was calculated per slide and was used to obtain the mean of each experimental group.

RNA isolation and RT-PCR analysis

RT-PCR was carried out to analyze the gene expression of a pan neuronal marker *Pgp 9.5*, a sensory fiber marker, substance P (*SP*), and a sympathetic fiber marker, tyrosine hydroxylase (*Th*). Total RNA was isolated from endometriotic-like lesions randomly selected using TRIzol reagent (Invitrogen Life Technologies), according to the manufacturer's instructions. Purified total RNAs were then quantified and assessed for purity by measurement of the 260/280 ratio using an UV spectrophotometer Beckman DU-640 B (Fullerton, CA, USA). Only samples with 260/280 ratio of 1.8-2.0 were used. The integrity was confirmed by running 2 µg RNA on a 0.8% (w/v) agarose gel. After GelRed staining (Biotium, Hayward, CA, USA), RNA bands were visualized with a UV transilluminator, and 28S and 18S rRNA band patterns were analyzed. Two micrograms of total RNA were reverse transcribed at 37 °C using random primers and M-MLV Reverse Transcriptase (Promega, Fitchburg, WI, USA) in a 26-µl reaction mixture. For amplification of the reverse transcription (RT) products, the reaction mixture consisted of 1× Green GoTaq reaction buffer, 0.2 mM deoxynucleoside triphosphate, 0.5 µM specific oligonucleotide primers, and 1.25 U GoTaq DNA polymerase (Promega) in a final volume of 50 µl. The PCR primers were designed using

Primer Express 3.0 software (Applied Biosystems, Waltham, MA, USA). The primers are described in **Table 1**.

The cDNA was amplified using a thermal cycler (My Cycler, BioRad, Hercules, CA, USA). Reaction products were electrophoresed on 2% (w/v) agarose gels, visualized with GelRed, and examined by ultraviolet transillumination. Band intensities of RT-PCR products were quantified using ImageJ (Image Processing and Analysis in Java from http://rsb.info.nih.gov/ij/). Relative levels of mRNA were expressed as the ratio of signal intensity for the target genes relative to that for the housekeeping gene *Actb* (β-actin).

Statistical analysis

Statistical analysis was performed using GraphPad Prism (Version 5, GraphPad Software Inc. San Diego CA). Values are presented as the mean ± SEM (n=8/group). Differences between groups were analyzed using two-tailed unpaired Student's *t*-test, or one-way analysis of variance (ANOVA) followed by the Tukey's multiple comparison test. In all comparisons, a *p-value* of less than 0.05 was considered statistically significant.

RESULTS

TNFα levels in peritoneal fluid are decreased in TNFRp55 deficient mice

We began by analyzing the levels of TNFα in the peritoneal fluid of mice with induced endometriosis to assess the general inflammatory environment surrounding the endometrial implants. In this regard, we observed a significant decrease in the levels of expression of this pro-inflammatory cytokine in KO mice compared to WT mice (p=0.0298, Figure 1), which suggests that the absence of TNFRp55 affects the expression of TNFα.

The number of COX2 immunoreactive cells is lower in TNFRp55$^{-/-}$ endometriotic implants

We continued our study by analyzing the expression of COX2, an enzyme expressed in pro-inflammatory conditions. The expression of the COX2 protein analyzed by western blot did not show statistically significant changes between the groups (p=0.1044, Figure 2A, B). However, when immunohistochemistry (IHC) was performed, a significant reduction in total positive cell staining

265 was observed in KO implants compared to WT implants (p=0.0063, Figure 2C, D). These results
266 indicate that in KO mice, COX2 is not expressed with the same intensity as in WT mice.

267 **Glucocorticoid receptor alpha levels are increased in TNFRp55$^{-/-}$ endometriotic implants**

268 To understand the inflammatory state in which the endometriotic-like lesions develop, we studied
269 not only pro-inflammatory markers but also glucocorticoid receptors, which are important anti-
270 inflammatory markers. Of these receptors, GRα in particular, mediates the anti-inflammatory
271 responses of glucocorticoids. We observed a statistically significant increase of GRα in KO lesions
272 compared to WT lesions (p=0.0223, Figure 3A, B), and a significant difference favoring the same
273 receptor when compared to GRβ among KO animals (p=0.0223, Figure 3B). Interestingly, when
274 IHC was performed, no difference between total cell percentages was found (Figure 3C), indicating
275 that the main difference is between expression of the different glucocorticoid receptor types. The
276 increase in the expression of GRα in the KO lesions possibly contributes to reduce the pro-
277 inflammatory activity.

