Luiza da Gama Coelho Riccio

O inibidor Btk Ibrutinib limita o desenvolvimento da endometriose em camundongos

Tese apresentada à Faculdade de Medicina da Universidade de São Paulo para obtenção do título de Doutor em Ciências

Programa de Obstetrícia e Ginecologia

Orientador: Prof. Dr. Maurício Simões Abrão

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Luiza da Gama Coelho Riccio

Btk inhibitor Ibrutinib limits endometriosis development in mice

Thesis presented to the *Faculdade de Medicina da Universidade de São Paulo* to obtain the degree of Doctor in Science

Program of Obstetrics and Gynecology

Supervisor: Prof. Dr. Maurício Simões Abrão

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To Augusto Riccio

in memoriam

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Luiza

« L’essentiel est invisible aux yeux »

Antoine de Saint-Exupéry

NORMATIZATION ADOPTED

This dissertation is in accordance with the following norms:

References: adapted from the International Committee of Medical Journals Editors (Vancouver).

University of São Paulo, Medical School. Division of Library and Documentation. “Guia de apresentação de dissertações, teses e monografias”. Elaborated by Anneliese Carneiro da Cunha, Maria Julia de A. L. Freddi, Maria F. Crestana, Marinalva de Souza Aragão, Suely Campos Cardoso, Valéria Vilhena. 3ª ed. São Paulo: Divisão de Biblioteca e Documentação; 2011.

Abbreviations of titles and journals in accordance with the List of Journals Indexed in Index Medicus.

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Immunology of endometriosis
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B lymphocytes inactivation by Ibrutinib limits endometriosis progression in mice

LIST OF ABBREVIATIONS, SYMBOLS AND ACRONYMS

%	percent
≤	less than or equal
±	plus-minus
αSMA	alpha smooth muscle actin
β-actin	beta-actin
µg	microgram
µm	micrometer
ANA	antinuclear antibodies
ANOVA	one-way analysis of variance
ANVISA	*Agência Nacional De Vigilância Sanitária*
BAFF	B cell activation factor
BAFF-R	B cell activation factor receptor
BALB/c	Bagg and Albino
Bcl-2	B-cell lymphoma 2
BCMA	B cell maturation antigen
BCR	B cell receptor
BLys	B lymphocyte stimulator
Breg	regulatory B cells
Btk	Bruton’s tyrosine kinase
C	celsius
CD	cluster of differentiation
COX-2	cyclooxygenase-2
D	day
DIE	deep infiltrating endometriosis
DNA	deoxyribonucleic acid
EDT	endometriosis
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay

FACS	fluorescence-activated cell sorting
FasL	Fas ligand
FDA	food and drug administration
Fizz-1	found in inflammatory zone 1
FSC	forward scatter
GnRH	gonadotropin-releasing hormone
H&E	Hematoxylin & Eosin
HGF	hepatocyte growth factor
IFN	interferon
Ig	immunoglobulin
IGF-1	insulin-like growth factor
IL	interleukin
iNOS	inducible nitric oxide synthase
INSERM	*Institut National de la Santé et de la Recherche Médicale*
Itk	interleukin-2-inducible T-cell kinase
KAR	killer activation receptor
kg	kilogram
KIR	killer immunoglobulin-like receptor
L	litre
LPMs	large peritoneal macrophages
MAC-1	macrophage-1 antigen
MAPK	mitogen-activated protein kinase
MFI	mean fluorescence intensity
mg	milligram
MHC	major histocompatibility complex
mL	millilitre
mm	millimeter
mmol	milimoles
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
NF-κB	nuclear factor kappa B
NK	natural killer
NS	non-significant

OMA	ovarian endometrioma
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PerC	peritoneal cavity
pg	picogram
PRISMA	preferred reporting items for systematic reviews and meta-analyses
rASRM	revised American Society of Reproductive Medicine
RNA	ribonucleic acid
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT-qPCR	reverse transcription quantitative polymerase chain reaction
SEM	standard error of the mean
sICAM-1	intercellular adhesion molecule soluble form
SNK	Student-Newman-Keuls
SPMs	small peritoneal macrophages
SR	Sirius Red
SSC	side-scattered light
TACI	transmembrane activator and calcium-modulator and cyclophilin ligand interactor
TCR	T-cell receptor
TEC	tec protein tyrosine kinase
TGF	transforming growth factor
Th	T helper
TLR4	toll-like receptor 4
TNF	tumor necrosis factor
Treg	regulatory T cell
USD	United States dollar
VEGF	vascular endothelial growth factor
vs	versus
XLA	X-linked agammaglobulinemia

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RESUMO

Riccio LGC. *O inibidor Btk Ibrutinib limita o desenvolvimento da endometriose em camundongos* [tese]. São Paulo: Faculdade de Medicina, Universidade de São Paulo; 2021.

Introdução: Endometriose é uma doença crônica ginecológica caracterizada pela presença e crescimento de células endometriais fora da cavidade uterina. É uma doença inflamatória benigna, porém frequente, afetando cerca de 5-15% das mulheres em idade reprodutiva, causando dor pélvica crônica e infertilidade. A fisiopatologia da endometriose ainda não está completamente esclarecida, e sua progressão está associada à inflamação crônica e a alterações na resposta imunológica. Células endometriais que alcançam a cavidade peritoneal através da menstruação retrógrada escapam dos mecanismos de imunovigilância, provavelmente devido a uma redução da atividade fagocítica dos macrófagos e da citotoxicidade das células *natural killer*. A imunidade adaptativa também contribui para o desenvolvimento da doença. A presença de uma ativação policlonal dos linfócitos B, com produção de autoanticorpos anti-endométrio, foi descrita em mulheres com endometriose. Porém, o papel exato destas células no mecanismo da doença ainda não é totalmente conhecido. Existe uma necessidade urgente de opções terapêuticas não-hormonais para o tratamento da endometriose e terapias imunes podem trazer novas perspectivas. **Objetivo:** Avaliar os efeitos da depleção com anti-CD20 e da inativação com o inibidor da *Bruton´s tyrosine kinase* (Btk) dos linfócitos B no desenvolvimento da endometriose em camundongos. **Métodos:** Neste estudo experimental, o desenvolvimento da endometriose foi comparado entre um grupo controle e animais tratados com o anticorpo anti-CD20, que causa a depleção dos linfócitos B, e com o inibidor Btk Ibrutinib, que leva à inativação destas células. Foram utilizados dez animais por grupo por experimento independente. Foi utilizado o modelo cirúrgico de endometriose, com transplante de tecido endometrial para a cavidade peritoneal dos camundongos. As lesões de endometriose foram comparadas através do volume, peso, medidas ultrassonográficas, histologia e

expressão gênica de genes alvo nos implantes. Os fenótipos dos linfócitos B, B ativados, B regulatórios (Breg), linfócitos T e macrófagos foram avaliados através da citometria de fluxo das células extraídas do baço e fluido peritoneal dos camundongos. Citocinas no soro e no fluido peritoneal foram quantificadas por ELISA. **Resultados:** O Ibrutinib preveniu o crescimento das lesões de endometriose, reduziu a expressão de ciclooxigenase-2, *alpha smooth muscle actine* e colágeno tipo I nas lesões, inativou os linfócitos B e aumentou o número de linfócitos Breg no baço de camundongos com endometriose. Além disso, o número de macrófagos M2 diminuiu na cavidade peritoneal dos animais tratados com Ibrutinib, comparados àqueles tratados com anti-CD20 e controles. A depleção dos linfócitos B com o anticorpo anti-CD20 não teve efeito sobre o crescimento e atividade dos implantes, nem sobre os macrófagos. **Conclusão:** O tratamento com Ibrutinib reduziu o tamanho e a atividade das lesões, bem como a expressão de marcadores de inflamação e fibrose, enquanto a depleção completa dos linfócitos B com o anti-CD20 não teve impacto sobre a doença. No entanto, ainda é incerto se a inativação dos linfócitos através do tratamento com o Ibrutinib poderia interferir no desenvolvimento da endometriose em humanos. Novos estudos podem ajudar a esclarecer o papel dos linfócitos B e seus subtipos na endometriose e contribuir para o desenvolvimento de novas estratégias terapêuticas.

Descritores: Endometriose; Linfócitos B; Imunologia; Linfócitos B reguladores; Macrófagos; Camundongos.

ABSTRACT

Riccio LGC. *Btk inhibitor Ibrutinib limits endometriosis development in mice* [thesis]. São Paulo: “Faculdade de Medicina, Universidade de São Paulo”; 2021.

Introduction: Endometriosis is a chronic gynecological disorder characterized by the presence and growth of endometrial tissue outside the uterine cavity. It is a benign but frequent inflammatory disease that occurs in about 5-15% of women in reproductive age, causing chronic pelvic pain and infertility. The physiopathology of endometriosis is not completely understood, and its progression is associated with chronic inflammation and aberrant immune response. Endometrial cells that reach peritoneal cavity through retrograde menstruation escape immunosurveillance probably due to reduced macrophagic phagocytosis and decreased natural killer cells cytotoxicity. Adaptive immune cells also contribute to disease progression. 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. There is an urgent need for new non-hormonal therapeutic strategies and targeting immune system cells can open new perspectives. **Objective:** To evaluate the effects of B lymphocyte depletion and inactivation on endometriosis development in mice. **Methods:** The experimental study included comparison of endometriosis development for 21 days in control mice versus animals treated with the anti-CD20 depleting antibody or with the Bruton´s tyrosine kinase (Btk) inhibitor Ibrutinib, that prevents B cell activation. Ten animals per group for each independent experiment were used. After syngeneic endometrial transplantation, murine endometriotic lesions were compared between treated and control mice using volume, weight, ultrasonographic measures, histology, and target genes expression in lesions. Phenotyping of activated and regulatory B cells (Breg), T lymphocytes and macrophages was performed by flow cytometry on isolated spleen and peritoneal cells of mice. Cytokines in the serum and peritoneal fluid were assayed by ELISA. **Results:** 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 Breg in the spleen 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, neither on the macrophages, compared to control mice. **Conclusion:** Ibrutinib treatment reduced the size and activity of the lesions, as well as the expression of inflammatory and fibrotic markers, whereas complete B lymphocyte depletion by anti-CD20 had no impact on the course of the disease. However, it is still unclear whether B cell inactivation by Ibrutinib can prevent establishment and/or progression of endometriosis in humans. Further investigation may contribute to clarifying the role of B cell subsets in human endometriosis and to developing new therapeutic strategies.

Descriptors: Endometriosis; B-Lymphocytes; Immunology; B-lymphocytes regulatory; Macrophages; Mice.

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1 INTRODUCTION

1 INTRODUCTION

Endometriosis is a chronic gynecological disorder, characterized by the presence and growth of endometrial tissue outside the uterine cavity (Giudice and Kao, 2004; Tosti et al., 2015). It is a benign but frequent inflammatory disease that occurs in about 5-15% of women in reproductive age (Giudice and Kao, 2004). The main symptoms of endometriosis are chronic pelvic pain (40-60%) and infertility (20-30%), with a major impact in the quality of life of the patients (Waller et al., 1993).

The current theory on endometriosis pathogenesis is based on the so-called retrograde menstruation phenomenon. It is defined as the reflux of endometrial fragments through the fallopian tubes during menstruation, with subsequent implantation in the peritoneal cavity (Sampson, 1927; McKinnon et al., 2018). However, as menstrual regurgitation occurs in 90% of women (Halme et al., 1984), and only a small part of them suffers from endometriosis, this theory is more likely to explain the initiation of the disorder. Additional inflammatory factors may be implicated in the development of endometriotic lesions (D'Hooghe and Debrock, 2002) and maintenance of the disease.

Hormonal abnormalities are also involved in endometriosis. The expression of aromatase in endometriotic implants induces local estrogen synthesis that, along with the progesterone resistance, favor proliferation of endometriotic lesions (McKinnon et al., 2018). However, hormonal interventions to treat endometriosis may control the symptoms, but have not been fully successful in controlling disease's progression, highlighting the role of other factors in endometriosis pathophysiology (Vercellini et al., 2014; Tosti et al., 2017).

Indeed, many studies have emphasized the role of chronic inflammation and aberrant immune response as major mechanisms of endometriosis development (Riccio et al., 2018). Several immunological abnormalities have been reported, such as decreased natural killer (NK) cytotoxicity and T cell reactivity, polyclonal activation of B cells and antibody production, modifications in inflammatory mediators, and increased number and activation of peritoneal macrophages

(Riccio et al., 2018). However, many aspects of the role of the immune system in endometriosis remains to be clarified.

Macrophages are the most prevalent type of immune cells in the peritoneal fluid (Ho et al., 1997), and their number, activation and cytokine production are increased in endometriosis (Oral et al., 1996; Berkkanoglu and Arici, 2003; Králíčková and Vetvicka, 2015). These cells induce inflammation, tissue repair, angiogenesis and the recruitment of fibroblasts and endothelial cells, through the production of immunomodulators (Oral et al., 1996; McLaren, 2000). The peritoneal environment is regulated by activated macrophages through the phagocytosis of red blood cells, tissue fragments and cellular debris (Králíčková and Vetvicka, 2015).

However, it seems that these immunosurveillance mechanisms are defective in endometriosis and the macrophages fail to eliminate ectopic endometrial cells that reach peritoneal cavity through retrograde menstruation (Králíčková and Vetvicka, 2015).

Endometriosis is associated with an imbalance towards pro-inflammatory cytokines mainly produced by innate immune cells, like macrophages and NK cells (Beste et al., 2014). This phenomenon leads to recruitment and activation of other immune cells like B lymphocytes, T lymphocytes and NK cells, that contribute to chronic local inflammation. In addition, they induce neo-angiogenesis and activation of stromal and epithelial endometrial cells, leading to endometriotic implants development (Riccio et al., 2018). Moreover, inflammation induces reactive oxygen species (ROS) production in endometriotic cells that activate various tyrosine kinases (Ngô et al., 2009; Santulli et al., 2015), contributing to this process.

In addition to innate immunity, cells from adaptive immunity also play a role in endometriosis (Riccio et al., 2018). Activation of $CD4^+$ T cells has been described with an imbalance towards a T helper (Th) 2 phenotype that drives the fibrosis of lesions (Podgaec et al., 2007; Chen et al., 2012, 2016) and a combined increase in Th17 cells maintains the inflammatory process (Gogacz et al., 2016). Regulatory $CD4^+FoxP3^+$ T cells have been investigated over the last few years with controversies regarding their number and their role (de Barros et al., 2017). Recent findings seem to support the major role of regulatory T cells in limiting

endometriosis development, such as fewer activated regulatory cells on active lesions and increased number and size of endometriotic lesions in mice depleted for regulatory T cells (Tanaka et al., 2017).

