

# B lymphocytes inactivation by Ibrutinib limits endometriosis progression in mice

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**STUDY QUESTION:** What are the effects of B lymphocyte inactivation or depletion on the progression of endometriosis?

**SUMMARY ANSWER:** Skewing activated B cells toward regulatory B cells (Bregs) by Bruton's tyrosine kinase (Btk) inhibition using Ibrutinib prevents endometriosis progression in mice while B cell depletion using an anti-CD20 antibody has no effect.

**WHAT IS KNOWN ALREADY:** A polyclonal activation of B cells and the presence of anti-endometrial autoantibodies have been described in a large proportion of women with endometriosis though their exact role in the disease mechanisms remains unclear.

**STUDY DESIGN, SIZE, DURATION:** This study included comparison of endometriosis progression for 21 days in control mice versus animals treated with the anti-CD20 depleting antibody or with the Btk inhibitor Ibrutinib that prevents B cell activation.

**PARTICIPANTS/MATERIALS, SETTING, METHODS:** After syngeneic endometrial transplantation, murine endometriotic lesions were compared between treated and control mice using volume, weight, ultrasonography, histology and target genes expression in lesions. Phenotyping of activated and regulatory B cells, T lymphocytes and macrophages was performed by flow cytometry on isolated spleen and peritoneal cells. Cytokines were assayed by ELISA.

**MAIN RESULTS AND THE ROLE OF CHANCE:** Btk inhibitor Ibrutinib prevented lesion growth, reduced mRNA expression of cyclooxygenase-2, alpha smooth muscle actin and type I collagen in the lesions and skewed activated B cells toward Bregs in the spleen and peritoneal cavity of mice with endometriosis. In addition, the number of M2 macrophages decreased in the peritoneal cavity of Ibrutinib-treated mice compared to anti-CD20 and control mice. Depletion of B cells using an anti-CD20 antibody had no effect on activity and growth of endometriotic lesions and neither on the macrophages, compared to control mice.

**LARGE SCALE DATA:** N/A.

**LIMITATIONS, REASONS FOR CAUTION:** It is still unclear whether B cell depletion by the anti-CD20 or inactivation by Ibrutinib can prevent establishment and/or progression of endometriosis in humans.

**WIDER IMPLICATIONS OF THE FINDINGS:** Further investigation may contribute to clarifying the role of B cell subsets in human endometriosis.

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**Key words:** endometriosis / B lymphocytes / Ibrutinib / Btk inhibitor / anti-CD20 / regulatory B cells / macrophages / mice

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## Introduction

Endometriosis is a gynecological disorder characterized by the presence and growth of endometrial tissue outside the uterine cavity (Giudice and Kao, 2004). It affects ~5–15% of women in reproductive age, causing chronic pelvic pain and infertility (Giudice and Kao, 2004). Menstrual regurgitation and implantation of endometrial fragments (Sampson, 1927) remain the most accepted theory to explain the initiation of the disease while decreased peritoneal immunosurveillance (Matarese et al., 2003), hormonal, inflammatory and fibrotic factors are implicated in the development of endometriotic lesions (D'Hooghe and Debrock, 2002; Viganò et al., 2017).

Endometriosis is associated with an imbalance toward pro-inflammatory cytokines mainly produced by innate immune cells like macrophages and natural killer cells (Beste et al., 2014). Moreover, inflammation induces reactive oxygen species production in endometriotic cells that stimulates lesion growth and neoangiogenesis through activation of various tyrosine kinases (Santulli et al., 2015).

In addition to innate immunity, cells from adaptive immunity also play a role in endometriosis (Riccio et al., 2018). Activation of CD4<sup>+</sup> T cells has been described with an imbalance toward a T helper 2 (Th2) phenotype that drives the fibrosis of lesions (Chen et al., 2016), and a combined increase in T helper 17 cells maintains the inflammatory process (Gogacz et al., 2016).

B cells are important players of innate and adaptive immune responses and their number is increased in the blood and peritoneal cavity of patients with endometriosis (Riccio et al., 2017). A polyclonal activation of B cells and the presence of anti-endometrial autoantibodies (Wild and Shivers, 1985; Fernández-Shaw et al., 1993) have been described in women with endometriosis though their exact role in the disease mechanisms remains unclear.

Thus, in this report, we hypothesized that B cell depletion with anti-CD20 antibody or inactivation with Bruton's tyrosine kinase (Btk) inhibitor Ibrutinib interfere with endometriosis progression. We have tested this hypothesis in a relevant mouse model to better characterize the role of B cells on this disease.

## Materials and Methods

### Mice

Six-week-old BALB/c female mice (Charles River Laboratories, L'Arbresle, France) weighing 16–20 g were used, 10 animals per experimental group for each independent experiment. Animals received humane care in compliance with institutional guidelines and were housed in autoclaved cages under standard 12 h photoperiod with food and water available *ad libitum*. The study was approved by the Ethics Committee of Paris Descartes University (CEEA 34), Paris (PROJET N° 2016040716219897 – V6 – APAFiS # 7283).

### Mice model of endometriosis

Endometriosis was surgically induced in mice by syngeneic transplantation of uterine tissue as previously described by Marcellin et al. (2017) (Supplementary Fig. S1).

### *In vivo* treatment of the operated mice

The operated mice were randomly separated into three groups: Ibrutinib, Anti-CD20 and Control. The Ibrutinib Group was treated with 15 mg/kg/day (Honigberg et al., 2010) of Ibrutinib (Pharmacyclics, Sunnyvale, USA). The drug was diluted in 0.06% carboxymethyl cellulose/H<sub>2</sub>O and administered by oral gavage with sesame oil daily, for 21 days, starting on the day of the surgery. The Anti-CD20 Group received an intraperitoneal 100 µg single dose of anti-CD20 antibody (clone 5D2, isotype IgG2a, kindly provided by Genentech, USA) the day after the surgery. The Control Group received vehicle by daily oral gavage for 21 days. Twenty-one days after implantation, animals were sacrificed by cervical dislocation, and retro-orbital blood sample was collected. Peritoneal cavity washing was performed with infusion and aspiration of 10 mL of phosphate buffered saline (PBS) to extract peritoneal cells, and spleens were surgically removed. Endometriotic implants were also collected, weighed and measured using a rule caliper. Tumors' volume (TV) were calculated as follows: TV (mm<sup>3</sup>) = (L × W<sup>2</sup>)/2, where L is the longest and W is the shortest measure of the lesion in mm (Tomayko and Reynolds, 1989). The right side implant of each mouse was fixed with 10% formaldehyde for subsequent histological analyses. The left side implant was frozen in liquid nitrogen for further RNA extraction and reverse transcription followed by Quantitative real-time PCR (RT-qPCR) analyses.

