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Galectin-3 plays an important role in endometriosis development and is a target to endometriosis treatment

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ABSTRACT

This study aimed to analyze galectin-3 importance in endometriotic lesions development and the effect of recombinant Gal-3 carbohydrate recognition domain (Gal3C) in experimental endometriosis treatment. Experimental endometriosis was induced in WT and Gal-3−/− mice. Initially developed lesions were macroscopically and histologically analyzed, including immunohistochemical analysis. Then, WT mice were treated with Gal3C for 15 days. Gal-3 deficiency and Gal3C treatment significantly impaired endometriosis development. A significant decrease in lesions implantation and size, *VEGF* and *VEGFR-2* expression, vascular density and macrophage distribution were observed in Gal-3 absence or inhibition. A greater presence of iNOS positive cells was observed in knockout mice lesions, while the presence of Arginase positive cells was higher in the WT animal lesions. In addition, COX-2 and *TGFb1* were reduced by Gal3C treatment. Data showed here indicate a relevant role of Gal-3 in endometriosis development and highlight a target of endometriosis treatment using Gal-3 inhibitor.

1. Introduction

Endometriosis is a benign gynecological disease associated with pelvic pain and infertility that affects 6–10% (over 176 million) women of reproductive age (Burney and Giudice, 2012; David Adamson et al., 2010) and 30–50% of infertile women worldwide (Holoch and Lessey, 2010). This disease is characterized by the presence of glands and/or stroma similar to endometrial tissue outside the uterine cavity (Giudice, 2010; Sasson and Taylor, 2008), more particularly ovaries and pelvic peritoneum (King et al., 2016).

Although endometriosis is widely studied, there is still no consensus on the genesis of the disease (Burney and Giudice, 2012). Retrograde menstruation is the most accepted theory for endometriosis development. It proposes the development of ectopic implants from fragments of endometrium that by retrograde menstrual flow extravasate into the peritoneal cavity and adhere to affected sites (Sampson, 1927). Thereafter, the formation of a vascular network is critical for cell proliferation and establishment of the endometriotic lesion. In this context,

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CD31, cluster of differentiation 31; COX-2, cyclooxygenase 2; Gal-3, galectin-3; Gal3C, recombinant Gal3 carbohydrate recognition domain; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HUVEC, human umbilical vein endothelial cells; iNOS, inducible nitric oxide synthase; KDR, kinase insert domain receptor; TGFb1, transforming growth factor-beta 1; VEGF, vascular endothelial growth factor; VEGFR-2, vascular endothelial growth factor receptor-2; WT, wild-type

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the angiogenesis is a key event contributing to development and maintenance of endometriosis (Marí-Alexandre et al., 2015).

Galectins is a β-galactoside-binding protein and has a significant influence on angiogenesis. Nangia-Makker et al. (2000) showed that Gal-3 can stimulate angiogenesis *in vitro* by a matrigel tube formation assay with HUVEC cells (human umbilical vein endothelial cells) and *in vivo* by increasing vascular density in murine tumors induced by lineages of breast tumor cells expressing this lectin. In addition, endothelial cells in tumors exhibit higher Gal-3 expression than endothelial cells present in non-tumor tissues, suggesting the participation of this molecule in vessel formation/maintenance (Jia et al., 2010; Ryschich et al., 2006).

Gal-3 also is related to cell embryogenesis, adhesion, differentiation and proliferation, apoptosis, mRNA splicing and regulation of the immune system response. The participation in all these processes has put Gal-3 in evidence for its study in diseases promotion and development, including endometriosis (Dumic et al., 2006; Newlaczyl and Yu, 2011). Noël et al. (2011) evaluated the expression of this lectin in human endometriotic lesions and showed that Gal-3 is overexpressed in peritoneal endometriosis, deep and infiltrating lesions, and the eutopic endometrium of women with endometriosis compared to the eutopic endometrium of women without endometriosis. These data suggest a possible role of Gal-3 in the development and maintenance of endometriosis.

The anti-angiogenic effect of Gal-3 inhibition was previously reported in tumor models. The treatment of human umbilical vein endothelial cells with a truncated Gal-3 Carbohydrate recognition domain was able to impair tubule formation and endothelial cells migration induced by multiple myeloma and ovarian cancer secreted factors (Mirandola et al., 2011, 2014). Recently, our group described an alternative method to produce human recombinant Gal-3 carbohydrate recognition domain (Gal3C) in *Escherichia coli*, using a less-expensive and more straightforward two-step chromatographic routine, able to produce high yields of recombinant protein free of carbohydrate ligands (Wiecikowski et al., 2018). In addition, purified recombinant Gal3C inhibited endothelial cells tubule formation *in vitro*, suggesting a potential use as an anti-angiogenic factor *in vivo* (Wiecikowski et al., 2018).

In this study, we showed that Gal-3 expression is involved in the development of endometriotic lesions, the inhibition of Gal-3 using a human recombinant Gal3C was able to diminish the endometriotic lesions by reducing the expression of angiogenic factors. Thus, Gal-3 may be a potential target for the treatment of endometriosis.

2. Materials and methods

2.1. Endometriosis experimental model

All experiments were conducted in accordance to the Ethical Guidelines from the Animal Care and Use Committee and the NIH Guidelines for the Care and Use of Laboratory Animals (http://oacu.od.nih.gov/regs/index.htm. 8th Edition, 2011). The Institutional Animal Care and Use Committee of Federal University of Rio de Janeiro (UFRJ) and West Zone State University (UEZO) approved the protocols used in this study (01200.001568/2013-87 and 009/2014, respectively).

Wild-type (WT) and Gal-3-deficient (Gal-3 −/−) female BALB/c mice (20 g and 8 weeks of age) were used in the experimental induction of endometriosis. Gal-3-deficient mice were obtained from the animal facilities of the Medical School of the University of São Paulo (USP). Endometriotic lesions induction was performed using the method described earlier by Vernon and Wilson (1985). Briefly, mice were anesthetized (ketamine and xylazine), the abdomen was opened, one uterine horn was removed, segmented, split longitudinally and sectioned in two pieces being implanted one piece on each side of the ventral abdominal peritoneum wall and the abdomen was closed. After twenty-eight days (day zero, D0), ventral midline laparotomy was performed to evaluate the viability and the area of endometrial explants macroscopically. Endometriotic implants of WT and *Gal-3* −/− BALB/c mice were analyzed for adhesion, development and size. The evaluation of lesion size was performed using a linear measure with the aid of a ruler. After this analysis, the lesions were collected and distributed in two groups, for histological analysis and protein extraction.

2.2. Recombinant Gal3C production and endometriosis treatment

The protocol to produce and purify recombinant Gal3C was described previously (Wiecikowski et al., 2018). In brief, cellular extracts of *E. coli* BL21 (DE3) bacteria transformed with plasmid pET25b(+) encoding the sequence between amino acids 103 to 250 of human Gal3 (Uniprot: P17931) were purified using two in tandem HiTrap SP HP columns, followed by a size exclusion chromatography using a Superdex 75 16/60 column. The purified protein was obtained in an elution buffer containing sodium phosphate 20 mM, sodium chloride 150 mM pH 7.4.