278 **Reinnervation is increased in TNFRp55$^{-/-}$ endometriotic implants**

279 Reinnervation of implants is a necessary process for lesion survival, and it is positively correlated to
280 pain symptoms in women with this disease. Inflammation can contribute to reinnervation by
281 neurotrophin production and release. In our TNFRp55 deficient model, we found that β-III TUBULIN
282 expression, a protein expressed and limited to neural tissues, was overexpressed in KO lesions
283 (p=0.0007, Figure 4A, B), indicating overall nerve fiber increase in this experimental group. We
284 completed the study with analysis of gene expression of different nerve fiber markers; we found
285 *Pgp 9.5*, a pan neuronal marker, overexpressed in KO lesions (p=0.0131, Figure 4D), thus
286 confirming β-III TUBULIN results. To assess the sensory and sympathetic nerve fiber density, we
287 studied gene expression of different nerve fiber markers: *SP* for sensory nerves and *Th* for
288 sympathetic nerves. Both markers where found to be overexpressed in KO lesions compared to
289 WT (*SP* p=0.0173, Figure 4E, F; *Th* p=0.0108, Figure 4G, H). Noteworthy, when the relation
290 between *SP* and *Th* was studied, an imbalance was found in KO mice favoring the sensory nerve

marker (p=0.0308, Figure 4I). These results suggest an overall increase of reinnervation in TNFRp55 deficient lesions, possibly in favor of sensory nerves.

BDNF and NGF are increased in ectopic implants of TNFRp55 deficient mice

Continuing the study of reinnervation, we analyzed possible contributing factors for enhanced reinnervation, focalizing on two neurotrophins known to promote nerve fiber growth: BDNF and NGF. Both neurotrophins increased in KO lesions compared to WT lesions, mature BDNF ($p<0.0001$, Figure 5A, B) and NGF ($p=0.0490$, Figure 5C, D). These results indicate a possible source for nerve fiber growth stimulation, which is increased in KO mice compared to WT mice.

Common neurotrophin receptor NTRp75 is overexpressed in TNFRp55$^{-/-}$ endometriotic implants

After studying BDNF and NGF, we continued to study by EIA which receptor is increased and, therefore, could be the probable target of these neurotrophins. We started analyzing TRKA, NGFs high affinity receptor and viewed no difference of protein expression between groups (Figure 6A). We continued by analyzing the low affinity receptor common to all neurotrophins, NTRp75. Interestingly, we found a significant increase in protein expression of this receptor in KO mice ($p=0.0212$, Figure 6B), suggesting that this receptor could be the main target of these neurotrophins in this study group. To complete these results, we assessed the activation of NFκB by phosphorylation (pNFκB), which is known to be induced by the activation of the NTRp75 pathway. We found that the relationship between pNFκB and NFκB is in favor of the first in KO mice with respect to WT mice ($p=0.0159$, Figure 6C), suggesting an increased activation of this transcription factor in this group.

DISCUSSION

The present work shows that the reduced inflammatory state, due to the deficiency of TNFRp55, favors the reinnervation of endometriotic-like lesions.

We first confirmed an increase in size of TNFRp55 deficient lesions (Vallcaneras *et al*., 2017) together with a decrease in TNFα level in the peritoneal fluid. Some researchers demonstrated that

TNFα levels decrease from minimal to severe stages of endometriosis (Cheong *et al*., 2002; Pizzo *et al*., 2002). In addition, Salmeri *et al*. (2015) found that TNFRp55 level decreases, whereas TNFRp75 increases, as the disease worsened. Therefore, the background suggests that our murine model of endometriosis in TNFRp55$^{-/-}$, might simulate an advanced stage of the disease with low levels of the proinflammatory cytokine TNFα, in comparison with the WT group. In fact, it has been shown that, in women with endometriosis, the peritoneal environment controls the differentiation of macrophage precursors, committing them toward an alternatively activated, reparatory phenotype (Bacci *et al*., 2009). Alternatively, activated macrophages show an anti-inflammatory phenotype that usually promotes the growth of tumors; this is what could be happening in our experimental model.