### Ultrasonography to evaluate implants size

The endometriotic implants were measured at Day 7 and Day 20 after the surgery through serial ultrasonography as previously described by Santulli et al. (2016) (Supplementary Fig. S2).

### Histology

Implants fixed with 10% formaldehyde were set in paraffin. Serial 4 µm sections were prepared and stained with hematoxylin & eosin (H&E) and Sirius Red (SR) prior to histological examination by light microscopy. Stained tissue sections were examined by pathologists experienced in endometriosis (P.S. and F.B.).

### RNA extraction and reverse transcription followed by RT-qPCR

Total RNA extraction was performed with Trizol Reagent (Invitrogen, Carlsbad, USA), according to the manufacturer's instructions, and it was followed by reverse transcription quantitative PCR reaction using Qiagen one-step kit. Eight target genes—Cyclooxygenase-2 (COX-2), alpha smooth muscle actin (ASMA), type I Collagen, CD3, CD19, inducible nitric oxide synthase (iNOS), CD86 and Found in inflammatory zone I (Fizz-1)—and one reference gene, Beta-actin (B-actin) as internal control, were analyzed by RT-qPCR (primers are listed in Supplementary Table S1).

### Cell stimulation and flow cytometry

Splenocytes and peritoneal cells were isolated and stained for surface receptors using standard flow cytometric protocols. Flow cytometry was performed using a FACS Fortessa II flow cytometer (BD Biosciences, USA) according to standard techniques, and data

were analyzed with FlowJo software (TreeStar, Ashland, USA). The panel of antibodies used for cell surface staining and the FACS gating strategies are described in [Supplementary Figs. S3-S5](#).

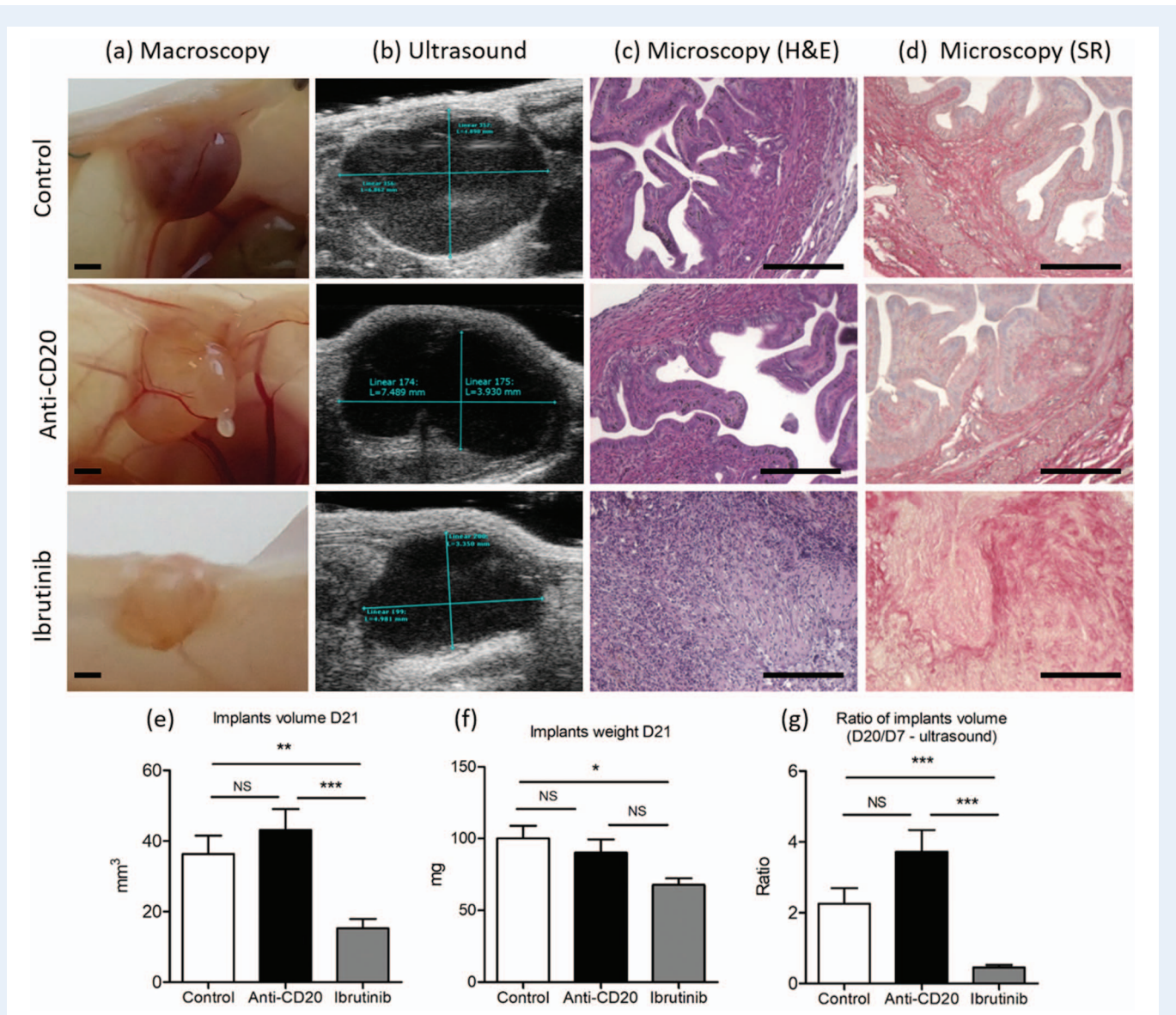
## ELISA assays

Serum and supernatant from cultured peritoneal cells were diluted (1:4) in ELISA/ELISPOT diluent 1× before being distributed on ELISA 96-well plates specific of tumor necrosis factor alpha (TNFA), interleukin 1 beta (IL-1B), interleukin 4 (IL-4), interleukin 6 (IL-6), interleukin 10 (IL-10), interleukin 13 (IL-13) and interferon gamma (IFNG) (Mouse ELISA Ready-SET-Go! eBioscience, Austria). Con-