Fourteen female BALB/c mice were used to test the recombinant Gal3C in the experimental endometriosis. After endometriotic lesion confirmation (D1), animals were divided randomly into two groups each containing seven animals: the Gal3C group was treated with 170 µL-containing 50 µg of recombinant Gal3C dissolved in elution buffer, and the control group received only the buffer. Both groups were administered daily by intraperitoneal injections for 15 consecutive days. The animals were housed in polyethylene cages at UEZO animal facility and were kept at a constant temperature (25 °C) under a 12-h light/dark cycle with free access to food and water.

One day after the treatment period (D16), the animals were euthanized by anesthesia overdose (Fig. 5A) and the abdomen was investigated for the presence of a lesion or suture alone. Peritoneal lesions, liver and blood were collected for analysis. The surface area of the explants was measured (length × width) to the nearest 0,1 mm using calipers and after excised were weighed and immediately divided for histological and PCR analyses. Body weight was measured before (D0) and after treatment (D16).

2.3. Histology, immunohistochemistry and morphometric analysis

Peritoneal lesions were fixed in 10% buffered formalin, embedded in paraffin and cut into 4-µm-thick sections for histological and immunohistochemical analyses. Part of the sections were stained with hematoxylin and eosin and examined microscopically for histological hallmarks of endometriosis and the other tissue sections were placed on silane-treated slides and incubated with the following antibodies: monoclonal antibody against VEGF, SC-57496 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:100 dilution; monoclonal antibody against VEGFR-2, SC-6251 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:100 dilution; anti-galectin-3, M3/38 hybridoma supernatant, (American Type Culture Collection) at 1:32 dilution; anti-CD31, DIA-310, Clone SZ31 (Dianova, Germany) at 1:100 dilution; polyclonal antibody against F4-80 macrophage antigen SC-26642 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:200 dilution; anti-arginase-1 SC-20150 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:100 dilution; anti-iNOS RB-9242-P1 (Thermo Scientific, MA, USA) at 1:100 dilution; and monoclonal antibody against COX-2 SC-376861 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:200 dilution. Incubations were carried out overnight and then revealed using LSAB2 Kit HRP, rat (Dako-Cytomation, Carpinteria, CA, USA) with diaminobenzidine (3,3′-diaminobenzidine tablets; Sigma, St. Louis, MO, USA) as the chromogen and counterstained with hematoxylin. For each case, negative control slides consisted of sections incubated with antibody vehicle or no immune rabbit or mouse serum were used. All tissues were examined by two blinded observers using a light microscope (Nikon, Tokyo, Japan) connected to a digital camera (Coolpix 990; Nikon, Tokyo, Japan). Ten fields of an immunostained section were

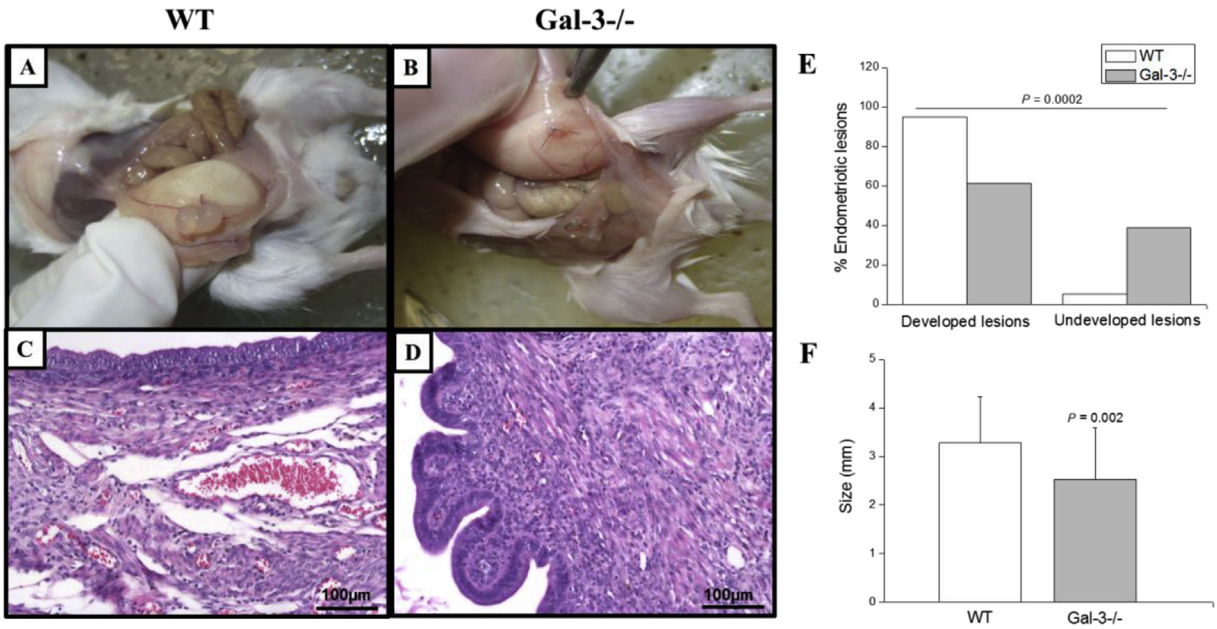

Fig. 1. Development of endometrial implants in WT and *Gal-3*−/− BALB/c mice. Macroscopic view of endometriotic lesions developed in peritoneum of WT (A) and *Gal-3*−/− mice (B) after 30 days of implant. C and D. Histological analyzes revealed that implants developed in both mice lines presented a simple columnar epithelium associated to an extensive, very cellular and abundantly vascularized stroma, similar to the characteristics of an eutopic endometrium, allowing the histopathological confirmation that the implants formed endometriotic lesions (C is WT group and D *Gal-3*−/− mice group). E. The distribution of developed endometriotic lesions was significantly different between two groups ($P = 0.0002$, $\chi 2$ test). F. Size of endometriotic lesions developed were significantly higher in WT compared with *Gal-3*−/− mice.

chosen at random and captured from each specimen. Quantification was assessed on captured high quality images (2048 × 1536 pixels buffer) using the Image Pro Plus 4.5.1 (Media Cybernetics, Silver Spring, MD, USA). Histologic scores (H) were calculated using formula H = ΣPi, where I is the intensity ranging from 0 (negative cells) to 3 (deeply staining cells) and P is the percentage of staining cells for each given i, with P values of 1, 2, 3, 4, and 5 showing < 15%, 15–50%, 50–85%, > 85%, and 100% positive-staining cells, respectively, as previously described (Machado et al., 2016). The staining result was expressed as mean ± standard deviations.