Another factor involved in the pathophysiology of endometriosis is the inducible enzyme COX2 (Jana *et al*., 2016). Women with endometriosis have an overexpression of COX2, possibly due to local inflammatory factors that stimulate its expression (Fagotti *et al*., 2004; Wu *et al*., 2010). In our experimental model, a significant reduction in the total number of cells staining positive for COX2 was observed in endometriotic-like lesions of KO mice compared with WT mice. This could be due to the low levels of TNFα in the peritoneal fluid of TNFRp55 deficient mice, since this cytokine can induce the expression of COX2 (Wu *et al*., 2002).

The study of the inflammatory state of TNFRp55 deficient mice was completed by glucocorticoid receptor expression analysis. These receptors mediate the actions of the glucocorticoids, which are powerful endogenous molecules that suppress inflammation by inhibiting cytokine transcription and modulating the expression of COX2 (Lim *et al*., 2014). In women with endometriosis, the pro-inflammatory state stimulates the production and action of glucocorticoids, mainly through TNFα (Monsivais *et al*., 2012). However, in our experimental model, due to the lack of TNFRp55 we have speculated a possible deregulation in the glucocorticoid system since previous reports demonstrate a crosstalk between TNFα and glucocorticoids (Van Bogaert *et al*., 2010). In fact, the results obtained demonstrate an increase in glucocorticoid receptor expression, mainly of GRα, against low levels of TNFα in our murine TNFRp55 deficient model of endometriosis, indicating the

possibility of glucocorticoid actions altering TNFα protein expression. Charmandari *et al*. demonstrated that GRα mediates the anti-inflammatory actions of the glucocorticoids, while GRβ is transcriptionally inactive, mediating its effects mainly by interfering mechanisms of GRα actions (Charmandari *et al*., 2005; Charmandari *et al.*, 2009). It is worth mentioning that glucocorticoid receptor activation can induce apoptosis in certain cell types such as lymphocytes, contributing to immunological escape, and that receptor activation can also induce survival signals, contributing to tumorigenesis (Wu *et al*., 2004; Wu *et al*., 2005). Therefore, the activation of glucocorticoid receptors possibly promotes anti-inflammatory and tumorigenic actions in KO animals, favoring endometriosis progression.

Previous results of our group (Vallcaneras *et al*., 2017), and those obtained in this work, prove that TNFRp55 deficiency contributes to worsen the pathology. This is evidenced by the increase in size of the lesions, the increased production of estradiol, the low levels of TNFα, the lower number of immunoreactive COX2 cells, and the greater expression of GRα. Furthermore, using the same experimental model, we found high antioxidant action in the peritoneal cavity of KO mice, without significant changes in the iNOS expression, and in correspondence with a decrease in lipid peroxidation in the endometriotic-like tissue (Delsouc *et al*., 2019). All these results suggest that the lesions grow and develop in the presence of a reduced inflammatory state. This hypothesis is supported by the findings of Mori *et al*. (2002), where they demonstrated that wound healing in TNFRp55 deficient mice occurs with lower cytokine expression, and reduced neutrophil and macrophage infiltration.

Interestingly, immune cells can produce various neurotrophic factors of various molecular families (Takei and Laskey, 2008). Therefore, we studied whether the characteristics of the peritoneal environment of TNFRp55 deficient mice influenced the reinnervation of uterine tissue in ectopic sites. In our model, we observed a significant increase of β-III TUBULIN in lesions of KO mice. The β-III TUBULIN is a microtubule protein normally expressed and restricted to cells of neuronal origin, but also implicated in uncontrolled proliferation, cancer development, and metastatic progression (Gao *et al*., 2008). Moreover, all neural markers assessed were higher in lesions of KO mice (*Pgp*

9.5, SP and Th). PGP 9.5 demonstrates the presence of nerve fibers in ectopic endometrium and has participation in nociceptive hypersensitivity induced by endometriosis (Tokushige *et al*., 2006; Al-Jefout *et al*., 2007). In women with endometriosis, PGP 9.5 positive nerve fiber density exhibits positive correlation with the gravity of pain symptoms (Zhang *et al*., 2009). Interestingly, in women with endometriosis, the sensory and sympathetic imbalance in favor of the first (Arnold *et al*., 2012) was also found in our TNFRp55 deficient model.