B lymphocytes are important players of the adaptive immune response, and they are responsible for the production of antibodies against antigens. In endometriosis, these cells seem to contribute to the progression of the disease through autoantibody secretion (Straub, 2007).

Several studies (Badawy et al., 1987, 1989; Gleicher et al., 1987; Odukoya et al., 1995, 1996a, 1996b; Lachapelle et al., 1996; Chishima et al., 2000; Antsiferova et al., 2005; Hever et al., 2007; Berbic et al., 2013; Scheerer et al., 2016; Walankiewicz et al., 2018) have described the presence of increased number and activation of B cells in the blood and peritoneal cavity of patients with endometriosis. A polyclonal activation of B cells is associated with an increased expression of activation markers like CD23 and Toll-like receptor 4 (TLR4), and of growth factors like B lymphocyte stimulator (BLys). BLys plays a key role in B cells survival, activation, and differentiation into plasma cells. It binds to B cell receptor (BCR) and through Bruton's tyrosine kinase (Btk) activates nuclear factor kappa B (NF-κB) pathway (Shinners et al., 2007).

Moreover, the presence of anti-endometrial autoantibodies (Wild and Shivers 1985; Fernández-Shaw et al., 1993) has been described in a large proportion of women with endometriosis, though its clinical significance remains to be determined. It has been hypothesized that infertility associated to endometriosis may be partly due to polyclonal B cell activation, associated with B-1 cell proliferation resulting in autoantibody abnormalities (Chishima et al., 2000).

Endometriosis is a chronic inflammatory disease that presents changes in both humoral and cellular immunity (Nothnick, 2001), leading to inflammatory reactions and proliferation of endometriotic cells (Osuga et al., 2011). There are some common aspects between endometriosis and autoimmune disorders, such as: tissue injury, polyclonal activation of B cells, abnormalities of B and T lymphocytes, changes in apoptosis, association with other autoimmune diseases, multiple organ involvement, familial occurrence and possible environmental and genetic factors associated (Nothnick, 2001).

The pathogenesis of endometriosis is multifactorial. Several authors have attempted to clarify the role of the immune system in endometriosis and various abnormalities have been detected, including increased B lymphocytes and excessive production of autoantibodies. The importance of the immune system in the pathogenesis of endometriosis supports the idea of using therapeutic strategies involving compounds that modulate specifically the functions of immune cells (Osuga et al., 2011).

Thus, in the present study, we have hypothesized that B lymphocytes contribute to endometriosis development and B cell depletion or inactivation can limit disease's progression. We have evaluated the effects of B cells through a double strategy – complete depletion with anti-CD20 antibody or inactivation with Btk inhibitor Ibrutinib – to characterize their effects on disease development in a relevant mice model of endometriosis.

There is an urgent need for new approaches to the medical treatment of endometriosis, especially non-hormonal therapies, and the better understanding of the immunological aspects of endometriosis could lead to the development of new therapeutic strategies targeting immune cells.

2 OBJECTIVES

2 OBJECTIVES

2.1 Primary

- To evaluate the effects of B lymphocyte depletion with anti-CD20 and inactivation with Btk inhibitor Ibrutinib on endometriosis development in mice.

2.2 Secondary

- To evaluate the effects of both strategies on M1 and M2 macrophage populations in the spleen and peritoneal fluid of endometriotic mice.
- To evaluate the effects of Ibrutinib treatment on T lymphocyte subsets in the spleen and peritoneal fluid of endometriotic mice.
- To evaluate the effects of Ibrutinib treatment on inflammatory and immunomodulatory cytokines in the serum and peritoneal fluid of endometriotic mice.

3 LITERATURE REVIEW

3 LITERATURE REVIEW

3.1 Endometriosis

Endometriosis is a gynecological disease currently defined as: “the presence of functional endometrial-like tissue outside the uterus, but in the pelvic cavity, or even outside, with evidence that lesions are cellularly active, or have an effect on normal physiology” (Audebert et al., 1992). Initially, peritoneal endometriosis, deep infiltrating endometriosis, endometrioma and adenomyosis where defined all together as “adenomyoma” (Benagiano et al., 2014).

The surgeon Thomas Cullen first described the morphology and clinic of “adenomyoma” in his book “Adenomyoma of the Uterus”, in the end of the 19th century (Benagiano et al., 2014). In 1860, Austrian pathologist Karl von Rokitansky described the microscopical aspects of endometriosis, although he did not appear to recognize that the disease he was examining was already being referred to by other names (Nezhat et al., 2012). In the 1920s, Sampson first introduced the term “endometriosis” and Frankl described “adenomyosis”, so the two diseases became separate entities, with different diagnosis criteria of the so-called “adenomyoma” (Benagiano et al., 2014).

The introduction of laparoscopy in the early 1960s made it possible to distinguish three different clinical presentations of endometriosis: a) peritoneal endometriosis, characterized by small superficial lesions, usually located in the pelvis; b) ovarian endometrioma (OMA), a cyst containing chocolate colored fluid derived from repeated hemorrhages of the endometriotic foci; c) deep infiltrating endometriosis (DIE), with lesions that invade the peritoneum deeper than 5mm (Nisolle and Donnez, 1997). These three phenotypes may co-exist in the same patient and the disease is usually multifocal (Andres et al., 2018).

Several authors have proposed different classifications systems to categorize endometriosis: Sampson (1921) proposed a classification for ovarian

cysts; Wicks and Larson (1949) classified endometriosis based on histological findings; Acosta et al. (1973) used surgical findings and Chapron et al. (2003) considered anatomical distribution of lesions in anterior and posterior compartments. However, none of these classifications systems have been widely accepted and used.

A score system was proposed in 1985 and revised in 1996 by the American Society of Reproductive Medicine (rASRM). This system classifies endometriosis in four stages: I (1-5 points), II (6-15 points), III (16-40 points), and IV (>40 points). rASRM has been widely used and accepted in different countries, but it has some limitations, concerning severity and location of deep infiltrating endometriosis.

The Enzian classification system was proposed in 2005 (Tuttlies et al., 2005) to improve rASRM and include location and severity of DIE, as well as its association with pain. It was revised and 2011 and it is considered a good complement do rASRM to describe DIE (Haas et al., 2013a). Unfortunately, the Enzian classification is more complex, does not have an international acceptance and it is mainly used in German-speaking countries (Haas et al., 2013b).

Histological analysis of endometriotic lesions show undifferentiated glandular and/or stromal cells surrounded by fibrotic tissue (Tosti et al., 2015). However, it has been shown that endometrial cells represent a minor component of the lesions and may be often absent, while a smooth muscle component and fibrosis represent consistent features of all disease forms. Therefore, the inclusion of fibrosis in the definition of endometriosis has been recently proposed (Vigano et al., 2018).

Abrão et al. (2003) compared the histological patterns with the stages of rASRM classification and showed that early disease (stages I and II) was associated with stromal and well-differentiated histological pattern and advanced disease (stages III and IV) was associated with pure or mixed undifferentiated disease.

The gold standard for the diagnosis of endometriosis is by laparoscopy with pathological confirmation (Dunselman et al., 2014). However, there is an increasing role of noninvasive diagnostic methods such as magnetic resonance imaging (MRI) (Barbisan et al., 2021) and transvaginal ultrasound with or without bowel preparation (Goncalves et al., 2010, 2021; Mattos et al., 2019).

The disease can be asymptomatic or cause six main symptoms: dysmenorrhea; dyspareunia; noncyclic pelvic pain; urinary or bowel cyclic symptoms and infertility. In most cases, the location and severity of the lesions are not clearly related to the symptoms, and the only strong association observed by most investigators is between deep posterior cul-de-sac lesions and dyspareunia (Vercellini et al., 2007).

Deep infiltrating endometriosis is related to more severe symptoms than peritoneal endometriosis and can affect 48% of all women with the disease. DIE can occur in multiple sites in the peritoneal cavity, including bowel, bladder, retrocervical area, vagina, and ureter (Bellelis et al., 2010). The treatment is often surgical, but complete excision of all DIE lesions is challenging, can have high rates of complications and often requires a multidisciplinary approach (de Paula Andres et al., 2017).

The clinical management of symptoms include painkillers and nonsteroidal anti-inflammatory drugs, but the most effective are hormonal compounds. The goal is to achieve inhibition of ovulation and abolition of menstruation, based on the concept that the response of the eutopic and ectopic endometrium is substantially similar (Vercellini et al., 2014). Medical therapy should be considered a long-term treatment, as the therapy for other chronic inflammatory conditions, and it has been consistently demonstrated that, as long as amenorrhea is achieved, there are no differences between the various available drugs in terms of pain relief (Vercellini et al., 2009). Moreover, the desire for a spontaneous pregnancy may contribute to the decision between clinical and surgical treatment, as the hormonal treatments are contraceptive.

3.1.1 Animal models in endometriosis

Although endometriosis has been described and studied for over a century, many aspects of its pathophysiology remain not completely understood. In this context, animal models of the disease could help clarifying its aspects and developing new therapeutic approaches. Many strategies have been tested to establish and validate research animal models of endometriosis. Several animal

models have been described, including non-human primates, rabbits, hamsters, rats, and mice, each one with its strengths and limitations.

The occurrence of spontaneous endometriosis – with lesions resembling human's disease in morphology and location – has been described in non-human primates (Story and Kennedy, 2004). It and has been suggested to result from increased exposure to retrograde menstruation due to controlled mating in captivity (D'Hooghe et al., 1996). The study of spontaneous endometriosis in primates would probably be the most suitable model to research purposes, but its low incidence and slow progression lead to the development of methods to artificially induce endometriotic lesions in these animals (Tirado-González et al., 2010).

These techniques include the use of different methods to occlude the uterine cervix to increase menstrual reflux through fallopian tubes (D'Hooghe et al., 1994) and the transplantation of endometrial tissue to ectopic sites in the peritoneal cavity (Yang et al., 2000; Fazleabas et al., 2002). Lesions observed after intraperitoneal inoculation of endometrium are similar to those observed in spontaneous disease in baboons (D'Hooghe et al., 1995). Primates offer a relevant preclinical model with many similarities to humans concerning reproductive anatomy, endocrinology, and physiology. (Tirado-González et al., 2010). However, the limitations of using primate models are ethical issues and high-cost development and maintenance (Van Duyne et al., 2009).

Laboratory mice have been widely used as endometriosis animal models, due to their low cost, possibility of studying endometrial lesions at different intervals of time, performing analysis in genetically similar animals, evaluating of drug and treatments effects, and introducing endometrial tissue into recipients (Becker et al., 2006). Although these models are the most used worldwide, they also present several limitations. The main physiological difference is the lack of menstruation in mice, so endometriosis must be induced surgically (Marcellin et al., 2017) or by peritoneal injection of endometrial tissue (Fainaru et al., 2008). Murine models are classified in homologous and heterologous models, accordingly to the origin of the endometrial tissue used for induction.

Homologous models are based on the transplantation of endometrial fragments from syngeneic animals in immunocompetent recipients. The

fragments can be surgically implanted in the peritoneal wall or other abdominal sites (Marcellin et al., 2017) or injected intraperitoneally (Somigliana et al., 1999). In these models, both donors and recipients receive exogenous estrogen treatment to prevent variation in the estrogenic cycle. In addition, estrogen therapy facilitates endometrial tissue growth in the donors to be suitable for transplantation and contributes to endometriosis development in recipients, as this hormone plays an important role in the disease's pathophysiology (Tirado-González et al., 2010).

In heterologous models, endometrial human tissue is transplanted into immunodeficient mice. The tissue can be obtained from menstrual fluid, endometrial biopsy (Story and Kennedy, 2004) or ovarian endometriomas (Santulli et al., 2016) and it can be transplanted by inoculation into the peritoneal cavity (Somigliana et al., 1999) or by laparotomy and suture of the fragments in the peritoneal wall (Santulli et al., 2016). The main strength of the heterologous model is the use of human endometrium, but the transplanted tissue has a limited lifespan, being unable to persist beyond four weeks (Grümmer et al., 2001). Other limitation is the lack of immune response of nude mice making it more susceptible for infections and unable to mimic the immune changes against endometrial tissue observed in endometriosis (Bruner-Tran et al., 2002).

Although none of the currently available models is perfect in simulating all aspects of the human disease, they are valuable tools to controlled studies that aim better understanding the disease's pathophysiology, developing new diagnostic methods and therapeutic interventions (Tirado-González et al., 2010).

3.2 Immunology of endometriosis

The pathophysiology of endometriosis is not completely understood. The main theories that have been proposed to explain the disease are: a) retrograde menstruation (Sampson, 1927): endometrial cells migrate through the fallopian tubes and reach the peritoneal cavity; b) celomic metaplasia (Bulun, 2009); c) blood and lymphatic dissemination of endometrial cells (Abrão et al., 2006); d)

endometrium-derived stem cells migrating to ectopic sites (Hufnagel et al., 2015); e) epigenetic changes leading to a pro-inflammatory microenvironment (Laganà et al., 2017); f) environmental toxicants acting as endocrine disrupters on the female reproductive system (Sofo et al., 2015). However, none of them can completely explain the disease in all its aspects, and its pathophysiology seems to be multifactorial.

The immune system also contributes to the development of endometriosis, and several abnormalities have been identified in women with the disease (Christodoulakos et al., 2007). Disturbances in immune homeostasis are associated with increase in implantation, proliferation, and angiogenesis of the ectopic endometrial tissue (Matarese et al., 2003).

3.2.1 Immunosurveillance

Retrograde menstruation described by Sampson (1927) is a phenomenon known to occur in most women in reproductive age with patent fallopian tubes (Halme et al., 1984). However, only 5-15% of them are affected with endometriosis. Endometrial cells that migrate through uterine tubes are not able to implant in the peritoneal cavity of healthy women, they are eliminated by immunosurveillance system and apoptosis. Changes in cell-mediated and humoral immunity probably prevent the clearance of the endometrial cells that reach the peritoneal cavity and allow their implantation and development (Senturk and Arici, 1999; Paul Dmowski and Braun, 2004).