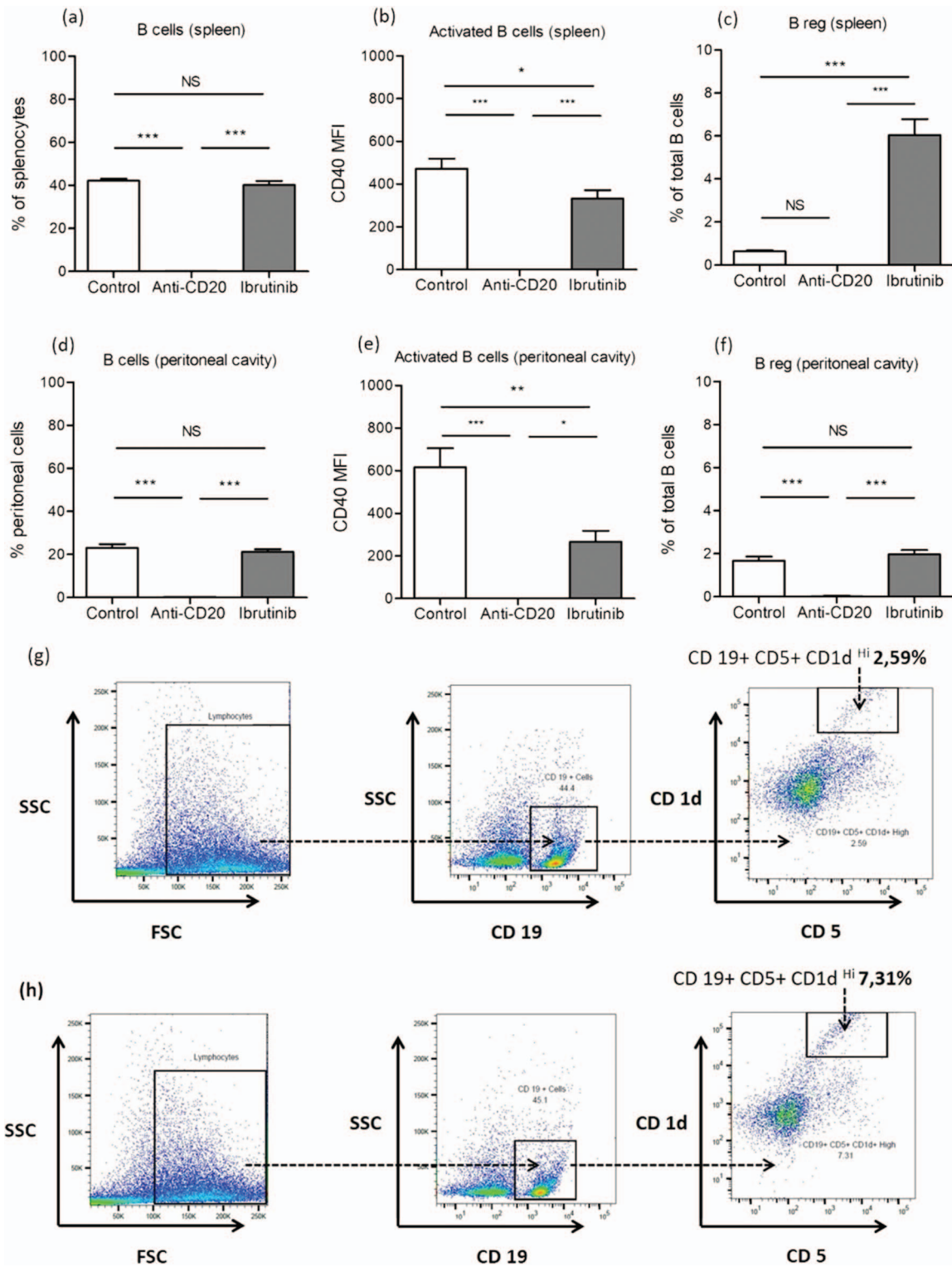
centrations were calculated from a standard curve according to the manufacturer's protocol.

## Statistical analysis

All data were analyzed using GraphPad Prism 5 software (GraphPad Software Inc., California, USA). A one-way analysis of variance (ANOVA) was performed to compare the three experimental groups. When group means were significantly different using the one-way ANOVA, pairwise comparisons were performed using Student–Newman–Keuls (SNK) *post hoc* test. The results from experiments comparing only two groups (Control and Ibrutinib) were analyzed with



**Figure 1** Effects of B cell modulating treatment on endometriotic implants development in mice. (a) Macroscopic view of the implants. (b) Ultrasonography images of peritoneal implants in mice on Day 20. (c) Coloration with H&E of implants at Day 21. (d) Coloration with SR of implants at Day 21. (e) Volume of the endometriotic implants on Day 21. (f) Weight of the implants on Day 21. (g) Ratio of the implants volume evaluated through ultrasound between Day 20 (D20) and Day 7 (D7). Data are mean ± SEM. Each group had  $n = 10$  mice. The one-way ANOVA was performed to detect significant differences among the three groups and further pairwise comparisons were performed using SNK test. NS, non-significant; \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ . Scale bar, 100  $\mu\text{m}$ .