Regarding nerve fiber growth and pain pathways in endometriosis progress, evidence indicates that neurotrophins have a crucial role (Chao, 2003; Rocha *et al*., 2017). We analyzed BDNF and NGF expression, and their common receptor NTRp75, and all of them were increased in the KO lesions. Both BDNF and NGF have a strong expression in ectopic implants and peritoneal fluid of patients with endometriosis, promoting neurite outgrowth (Barcena de Arellano *et al*., 2013; Wu *et al*., 2017; Ding *et al*., 2018). In our model system, we demonstrated an increase in the expression of neurotrophic factors (BDNF, NGF, and NTRp75 receptor) in lesions from KO mice, suggesting that BDNF and NGF have an essential role in lesion innervation development in TNFRp55 absent conditions. Therefore, we can imply that the imbalance of TNF receptors that occurs while the disease progresses (Salmeri *et al.*, 2015) could contribute to the development of nerve fibers due to the overexpression of BDNF and NGF in endometriotic-like lesions. Regarding this, Li *et al.* (2011) found a gradual increase in NGF levels and its receptors with adenomyosis disease progression, another gynecological disease involving endometrial tissue. In addition, there is evidence to show that tumor neo-neurogenesis plays an active role in tumor growth and metastasis (Jobling *et al*., 2015).

Lastly, we demonstrated a significant increase in NTRp75 protein expression in KO mice lesions. Traditionally, activated NTRp75 exhibits an inhibitory effect upon cell proliferation. However, this receptor has been found activated in various cancers, inducing the proliferation of cancer cells (Meldolesi, 2018) through activation of the NFκB transcription factor (Dollé *et al*., 2004). Interestingly, we found a higher pNFκB/NFκB ratio in the endometriotic-like lesions of KO mice.

However, NFκB is a widely expressed transcription factor, important in the production of many inflammatory mediators, so additional studies are necessary to determine its precise role.

In summary, these results suggest that a subexpression of TNFRp55 can have serious consequences for women with advanced endometriosis. Interestingly, it has been shown that etanercept (recombinant human tumor necrosis factor receptor [p75]: Fc fusion protein) is effective in reducing endometriotic lesions in animal models (Barrier *et al*., 2004; Islimye *et al*., 2011; Zulfikaroglu *et al*., 2011). However, in patients with an advanced stage of endometriosis, etanercept increased pain symptoms (dysmenorrhea, deep dyspareunia and intermenstrual pain) (Shakiba and Falcone, 2006). Therefore, Shakiba and Falcone (2006) suggested that the suppression of TNFα by etanercept might not be beneficial for patients with advanced endometriosis. Our results suggest something similar. We obtained larger endometriotic-like lesions with more reinnervation in TNFRp55$^{-/-}$ mice where the TNFα levels were lower, which could indicate a greater sensitivity to pain (unmeasured variable). In addition, there is evidence showing that tumor neoneurogenesis plays an active role in tumor growth and metastasis (Jobling *et al*., 2015). Therefore, studies oriented towards treatments that could return the TNF system to physiological conditions should be a priority to prevent the progression of the disease.

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Authors' roles

Casais M and Meresman G conceived and designed the study. Ghersa F, Delsouc MB, Goyeneche AA and Vallcaneras SS performed the experiments. Experiments were done in the laboratories of Dr. Casais and Dr. Telleria under their tutelage. Casais M, Ghersa F and Delsouc MB analyzed and interpreted the data and wrote the manuscript. All authors participated in the revision of the article

and approved the manuscript for publication. All persons designated as authors qualify for authorship.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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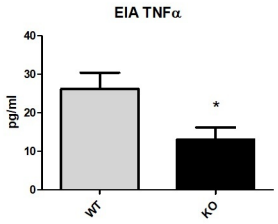
638

639 **Figure captions**

640 **Figure 1. Protein levels of TNFα in peritoneal fluid.** TNFα levels were assessed in peritoneal
641 fluid of wild type (WT) and TNFRp55 knock-out (KO) mice by enzyme immunoassay (EIA). Results
642 are expressed as mean ± SEM. of eight animals per experimental group. Student's *t*-test was used.
643 *p<0.05.