It is not clear how ectopic endometrial cells perform immunosurveillance evasion, and some hypotheses have been proposed to explain this phenomenon. The endometriotic implants produce proteins that interfere in their recognition by the leukocytes, such as the intercellular adhesion molecule soluble form (sICAM-1). This circulating protein binds to leukocyte function antigen (LFA-1) and the leukocytes become less available to identify the aberrant endometrial cells through their ICAM-1. The messenger ribonucleic acid (mRNA) expression of sICAM-1 was shown to be increased in endometriotic stromal cells, compared to stromal cells of eutopic endometrium (Vigano et al., 2018).

In addition, dysfunctional or aberrant cells of the normal endometrium are usually eliminated by apoptosis as part of a tissue repair mechanism during menstrual cycle. Overexpression of antiapoptotic factors and decreased expression of proapoptotic factors interfere in this mechanism of programmed cell death of the endometrial cells that reach peritoneal cavity, leading to the development of the disease (Tosti et al., 2015).

The Fas-Fas ligand (FasL) apoptosis pathway seems to be involved in peritoneal immunosurveillance (Vetvicka et al., 2016). In endometriosis, increased FasL expression in stromal cells leads to Fas-mediated apoptosis of activated immune cells that express Fas, such as T lymphocytes and NK cells. Through this mechanism, ectopic endometrial cells escape immunosurveillance (Selam et al., 2002), so apoptosis pathways could be a therapeutic target for endometriosis. The use of gonadotropin-releasing hormone (GnRH) analogs to increase proapoptotic protein Bax and decrease antiapoptotic protein B cell lymphoma 2 (Bcl-2) in endometrial cell cultures has been described (Bilotas et al., 2007).

Ectopic endometrial cells escape immunosurveillance and it has been proposed that stromal cells are involved in cellular adhesion to intraperitoneal surface, whereas glandular cells play a role in invasion and growth of the lesion (Ahn et al., 2015). The growth of endometrial implants starts an intense inflammatory response, with immune cells recruitment, angiogenesis and proinflammatory cytokines and growth factors increasing. In addition, tissue-repair mechanisms are also activated, with fibroblasts mobilization and proliferation of connective tissue (Paul Dmowski and Braun, 2004).

Endometriosis is a chronic inflammatory disease, and inflammation plays a key role through mitogen-activated protein kinase (MAPK) signaling pathways leading to increased cyclooxygenase-2 (COX-2), interleukins and oxidative stress (Santulli et al., 2015). MAPK are altered in endometriotic lesions, and it was shown that their inhibitors can control disease progression both *in vitro* and in animal models (Leconte et al., 2015). However, the use of MAPK inhibitors in the treatment of endometriosis is still limited due to their teratogenicity and side effects (Santulli et al., 2015).

The MAPK pathway can increase inflammation and endometriosis clinic repercussion by: recruitment of immune cells and amplification of the inflammatory response (Kaminska, 2005); generation of an antiapoptotic signal (Harada et al., 2004); increased growth factors expression leading to angiogenesis (Hamden et al., 2005); playing a role in the development of pain and hypersensitivity to pain (Ji and Suter, 2007); or acting as intracellular and extracellular signal transducers in endometriotic cells (Santulli et al., 2015).

Many functional changes in the immunological components of the peritoneal fluid of women with endometriosis have been described. Macrophages, NK cells, T lymphocytes, B lymphocytes and cytokines are altered (Ho et al., 1997), but the exact role of these changes in the progression of the disease has not been completely clarified (Gazvani and Templeton, 2002).

3.2.2 Innate immunity

Macrophages and NK cells are important players of innate immunity, and alterations in both types of cells have been described in endometriosis. Macrophages number and activation are increased in endometriosis (Oral et al., 1996), as well as their cytokines production (Berkkanoglu and Arici, 2003; Králíčková and Vetvicka, 2015).

3.2.2.1 The role of macrophages

Activated macrophages can regulate the peritoneal environment by phagocyting red blood cells, damaged tissue fragments and cellular debris (Králíčková and Vetvicka, 2015) or by producing soluble mediators like cytokines, prostaglandins, complement components and enzymes. Through the secretion of these immune mediators, macrophages can induce inflammation, tissue repair, and neovascularization and may favor the recruitment of fibroblasts and endothelial cells (Oral et al., 1996; McLaren, 2000). The macrophage-derived

cytokines stimulate the activation of other immune cells such as T and B lymphocytes.

Despite of their increased activation, the phagocytic activity of macrophages is reduced in endometriosis (Králíčková and Vetvicka, 2015), as they fail to eliminate the ectopic endometrial cells that reach the cavity through retrograde menstruation. The expression of CD36 receptor and the activation of matrix metalloproteinases regulate macrophages' phagocytic function and both mechanisms are suppressed by prostaglandin E2, which is overexpressed in patients with endometriosis (Wu et al., 2005).

The scavenger function of the peritoneal macrophages depends on their attachment to extracellular matrix components. Increased nonadherent macrophages have been described in the peritoneal fluid of women with endometriosis, suggesting a defective scavenger function that could lead to survival of ectopic endometrial cells (Berkkanoglu and Arici, 2003).

Macrophages exhibit a phenotypic plasticity in their various microenvironments and are classified in two main groups, with different functions. The M1 macrophages produce high quantities of inflammatory cytokines and nitric oxide (NO) through inducible nitric oxide synthase (iNOS) (Orecchioni et al., 2019) and are specialized in the elimination of microorganisms and defective cells. Interferon (IFN)- γ and tumor necrosis factor (TNF) are associated with the induction of "classically activated" or M1-like macrophages, expressing anti-microbial effector functions (Schleicher et al., 2016).

Cytokines such as interleukin (IL)-4, IL-10, IL-13, or transforming growth factor (TGF)-β limit the release of proinflammatory factors by macrophages and promote macrophage phenotypes that suppress T cell responses and/or support tissue repair, named "alternatively activated" or M2 macrophages. These macrophages present a distinct profile and have distinct functions: they modulate adaptive immune response, promote angiogenesis and tissue repair, and scavenge cellular debris (Cominelli et al., 2014). M2 macrophages are related to upregulation of the resistin-like molecule alpha (Relma, Retnla; also termed ''found in inflammatory zone-1'', Fizz1) and arginase 1 (Arg1) (Schleicher et al., 2016). Arg1 expression is characteristic for wound healing and tissue regeneration (Schleicher et al., 2016).

An imbalance in M1 macrophages was shown in the eutopic endometrium of women with endometriosis (Takebayashi et al., 2015). However, M2 macrophages are significantly upregulated in the peritoneum and lesions of women (Bacci et al., 2009) and rhesus macaques with the disease (Smith et al., 2012). Experiments with macrophage depletion further demonstrated the key role of M2 macrophages in endometriotic grafting, development, and persistence (Bacci et al., 2009; Haber et al., 2009). In addition, selective adoptive transfer of M2 macrophages indicated that they promote endometriosis progression (Bacci et al., 2009).

The imbalance in macrophage subtypes was evaluated in a murine model of endometriosis, considering the classification in large peritoneal macrophages (LPMs) and small peritoneal macrophages (SPMs) (Yuan et al., 2017). The authors have shown an increased proportion of SPMs and an opposite trend for the LPMs. They proposed that this new classification of macrophages should be included in further studies in endometriosis field.

To summarize, macrophages play a key role in the development of endometriosis once they fail to eliminate the ectopic endometrial cells that reach the peritoneal cavity by retrograde menstruation. In addition, tissue-repair mechanisms through M2 macrophages in the peritoneal cavity contribute to the implantation and proliferation of endometrial cells, resulting in the development of endometriotic lesions.

3.2.2.2 The role of NK cells

Natural killer (NK) cells can kill target cells and secrete cytokines that participate in the adaptive immune response and tissue repair. NK cells are able to distinguish stressed cells that have undergone some degree of injuries from normal cells. Ectopic endometrial cells that reach the peritoneal cavity achieve to escape the clearance and are not targeted or removed by NK cells in a not completely understood mechanism called “immunoescape” (Vetvicka et al., 2016). Decreased NK cell’s function could explain this mechanism, leading to adhesion and proliferation of endometrial cells, resulting in endometriotic lesions.

However, it is also possible that this aberrant NK cell function is a consequence of the chronic inflammatory environment provided by the disease (Kikuchi et al., 1993).

Decreased NK cytotoxicity against endometrial cells in women with endometriosis was first described in 1991 (Oosterlynck et al., 1991) and it has been well established since then (Wilson et al., 1994). This phenomenon is more evident in the peritoneal cavity (Oosterlynck et al., 1993b; Ho et al., 1997), but has also been observed in peripheral blood of women with endometriosis (Oosterlynck et al., 1993a; Dias et al., 2012) and it can be correlated to advanced stages of the disease (Oosterlynck et al., 1993a).

NK cells have different subsets, and they play a role in the interface between innate and adaptive immune response. The NK T cells represent 15-20% of these cells and express T-cell receptor (TCR) and CD3 membrane complex, in addition to classical CD16 expression. They can both kill target cells and secrete cytokines such as IL-4 and IL-10, which are important in the control of autoimmunity (Moretta, 2002).

The NK cell detection system includes a variety of cell surface activating (KAR) and inhibitory (KIR) receptors, that regulate NK cell activities. Among the cell surface activating receptors, two main receptors that trigger a cytotoxic response can be distinguished: NKG2D and CD16 (FcgRIIIa). The second can bind and destroy immunoglobulin G (IgG)-coated stressed cells by a mechanism called antibody-dependent cell-mediated cytotoxicity. In addition, the cytotoxic activity of the NK cells can be increased by cytokines such as IL-2 (Paul Dmowski and Braun 2004).

González-Foruria et al. (2015) evaluated ligands for NKG2D in the peritoneal fluid of women with endometriosis and demonstrated a significant increase in soluble NKG2D ligands. These soluble forms act as decoy receptors, representing a lower expression of NKG2D in ectopic endometrial cell surface, heading toward greater evasion from NK cell recognition.

Despite the decreased NK cell function in endometriosis, the mechanisms of this suppression are not clear. There is also no consensus regarding the number of NK cells in endometriosis, neither in the blood nor in the peritoneal cavity (Oosterlynck et al., 1993a, 1993b; Hsu et al., 1997; Dias et al., 2012).

Qualitatively, an increased expression of KIR on peritoneal NK cells from women with endometriosis was reported, which could explain the decreased peritoneal NK cell activity in these patients (Wu et al., 2000; Maeda et al., 2002).

Prostaglandins and cytokines derived from macrophages in the inflammatory peritoneal environment in endometriosis may also modulate NK activity. This hypothesis is corroborated by studies showing that serum and peritoneal fluid of women with endometriosis suppressed NK cytotoxic activities when compared to serum and peritoneal fluid of controls (Oosterlynck et al., 1993b).

NK cells contribute to the balance of immune self-tolerance by targeting cells that present self-antigens. Therefore, their reduced activity in endometriosis could explain the increased autoimmune reactivity observed in the disease (Matarese et al., 2003).

3.2.3 Adaptive cell-mediated immunity: T lymphocytes

Adaptive immunity plays an essential role in the survival and proliferation of ectopic endometrial cells. Indeed, endometriosis is characterized by the reduced activity of cytotoxic T cells; modulation of cytokine secretion by T helper cells and autoantibody production by B lymphocytes (Osuga et al., 2011; Králíčková and Vetvicka, 2015).

T lymphocytes are derived from stem cells in the bone marrow and fetal liver, completing their development in the thymus. The main T cells subsets are those that express glycoproteins CD4 and CD8, which function as co-receptors for major histocompatibility complex (MHC) class II and class I molecules, respectively (Startseva, 1980; Paul Dmowski and Braun, 2004).

The $CD8^+$ T cells can activate macrophages and kill cells that are infected by virus or intracellular pathogens (Startseva, 1980; Paul Dmowski and Braun, 2004). The $CD4^+$ T cells can be classified in Th1 and Th2, with different functions: Th1 cells promote the differentiation of the $CD8^+$ T cells and facilitate cell-mediated immunity by activating monocytes and macrophages; Th2 cells lead to the differentiation of B cells into plasma cells that secrete antibodies. The two

groups of lymphocytes secrete different cytokines: Th1: IL-2, IL-12, interferon (IFN)-γ, TNF-α and TNF-β; Th2: IL-4, IL-5, IL-6, IL-10, and IL-13 (Gazvani and Templeton, 2002).

Studies that have evaluated T lymphocytes in patients with endometriosis showed higher $CD4^+/CD8^+$ ratio and increased concentration of each subset in the peritoneal fluid of the patients, but with a relative reduction in Th1 cells (Ho et al., 1997). The endometriotic lesions showed higher concentration of T lymphocytes when compared to eutopic endometrium, but with a similar $CD4^+/CD8^+$ ratio. There were no changes in the peripheral blood (Startseva, 1980; Paul Dmowski and Braun, 2004) and endometriotic lesions also showed higher Th17 lymphocyte fraction when compared to eutopic endometrium (Takamura et al., 2015).

The mechanism of implantation of the ectopic endometrial cells in the peritoneal cavity depends on altered macrophages. These cells also produce inflammatory cytokines that recruit and activate Th1 and Th2 T cells (Ho et al., 1997).

Another important subset of the T lymphocytes is the regulatory T cells (Treg). They are potent suppressors of inflammatory immune responses and are responsible for maintaining antigen-specific T-cell tolerance and immune homeostasis. A systematic review (de Barros et al., 2017) evaluated the role of Treg in endometriosis. The authors concluded that there is a higher concentration of Treg cells and/or their expression markers in the peritoneal fluid and in the endometriotic lesions of women with endometriosis, when compared to controls. However, there is no consensus about the concentration of Treg cells in the eutopic endometrium and peripheral blood of these patients.

3.2.4 Inflammatory mediators

Increased soluble factors such as autoantibodies, cytokines, growth factors, adhesion molecules, enzymes, hormones, prostaglandins, and ROS have been described in the blood, peritoneal fluid, and lesions of patients with endometriosis (Oral et al., 1996; Koninckx et al., 1998; Mathur, 2000; Harada et al., 2004). This

fact is probably a consequence of the high number of leukocytes, macrophages, and other immune cells in the peritoneal cavity of these patients.

These proteins work as mediators of the immune system (Kayisli et al., 2002), regulating: proliferation and differentiation of immune cells; immunoglobulin secretion; cytotoxic activities and enzymes and acute phase proteins secretion (Gazvani and Templeton, 2002).

Studies have shown that the high concentration of inflammatory mediators in the peritoneal fluid in endometriosis has toxic effects on oocyte pick up by the fimbria, sperm-oocyte interaction, and embryo implantation, leading to an aberrant reproductive function in these women (Paul Dmowski and Braun, 2004).

