


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

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Submitted on January 3, 2019; resubmitted on April 15, 2019; editorial decision on May 1, 2019

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 4 weeks, the lesions and the peritoneal fluid were collected. The level of TNF α in the peritoneal fluid was evaluated by enzyme-linked immunosorbent assay (EIA). The expressions of COX2, GR α and GR β 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 NF κ B (pNF κ B) and total NF κ B in lesions were measured by EIA. Compared with the WT group, the KO mice showed lower TNF α levels in the peritoneal fluid and lower numbers of COX2 immunoreactive cells along with increased expression of GR α , β -III TUBULIN, *Pgp 9.5*, *SP*, *Th*, BDNF, NGF, NTRp75 and pNF κ B 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.

Key words: 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 (TNF α) 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 TNF α 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.

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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 et al., 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 TNF α (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 TNF α 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 (GR α and GR β , 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 TNF α —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 et al., 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 Third 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—07:00–19:00 h—and 12 h of darkness), controlled temperature (22°C \pm 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 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 \pm 0.21 (n = 12) vs. 3 (n = 11), P < 0.05] and exhibited greater volume [11.77 \pm 0.49 mm³ (n = 12) vs. 28.94 \pm 3.30 mm³ (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 \sim 4 mm². 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 behavior and daily activity. No alteration in their behavior 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 (KH₂PO₄ 0.015 M, NaH₂PO₄ 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 250g 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 completely opened to have access to the endometriotic-like lesions, which were later randomly selected to perform different analyses.

Western blot

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

facturer's instructions. The rest of enzyme-linked immunosorbent assays (EIAs) were done as follows: 20 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 1 h 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-phospho-NFκBp65 (1:1000, 3033S, Cell Signaling Technology) overnight at 4°C. After three washes, 50 µl of goat anti-rabbit IgG-horse-radish peroxidase (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. The oxidation reaction of the substrate was stopped with 2 M sulfuric acid, and the optical density 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 were 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°C overnight. Endogenous peroxidase activity was blocked with 3% H₂O₂ 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 200×. A total of 100 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

Table 1 Primers used for PCR amplification.

Gene name	Primers 5'-3'	GenBank accession #	Fragment size (bp)	N° cycles
<i>Pgp 9.5</i>	ACGGCCATCTGTACGAGCTCCGGCAGAGAAGCGGACCTCC	AF_172334	144	35
<i>SP</i>	GGCCAAGGAGAGCAAAGACGAGGATTTTCATGTTCCGATT	NM_009311	88	35
<i>Th</i>	CCTTCCGTGTGTTTCAGTGCTCAGCCAACATGGGTACGTG	NM_009377	112	35
<i>Actb</i>	CGGAACCGCTCATTGCCACCCACACTGTGCCCATCTA	NM_007393	289	35

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 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 \pm SEM ($n = 8$ /group). Differences between groups were analyzed using two-tailed unpaired Student's *t*-test or one-way ANOVA followed by the Tukey's multiple comparison test. In all comparisons, a *P*-value of <0.05 was considered statistically significant.

Results

TNF α levels in peritoneal fluid are decreased in TNFRp55 deficient mice

We began by analyzing the levels of TNF α 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

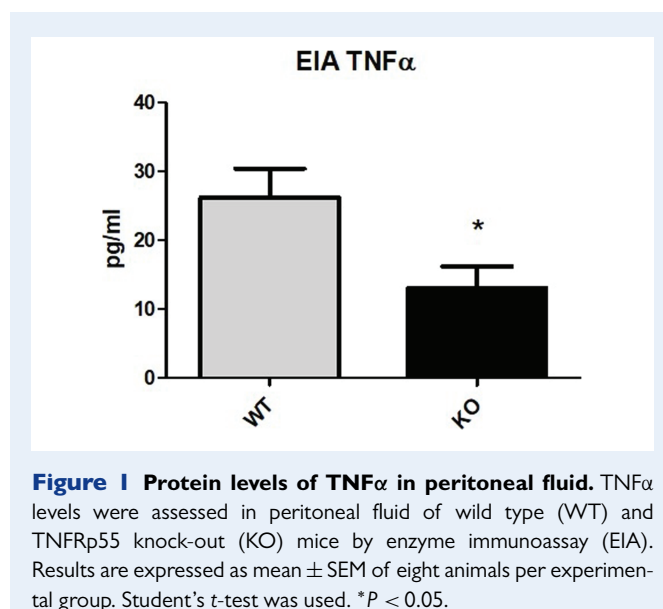


Figure 1 Protein levels of TNF α in peritoneal fluid. TNF α levels were assessed in peritoneal fluid of wild type (WT) and TNFRp55 knock-out (KO) mice by enzyme immunoassay (EIA). Results are expressed as mean \pm SEM of eight animals per experimental group. Student's *t*-test was used. **P* < 0.05.

this pro-inflammatory cytokine in KO mice compared to WT mice ($P = 0.0298$, Fig. 1), which suggests that the absence of TNFRp55 affects the expression of TNF α .