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646 **Figure 2. Expression of COX2 enzyme in endometriotic implants.** A) COX2 and ACTIN western
647 blot images; two bands are shown as representatives per experimental group. Images were
648 quantified using ImageJ and expressed in relative units. B) Western blot semi-quantification results
649 are expressed as mean ± SEM. of eight lesions per experimental group. Student's *t*-test was used.
650 C) Total positive cell percentages of COX2 immunohistochemistry (IHC). D) Micrographs show
651 representative histological sections of endometriotic-like lesions of wild type (WT) (n = 5) (I) and
652 knock-out (KO) (n = 5) (II). As a negative control, one section of each slide was assayed without

the primary antibody (III). Arrows indicate representative positive cells. Statistical comparisons were performed by Student's *t*-test. **p < 0.01. Magnification 200X. ACTIN: β-actin.

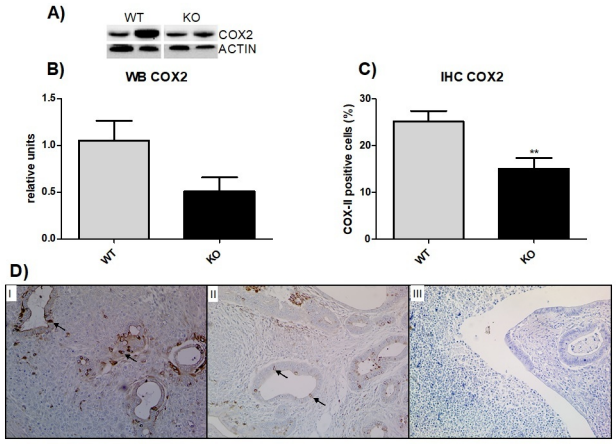


Figure 3. Glucocorticoid receptor expression in endometriotic implants. A) GRα, GRβ and ACTIN images display representative bands per experimental group. The images were semi-quantified using ImageJ and expressed in relative units. B) Western blot semi-quantification results are expressed as mean ± SEM. of eight lesions per experimental group. ANOVA followed by Tukey's Test was used. *p<0.05. C) Total positive cell percentages of GR by immunohistochemistry (IHC). D) Micrographs show representative histological sections of endometriotic-like lesions of wild type (WT) (n = 5) (I) and knock out (KO) (n = 5) (II). As a negative control, one section of each slide was assayed without the primary antibody (III). Arrows indicate representative positive cells. Statistical comparisons were performed by Student's *t*-test. Magnification 200X.

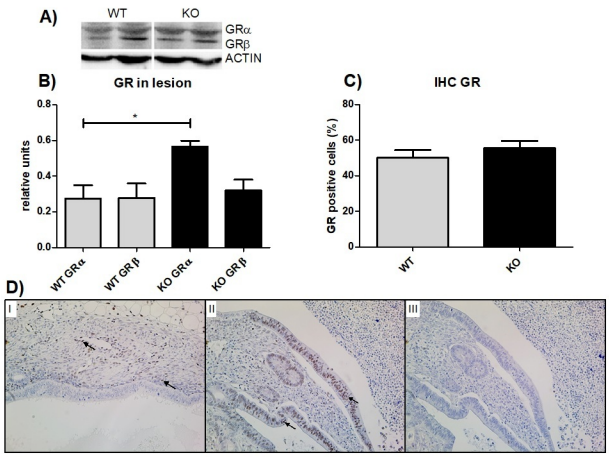


Figure 4. Nerve fiber markers in endometriotic implants. A) β-III TUBULIN and ACTIN western blot show images of two representative bands per experimental group. The images were semi-quantified using ImageJ and expressed in relative units. B) Western blot semi-quantification results are expressed as mean ± SEM. of eight lesions per experimental group. Student's *t*-test was used. ***p<0.001. Photograph and quantification results of RT-PCR (mRNA) of *Pgp 9.5* (C and D), *SP* (E and F) and *Th* (G and H). *Actb* was used as housekeeping gene. Four bands are shown as representatives per experimental group. The gel photographs were quantified using ImageJ and expressed in relative units. Results are expressed as mean ± S.E.M. of eight animals per experimental group. Student's *t*-test was used. *p<0.05. I) Relation between *SP* and *Th* is shown as mean ± S.E.M. of eight lesions per experimental group. Student's *t*-test was used. *p<0.05. β-IIITub: β-III TUBULIN.