Many cytokines – IL-1 (Sikora et al., 2018; Malvezzi et al., 2019), IL-4 (OuYang et al., 2008), IL-6 (Podgaec et al., 2012; Malvezzi et al., 2019), IL-8 (Arici et al., 1996; Iwabe et al., 1998; Malvezzi et al., 2019), IL-10 (Ho et al., 1997), IL-33 (Santulli et al., 2012), and TNF-α (Richter et al., 1998; Arlıer et al., 2018) – and growth factors –TGF-β (Podgaec et al., 2012), insulin-like growth factor (IGF-1) (Chang and Ho, 1997; Kim et al., 2000), hepatocyte growth factor (HGF) (Osuga et al., 1999), epidermal growth factor (EGF) (Laschke et al., 2006), platelet-derived growth factor (PDGF) (Laschke et al., 2006), and vascular endothelial growth factor (VEGF) (Mahnke et al., 2000; McLaren, 2000; Laschke et al., 2006) – are significantly increased in endometriosis. In addition, studies have shown that there are changes in the Th1/Th2 balance toward Th2 in endometriosis (Moretta, 2002; Podgaec et al., 2007; Králíčková and Vetvicka, 2015).

In endometriotic lesions, VEGF induces angiogenesis, and its immunostaining was observed in the epithelium of endometriotic implants (Shifren et al., 1996), particularly in hemorrhagic red implants (Donnez et al., 1998). VEGF is also increased in the peritoneal fluid of women with endometriosis (Lebovic et al., 2001; Laschke et al., 2006). However, is not yet clarified whether it is produced by endometriotic lesions (Shifren et al., 1996; Lebovic et al., 2000) or by activated peritoneal macrophages (McLaren, 2000).

IL-6 is one of the main cytokines in the inflammatory cascade in endometriosis. It is elevated in the peritoneal cavity and blood of these patients, and it is correlated with disease activity (Oral and Arici, 1996; Harada et al., 2001)

and infertility (Malvezzi et al., 2019). IL-10 is a potent down modulator of inflammatory responses and immune cell function – like B cells and macrophages – so it is probable that both IL-6 and IL-10 are partially responsible for the aberrant immune regulation observed in endometriosis (Gazvani and Templeton, 2002).

IL-6 can inhibit the proliferation of eutopic endometrial stromal cells (Zarmakoupis et al., 1995), but it has been shown that ectopic stromal cells are resistant to IL-6, showing no inhibitory response (Rier et al., 1995). This cytokine induces T cell activation and differentiation of B lymphocytes into antibody-producing plasma cells, and it can lead to polyclonal B cell stimulation in autoimmune diseases (Paul Dmowski and Braun, 2004). IL-1 is another cytokine that affects B cells and production of antibodies in addition to increasing prostaglandins, collagen, and tissue repair (Ho et al., 1997; Senturk and Arici, 1999).

IL-1 and TNF-α usually initiate the cascade of cytokines and inflammatory response. TNF-α is increased in the peritoneal fluid of women with endometriosis, with higher concentrations in the later stages of the disease (Funamizu et al., 2014). It has been suggested that it may contribute to the adhesion of endometrial cells to the peritoneal cavity (Zhang et al., 1993) and regulation of inhibitory κB protein (Arlıer et al., 2018).

IL-8 is also increased in endometriosis (Arici et al., 1996; Iwabe et al., 1998), contributes to cell adhesion (Garcia-Velasco and Arici, 1999) and is a potent angiogenic factor (Paul Dmowski and Braun, 2004). IL-8 stimulates the growth of topic and ectopic endometrial cells (Iwabe et al., 1998), probably through TNF-α activation (Iwabe et al., 2000). It is produced by the mesothelium as a response to proinflammatory cytokine stimuli and IL-8 levels can be correlated to the severity of the disease (Arici et al., 1996) and infertility (Malvezzi et al., 2019).

Concerning the IL-10 family, IL-19 and IL-22 were demonstrated to be significantly decreased in the sera of women with ovarian endometrioma (Santulli et al., 2013). In addition, there was a reverse correlation between levels of these cytokines and the occurrence of deep dyspareunia in those patients. The authors concluded that the low levels of these anti-inflammatory cytokines may exert effects favorable to the development of ovarian endometrioma.

IL-13 is another anti-inflammatory cytokine that was shown to be decreased in endometriosis. It is a potent regulator of macrophage activation and its reduction in the peritoneal fluid of women with endometriosis could contribute to the pathogenesis of the disease (Gallinelli et al., 2004).

The cytokine production in the immune system works in a cascade mode: the biosynthesis of one type of cytokine activates the production of a whole group of inflammatory mediators. In addition, each cytokine has various target tissues and biologic effects, which makes more difficult to clarify the role of a specific mediator in the development of endometriosis. It has also been shown that they can be produced by endometriotic cells, mesothelium, and other resident cells in the peritoneal cavity (Harada et al., 2001; Song et al., 2003). Cytokines are also deregulated in the peripheral blood of women with endometriosis, suggesting a systemic effect of the disease (Paul Dmowski and Braun, 2004; Carmona et al., 2012; Santulli et al., 2013).

3.3 B lymphocytes and endometriosis

The immune cells of lymphoid lineage play a key role in the survival and proliferation of endometrial cells and many lymphocytes have been identified in endometriotic implants (Klentzeris et al., 1995). An aberrant function of these immune cells has been described in endometriosis, with reduced activity of cytotoxic T cells, secretion of cytokines by T helper cells and autoantibody production by B lymphocytes (Osuga et al., 2011; Králíčková and Vetvicka, 2015).

Bone-marrow derived lymphocytes, or simply B lymphocytes, are players of humoral immune response and produce antibodies against antigens. The major subsets of B cells are follicular B cells, marginal zone B cells and B-1 B cells, each of which is found in distinct anatomic locations within lymphoid tissues (Abbas et al., 2011). In the pathogenesis of endometriosis, these cells seem to contribute to the occurrence of the disease by autoantibody secretion (Straub, 2007).

Table 1 summarizes the results of 23 studies selected by systematic review of literature concerning the role of B lymphocytes in endometriosis (Riccio et al., 2017).

Table 1 – Studies that evaluated the role of B lymphocytes in endometriosis.

Samples	Study design	Population	Methods	Markers	B lymphocytes in EDT	References
	Case-control	EDT x controls	IBT	Monoclonal antibodies	Increased B cells	Badawy et al., 1987
Blood/ serum	Descriptive	59 EDT	ELISA	FAN; IgG; IgM lupus anticoagulant	Abnormal polyclonal B cells activation	Gleicher et al., 1987
	Case-control	19 EDT x 26 infertile	IBT; ELISA	B cells; IgA; IgG.	Increased B cells and IgG	Badawy et al., 1989
	Case-control	42 EDT x 20 infertile x 22 controls	*In vitro* stimulation with polyclonal B-cell activators	IgG1; IgG2; IgG3	No difference in B cells. ↓ polyclonal IgG2 production in EDT stages III and IV	Gebel et al., 1993
	Case-control	21 EDT x 18 controls	ELISA	sCD23	Activation of B cells	Odukoya et al., 1995
	Case-control	25 EDT and idiopathic infertility	Flow cytometry	CD19	No difference	Nava-Loya et al., 1996
	Case-control	57 EDT x 40 controls	ELISA	sCD23; IgG	Increased amount and activation of B cells	Odukoya et al., 1996a
	Case-control	31 EDT x 14 controls	Flow cytometry; IF	CD5; ANA	B cells are related to ANA production.	Chishima et al., 2000
	Case-control	175 EDT x 131 controls	Flow cytometry	CD20	Decreased B cells	Gagné et al., 2003
	Case-control	15 EDT x 20 controls	Flow cytometry	CD20; CD5	No difference	Antsiferova et al., 2005
	Case-control	10 OMA x 10 adenomyosis x 10 leiomyoma	IHC; PCR; ELISA	BlyS; Plasma cells	Increased BlyS	Hever et al., 2007

Table 1 – Studies that evaluated the role of B lymphocytes in endometriosis (continuation).

Samples	Study design	Population	Methods	Markers	B lymphocytes in EDT	References
Blood/ serum	Case-control	87 EDT x 33 adenomyosis x 205 controls	PCR	BlyS 817C/T polymorphism	Heterozygosity ↓ risk of DIE; BlyS may play a role in the pathogenesis.	de Graaff et al., 2010
	Case-control	165 infertile EDT x 83 idiopathic infertility x 145 controls	PCR	BlyS 817C/T polymorphism	No difference	Christofolini et al., 2011
	Case-control	25 EDT x 20 controls	Flow cytometry	PD-1$^+$/PD-L1$^+$ CD19$^+$	Increased PD-1$^+$/PD-L1$^+$ B cells	Walankiewicz et al., 2018
Peritoneal fluid	Case-control	EDT x controls	IBT	Monoclonal antibodies	Increased B cells	Badawy et al., 1987
	Case-control	19 EDT x 26 infertile	IBT; ELISA	B cells; IgA; IgG	Increased B cells, IgA and IgG	Badawy et al., 1989
	Case-control	25 EDT and idiopathic infertility	Flow cytometry	CD 19	No difference	Nava-Loya et al., 1996
	Case-control	47 EDT x 35 controls	ELISA	sCD23	↑ B cell activation; higher in stages I and II	Odukoya et al., 1996b
	Case-control	31 EDT x 14 controls	Flow cytometry; IF	CD5; ANA	Increased B-1 cells	Chishima et al., 2000
	Case-control	46 EDT x 52 controls	ELISA; PCR	IgG; IgA; Bcl-6; Blimp-1	↓ Bcl-6 and ↑ Blimp-1 No difference in Ig	Yeol et al., 2015
Endometrium (eutopic and ectopic)	Descriptive	15 EDT	ABC; IHC	anti-leu-12	Very few B cells in the lesions	Oosterlynck et al., 1993a
	Case-control	12 EDT x 23 controls	IHC	CD22	No difference	Witz et al., 1994

Table 1 – Studies that evaluated the role of B lymphocytes in endometriosis (conclusion).

Samples	Study design	Population	Methods	Markers	B lymphocytes in EDT	References
Endometrium (eutopic and ectopic)	Case-control	21 infertile EDT x 18 controls	IHC	CD22	No difference in eutopic endometrium	Klentzeris et al., 1995
	Case-control	30 infertile EDT x 10 controls	IHC	IgG	No difference	Nomiyama et al., 1997
	Case-control	15 EDT x 20 controls	Flow cytometry	CD20; CD5	Increased B cells. ↑ activation in ectopic endometrium	Antsiferova et al., 2005
	Case-control	10 OMA x 10 adenomyosis x 10 leiomyoma	IHC; PCR; ELISA	BlyS; Plasma cells	↑ BlyS and plasma cells	Hever et al., 2007
	Case-control	87 EDT x 33 adenomyosis x 205 controls	PCR	BlyS 817C/T polymorphism	Heterozygosity ↓ risk of DIE; BlyS may play a role in the pathogenesis	de Graaff et al., 2010
	Case-control	48 EDT X 24 adenomyosis X 12 controls	IHC	CD20	↑ B cells in EDT lesions, adenomyosis and endometrium	Scheerer et al., 2016
Follicular fluid	Case-control	12 infertile EDT x 35 tubal factor x 13 idiopathic	Flow cytometry	CD3; CD4; CD8; CD14; CD20; CD45; CD56	Increased B cells	Lachapelle et al., 1996
Pelvic lymph nodes	Case-control	7 EDT x 9 controls	IHC	CD 20; CD79; plasma cells	Increased B cells during proliferative phase	Berbic et al., 2013

ABC: avidin-biotin immunoperoxidase technique; ANA: antinuclear antibodies; Bcl-6: B cell leukemia lymphoma-6; Blimp-1: B lymphocyte inducer of maturation program-1; BlyS: B lymphocyte stimulator; DIE: deep infiltrating endometriosis; EDT: Endometriosis; ELISA: enzyme-linked immunosorbent assay; IBT: Immunobead rosette technique; IF: Immunofluorescence; IHC: Immunohistochemistry; OMA: ovarian endometrioma; PD-1: Programmed cell death 1; PD-L1: Programmed cell death 1 ligand; PCR: protein chain reaction. SOURCE: Riccio et al., 2017 – Updated

Different markers and samples were assessed by the authors to evaluate the direct or indirect role of B cells in endometriosis. Most of the selected studies have reported increased number and/or activation of B lymphocytes or higher concentration of antibodies in endometriosis (Badawy et al., 1987, 1989; Gleicher et al., 1987; Gebel et al., 1993; Odukoya et al., 1995, 1996a, 1996b; Lachapelle et al., 1996; Chishima et al., 2000; Antsiferova et al., 2005; Hever et al., 2007; de Graaff et al., 2010; Berbic et al., 2013; Scheerer et al., 2016; Walankiewicz et al., 2018).

An increase in the reactivity of B lymphocytes in endometriosis was first suggested in 1980 (Startseva, 1980). In the same year, another study (Weed and Arquembourg, 1980) demonstrated IgG and complement deposits in the endometrium and decreased serum complement, suggesting an autoimmune response with complement consumption by the antigen-antibody complex.

A few years later, the presence of anti-endometrial antibodies in the serum of women with endometriosis was described (Wild and Shivers, 1985). Immunohistochemical analysis revealed that these anti-endometrial antibodies bind to endometrial glands and to the ectopic tissue (Fernández-Shaw et al., 1993). A subsequent western blot analysis demonstrated that autoantibodies react with membrane proteins of the endometrial cells and that the immunoreactivity increases with disease progression (Bohler et al., 2007).

While evaluating the role of B cells through soluble CD23 and IgG autoantibodies, Odukoya et al. (1995, 1996a, 1996b) demonstrated increased amount and activation of B cells in the blood and peritoneal fluid of women with endometriosis. They also described higher concentration of soluble CD23 in patients with stage I and II endometriosis, suggesting that mild endometriosis may be immunologically more active than severe endometriosis. Gebel et al. (1993) findings also agree with this statement as they have reported reduced polyclonal IgG2 production in stage III and IV endometriosis.

It is speculated that the infertility associated endometriosis is partly due to autoantibody abnormalities regarded as the result of polyclonal B-cell activation associated with B-1-cell proliferation. Hever et al. (2007) analyzed significantly upregulated genes in endometriosis versus control endometrium and concluded that 53 genes associated with immune responses had altered expression.