2.4. Protein extraction and western blot

Total protein was extracted from ectopic tissues using protein extraction buffer and protein concentration was quantified using Bradford method (Bradford, 1976). Protein extracts were resolved by SDS-PAGE and transferred to a polyvinylidene membrane (Bio-Rad, USA) using an electroblotter (Bio-Rad). Membranes were blocked with 5.0% nonfat milk, followed by overnight incubation with primary antibodies to VEGF (ab46154, abcam), VEGFR-2 (ab39256, abcam), Galectin-3 (M3/38 hybridoma supernatant, American Type Culture Collection) e lamin A/C (4777S, Uniscience). Primary antibody binding was detected using an HRP-conjugated secondary antibody (anti-rat, Vector Laboratories BA-40011:2000); anti-rabbit (Southern Biotech 4010-05). Western Blot films were scanned and the images obtained were quantified using ImageJ 1.38x image analysis software (Wayne Rasband, NIH, USA). The values obtained by densitometry were used for the statistical analyzes.

2.5. Real-time RT PCR

The mRNA levels of *VEGF*, kinase insert domain receptor (*KDR*), gene which encodes VEGF receptor 2 and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were determined using TaqMan® real-time polymerase chain reaction. Transforming growth factor, beta 1 (*TGFB1*) mRNA levels were quantified using Hot FirePol Evagreen® qPCR mix (Solys Biodyne). Total RNA was isolated from endometriosis samples using Trizol® reagent (Invitrogen) according to the manufacturer's instructions and quantified using Nanodrop® spectrophotometer. Two micrograms of total RNA were used for cDNA synthesis, using RevertAid cDNA synthesis kit (Thermo Fisher Scientific). TaqMan Universal PCR Master Mix and validated TaqMan assays were purchased from Applied Biosystems (*VEGF*: Mm01281449_m1; *KDR*: Mm01222421_m1; and *GAPDH:* Mm99999915_g1). *TGFB1* primers Fw 5′TACCATGCCAACTTCTGTCTGGGA-3′ and Rv 5′ATGTTGGACAACTG CTC CACC-3′ were purchased from Thermo Fisher Scientific. Each sample was analyzed in triplicate using a 7500 Real-Time thermocycler (Applied Biosystems®). The relative quantification of target genes was performed by ΔΔCt method using *GAPDH* as endogenous control.

2.6. Biochemical analysis

Glycemia (G-Tech Free, SD Biosensor Inc, Duque de Caxias, RJ, Brazil), creatinine, urea, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were evaluated from the blood samples taken using respective kits (K067, K056, K048 and K049, Bioclin, Belo Horizonte, MG, Brazil), according to the manufacturer's instructions, and quantified by spectrophotometer.

2.7. Statistical analysis

Data were expressed as mean ± standard deviations (SD). Statistical analyses were performed using Student's t-test. For morphometric analysis, statistical calculations were carried out with use of the Stat-Xact-5 software program (CYTEL Software Corporation, Cambridge, MA). The association of endometriosis development in WT and Gal-3−/− animals was estimated by the odds ratio (OR) and 95% confidence interval (CI). Data were expressed as percentages and compared between two groups with the Chi-Square ($\chi 2$) test. Differences were considered significant when the P values were < 0.05.

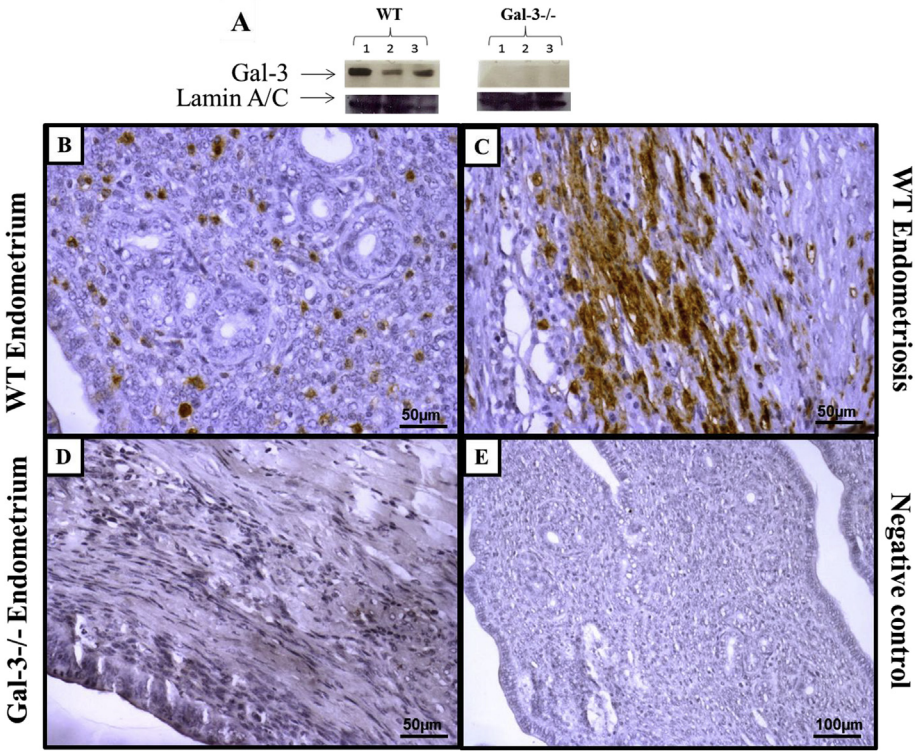

Fig. 2. Galectin-3 expression and distribution in the eutopic endometrium and peritoneal endometriosis of WT and *Gal-3*−/− mice. Gal-3 expression in eutopic endometrium of WT and *Gal-3*−/− mice by Western Blot (A). Gal-3 immunodistribution in eutopic endometrial sections of WT and *Gal-3*−/− mice (B and D, respectively) and peritoneal endometriosis of WT (C). Gal-3 distribution was not observed in tissues obtained from *Gal-3*−/− mice, while the expression was positive in WT animal tissues, with a greater distribution in endometriotic lesions. Negative control of the immunohistochemical reaction (E).