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 ($P = 0.1044$, Fig. 2A and B). However, when immunohistochemistry (IHC) was performed, a significant reduction in total positive cell staining was observed in KO implants compared to WT implants ($P = 0.0063$, Fig. 2C and D). These results indicate that in KO mice, COX2 is not expressed with the same intensity as in WT mice.

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

To understand the inflammatory state in which the endometriotic-like lesions develop, we studied not only pro-inflammatory markers but also glucocorticoid receptors, which are important anti-inflammatory

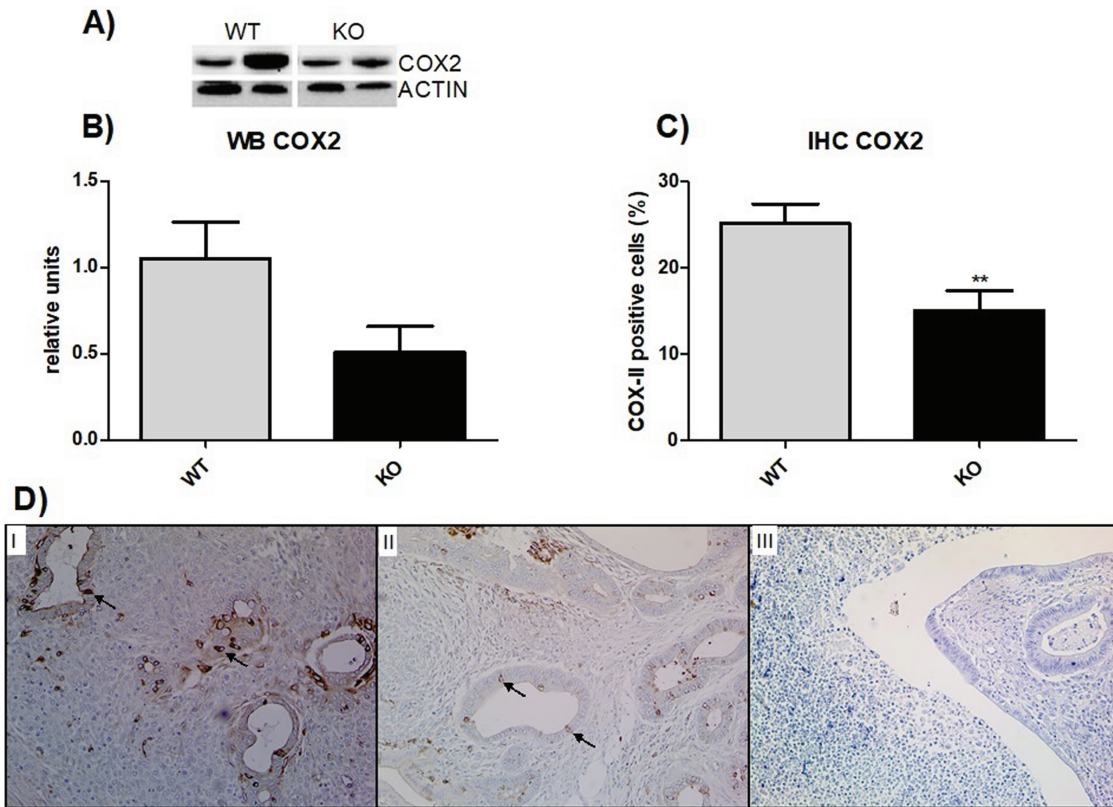


Figure 2 Expression of COX2 enzyme in endometriotic implants. (A) COX2 and ACTIN western blot images; two bands are shown as representatives per experimental group. Images were quantified using ImageJ and expressed in relative units. (B) Western blot semi-quantification results are expressed as mean \pm SEM of eight lesions per experimental group. Student's *t*-test was used. (C) Total positive cell percentages of COX2 immunohistochemistry (IHC). (D) Micrographs show representative histological sections of endometriotic-like lesions of WT ($n = 5$) (I) and 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 $\times 200$. ACTIN: β -actin.