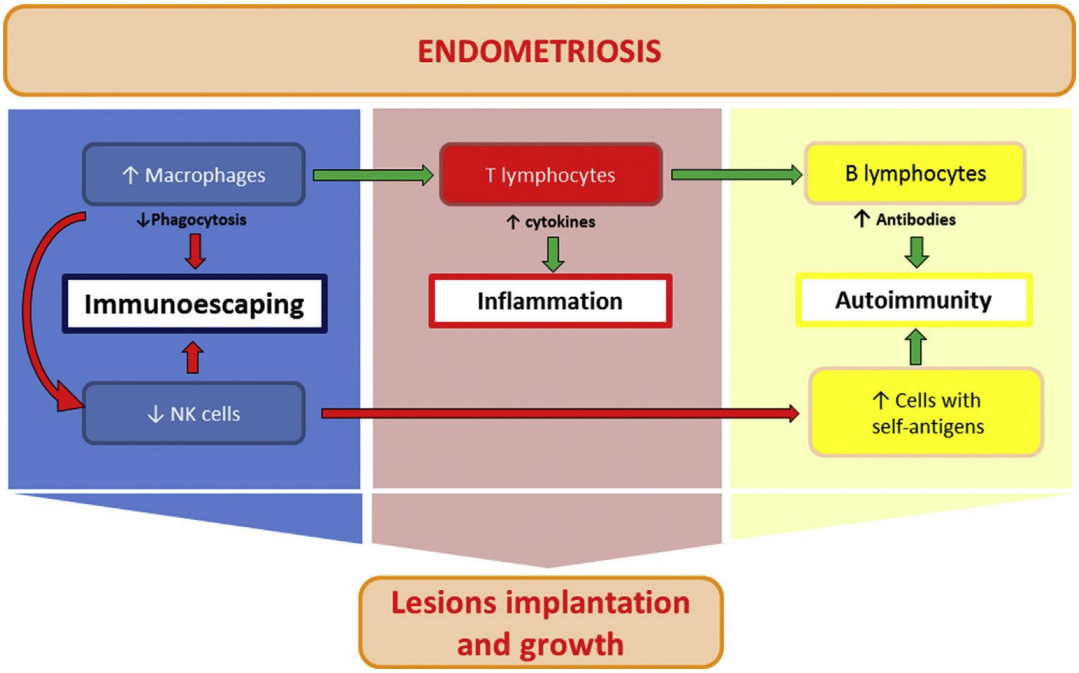
Increased B cells were described in the follicular fluid of infertile patients with endometriosis (Lachapelle et al., 1996), suggesting that this could be one of the factors impairing their fertility.

Besides anti-endometrium antibodies, B lymphocytes seem to contribute to the pathogenesis of endometriosis by producing anti-deoxyribonucleic acid (anti-DNA), antiphospholipid and antinuclear antibodies (ANA), usually observed in autoimmune diseases (Osuga et al., 2011). ANA antibodies have been detected in 29-47% of women with endometriosis (Iborra et al., 2000). However, ANA positivity does not seem to be an aggravating factor in patients with pelvic endometriosis (Dias et al., 2006).

Some authors have proposed that endometriosis has an autoimmune etiology, presenting changes in both humoral and cellular immunity (Nothnick, 2001) that lead to inflammatory reactions and proliferation of endometriotic cells (Osuga et al., 2011). Nothnick (2001) lists common characteristics between endometriosis and autoimmune diseases: tissue injury, polyclonal activation of B cells, abnormalities of B and T lymphocytes, changes in apoptosis, association with other autoimmune disorders, multiple organ involvement, familial occurrence and possible environmental and genetic factors associated.

Possible common backgrounds of immune dysfunctions between autoimmune diseases and endometriosis were also proposed by Chishima et al. (2000). They reported that B cells are related to ANA production in the blood of patients with endometriosis and also found increased B-1 cells in peritoneal exudate cells of these women.

Figure 1 summarizes the hypothesis on role of the immune system in endometriosis (Riccio et al., 2018).



SOURCE: Riccio et al., 2018.
Figure 1 – Immune response in endometriosis

3.3.1 Regulatory B cells

Inflammation is the key response to infections and after the pathogens are cleared, this response must be controlled to avoid damaging host tissues. The release of anti-inflammatory mediators and cytokines limits inflammation and the cells that produce these factors are named “regulatory” or “suppressive”.

A population of suppressor B cells, collectively known as regulatory B cells (Breg), has been associated with the inhibition of excessive inflammation. They control the expansion of pathogenic T cells and other pro-inflammatory lymphocytes through the production of IL-10, IL-35 and TGF-β. Distinct Breg populations can be induced by different inflammatory environments (Rosser and Mauri, 2015). The study of mice lacking Breg cells that produce IL-10 has shown that defective Breg can lead to chronic inflammation (Fillatreau et al., 2002; Honigberg et al., 2010; Herman et al., 2011) and that these animals were unable to recover from autoimmune diseases.

Many subsets of Breg cells have been described, but it is still unclear how they are developmentally linked (Rosser and Mauri, 2015). In humans, two main Breg phenotypes have been described: $CD19^{+}CD24^{high}CD38^{high}CD1d^{high}$ and $CD19^{+}CD24^{high}CD27^{+}$ (Iwata et al., 2011).

Currently, there are two theories for Breg development: they consist in a specific B cells lineage with a factor that controls the expression of their regulatory characteristics; or any B cell can be induced by inflammatory factors to develop a suppressive nature and become a Breg (Rosser and Mauri, 2015).

So far, a Breg-cell-specific transcription factor, like Foxp3 in Treg cells (Rudensky, 2011) has not been identified. This fact, in addition to the heterogeneity of Breg phenotypes, supports the theory that these cells are not a specific lineage, but a more “reactive” form, induced by an inflammatory environment (Rosser and Mauri, 2015). It has been shown, in both mice and human, that immature B cells, mature B cells, and plasmablasts are able to differentiate into Breg that produce IL-10.

Regulatory B cells, through the production of IL-10, TGF-β, and IL-35, can interact with different immune cells to suppress immune responses. They can induce the differentiation of other regulatory cells, such as Treg, and suppress pro-inflammatory cells: monocytes that produce TNF-α; Th1 cells, cytotoxic CD8+ T cells and IL-12-producing dendritic cells (Rosser and Mauri, 2015).

3.3.2 Anti-CD20 and B lymphocytes depletion

Cluster of differentiation 20 (CD 20) is a membrane antigen present on the surface of all B lymphocytes: pre-B cells, mature B cells and even malignant B cells (Payandeh et al., 2019). This protein regulates B cells to proceed from a resting phase (G0) on to G1 phase and regulate the cell cycle from the S phase to mitosis. It plays a role in regulation of growth and differentiation of B lymphocytes (Stamenkovic and Seed, 1988; Tedder et al., 1988).

The increased expression of CD20 has been detected in patients with certain types of B-cell lymphoma and leukemia. High expression of CD20 molecule on the surface of B cells make antibodies-based therapy a good

strategy (Payandeh et al., 2019). Anti-CD20 antibodies had been effectively used in the treatment of many diseases including cancer and immune related disorders (Du et al., 2017; Salles et al., 2017).

Anti-CD20 antibodies exert their effects on B cells via several molecular mechanisms: a) complement-dependent cytotoxicity: plasma membranes are damaged without involvement of immune system cells or antibodies; b) antibody-dependent cell-mediated cytotoxicity: cell-mediated lytic mechanism with autoreactive antibodies; c) programmed cell death, including apoptosis; d) antibody dependent cellular phagocytosis, catalyzed by macrophages, neutrophils and mature dendritic cells; e) ROS dependent non-apoptotic cell death; and f) homotypic adhesion and lysosome mediated non-apoptotic cell death, through the dispersion of lysosomes content in the cytoplasm (Payandeh et al., 2019).

3.3.3 Ibrutinib: a Bruton´s tyrosine kinase (Btk) inhibitor

The B cell activation factor (BAFF) also known as B lymphocyte stimulator (Blys) belongs to TNF family and it is a key factor to B cells survival. BAFF is produced by macrophages and plays a role in B lymphocytes development and differentiation into plasma cells (Schiemann et al., 2001). Regulation defects in BAFF expression led to its excessive production, increasing B cells activation and autoantibody production, causing autoimmune phenomena. High concentrations of BAFF were identified in the plasma of patients with autoimmune diseases such as lupus, Sjögren syndrome and rheumatoid arthritis (Zhang and Bridges, 2001; Groom et al., 2002; Stohl et al., 2003; Ramos-Casals et al., 2005). Its concentration is also elevated in endometriotic lesions (Hever et al., 2007).

BAFF can bind to three different receptors of TNF superfamily: BAFF receptor (BAFF-R), transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI) and B cell maturation antigen (BCMA) (O'Connor et al., 2004). BCMA receptor is responsible for plasmocyte survival (Tarte et al., 2003) and its expression is increased in endometriotic lesions (Hever et al., 2007).

BAFF binds to BAFF-R, activates NF-κB pathway through Btk, leading to B lymphocyte survival, development, and function (Shinners et al., 2007). Btk was initially shown to be defective in the primary immunodeficiency X-linked agammaglobulinemia (XLA) and shortly after its discovery, it 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 and antibody production (Herman et al., 2011).

Btk has a crucial function in oncogenic signaling that is critical for proliferation and survival of leukemic cells in many B cell malignancies. Inhibitors of Btk have shown anti-tumor activity, first in animal models and later in humans, with durable remissions against a variety of B-cell malignancies, including mantle cell lymphoma, follicular lymphoma, and chronic lymphocytic leukemia (Harrison, 2012; Byrd et al., 2013; Jain et al., 2018).

Ibrutinib is an orally bioavailable covalent Btk inhibitor that irreversibly binds to Btk at Cysteine-481 residue (Honigberg et al., 2010; Herman et al., 2011). This drug blocks NF-κB pathway leading to B lymphocytes inactivation. Several studies have shown that ibrutinib binds to Btk and leads to inhibition of BCR signaling, reducing the activation of malignant B cells and B cells involved in autoimmunity and infectious disease pathogenesis (Hutcheson et al., 2012; Kil et al., 2012; Vargas et al., 2013).

However, Ibrutinib is not a selective inhibitor, as its binding profile includes other kinases, such as interleukin-2-inducible T-cell kinase (Itk); tec protein tyrosine kinase (TEC) and epidermal growth factor receptor (EGFR) (Dubovsky et al., 2013; Cheng et al., 2014).

Ibrutinib was approved by Food and Drug Administration (FDA) in 2013, initially for the treatment of mantle cell lymphoma (Wang et al., 2013), and has been used in the treatment of B cells autoimmune disfunctions and malignancies since (Shinners et al., 2007; Rushworth et al., 2013; Kokhaei et al., 2016; Miklos et al., 2017). The drug is generally well tolerated, with rapid and durable responses, but can have some side effects: diarrhea, upper respiratory tract infection, fatigue; and more severe but rare, bleeding, and atrial fibrillation (Tang et al., 2018; Paydas, 2019).

4 METHODS

4 METHODS

An experimental study was performed in scientific collaboration between the Endometriosis Division of Obstetrics and Gynecology Department of *Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo*, São Paulo, Brazil and U1016-Batteux of *Institut National de la Santé et de la Recherche Médicale* (INSERM), *Hôpital Cochin, Université Paris-Descartes*, Paris, France, between 2016 and 2018.

4.1 Mice

The present study was reviewed and approved by Ethics Committees *Comité d'Ethique en matière d'Expérimentation Animale*, Paris Descartes University (CEEA 34), Paris (*PROJET Nº 2016040716219897 – V6 – APAFiS # 7283*) and *Comitê de Ética em Pesquisa do Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo* (*Parecer nº 1.532.975*).

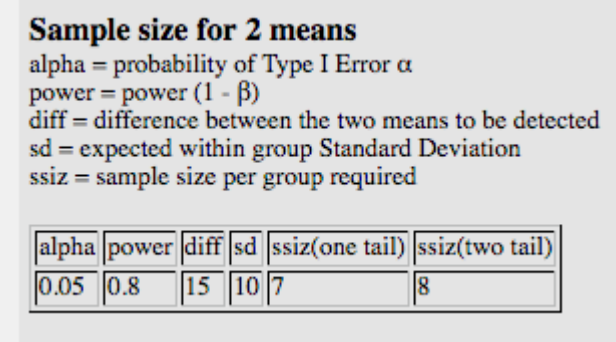
Six-week-old BALB/c female mice (Charles River Laboratories, L´Arbresle, France) weighing 16-20g were used. Animals received humane care in compliance with institutional guidelines and were housed in clean cages under standard 12h photoperiod with food and water available *ad libitum*. All experimental procedures and animal care were approved by the institutional board.

4.1.1 Sample Size

The volume of implants in mm^3 was considered as the primary outcome. Sample size was calculated to allow detection of a minimum $15mm^3$ difference between groups (based on hypothesis and preliminary data) with a 95% confidence interval and power of 80% (0.8).

The expected standard deviation was 10mm^3, based on a previous study (Leconte et al., 2015). As data were reported in means and standard error, we have multiplied the standard error (2.65, mean of 3.9 and 1.4, the provided values in the study) by the square root (3.8) of the number of animals (15) to obtain the standard deviation (10).

Then, we inserted these data in an online calculator (available at http://www.obg.cuhk.edu.hk) and obtained a sample size of eight mice per group. The results provided are shown in Figure 2.



Sample size for 2 means

alpha = probability of Type I Error α
power = power (1 - β)
diff = difference between the two means to be detected
sd = expected within group Standard Deviation
ssiz = sample size per group required

alpha	power	diff	sd	ssiz(one tail)	ssiz(two tail)
0.05	0.8	15	10	7	8

Figure 2 – Results provided by sample size calculator. To calculate sample size, data of confidence interval, power, difference to be detected between groups and expected standard deviation were inserted in an online calculator. The result was eight mice per group

To account for any potential losses occurring over the course of the experiments we further added two mice in each group, so ten animals per group for each independent experiment were included. Each experiment had three groups: Control, that received only vehicle; Anti-CD20, treated with anti-CD20 antibody to achieve complete B lymphocyte depletion; and Ibrutinib, treated with the Btk inhibitor to inactivate B lymphocytes.