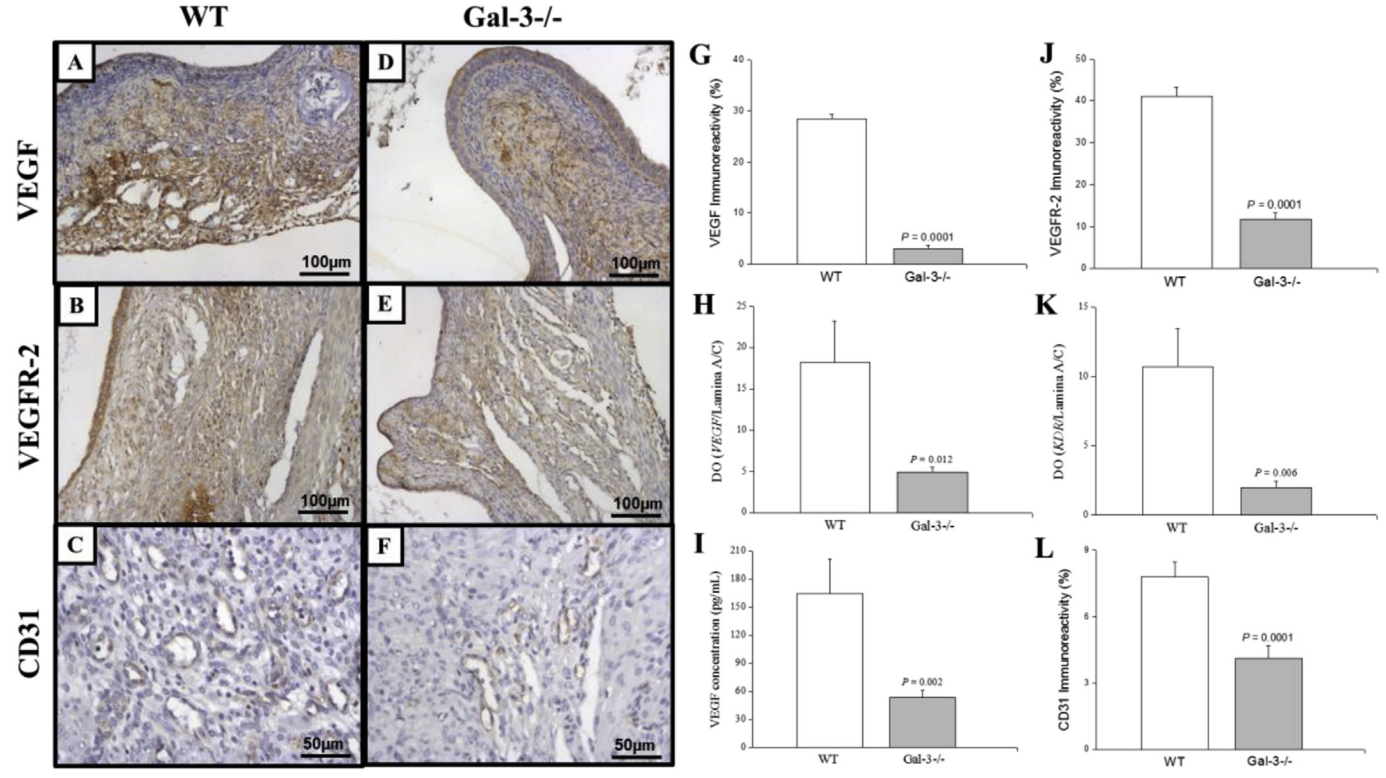

Fig. 3. Angiogenic process analysis in WT and Gal-3−/− mice. Immunodistribution of VEGF and VEGFR-2 in WT (A and B, respectively) and Gal-3−/− mice (D and E, respectively) endometriosis. Quantification of VEGF and VEGFR-2 immunodistribution (G and J, respectively). Analysis of VEGF and VEGFR-2 expression in WT and Gal-3−/− mice endometriosis (H and K, respectively). VEGF concentrations were measured by ELISA in peritoneal fluid of WT and Gal-3−/− (I). Immunodistribution of CD31 in WT (C) and Gal-3−/− mice (F) endometriosis. A greater number of CD31 positive vessels were observed in WT endometriosis (L).

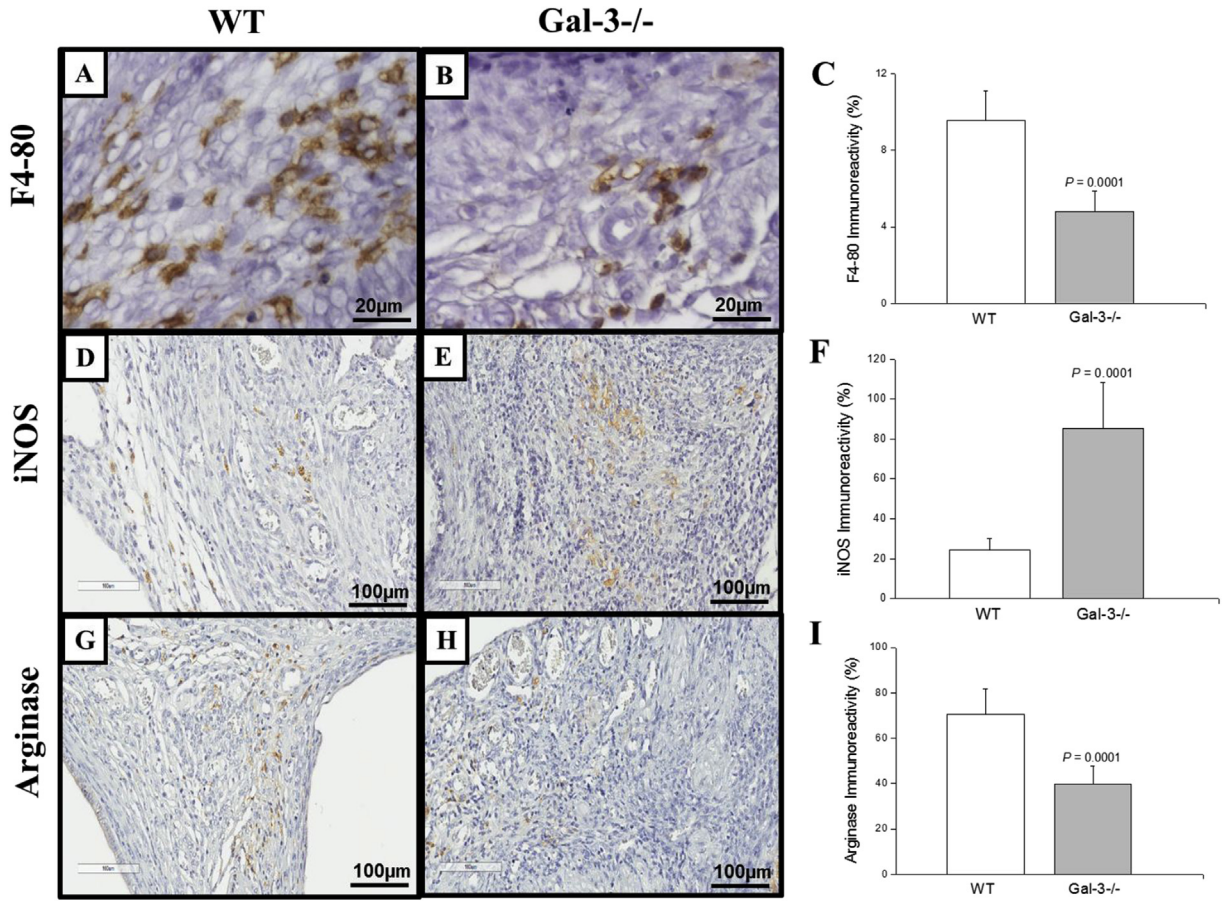

Fig. 4. Macrophage population analysis in WT and *Gal-3*−/− mice. Immunodistribution of F4-80 positive cells in WT (A) and *Gal-3*−/− mice (B) endometriosis. Quantification of F4-80 positive cells (C). Immunodistribution of iNOS and arginase positive cells in WT (D and G) and *Gal-3*−/− mice (E and H) endometriosis. Quantification of iNOS and arginase positive cells (F and I, respectively).