**Figure 2 B cell phenotype analysis in spleen and peritoneal cavity of endometriotic mice.** Frequency of B cells (B220<sup>+</sup>CD19<sup>+</sup>) in spleen (a) and peritoneal cavity (d) of mice. Data represent mean  $\pm$  SEM. Surface CD40 expression in B cells (activated B cells) in spleen (b) and peritoneal cavity (e). Data represent the MFI of CD40 expression  $\pm$  SEM. Frequency of Breg (B220<sup>+</sup>CD19<sup>+</sup>CD5<sup>+</sup>CD1d<sup>high</sup>) in spleen (c) and peritoneal cavity (f). Data represent mean  $\pm$  SEM. Gating strategy for identification of B regulatory cell frequency in Control Group (g) and Ibrutinib Group (h). Each group had  $n = 10$  mice. The one-way ANOVA was performed to detect significant differences among the three groups and further pairwise comparisons were performed using SNK test. NS, non-significant; \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ .

the Mann–Whitney test. In the figures, the error bars represent the standard error of the mean (SEM). A  $P$ -value of  $<0.05$  was accepted as significant.

## Results

### Effects of B cells treatments on endometriotic implants development in mice

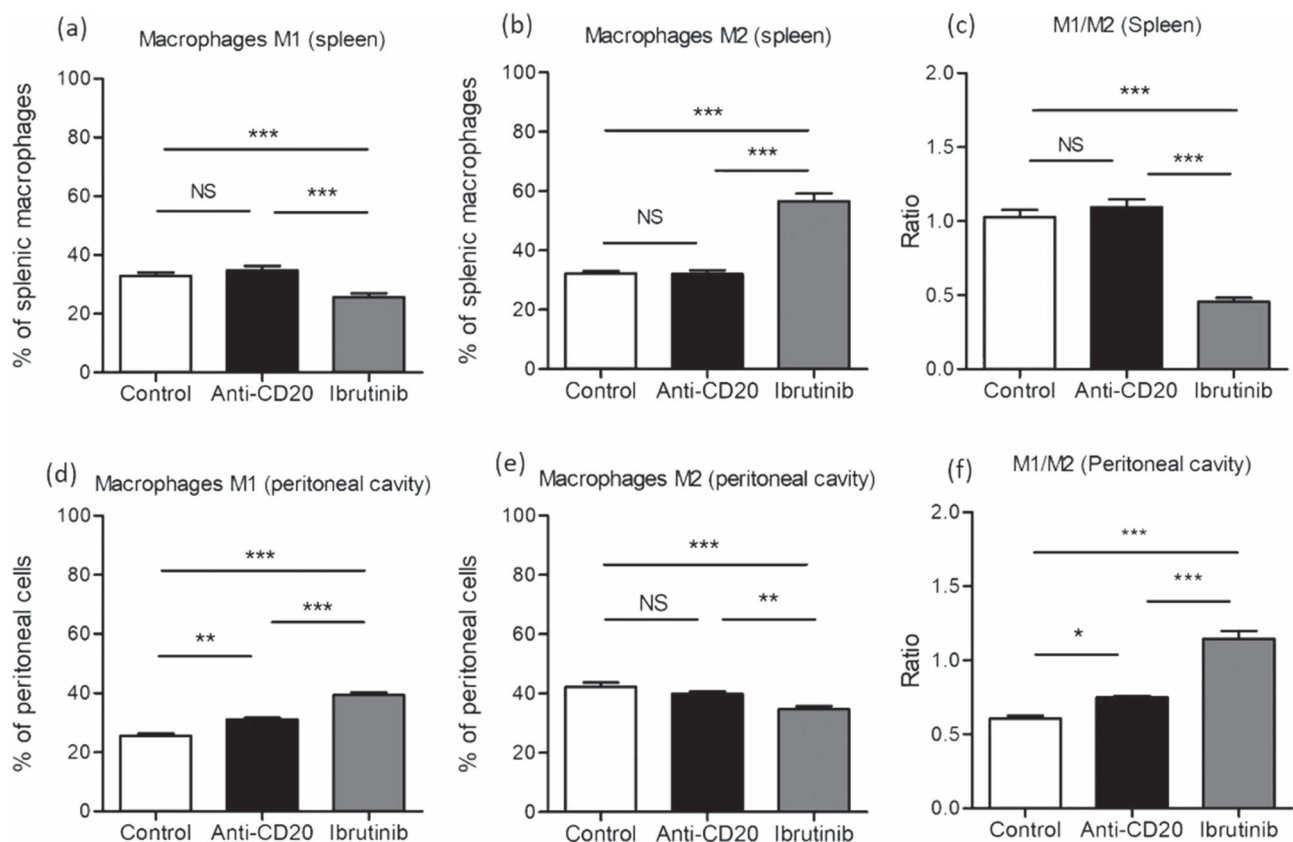
After 21 days of treatment, the Btk inhibitor Ibrutinib was effective in reducing the development of endometriosis in mice (Fig. 1). Animals in this group had smaller and less active implants (no fresh blood, no angiogenesis and few glands), whereas Control and Anti-CD20 Groups showed persistent, larger and more active lesions through macroscopic (Fig. 1a) and microscopic (Fig. 1c and d) evaluations. Implant volume (Fig. 1e) and weight (Fig. 1f) from Ibrutinib Group were significantly reduced at Day 21 compared to Control and Anti-CD20 Groups. An ultrasound imaging analysis of the implants was also performed at Day 7 and at Day 20 after the procedure (Fig. 1b), demonstrating a reduced volume in the Ibrutinib Group compared to Control and Anti-CD20 Groups (Fig. 1g).

### Flow cytometry analysis of B cells populations

Anti-CD20 treatment depleted all B cells (defined as B220<sup>+</sup>CD19<sup>+</sup>) in the spleen and peritoneum compared to Control Group (Fig. 2) while Ibrutinib treatment did not affect the percentage of splenic (Fig. 2a) or peritoneal B cells (Fig. 2d). Activation of B cells was assessed by mean fluorescence intensity (MFI) of the co-stimulatory CD40 marker expression within the B cell population. Ibrutinib treatment decreased B cells activation in the spleen (Fig. 2b) and peritoneal cavity (Fig. 2e) compared to Control Group. When gating (Fig. 2g and h) on the CD19<sup>+</sup>CD5<sup>+</sup>CD1d<sup>high</sup> subset, known as regulatory B cells (Bregs) (Rosser and Mauri, 2015), we observed a total depletion with the Anti-CD20 treatment. Interestingly, Ibrutinib treatment induced an important increase in the frequency of splenic Breg population compared to the Control Group (Fig. 2c). No significant difference was observed in the Breg population in the peritoneal cavity between Ibrutinib and Control Groups (Fig. 2f).