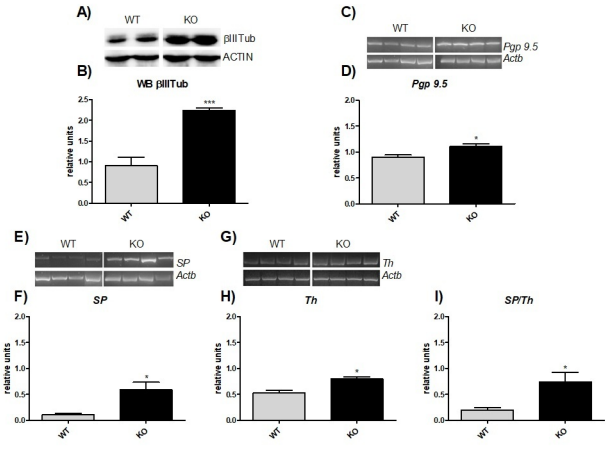
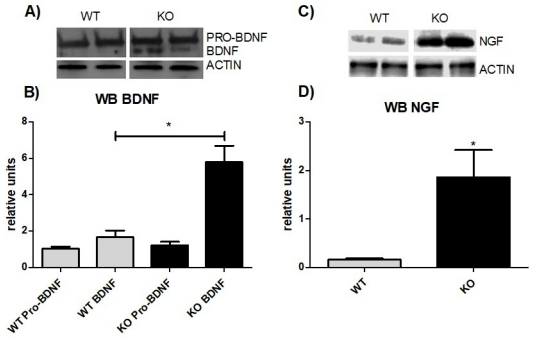


Figure 5. Neurotrophin expression in endometriotic implants. Western blot images of A) BDNF and C) NGF both with ACTIN used as loading control. Bands are shown as representatives per experimental group. Images were semi-quantified using ImageJ and expressed in relative units. Western blot semi-quantification results of BDNF (B) and NGF (D) are expressed as mean ± SEM. of eight lesions per experimental group. For BDNF one-way analysis of variance (ANOVA) followed by Tukey's Test was used. For NGF Student's *t*-test was used. *p<0.05.



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689 **Figure 6. Neurotrophin receptor expression in endometriotic implants.** Enzyme immunoassay
690 (EIA) of A) TRKA and B) NTRp75. Results are expressed as mean ± SEM. of eight lesions per
691 experimental group. Student's *t*-test was used. *p<0.05. C) Relation between pNFκB and NFκB
692 results obtained by EIA is shown as mean ± SEM. of eight animals per experimental group.
693 Student's *t*-test was used. *p<0.05.

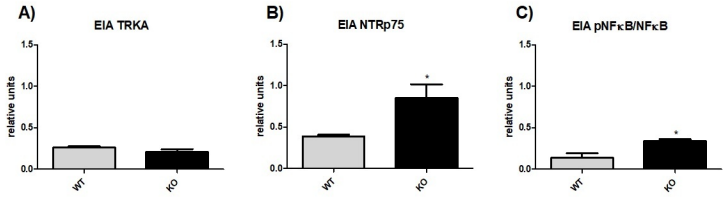

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Table 1: Primers used for PCR amplification

Gene name	Primers 5'-3'	GenBank accession #	Fragment size (bp)	Nº cycles
Pgp 9.5	ACGGCCATCTGTACGAGCTC CGGCAGAGAAGCGGACCTCC	AF_172334	144	35
SP	GGCCAAGGAGAGCAAAGA CGAGGATTTTCATGTTCGATT	NM_009311	88	35
Th	CCTTCCGTGTGTTTCAGTGC TCAGCCAACATGGGTACGTG	NM_009377	112	35
Actb	CGGAACCGCTCATTGCC ACCCACACTGTGCCCATCTA	NM_007393	289	35

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Table 2: Previous published results by Vallcaneras *et al*. (2017) relevant to this study.

	WT	**TNFRp55 $^{-/-}$**	***p-value***
N° developed lesions/mouse	2.47 ± 0.21 (n=12)	3 (n=11)	<0.05
Lesion volume (mm^3)	11.77 ± 0.49 (n=12)	28.94 ± 3.30 (n=11)	<0.05

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