3. Results

3.1. Development of endometrial implants in WT and Gal-3 knockout BALB/c mice

Macroscopic analysis revealed a success rate of 94.7% in WT lesions development, while in Gal-3−/− the success rate was significantly lower (61.2%) (Fig. 1E). Gal-3 expression was associated with a higher risk (approximate 11-fold) of endometriosis development (OR: 11.4, 95% CI: 2.5–52.9, $P = 0.0002$, $\chi 2$ test). Macroscopic evaluation (Fig. 1A and B) also showed that the size of developed lesions significantly differed between the two experimental models, with larger lesions in WT animals (Fig. 1F).

Morphologically the developed implants were similar to human endometriosis, with cystic characteristic and presence of inflammatory liquid in its interior. Histological analyzes revealed that implants developed in both mice lines presented a simple columnar epithelium associated to an extensive, very cellular and abundantly vascularized stroma (Fig. 1C and D).

In addition, Gal-3 distribution was not observed in tissues obtained from Gal-3−/−, while the expression was positive in WT animal tissues, with a greater distribution in endometriotic lesions (Fig. 2).

3.2. Angiogenic factors expression and vascular density were higher in Gal-3 knockout mice endometriosis

VEGF and VEGFR-2 immunoreactivity were observed in the endometriotic lesions, mainly in stromal and vascular endothelial cells. Immunohistochemical analysis showed a higher VEGF and VEGFR-2 expression in WT endometriotic lesions (Fig. 3A and B, respectively) compared to Gal-3−/− (Fig. 3D and E, respectively). These results were confirmed by histomorphometry evaluation (Fig. 3G and J), protein expression analysis (Fig. 3H and K) and ELISA analysis of peritoneal fluid (Fig. 3I).

Moreover, CD31 immunodistribution analysis revealed a greater vascular density in WT endometriosis compared to Gal-3−/− lesions (Fig. 3C and F, respectively). Histomorphometric analysis showed a significant higher vascular density in WT lesions (Fig. 3L).

Since endometriosis is an inflammatory disease and macrophages are important inducers of angiogenesis, we evaluated the presence of these cells in developed lesions. F4/80 positive cells analysis revealed a greater amount of macrophages in WT lesions (Fig. 4A) compared to Gal-3−/− lesions (Fig. 4B). This result was confirmed by histomorphometric analysis (Fig. 4C).

After these findings, the subpopulations of these cells were investigated. For this purpose, iNOS, a marker of M1 macrophages, and arginase-1, a marker of M2 macrophages, distribution were evaluated. This analysis revealed a significant greater presence of iNOS positive cells in the endometriotic lesions of Gal-3−/− animals (Fig. 4E and F), while the number of arginase positive cells was higher in the WT lesions (Fig. 4G and I).

3.3. Gal3C treatment reduced endometriotic lesions without toxicity

Since the lesions were lower in Gal-3−/−, we tested the use of a recombinant Gal3C in the endometriosis model (Fig. 5A). All 14 BALB/c mice used in this analysis presented viable cystic and well-vascularized lesions 28 days after endometrial tissue transplantation (Fig. 5B and C).