### B cell blockade impacted the distribution of M1 and M2 macrophage subsets

Concerning macrophage distribution, Ibrutinib treatment induced, in the spleen, an important decrease in the frequency of the M1 subset



**Figure 3** Macrophage M1/M2 distribution in spleen and peritoneal cavity in endometriotic mice. Macrophages were gated on CD11b<sup>+</sup>F4/80<sup>+</sup> cells isolated from spleen (a and b) and from the peritoneal cavity (d and e). M1 macrophages (a and d) were further characterized as CD43<sup>+</sup> and Ly6C<sup>high</sup> and M2 macrophages (b and e) as CD206<sup>+</sup> and Ly6C<sup>low</sup>. The ratio of M1/M2 population was calculated (c and f). Data represent mean  $\pm$  SEM. Each group had  $n = 10$  mice. The one-way ANOVA was performed to detect significant differences among the three groups and further pairwise comparisons were performed using SNK test. NS, non-significant; \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ .

(Fig. 3a) and a significant increase in the M2 subset (Fig. 3b) compared to Control and Anti-CD20 Groups, resulting in a decreased M1/M2 ratio (Fig. 3c). An opposite variation was observed in the peritoneal cavity, where Ibrutinib increased the frequency of M1 (Fig. 3d) while reducing M2 (Fig. 3e) compared to Control and Anti-CD20 Groups, resulting in an increased M1/M2 ratio (Fig. 3f). There was no significant difference in M1 or M2 frequency or M1/M2 ratio in the spleen (Fig. 3a–c) between Anti-CD20 and Control Groups. However, in the peritoneal cavity, an increased M1 frequency (Fig. 3d) was observed in Anti-CD20 Group compared to Controls, leading to a significant difference in the M1/M2 ratio (Fig. 3f) between the two groups.

### Effects of Ibrutinib in quantitative expression of genes in endometriotic implants of mice

Ibrutinib Group showed a 5-fold reduction of mRNA expression of COX-2 in the lesions compared to Control Group (Fig. 4a). The effects of Ibrutinib treatment on fibrosis were assessed by ASMA (Fig. 4b) and type I collagen (Fig. 4c) mRNA expression in the implants, and both were significantly reduced in this group, compared to controls. There were no differences in these inflammatory and fibrotic markers between the Anti-CD20 Group and the controls. To evaluate the immune cells infiltration in the implants, we have analyzed the mRNA expression of the following markers as a proxy: CD19 (for B cells, Fig. 4d), iNOS and CD86 expression (for M1 macrophages, Fig. 4e and f, respectively) were increased in the Ibrutinib Group while Fizz-1 expression (for M2 macrophages, Fig. 4g) was decreased in Ibrutinib Group, compared to Control Group. CD3 expression (for T lymphocytes, Fig. 4h) was not significantly different between Ibrutinib and Control groups.

phocytes, Fig. 4h) was not significantly different between Ibrutinib and Control groups.

### Effects of Ibrutinib on T lymphocytes

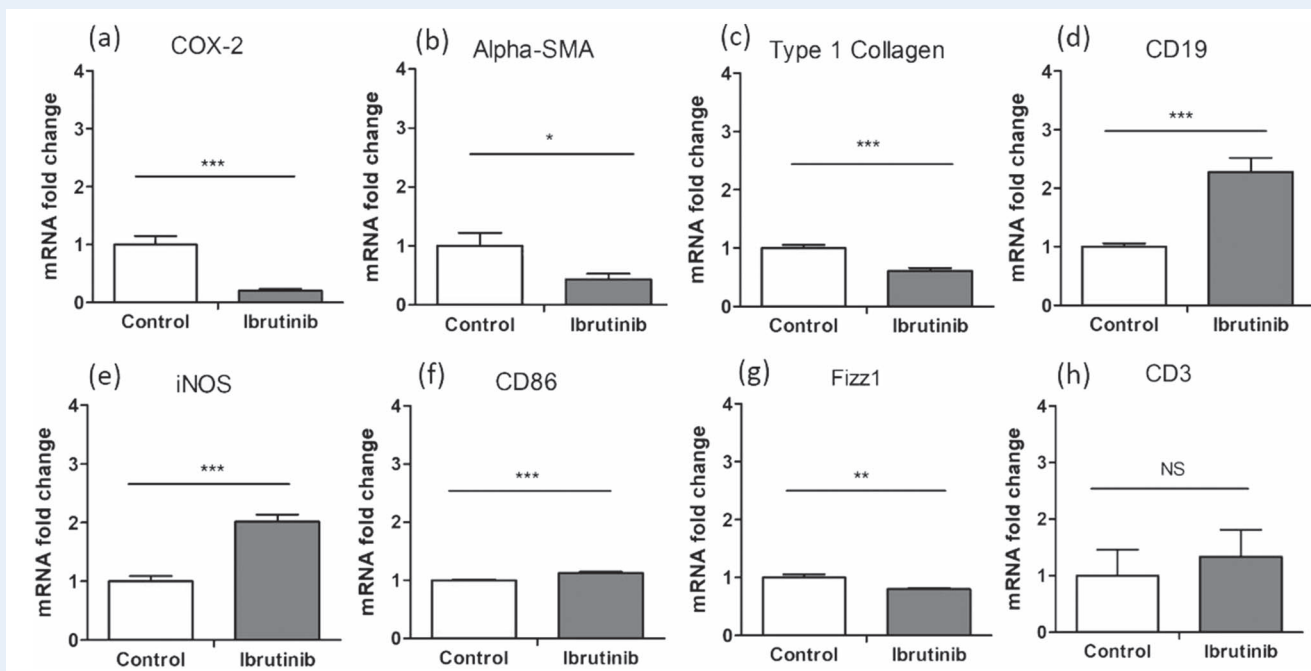
There were no significant differences in T lymphocytes subsets number or activation. Indeed, the total number or proportion of naïve (defined as CD62L<sup>high</sup> CD44<sup>low</sup>) or memory (CD62L<sup>low</sup> CD44<sup>high</sup>) CD4<sup>+</sup> and CD8<sup>+</sup> T cells were not significantly different in the peritoneum or in the spleen of mice between Ibrutinib and Control Group (Supplementary Fig. S6).