markers. Of these receptors, GR α in particular mediates the anti-inflammatory responses of glucocorticoids. We observed a statistically significant increase of GR α in KO lesions compared to WT lesions ($P = 0.0223$, Fig. 3A and B) and a significant difference favoring the same receptor when compared to GR β among KO animals ($P = 0.0223$, Fig. 3B). Interestingly, when IHC was performed, no difference between total cell percentages was found (Fig. 3C), indicating that the main difference is between expression of the different glucocorticoid receptor types. The increase in the expression of GR α in the KO lesions possibly contributes to reduce the pro-inflammatory activity.

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

Reinnervation of implants is a necessary process for lesion survival, and it is positively correlated to pain symptoms in women with this disease. Inflammation can contribute to reinnervation by neurotrophin production and release. In our TNFRp55 deficient model, we found that β -III TUBULIN expression, a protein expressed and limited to neural tissues, was overexpressed in KO lesions ($P = 0.0007$, Fig. 4A and B),

indicating overall nerve fiber increase in this experimental group. We completed the study with analysis of gene expression of different nerve fiber markers; we found *Pgp 9.5*, a pan neuronal marker, overexpressed in KO lesions ($P = 0.0131$, Fig. 4D), thus confirming β -III TUBULIN results. To assess the sensory and sympathetic nerve fiber density, we studied gene expression of different nerve fiber markers: *SP* for sensory nerves and *Th* for sympathetic nerves. Both markers were found to be overexpressed in KO lesions compared to WT (*SP* $P = 0.0173$, Fig. 4E and F; *Th* $P = 0.0108$, Fig. 4G and H). Noteworthy, when the relation between *SP* and *Th* was studied, an imbalance was found in KO mice favoring the sensory nerve marker ($P = 0.0308$, Fig. 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,