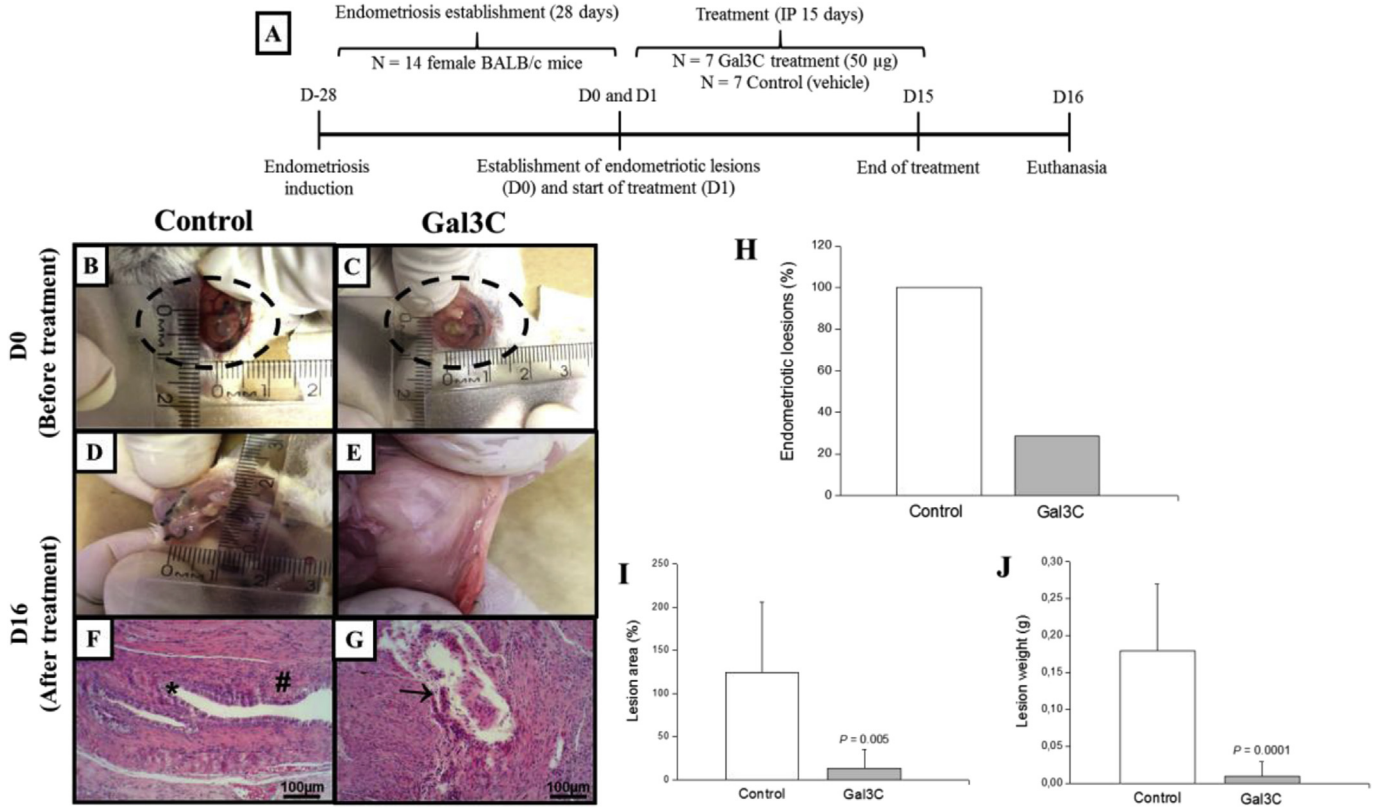

Fig. 5. Morphological characteristics of mice peritoneal endometriotic lesions in control and Gal3C treated groups. Schematic representation of endometriosis experimental mice model and Gal3C treatment (A). Macroscopic view of implants in the peritoneal cavity wall before treatment (B and C). Macroscopic view of lesions after treatments (D and E). In the control group (D), the endometriotic lesions were cystic, resembling human peritoneal endometriosis. In the Gal3C group (E), a marked decrease in the growth of the lesions were visualized. Histologically, in the control group (F) were observed the presence of endometrial glands (*) and stromal cells (#). In the Gal3C group (G), showed a tissue atrophy and regression of lesions (→). (H) Percentage of endometriotic lesions before and after treatment in the control and Gal3C groups. Measurements of the lesion area (I) and weight (J) demonstrated a statistically significant difference between the groups. Gal3C = Recombinant galectin-3 carbohydrate domain.

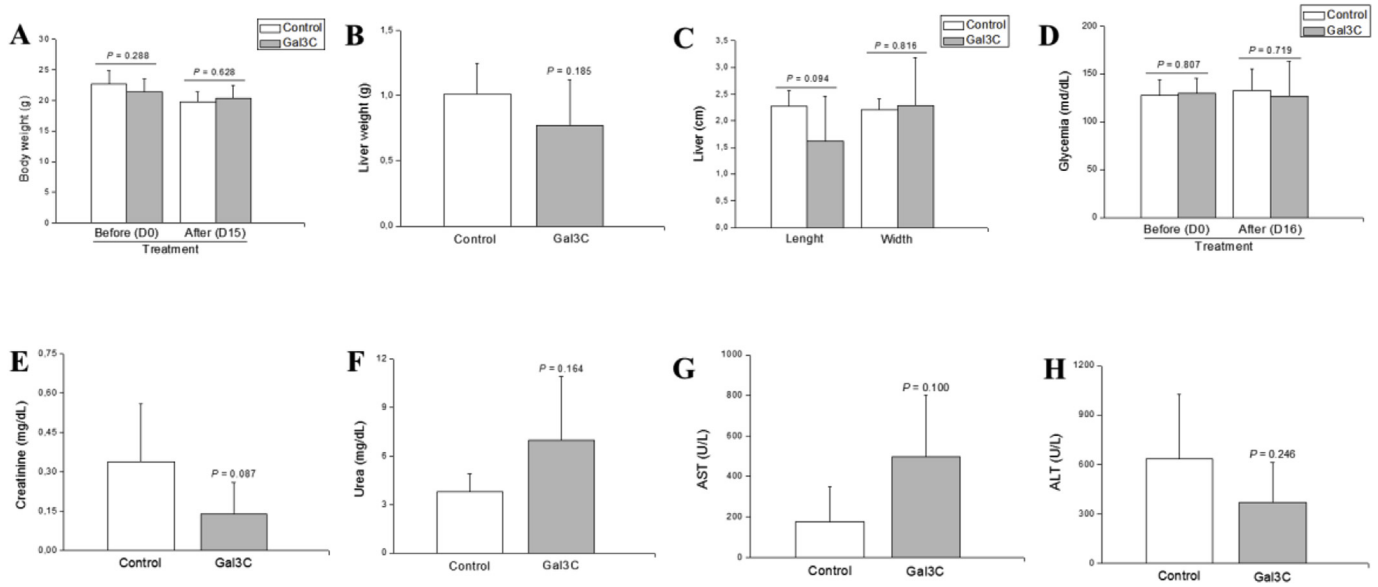

Fig. 6. No toxicity was observed in Gal3C treated group. No evidence of toxicity was noted between the treated Gal3C animals and the control based on body weight (A), liver weight (B) and size (C), glycemic (D), serum creatinine (E), serum urea (F), serum AST (G) and serum ALT (H).

The endometriotic implants were suppressed in X/7 animals treated with Gal3C (Fig. 5E and H) compared to the control group (Fig. 5D). In addition, lesions area and weight were significantly reduced in Gal3C group (Fig. 5I and J). The histopathological analysis of control group showed stroma and endometrial glands with a remarkable vascularization (Fig. 5F), while the treatment with Gal3C notably promoted the atrophy and regression of the lesions (Fig. 5G).