### Ibrutinib treatment effects in cytokine balance

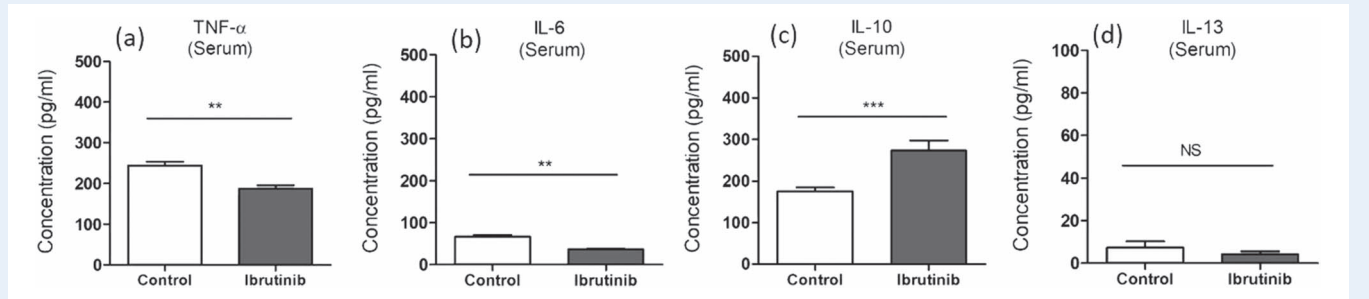
We have measured cytokine concentration in the serum (Fig. 5) and peritoneal fluid (Fig. 6) of endometriotic mice. In the sera, treatment of animals with Ibrutinib decreased TNFA (Fig. 5a) and IL-6 concentrations (Fig. 5b) and increased IL-10 levels compared to Control Group (Fig. 5c); no significant difference was observed for IL-13 levels (Fig. 5d). In the peritoneal fluid, Ibrutinib treatment increased IFNG concentration (Fig. 6d) and decreased IL-13 (Fig. 6e) and IL-4 concentrations (Fig. 6f) when compared to controls. No differences in peritoneal concentrations of TNFA (Fig. 6a), IL-6 (Fig. 6b), IL-10 (Fig. 6c) or IL-1B (Fig. 6g) were observed with Ibrutinib treatment compared to controls.

## Discussion

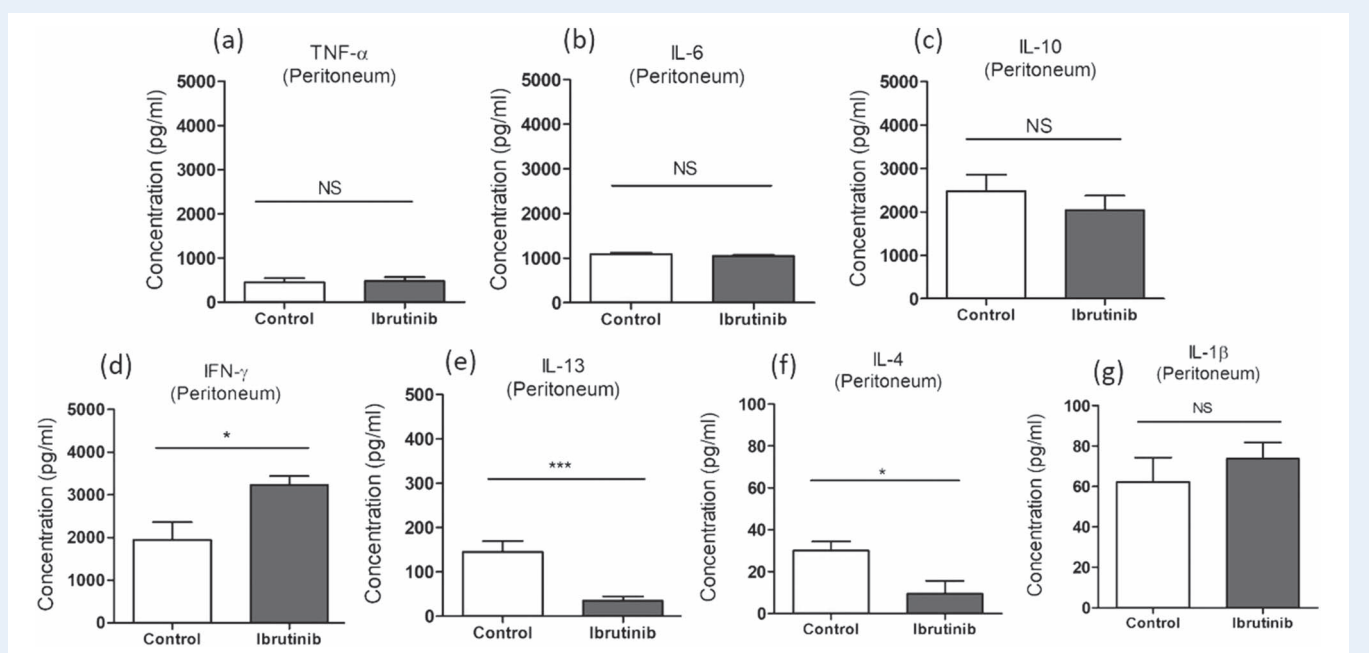
Many studies have attempted to clarify the role of the immune system in endometriosis and various abnormalities have been detected, includ-



**Figure 4** Effects of Ibrutinib in quantitative expression of genes in endometriotic implants of mice. (a) COX-2; (b) ASMA; (c) Type I collagen; (d) CD19 (for B lymphocytes); (e) iNOS (for M1 macrophages); (f) CD86 (for M1 macrophages); (g) Fizz-1 (for M2 macrophages) and (h) CD3 (for T lymphocytes) mRNA levels. Data are normalized to the reference gene (B-actin) and are expressed as ratio versus Control Group. Each group had  $n = 10$  mice. Mean values were compared by using the Mann–Whitney test. NS, non-significant; \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ .



**Figure 5** Effects of Ibrutinib on systemic cytokines of endometriotic mice. (a) TNFA; (b) IL-6; (c) IL-10 and (d) IL-13 concentrations in the sera of mice measured by ELISA. Data represent mean  $\pm$  SEM. Each group had  $n = 10$  mice. The Mann–Whitney test was used to detect significant differences. NS, non-significant; \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ .



**Figure 6** Effects of Ibrutinib on peritoneal cytokines of endometriotic mice. (a) TNFA; (b) IL-6; (c) IL-10; (d) IFNG; (e) IL-13; (f) IL-4 and (g) IL-1B concentrations in peritoneal fluid of mice measured by ELISA. Data represent mean  $\pm$  SEM. Each group had  $n = 10$  mice. The Mann–Whitney test was used to detect significant differences. NS, non-significant; \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ .

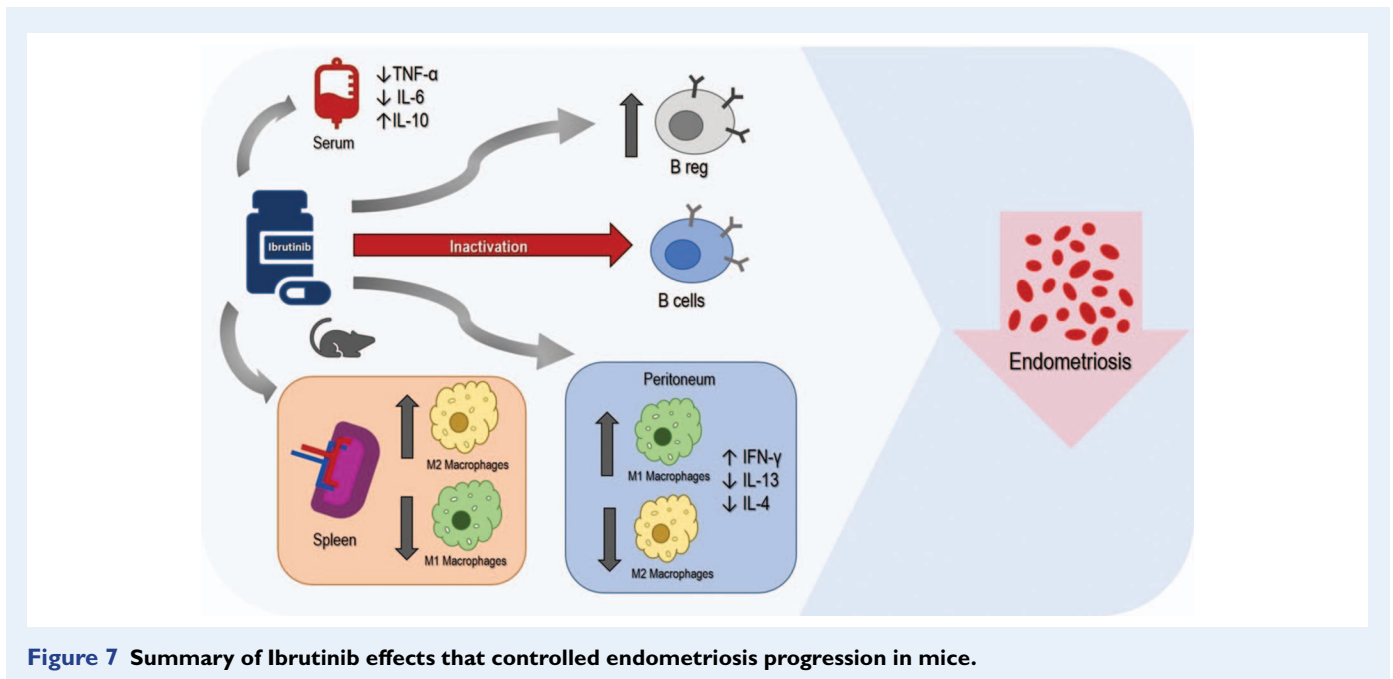
ing increased B lymphocytes number and activation with excessive production of autoantibodies. In order to evaluate the role of B cells on endometriosis we have used a dual strategy: a complete depletion of B cells using anti-CD20 treatment or an immunomodulatory strategy using a Btk inhibitor that blocks B cells activation.

In this report, we have shown that anti-CD20 had no impact on the course of the disease with no differences in the size of lesions despite a confirmed complete B cells depletion, maintained 3 weeks after the injection of the antibody. Anti-CD20-mediated depletion of B cells has been widely used in humans for the treatment of both B cell malignancies and autoimmune and systemic inflammatory diseases (Edwards et al., 2004; Harrison, 2012).

By contrast, treatment with Ibrutinib reduced the size and the activity of the lesions, as well as the expression of inflammatory and fibrotic markers. Progression of endometriotic lesions has been

associated with chronic inflammation and fibrosis leading to an altered tissue function. COX-2 produces prostaglandins and is involved in the inflammatory response that contributes to lesion activity and growth in endometriosis (Santulli et al., 2016). Moreover, increased expression of type I collagen and ASMA, marking myofibroblast differentiation, has also been associated with severe forms of endometriosis (González-Foruria et al., 2017; Viganò et al., 2017).

Ibrutinib is a selective covalent and irreversible inhibitor of Btk, a non-receptor kinase essential for B cells development and function of mature B cells. Shortly after its discovery, Btk was placed in the signal transduction pathway downstream of the B cell antigen receptor and was found to have a major role in the control of B cell activation (Herman et al., 2011). Many *in vitro* and *in vivo* studies confirm the specific activity of Ibrutinib against Btk-restricted targets (Honigberg et al., 2010; Woyach et al., 2012). Inhibitors of Btk have shown



**Figure 7** Summary of Ibrutinib effects that controlled endometriosis progression in mice.

anti-tumor activity, first in animal models and subsequently in the clinics, with durable remissions against a variety of B cell malignancies (Harrison, 2012).

The role of Btk in the development of Bregs is unclear (Rosser and Mauri, 2015). However, in mice lacking B cell linker, a Btk adaptor molecule also implicated in B cell signaling; the percentages of CD1d<sup>hi</sup>CD5<sup>+</sup> Bregs were markedly increased (Jin et al., 2013) as observed in the present study in Ibrutinib-treated endometriotic mice.