4.1.2 Murine model of endometriosis

Endometriosis was surgically induced in mice by syngeneic transplantation of uterine tissue as previously described by Marcellin et al., (2017). Five donor BALB/c mice provided uterine horns to generate endometriosis-like lesions in

each group of 10 mice in each experiment performed. All mice (donors and recipients) received 56 μg/kg of 17β-estradiol (Provames®, Sanofi-Aventis, France) daily by oral gavage, during three days before the procedures. Figure 3 shows the steps of surgically induced murine model of endometriosis. A post-operative oral gavage of all recipient mice with 56 μg/kg/day of 17β-estradiol (Provames®, Sanofi-Aventis, France) was performed daily for three days after implantation.

The animals were euthanized 21 days after the surgery, and Figure 4 shows the surgical findings, representative of endometriosis: solid-cystic peritoneal lesions, adhesions to other organs, and cysts with hemorrhagic fluid inside.

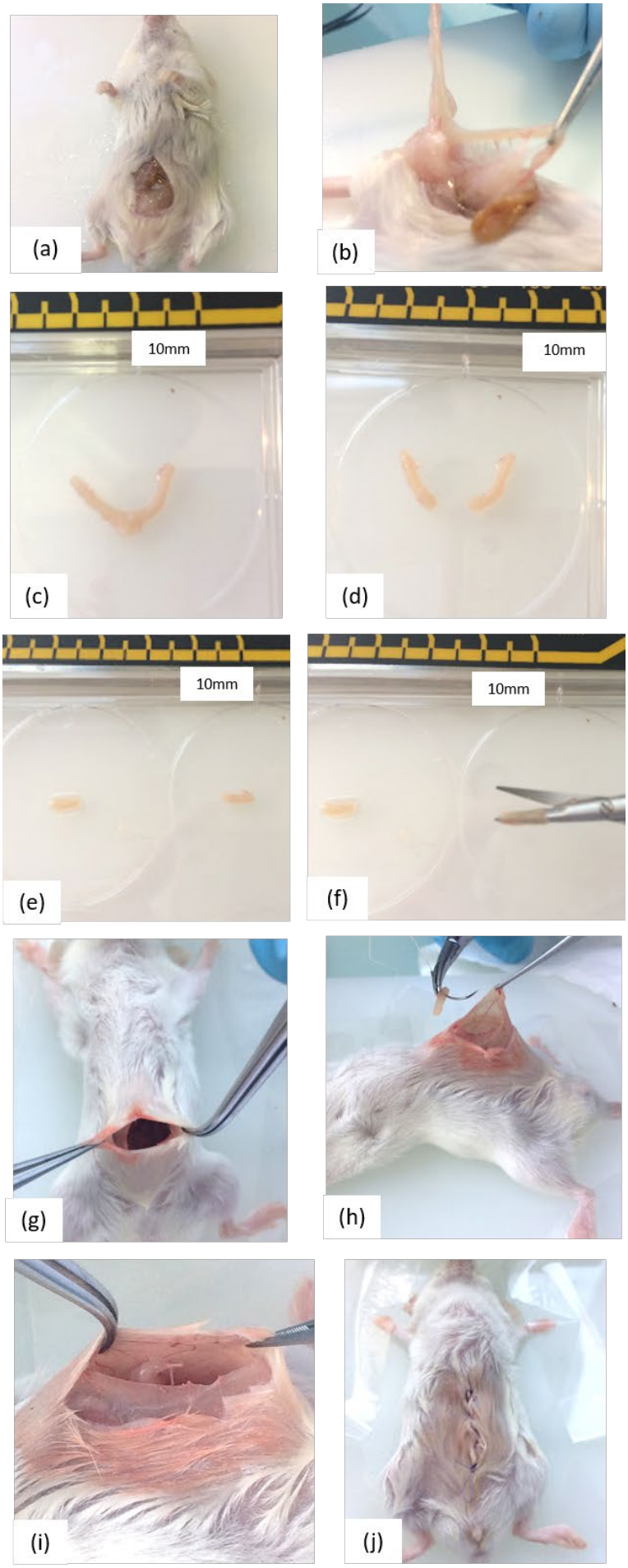

(a)
(b)
10mm
(c)
10mm
(d)
10mm
(e)
10mm
(f)
(g)
(h)
(i)
(j)

Figure 3 – Surgically induced endometriosis murine model. Donor mice were euthanized by cervical dislocation **(a)** and uterine horns were surgically extracted **(b)** and transferred into a Petri dish containing 37°C-warm phosphate buffered saline (PBS) **(c)**. The uterine horns were separated **(d)**, cut in half **(e)** and opened longitudinally with micro scissors **(f)**, so each uterus provided four samples that were prepared for grafting onto the peritoneal cavity of recipient mice. The samples had 7.72 ± 1.8 mm in the longest measure, with no significant difference between the groups (p=0.8182). BALB/c recipient mice were anesthetized with isoflurane and mechanically ventilated. An incision was made on the ventral midline **(g)** and half horn fragments were sutured onto the parietal peritoneum **(h)** with two 7/0 polypropylene stitches (Prolen®, Ethicon, Somerville, NJ), one sample on the right side and another on the left side **(i)**. In all mice, tissue samples were sutured at similar positions of the abdominal wall to ensure that host tissue sites exhibited a comparable vascularization. The cutis was sutured with a 6/0 nylon thread **(j)**

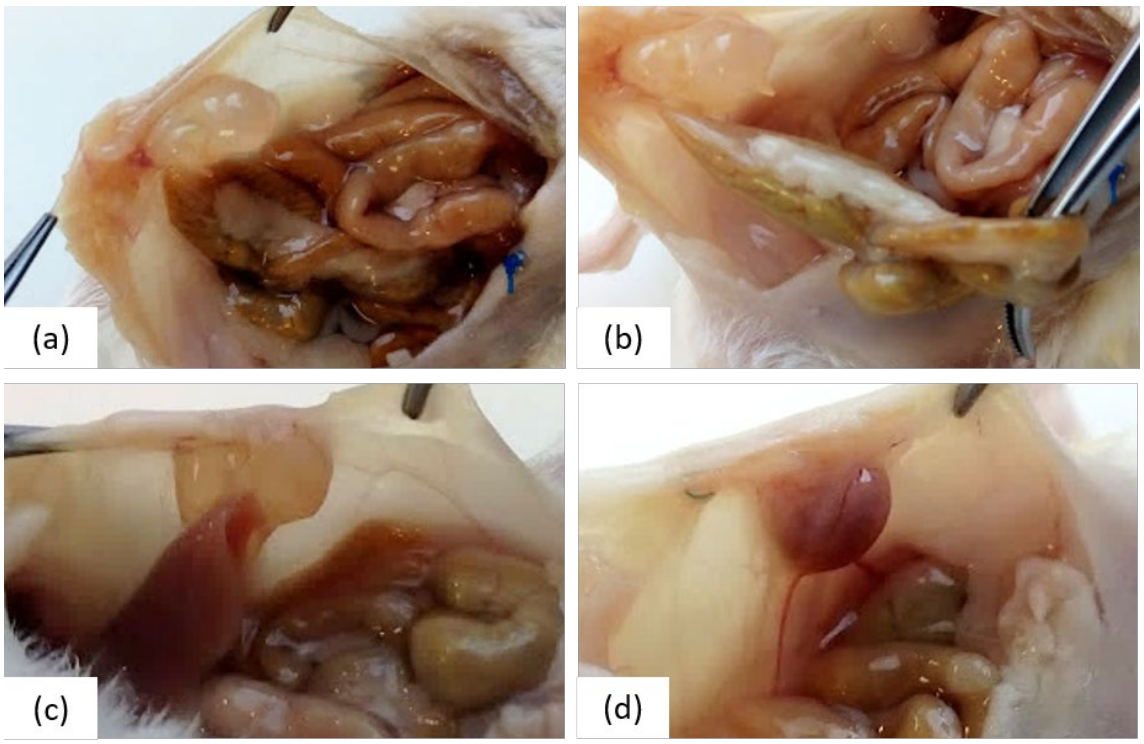


Figure 4 – Surgical findings in endometriosis murine model. Adhesions between bowel and solid-cystic peritoneal lesions **(a;b)**; Splenic adhesions **(c)**; Cyst with hemorrhagic fluid inside **(d)**

4.1.3 *In vivo* treatment of the operated mice

The operated mice were randomly separated into three groups: Control, Anti-CD20 and Ibrutinib, with 10 animals per group for each independent experiment. The *in vivo* treatment protocol is shown in Figure 5.

The Ibrutinib Group was treated with 15 mg/kg/day (Honigberg et al., 2010) of Ibrutinib (Pharmacyclics, Sunnyvale, USA). The dose of Ibrutinib was previously tested in mice, and it was shown that 5mg/kg/day was an underdose, with only partial Btk inhibition, and the drug became effective when the dose reached 10mg/kg/day (Schutt et al., 2015) and was fully effective in 12.5 mg/kg/day (Honigberg et al., 2010). It was reported in Ibrutinib´s FDA approval document (Leighton 2013) that it becomes toxic in mice in 80mg/kg/day dose.

In this study a 15mg/kg/day dose was chosen, considering that part of the substance could be lost in the canula during oral gavage procedures. The drug was diluted in 0.06% carboxymethyl cellulose/H_2O 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 (D1). The Control Group received vehicle by daily oral gavage for 21 days.

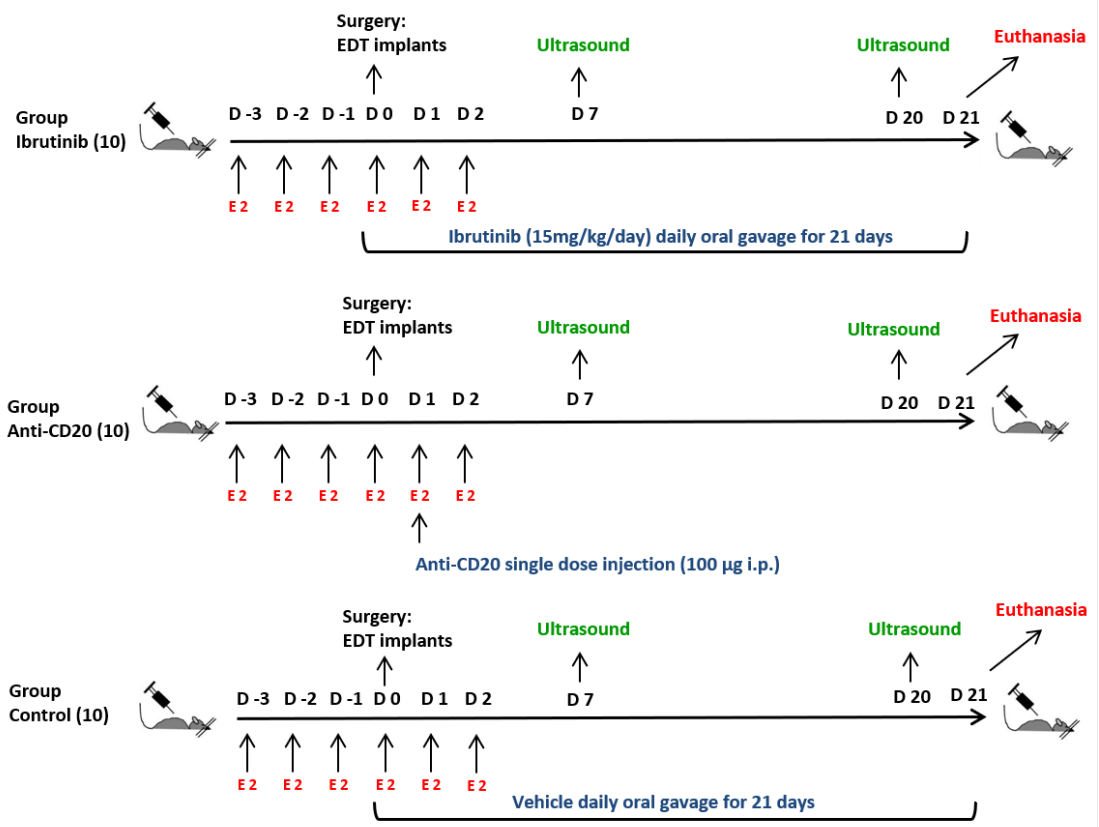


Figure 5 – *In vivo* treatment of the operated mice. Representation of the steps of the experiments and the treatment protocols for the three groups: Ibrutinib, Anti-CD20 and Control. E2: 17β-estradiol; EDT: endometriosis; D: day; i.p.: intraperitoneal; mg: miligram; µg: microgram

Twenty-one days after implantation, animals were euthanized by cervical dislocation. Retro-orbital blood sample was collected for cytokine analysis. Peritoneal cavity washing was performed with infusion and aspiration of 10mL of PBS to extract peritoneal cells to perform flow cytometry. Spleens were also collected for flow cytometry analysis.

Endometriotic implants were surgically removed, weighed, and measured using a rule caliper. Implants' volume (TV) was calculated as follows: TV (mm^3) = (L x W^2)/2, where L is the longest and W 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 analysis. The left-side implant was frozen in liquid nitrogen for further RNA extraction and quantitative real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR).

4.2 Ultrasonography to evaluate implants' size

The endometriotic implants were measured through serial ultrasonography, at Day 7 and Day 20 after the surgery, as previously described by Santulli et al., (2016). The Vevo 2100 high-frequency ultrasound imaging system (VisualSonics; Toronto, Canada) was used. The probe has a 40-MHz center frequency (MS550) and an adaptable focal depth. The spatial resolution at the focus is 40 x 80 x 80 μm^3.

For the exam, the mouse was kept under anesthesia with 1.5% isoflurane, restrained on a heated stage and had the abdomen shaved with depilatory cream. Ultrasound contact gel was applied on the abdomen (Figure 6a), and an image sequence with two-dimensional axial views of the endometriotic implant was acquired (Figure 6b), as the probe was swept from the upper to the lower abdominal wall of the mouse. The implant volume was calculated as described [TV (mm^3) = (L x W^2)/2]. The exams were performed at *Plateforme Imageries du Vivant (PIV) de l'Université Paris Descartes, INSERM U1016*, Paris, France, and all the image acquisitions were performed by the same blinded operator.