The animals receiving Gal3C treatment did not exhibit signals of

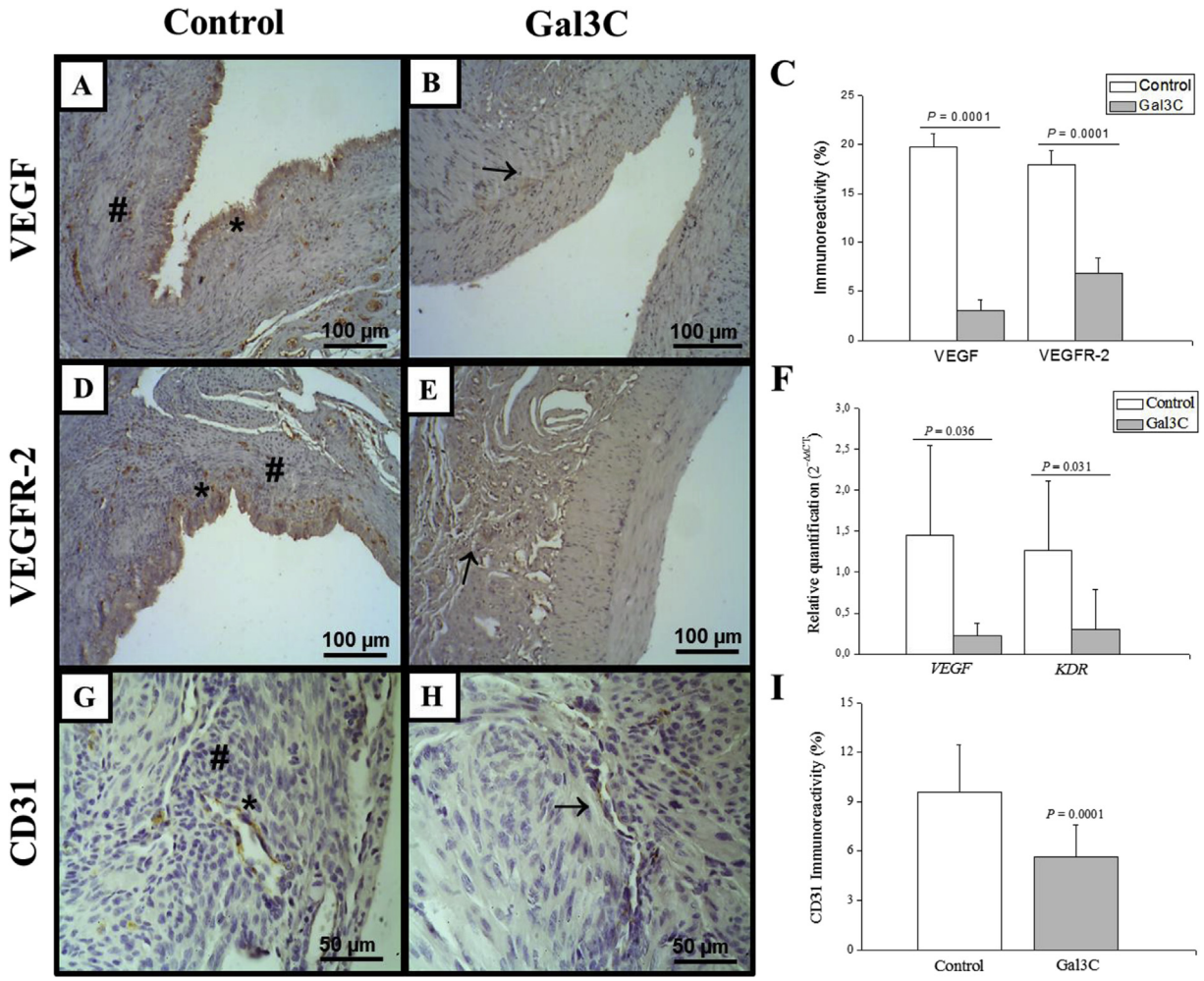

Fig. 7. Gal3C decreases angiogenesis markers in endometriotic mice. The immunoreactivity of VEGF and VEGFR-2 were detected predominantly in the glands (*) and surrounding the stroma (#) in control group (A and D). Treated group (B and E) exhibited a significant decrease in immunoreactivity of VEGF and VEGFR-2 (→). C. Histomorphometry evaluations of VEGF and VEGFR-2. F. *VEGF* and *KDR* mRNA transcripts in control group were significantly higher than the Gal3C group. Immunodistribution of CD31 (→) in control (G) and treated group (H). A significantly decreased number of CD31 positive vessels were observed in treated group lesions (I).

induced toxicity, based on food consumption, body weight, weight and size of liver, glycemia, creatinine, urea, AST and ALT plasma levels (Fig. 6).

3.4. Gal3C treatment inhibits angiogenesis and inflammatory process in endometriosis

VEGF and VEGFR-2 immunoreactivity were located in the endometriotic lesions, notably in the glandular epithelium, stromal cellular cytoplasm and in the vascular endothelial cells. Furthermore, the expression of these angiogenic markers were reduced in Gal3C treated group (Fig. 7B and E) compared to the control (Fig. 7A and D). These data were confirmed by histomorphometric evaluations (Fig. 7C) and quantitative real-time PCR of *VEGF* and *KDR* mRNA transcripts (Fig. 7F). CD31 immunostaining was also performed and a decrease of vascular density was observed in Gal3C treated group (Fig. 7H). These data were confirmed by histomorphometric evaluations (7I).

Finally, there is a significant decrease of F4/80-positive cells and COX-2 and TGFb1 expression in endometriotic lesions of Gal3C treated group (Fig. 8B, E and G) compared to the control group (Fig. 8A, D and G). The histological scores of F4-80 and COX-2 immunostaining confirmed these data (Fig. 8C and F).

4. Discussion

In this study, we evaluated the influence of Gal-3 on the development of endometriotic lesions and as a potential treatment target for endometriosis. In a previous study, our group demonstrated that galectin-3 expression was increased in experimental peritoneal endometriotic lesions (de Mattos et al., 2016). This data is in agreement with Noël et al. (2011) which demonstrated an overexpression of Gal-3 in peritoneal, ovarian and infiltrative endometriotic lesions in patients compared to the eutopic endometrium samples of the control group. In addition, Caserta et al. (2014) found significantly higher levels of Gal-3 in the peritoneal fluid of women with endometriosis when compared to women without the disease. Gal-3 levels also presented a positive correlation with disease stage and symptoms duration. Our results support these data, showing an overexpression of Gal-3 in the endometriotic tissue of wild-type animals and correlates with the observation that Gal-3 knockout mice presented markedly lower frequency of endometriotic lesions development. In fact, Gal-3 expressing animals were more than 11 times more prone to endometriosis.

Angiogenesis is a pivotal event in several pathologies, including endometriosis, and Gal-3 seems to be involved with the angiogenic factors regulation. Park et al. (2015) demonstrated that VEGF secretion was diminished when Gal-3 expression was silenced in osteosarcoma cells. However, when Gal-3 expression was induced, an increased