The effect of Ibrutinib on the course of endometriosis compared to anti-CD20 treatment led us to investigate extra B cell-mediated effects of Btk. Growing evidence also suggests roles for Btk in mononuclear cells of the innate immune system, especially macrophages (Weber et al., 2017). Macrophages play a central role in the orchestration of inflammation and fibrosis in endometriosis and undergo equally polarized activation into the M1 (classically) and M2 (alternatively) activated subsets (Bacci et al., 2009). Btk has been shown to regulate macrophage polarization in response to various stimuli with a skew from M1 to M2 macrophages (Ní Gabhann et al., 2014).

The discrepancy between the profile of macrophages in the spleen and in the peritoneal cavity can be related to the role of Btk in cellular migration (de Gorter et al., 2007). Btk combines with Rac to modulate actin polymerization and cytoskeleton rearrangement, impacting on inflammatory mast cells or neutrophils recruitment (Kuehn et al., 2010), through macrophage-1 antigen (MAC-1) activation. Since MAC-1 is also expressed on macrophages, such phenomenon may explain the inhibition of M2 cells migration into the peritoneal cavity in endometriotic mice treated with Ibrutinib. Interestingly, the increase in the peritoneal M1/M2 ratio may participate of the therapeutic effect of Ibrutinib. Bacci et al. (2009) have shown a correlation between active endometriosis and an increased number of M2 cells in the peritoneal cavity of women and mice and that early injections of M2 cells aggravate endometriosis in mice while injections of M1 cells prevent it.

T lymphocytes play an important role in the development of endometriosis (Riccio et al., 2018). In the present study, Ibrutinib had no effect on peritoneal or splenic T lymphocytes number or activation, and there was no difference in CD3 gene expression in the endometriotic implants from endometriotic mice treated or not with Ibrutinib. Ibrutinib impacts mainly B cell through interaction with Btk, but investigators (Kokhaei et al., 2016; Long et al., 2017) have described Ibrutinib also as an Interleukin-2-inducible T-cell kinase (Itk) inhibitor, subverting Th2 immunity and potentializing T helper 1-based immune responses (Dubovsky et al., 2013). However, the affinity of Ibrutinib for Btk is 20 times higher than the one for Itk (Honigberg et al., 2010), and the double Btk-Itk inhibition was achieved with a 25 mg/kg/day dose (Dubovsky et al., 2014), much higher than the one used in our study. Thus, the findings of effective control of endometriosis progression by Ibrutinib seem to be due to its Btk inhibition pathway and to its effects on Bregs rather than its role on T cells. The use of a more selective Btk inhibitor such as Acalabrutinib could confirm the mechanisms behind Ibrutinib's effects on endometriosis progression.

Regarding cytokine production, B cells overexpressing wild-type Btk were selectively hyper responsive to B cell receptor stimulation and showed enhanced Ca<sup>2+</sup> influx, nuclear factor- $\kappa$ B activation, resistance to Fas-mediated apoptosis and defective elimination of self-reactive B cells *in vivo*, consistent with the pro-inflammatory and autoimmune role of Btk (Kil et al., 2012). As a result, the high production of IL-6 by B cells from CD19-hBtk transgenic mice (Corneth et al., 2016) fits with the decrease in inflammatory cytokines IL-6 and TNFA induced by Ibrutinib in our experiments. The role of Btk in IL-10 production is more complex as Btk<sup>-/-</sup> mice have been shown to overproduce IL-10 but not IL-6 upon allergic challenge. That means that Btk may support IL-10 secretion upon an immuno-inflammatory challenge as observed in Ibrutinib-treated endometriotic mice, further supporting the anti-inflammatory role of this molecule (Lundy et al., 2005). In endometriosis, decreased levels of IL-6 and increased IL-10 have been



associated with an amelioration of endometriosis as observed in our model (Schwager *et al.*, 2011).

Treatment of endometriotic animals with Ibrutinib led to high concentration of peritoneal IFNG and low concentrations of peritoneal IL-4 and IL-13 compared to controls. Those results are compatible with the M1/M2 macrophages findings as M1 macrophages emerge from an environment rich in IFNG and M2 macrophages produce high amounts of IL-4 and IL-13 (Ni Gabhann *et al.*, 2014).

## Strengths and limitations

Infertility is one of the main issues of endometriosis; however, most of the non-surgical treatment options available are contraceptive, leaving women affected by the disease with the difficult choice between controlling the pain and trying to conceive. Many studies have demonstrated the important role of the immune system in the progression of endometriosis, so this could be a main target for the development of new non-hormonal therapeutic strategies. In the present study we have tested a drug approved by the Food and Drug Administration that target immune cells and it was effective in controlling the disease's progression in mice. The effects of Ibrutinib's treatment are summarized in Fig. 7. This study has the limitations of using an animal model and perhaps not completely clarifying the mechanisms and pathways of the drug efficacy observed. There is still a long path before applying these findings for human treatment; however, they can open a door to further studies and the development of new immunoregulatory therapeutic strategies for endometriosis.

## Conclusion

We conclude that Btk inhibitor Ibrutinib controlled endometriosis progression in mice while total B cell depletion using an anti-CD20 antibody had no effect on the course of the disease. In addition, our findings suggest that Bregs might help blocking the development of lesions, as these cells were depleted by anti-CD20 antibody and preserved by Ibrutinib. The use of Ibrutinib to skew activated B cells toward Bregs and increase the M1/M2 ratio into the peritoneal cavity opens new perspectives in both understanding and treating endometriosis.

## Supplementary data

Supplementary data are available at *Human Reproduction* online.

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## Authors' roles

F.B. and C.C. conceived and designed the study. L.G.C.R., M.J., S.C. and P.S. executed the experiments. All the authors analyzed and interpreted the data. P.S., L.D., M.S.A. and F.B. supervised and reviewed the

statistical analysis. S.C. and L.D. contributed to the data collection. L.G.C.R., M.J., F.B., F.R., P.S., M.S.A. and C.C. drafted the manuscript. All the authors read and approved the final version of the manuscript.

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## Conflict of interest

The authors state no conflict of interest.

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