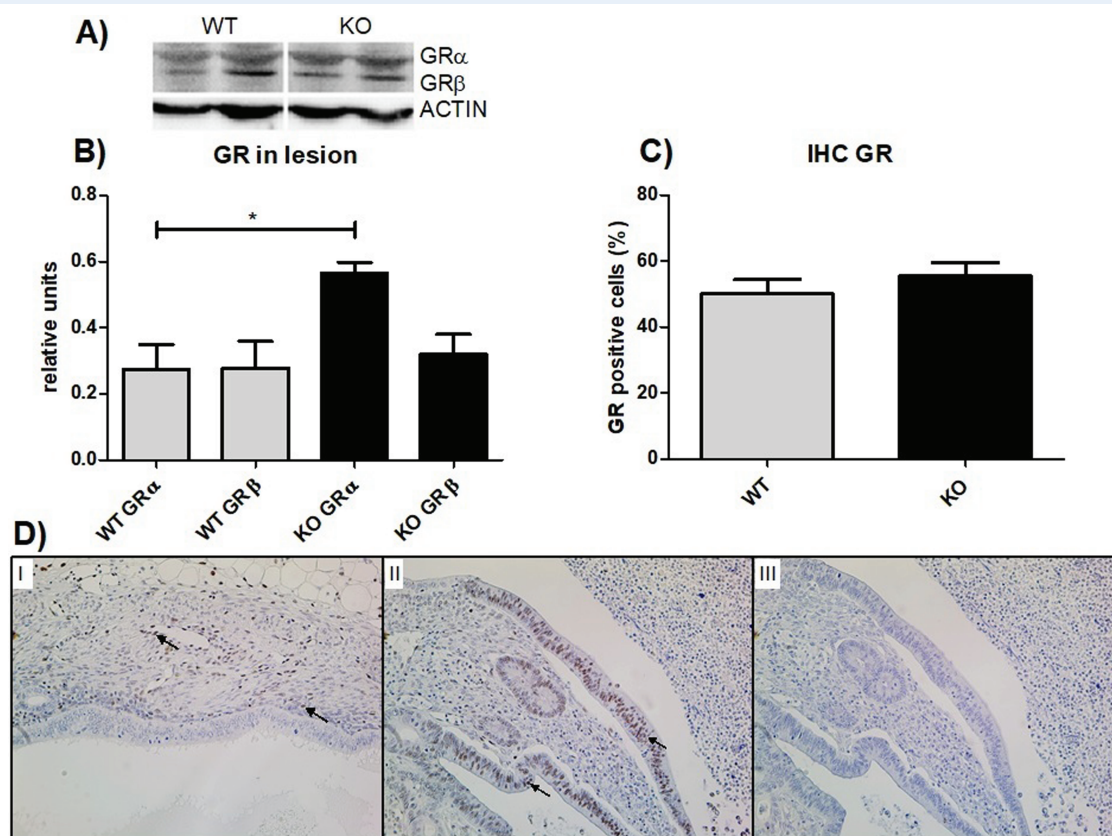


Figure 3 Glucocorticoid receptor expression in endometriotic implants. **(A)** GR α , GR β and ACTIN images display representative bands per experimental group. The images were semi-quantified using ImageJ and expressed in relative units. **(B)** Western blot semi-quantification results are expressed as mean \pm 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 IHC. **(D)** Micrographs show representative histological sections of endometriotic-like lesions of WT ($n = 5$) (I) and 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 $\times 200$.

mature BDNF ($P < 0.0001$, Fig. 5A and B) and NGF ($P = 0.0490$, Fig. 5C and 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 (Fig. 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 ($P = 0.0212$, Fig. 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 NF κ B by phosphorylation (pNF κ B), which is known to be induced by the activation of the

NTRp75 pathway. We found that the relationship between pNF κ B and NF κ B is in favor of the first in KO mice with respect to WT mice ($P = 0.0159$, Fig. 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 TNF α level in the peritoneal fluid. Some researchers demonstrated that TNF α 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 pro-inflammatory cytokine TNF α , in comparison with the WT group. In fact, it has been