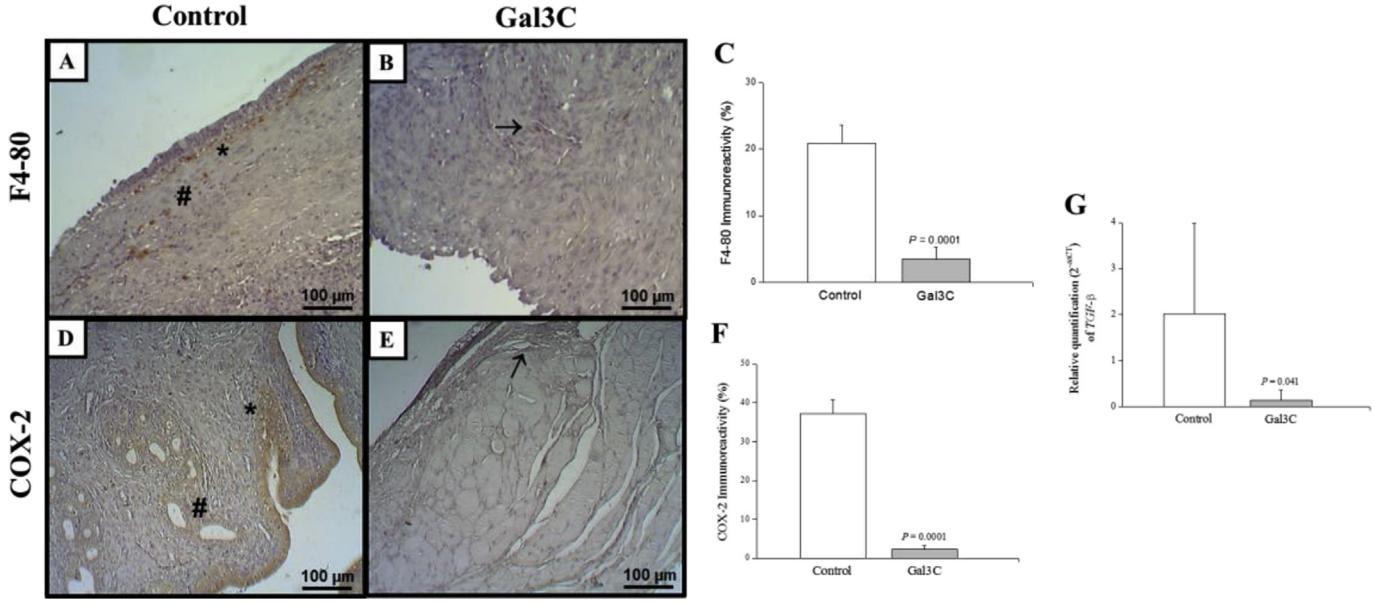

Fig. 8. Gal3C decreases macrophages number and inflammatory markers in endometriotic mice. F4-80 immunoreactivity (A and B) revealed a higher number of macrophages in the stroma (#), primarily around the glandular epithelium of control lesions (*) and was significantly reduced in Gal3C treated lesions (→). C. Histomorphometry evaluations of F4-80. COX-2 immunoreactivity was detected predominantly in the glands (*) and surrounding the stroma (#) in control group (D) and was reduced in Gal3C treated lesions (→) (E). F. Histomorphometry evaluations of COX-2. **G.** *TGF-β* mRNA transcripts in control group were significantly higher than the Gal3C group.

vascular density and VEGF expression was observed in murine melanoma tumors (Machado et al., 2014). VEGF signaling seems to be the main responsible for Gal-3 pro-angiogenic effect, since Gal-3-deficient endothelial cells internalize the VEGF receptor type 2 (VEGFR-2), which prevents VEGF-A induced angiogenesis (Markowska et al., 2010). In addition, tumor-endothelial cells have a higher Gal-3 expression than endothelial cells present in non-tumor tissues, suggesting the participation of this molecule in vessel formation/maintenance (Jia et al., 2010; Ryschich et al., 2006). In accordance, we observed that vascular density and expression of pro-angiogenic components, such as VEGF and VEGFR-2, was increased in endometriotic lesions of wild-type animals when compared to Gal-3 deficient animals. This result suggests that high expression of Gal-3, observed in endometriotic lesions, contributes to the extensive vascular formation observed in this disease.

Endometriotic lesions microenvironment was also modified by the absence of Gal-3, contributing to the decreased angiogenesis. As shown in Fig. 4, the macrophage population in the site of endometriosis implant was markedly decreased in Gal-3 knockout mice. Macrophages are the largest population of leukocytes found in the peritoneal fluid of patients with endometriosis, reaching 60% of the total leukocytes (Chang et al., 2017) and play an important role in angiogenesis. In hypoxia conditions, high levels of lactate and in inflammatory processes, these cells produce a large amount of pro-angiogenic cytokines (Coffelt et al., 2010; Itaya et al., 2001; Riabov et al., 2014). Bacci et al. (2009) used a macrophage depletion model to study the influence of these cells on the development of endometriosis. It was observed that in absence of macrophages, the endometrial implant still able to adhere to the peritoneal cavity and to infiltrate the serous membrane. Moreover, when macrophage depletion was performed after the establishment of endometriotic lesions, they stopped the growth and the blood vessels did not extend to the tissue core. In this way, Capobianco et al. (2011) shown that the macrophages were close to newly formed vessels using endometriosis animal model, and that when these cells were depleted, the endothelial cells did not organize effectively.

Several authors demonstrated the abnormal distribution of macrophages within the endometriotic lesion (Hutter et al., 2013; Laschke and Menger, 2007; Machado et al., 2016; Scheerer et al., 2016; Wickström et al., 2017). In addition, studies showed that these macrophages can exhibit high plasticity and different functional profiles (M1 or M2 phenotype) once stimulated by the signals released in the microenvironment (Ruffell and Coussens, 2015; Pyonteck et al., 2013; Qian and Pollard, 2010; Rolny et al., 2011). Typically, M2 macrophages are source of several pro-angiogenic factors such as transforming growth factor beta (TGF-b), VEGF and Platelet-derived growth factor (PDGF). The release of these factors favors the establishment of vascular network (Grivennikov et al., 2011; Leek et al., 1996; Murray et al., 2014; Yang and Zhang, 2017). It was demonstrated that macrophages from patients with endometriosis, as well as those from experimental models, express alternative activation markers (M2 polarization), while their respective controls did not express these markers (Bacci et al., 2009). Wang et al. (2014) have demonstrated that endometrial-derived stromal cells from women with endometriosis are able to induce M2 polarization of macrophages *in vitro*, and when these cells are indirectly co-cultured, M2 macrophages are able to stimulate the invasive ability of stromal cells derived from women with endometriosis. In addition, it has been demonstrated that exposure to estradiol may favor the development of endometriosis by inducing M2 polarization of macrophages (Wang et al., 2015). In our study, we observed that the presence of iNOS positive cells, a marker of M1 macrophages, was higher in endometriotic lesions developed in knockout animals, whereas more arginase positive cells (an M2 cell marker) were found in WT animal lesions. These findings suggest that Gal-3 expression favors macrophage migration and M2 polarization in endometriotic lesions.

We also characterized Gal-3 as a potential target for endometriosis treatment. Using a previously reported recombinant Gal3C we show that Gal-3 inhibition was able to reproduce the results observed in Gal-3 knockout mice, exhibiting the regression of lesions, associated with the decrease of angiogenic markers VEGF and VEGFR2, vascular density and macrophage population. Chen et al. (2017), using a corneal neovascularization model showed that pharmacological inhibition of Gal-3 significantly decreased angiogenesis by approximately 30% as well as *in vitro* migration and vascular structures formation induced by VEGF. Moreover, we demonstrated in the GAL3C treated group a decreased expression of TGFb1, which correlates with the VEGF expression reduction and macrophages M1 polarization. These results corroborate

with Machado et al. (2014) that proposed that galectin-3 absence down-regulated angiogenesis by modulating macrophages responses to VEGF and TGFβ1 signaling pathways. Previously, our group showed that COX-2 expression was increased in endometriotic lesions and that its inhibition led to endometriosis regression by VEGF downregulation (Machado et al., 2010). Here, we also showed that GAL3C treatment decreased COX-2 expression, what reinforce the role of Gal-3 in angiogenesis and inflammation modulation in endometriosis.

5. Conclusion

In this study, we demonstrated that Gal-3 significantly contributes to the establishment, growth and development of endometriotic lesions. This effect was associated to the modulation of angiogenic factors, such as VEGF and its receptor VEGFR-2, and the enrichment of M2 polarized macrophages in the endometriotic microenvironment. Moreover, Gal-3 inhibition with human recombinant Gal3C was able to reduce endometriotic lesions by downregulation of angiogenic and inflammatory factors. Taken together, the data reported here demonstrate that Gal-3 can be a pharmacological target in endometriosis treatment, inhibiting the lesions development by reducing angiogenesis and inflammation process.

Declarations of interest

None.

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