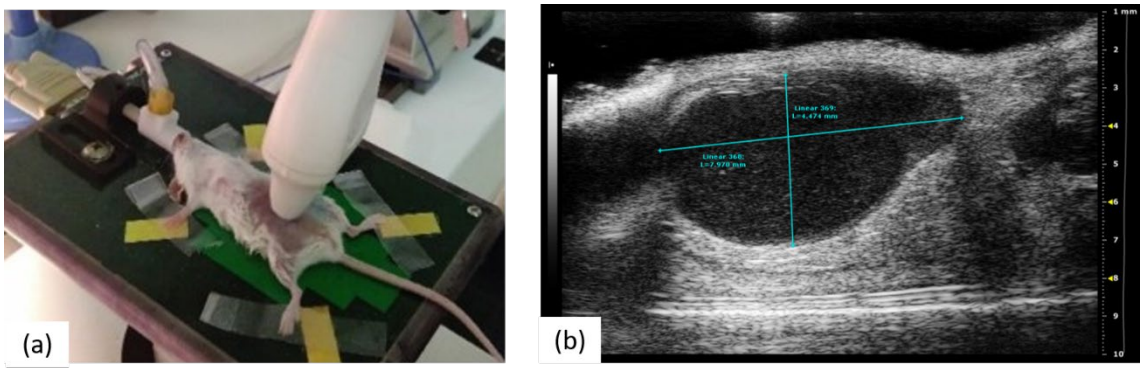


Figure 6 – Ultrasonography to evaluate implants' size in mice. **(a)** Mice were anesthetized and submitted to ultrasonography at Day 7 and Day 20 after the surgery to induce endometriosis; **(b)** Example image of endometriotic implant

4.3 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 two blinded pathologists experienced in endometriosis, to confirm the presence of the disease in the samples.

4.4 RNA extraction and reverse transcription followed by quantitative real-time polymerase chain reaction (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 polymerase chain reaction (RT-qPCR) using Qiagen one step kit. Seven target genes – COX-2, alpha smooth muscle actin (αSMA), Type I Collagen, CD3, inducible nitric oxide synthase (iNOS), CD86 and Found in inflammatory zone 1 (Fizz-1) – and one reference gene, Beta-actin (β-actin) as internal control, were analyzed by RT-qPCR. Quantitative PCR was carried out on a Light Cycler® 480, 96-well apparatus (Roche Molecular Systems, Switzerland), with 160 ng of cDNA as template. We used the amplification kit Light Cycler 480 SYBR Green I Master (Roche Molecular Systems, Switzerland), according to the manufacturer's instructions. The relative fold-changes of each target gene compared with the reference gene, was determined by the formula $2^{-\Delta\Delta Ct}$. The used primers are listed in Table 2.

Table 2 – List of murine primers used for quantitative real-time polymerase chain reaction (RT-qPCR) analysis for tissues and cells.

Gene	Primer sequence 5'-3' (F)	Primer sequence 5'-3' (R)
β-actin	ACCACCATGTACCCAGGCATT	CCACACAGAGTACTTGCGCTCA
Asma	CTACGAACTGCCTGACGGG	GCTGTTATAGGTGGTTTCGTGG
Type I collagen	TGTTCGTGGTTCTCAGGGTAG	TTGTCGTAGCAGGGTTCTTTC
COX-2	GCCTACTACAAGTGTTTCTTTTTGCA	CATTTTGTTTGATTGTTCACACCAT
CD3	CCCTGAGTCCCCTCTACACTT	TGCCCCAGAAAGTGTTCCAC
iNOS	GCCCAGCCAGGTACAGAG	CCTTGGTGCAGAAAACCCTTA
CD86	ACGGACTTGAACAACCAGACT	CGTCTCCACGGAAACAGCAT
Fizz-1	TATGAACAGATGGGCCTCCT	CCACTCTGGATCTCCCAAGA

αSMA: alpha smooth muscle actin; β -actin: Beta-actin; COX-2: cyclooxygenase-2; Fizz-1: Found in inflammatory zone 1; iNOS: inducible nitric oxide synthase

4.5 Isolation and stimulation of spleen and peritoneal cells

Spleen were extracted from mice and crushed in complete Roswell Park Memorial Institute (RPMI) medium. Erythrocytes were lysed in potassium acetate solution and spleen cells suspension was obtained after three times washes in complete medium. For each mouse, splenocytes were enumerated using a Malassez counting chamber. Isolation of peritoneal cells was performed as described before (Ray and Dittel, 2010). Briefly, peritoneal cells were retrieved by peritoneal lavage with 8mL of cold PBS containing 2mmol/L of ethylenediaminetetraacetic acid (EDTA). Cells were counted and viability – verified by trypan blue exclusion – was typically >98%. Peritoneal cells stimulation was performed by 24h of incubation at 37°C in complete RPMI medium with 10µg/mL of concanavalin A (Sigma Aldrich C5275). Then, supernatant was collected and stored at -80°C for further cytokine assessment.

4.6 Flow cytometry

Flow cytometry was performed using a fluorescence-activated cell sorting (FACS) Fortessa II flow cytometer (BD Biosciences, USA), according to standard techniques and data were analyzed with FlowJo software (TreeStar, Ashland,

USA). The following antibodies were used for cell-surface staining: Panel A for B cell phenotyping and regulatory B cells characterization: B200-Alexa Fluor 700, CD5-PercP-Cy5, CD19-APC, CD1d-PE, and CD40-FITC. Panel B for macrophage phenotyping and M1/M2 characterization: F4/80-BV711, CD11b-BV51, CD43-BV421, CD206-Alexa Fluor 647, Ly6C-PeCy7, CD62L-FITC and CD80-PE, purchased from BioLegend, Ozyme (Montigny-le-Bretonneux, France). TCD4^{+} and TCD8^{+} cells and their subsets were identified using the following antibodies: CD3-PE, CD4-BV421, CD8-PeCy7, CD44-APC, CD62L-FITC, CD69-PercP-Cy5.

M1 macrophages were defined as F4/80^{+}CD11b^{+}Ly6C^{High}CD206^{-}CD43^{+} CD62L^{-} and M2 macrophages as F4/80^{+}CD11b^{+}Ly6c^{Low}CD206^{+}CD43^{-}CD62L^{+} (Italiani and Boraschi, 2014). Regulatory B cells were defined as CD19^{+}CD5^{+}CD1d^{High} (Mauri and Menon, 2015). Figure 7 shows an example of the FACS gating strategy used.

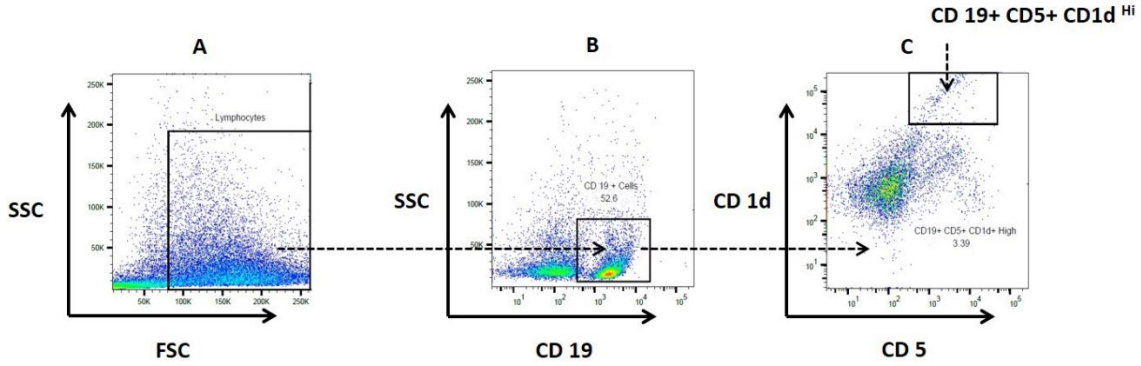


Figure 7 – Gating strategy for identification of mouse regulatory B cells (Breg). Breg were defined as CD19^{+}CD5^{+}CD1d^{High}

4.7 Cytokine assessment by Enzyme-Linked Immunosorbent Assay (ELISA)

Serum and supernatant from cultured peritoneal cells were diluted (1:4) in ELISA/ELISPOT diluent 1x before being distributed on ELISA 96-well plates specific of TNF-α, IL-1β, IL-4, IL-6, IL-10, IL-13 and IFN-γ (Mouse ELISA Ready-

SET-Go! eBioscience, Austria). Concentrations were calculated from a standard curve according to the manufacturer's protocol.

4.8 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 the Mann Whitney test. In the figures, the error bars represent the standard error of the mean. A p value <0.05 was accepted as significant.

5 RESULTS

5 RESULTS

5.1 Effects of B cell modulating treatment on endometriotic implants' size in mice

The analysis of the endometriotic implants in the peritoneal cavity of mice is shown in Figure 8. After 21 days of treatment, the Btk inhibitor Ibrutinib was effective in reducing the development of endometriosis in mice. Mice 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 (Figure 8a) and microscopic (Figures 8c and 8d) evaluations.

Implant volume (Figure 8e) from Ibrutinib Group was significantly reduced at Day 21 compared to Control Group (15.27 ± 2.67 *vs* 36.35 ± 5.16 mm^3, p=0.001) and to Anti-CD20 Group (15.27 ± 2.67 *vs* 43.12 ± 5.95 mm^3, p=0.0004). Also, implant weight (Figure 8f) was significantly decreased in Ibrutinib Group when compared to Control (67.87 ± 4.43 mg *vs* 100.1± 8.84 mg, p=0.006) and to a lesser extent to the Anti-CD20 Group (67.87 ± 4.43mg *vs* 90.35 ± 9.04 mg, p=0.13), but not significant.

Ultrasound imaging analyses of the implants' volume was also performed at D7 and at D20 after the procedure (Figure 8b), demonstrating a reduced volume in the Ibrutinib Group compared to Control and Anti-CD20 Groups (Figure 8g). The ratio between D20 and D7 measures was 0.45 ± 0.06 for Ibrutinib Group versus 3.72 ± 0.61 for Anti-CD20 Group (p<0.0001) and 2.25 ± 0.43 for Control Group (p=0.0002).

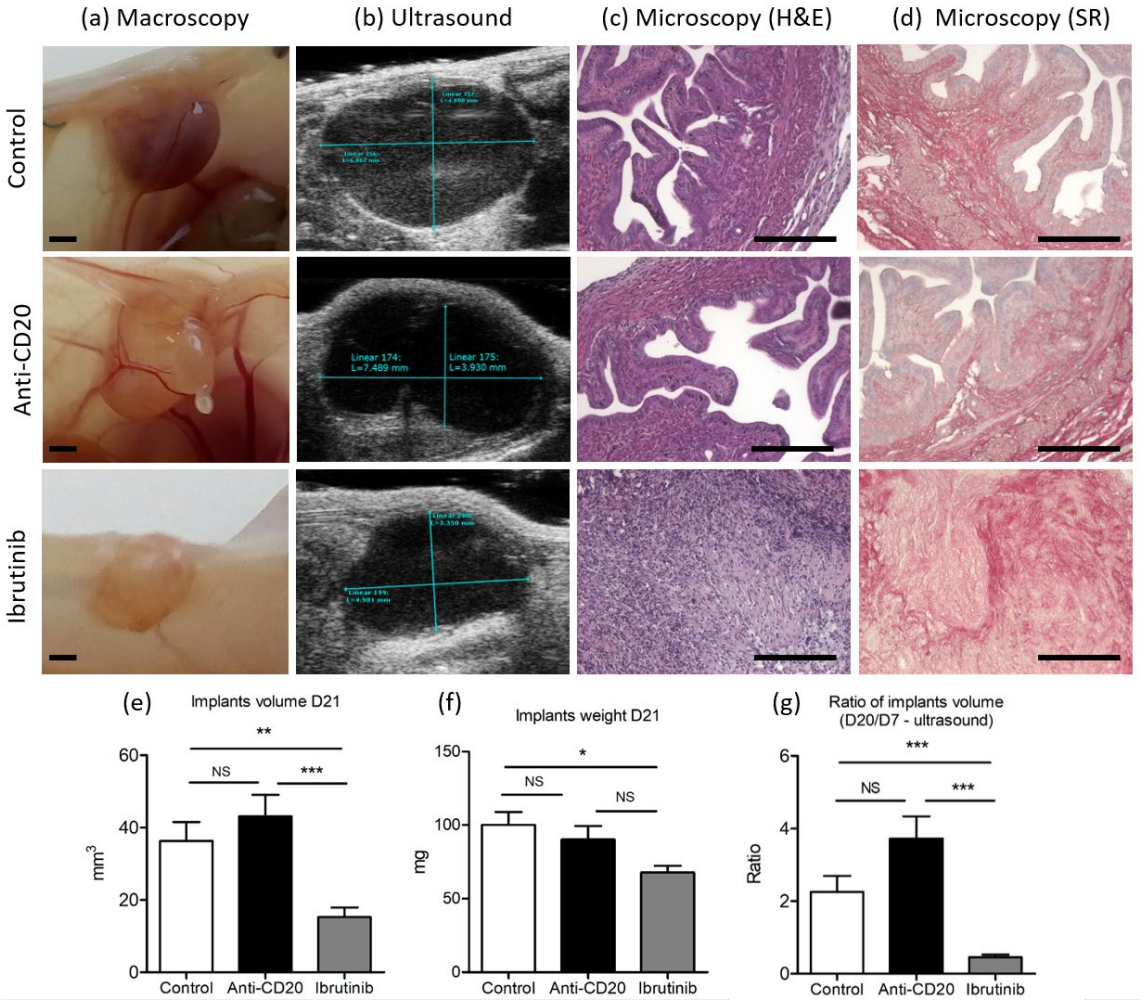


Figure 8 – Effects of B cell modulating treatment on endometriotic implants development in mice. **(a)** Macroscopic view of the implants. **(b)** Ultrasonography images of peritoneal implants in mice on Day 20. **(c)** Staining with Hematoxylin & Eosin of implants at Day 21. **(d)** Staining with Sirius Red 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 ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001. Scale bar: 100 µm

5.2 Flow cytometry analysis of the B cells populations

Anti-CD20 treatment depleted all B cells (defined as $B220^{+}CD19^{+}$) in the spleen and peritoneum compared to Control Group (0.19 *vs* 42 %; 0.17 *vs* 23 %, respectively, $p<0.001$, Figures 9a and 9d), while Ibrutinib treatment did not affect the percentage of splenic (Figure 9a) or peritoneal B cells (Figure 9d). Activation

of B cells was assessed by mean fluorescence intensity (MFI) of the co-stimulatory CD40 marker expression within the B cells population ($B220^{+}CD19^{+}$). Ibrutinib treatment decreased B cells activation in the spleen (MFI = 332 ± 40 *vs* 472 ± 47, p= 0.01, Figure 9b) and in the peritoneum (MFI = 267 ± 50 *vs* 617 ± 88, p=0.01, Figure 9e) compared to Control Group.