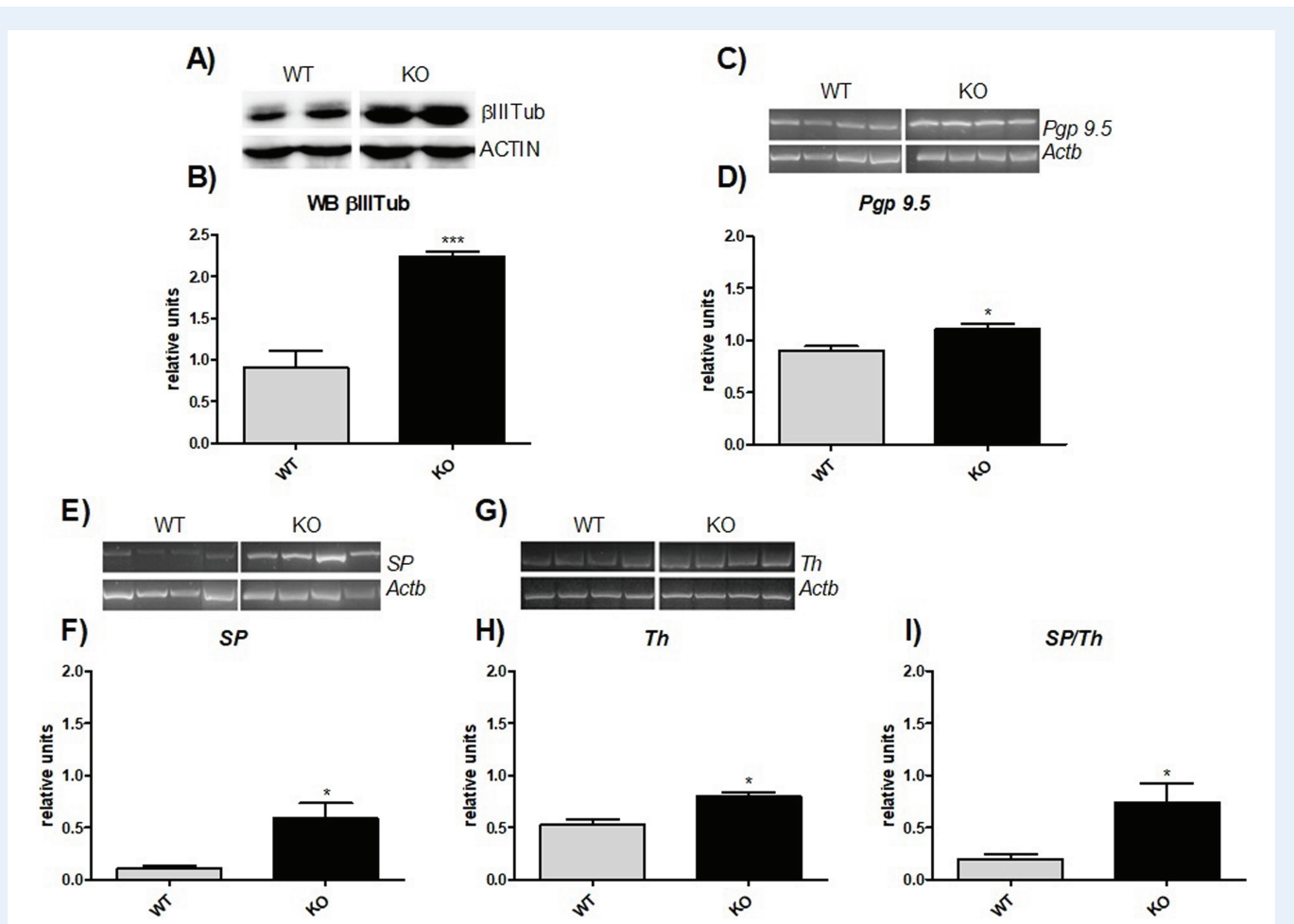


Figure 4 Nerve fiber markers in endometriotic implants. (A) β -III TUBULIN and ACTIN western blot show images of two representative bands per experimental group. The images were semi-quantified using ImageJ and expressed in relative units. (B) Western blot semi-quantification results are expressed as mean \pm 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 \pm SEM 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 \pm SEM of eight lesions per experimental group. Student's *t*-test was used. **P* < 0.05. β -III Tub: β -III TUBULIN.

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 TNF α 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 TNF α (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 TNF α and glucocorticoids (Van Bogaert *et al.*, 2010). In fact, the results obtained demonstrate an increase in glucocorticoid receptor expression, mainly of GR α , against low levels of TNF α in our murine TNFRp55 deficient model of endometriosis, indicating the possibility of glucocorticoid actions altering TNF α protein expression. Charmandari *et al.* (2005, 2009) demonstrated that GR α mediates the anti-inflammatory actions of the glucocorticoids,

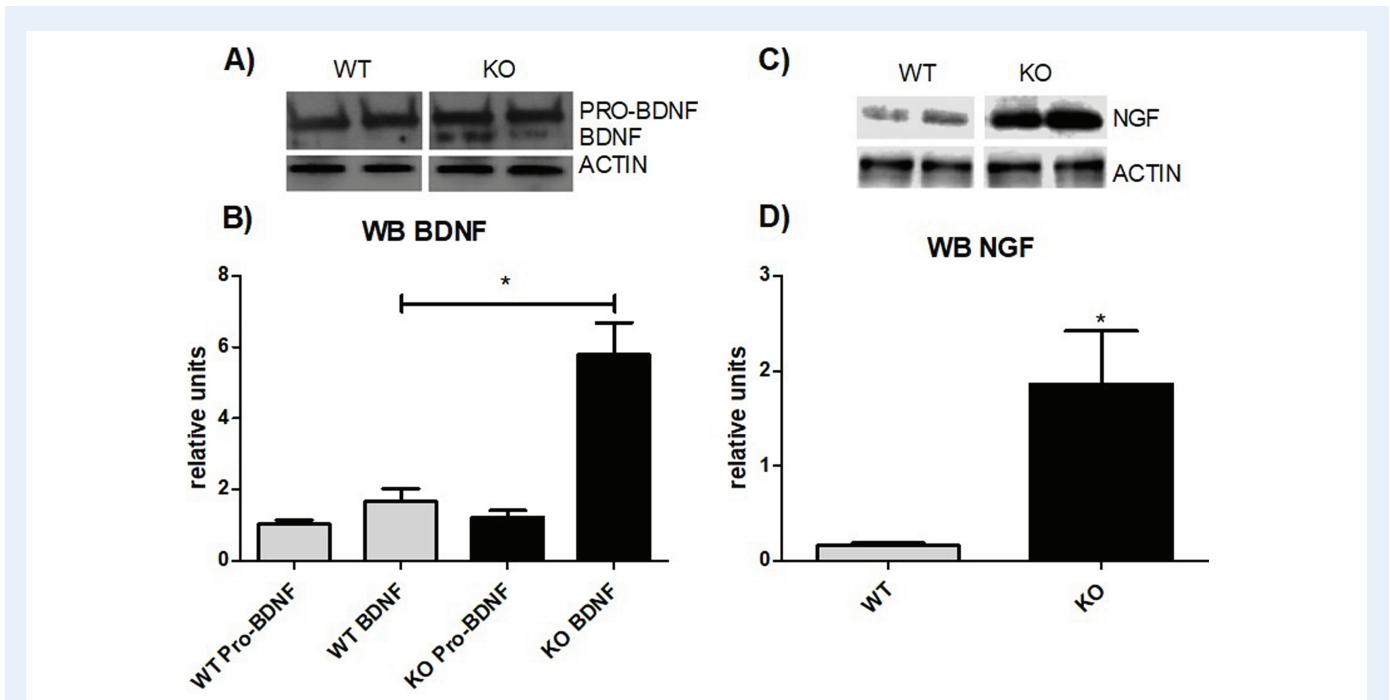


Figure 5 Neurotrophin expression in endometriotic implants. Western blot images of (A) BDNF and (C) NGF both with ACTIN used as loading control. Bands are shown as representatives per experimental group. Images were semi-quantified using ImageJ and expressed in relative units. Western blot semi-quantification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

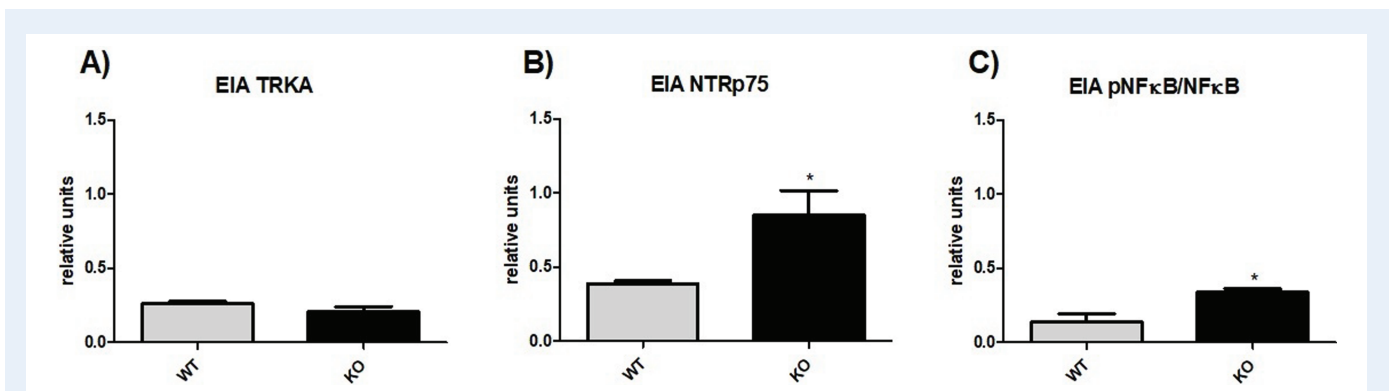


Figure 6 Neurotrophin receptor expression in endometriotic implants. EIA of (A) TRKA and (B) NTRp75. Results are expressed as mean \pm SEM of eight lesions per experimental group. Student's *t*-test was used. **P* < 0.05. (C) Relation between pNFκB and NFκB results obtained by EIA is shown as mean \pm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

while GR β is transcriptionally inactive, mediating its effects mainly by interfering mechanisms of GR α actions. 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, 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 (Valcaneras 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 TNF α , the lower number of immunoreactive COX2 cells and the greater expression of GR α . 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 neoneurogenesis 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 NF κ B transcription factor (Dollé et al., 2004). Interestingly, we found a higher pNF κ B/NF κ B ratio in the endometriotic-like lesions of KO mice. However, NF κ B 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 TNF α 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 TNF α 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 toward treatments that could return the TNF system to physiological conditions should be a priority to prevent the progression of the disease.

Acknowledgments

We gratefully thank the Laboratorio de Inmunopatología (IMIBIO-SL CONICET, UNSL) for providing TNFRp55 deficient mice and PhD Magdalena Mont Guevara for facilitating some of the reagents used for this study. This work is part of the Doctoral thesis of Federica Ghersa.

Authors' roles

C.M. and M.G. conceived and designed the study. G.F., D.M.B., G.A.A. and V.S.S. performed the experiments. Experiments were done in the laboratories of Dr Casais and Dr Telleria under their tutelage. C.M., G.F. and D.M.B. 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.

Funding

Universidad Nacional de San Luis, Argentina (PROICO-CyT number 2-2916) and Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina (PIP number 112 201501 00391 CO). Federica Ghersa was the recipient of a scholarship from the Emerging Leaders in the Americas Program, Global Affairs Canada.

Conflict of interest

The authors declare that there are no conflicts of interest.

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