When gating on the $CD19^{+}CD5^{+}CD1d^{high}$ subset, known as regulatory B cells (Breg) (Rosser and Mauri, 2015), a total depletion of these cells with the Anti-CD20 was observed (Figures 9c and 9f). Interestingly, Ibrutinib treatment induced an important increase in the frequency of splenic Breg population compared to the Control Group (6.04 ± 0.73 % *vs* 0.63 ± 0.05 %, p<0.0001, Figure 9c). No significant difference was observed in the Breg population in the peritoneal cavity between Ibrutinib and Control Groups (Figure 9f). The gating strategy for identification of Breg frequency in Control Group and Ibrutinib Group is shown in Figures 9g and 9h, respectively.

5.3 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 10a) and a significant increase in the M2 subset (Figure 10b) compared to Control (M1 frequency: 25.70 ± 4.00 % *vs* 32.89 ± 3.86 %, p=0.0006; M2 frequency: 56.66 ± 7.66 % *vs* 32.27 ± 2.83 %, p<0.0001) and Anti-CD20 Groups (M1 frequency: 25.70 ± 4.00 % *vs* 34.80 ± 4.46 %, p=0.0002; M2 frequency: 56.66 ± 7.66 % *vs* 32.05 ± 3.82 %, p< 0.0001), resulting in a decreased M1/M2 ratio (p<0.0001, Figure 10c).

An opposite variation was observed in the peritoneal cavity, where Ibrutinib increased the frequency of M1 (Figure 10d) while reducing M2 (Figure 10e) compared to Control (M1 frequency: 39.39 ± 2.73 % *vs* 25.59 ± 2.70 %, p<0.0001; M2 frequency: 34.24 ± 3.08 % *vs* 42.26 ± 4.58 %, p=0.0006) and Anti-CD20 Groups (M1 frequency: 39.39 ± 2.73 % *vs* 31.15 ± 1.66 %, p<0.0001; M2 frequency: 34.74 ± 3.08 % *vs* 39.88 ± 2.44 %, p=0.0012), resulting in an increased M1/M2 ratio (p<0.0001, Figure 10f).

There was no significant difference in M1 or M2 frequency or M1/M2 ratio in the spleen (Figures 10a-c) between Anti-CD20 and Control Groups (M1 frequency: 34.80 ± 4.46 % *vs* 32.89 ± 3.86 %, p=0.3154; M2 frequency: 32.05 ± 3.82 % *vs* 32.27 ± 2.83 %, p=1.000; M1/M2 ratio p=0.07). However, in the peritoneal cavity, an increased M1 frequency (Figure 10d) was observed in Anti-CD20 Group compared to Controls (31.15 ± 1.66 % *vs* 25.59 ± 2.70 %, p=0.0004), leading to a significant difference in the M1/M2 ratio (Figure 10f) between the two Groups (p=0.0002).

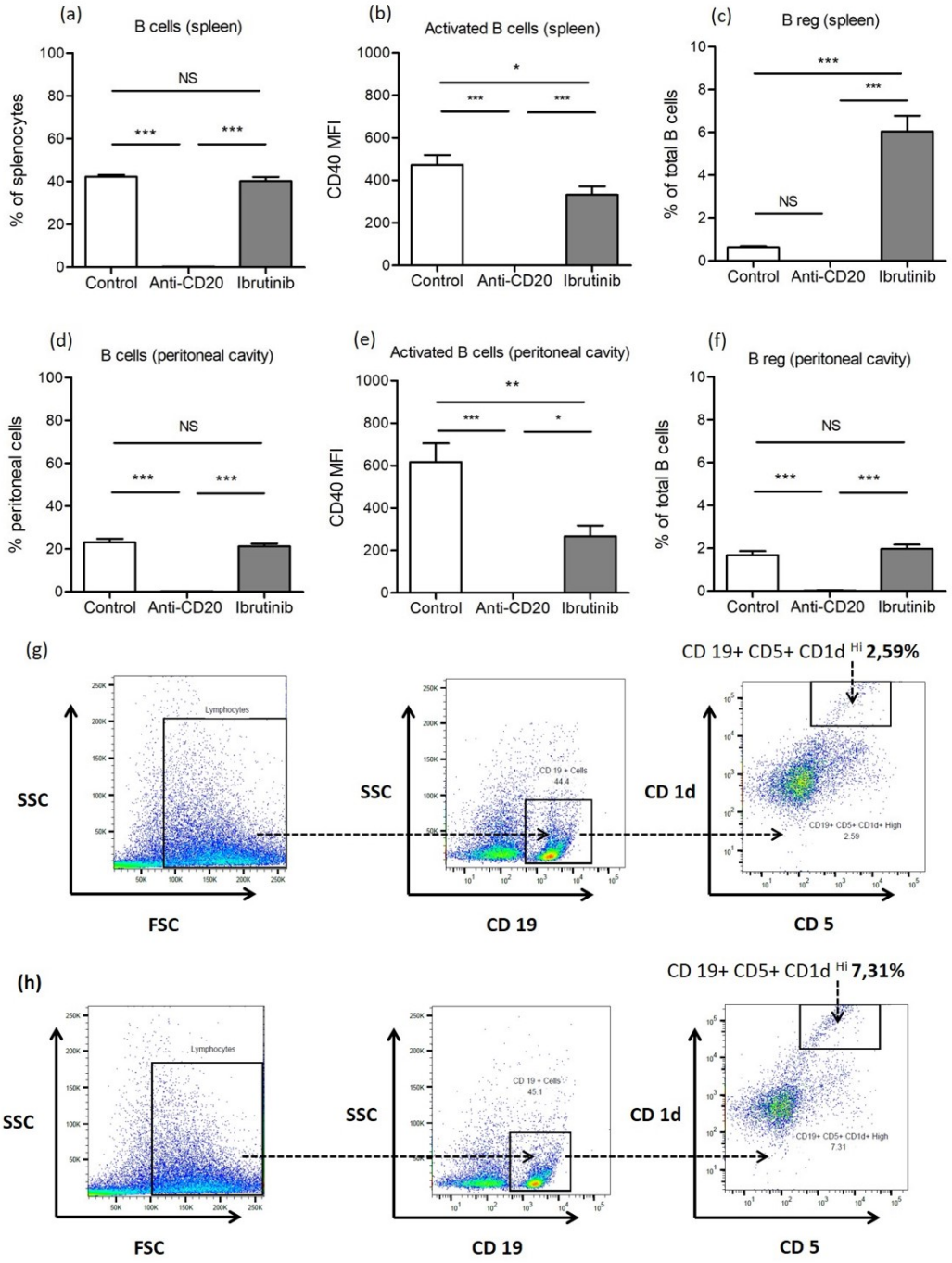


Figure 9 – B cell phenotype analysis in spleen and peritoneal cavity of endometriotic mice. Frequency of B cells ($B220^{+}CD19^{+}$) in spleen **(a)** and peritoneal cavity **(d)** of mice. Data represent mean ± SEM. Surface CD40 expression in B cells (activated B cells) in spleen **(b)** and peritoneal cavity **(e)**. Data represent the MFI of CD40 expression ± SEM. Frequency of Breg ($B220^{+}CD19^{+}CD5^{+}CD1d^{high}$) in spleen **(c)** and peritoneal cavity **(f)**. Data represent mean ± SEM. Gating strategy for identification of regulatory B 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; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$

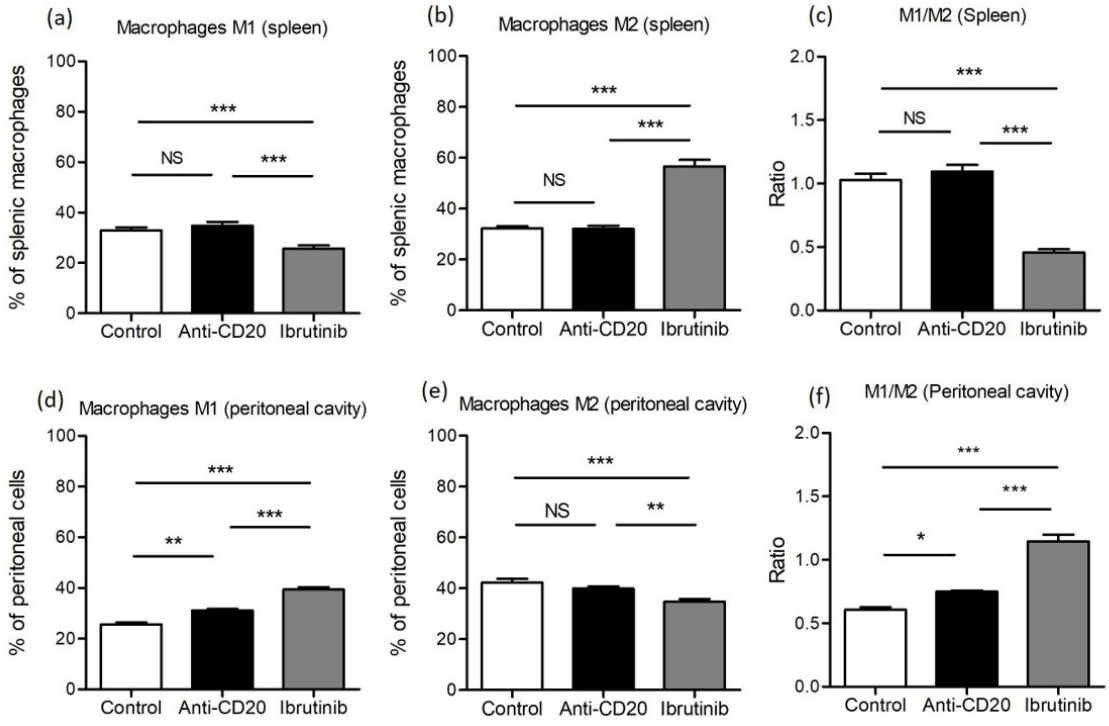


Figure 10 – Macrophage M1/M2 distribution in spleen and peritoneal cavity in endometriotic mice. Macrophages were gated on $CD11b^{+}F4/80^{+}$ cells isolated from spleen **(a;b)** and from the peritoneal cavity **(d;e)**. M1 macrophages **(a;d)** were further characterized as $CD43^{+}$ and $Ly6C^{high}$ and M2 macrophages **(b;e)** as $CD206^{+}$ and $Ly6C^{low}$. The ratio of M1/M2 population was calculated **(c;f)**. Data represent 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 ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001

5.4 Effects of Ibrutinib on 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 the Control Group ($p<0.0001$, Figure 11a). The effects of Ibrutinib treatment on fibrosis were assessed by αSMA (Figure 11b) and type I collagen (Figure 11c) mRNA expression in the implants, and both were significantly reduced in this group, compared to controls ($p=0.0002$ and $p=0.04$, respectively). There were no differences in these inflammatory and fibrotic markers between the Anti-CD20 Group and the controls.

Immune cells infiltration in the implants was evaluated through mRNA expression of each cell subtype marker. iNOS and CD86 expression (for M1 macrophages, $p<0.0001$, Figure 11d; and $p=0.0003$, Figure 11e, respectively) were increased in the Ibrutinib Group, while Fizz-1 expression (for M2 macrophages, $p=0.0042$, Figure 11f) was decreased in Ibrutinib Group, compared to Control Group. CD3 expression (for T lymphocytes, $p=0.6133$, Figure 11g) was not significantly different between Ibrutinib and Control groups.

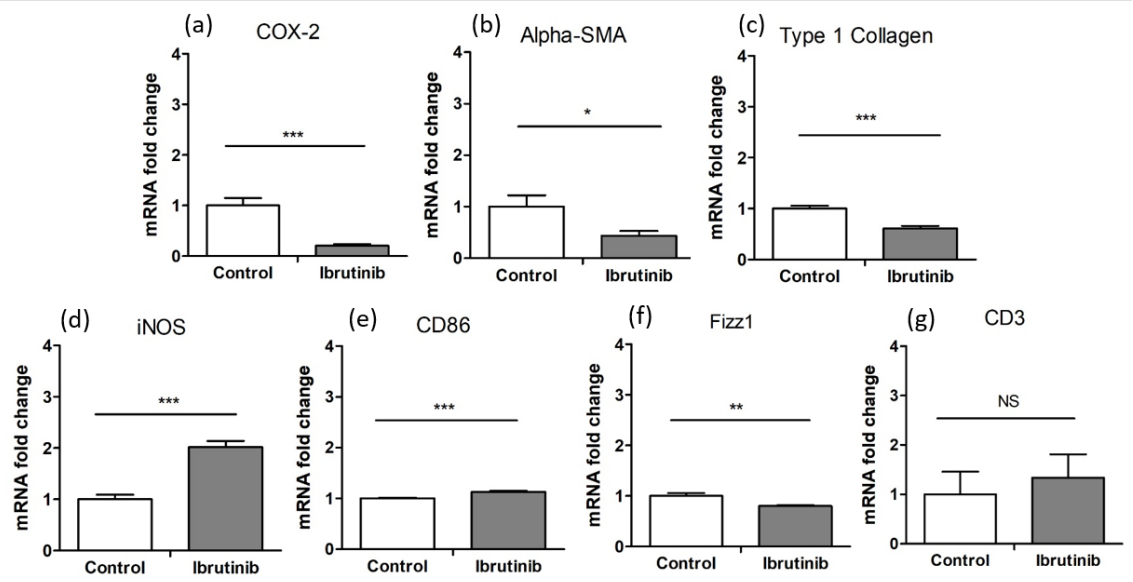


Figure 11 – Effects of Ibrutinib on quantitative expression of genes in endometriotic implants of mice. **(a)** COX-2; **(b)** αSMA; **(c)** Type 1 collagen; **(d)** iNOS (for M1 macrophages); **(e)** CD86 (for M1 macrophages); **(f)** Fizz-1 (for M2 macrophages) and **(g)** CD3 (for T lymphocytes) mRNA levels. Data are normalized to the reference gene (β-actin) and are expressed as ratio versus Control Group. Each group had n=10 mice. The Mann Whitney test was used to detect significant differences. NS, non-significant; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$

5.5 Effects of Ibrutinib on T lymphocytes

There were no significant differences in T lymphocytes subsets number or activation. Indeed, total number or proportion of naïve (defined as $CD62L^{high}$ $CD44^{low}$) or memory ($CD62L^{low}CD44^{high}$) $CD4^+$ and $CD8^+$ T cells were not significantly different in the peritoneum (Figure 12) or in the spleen of mice between Ibrutinib and Control Group (Figure 13).

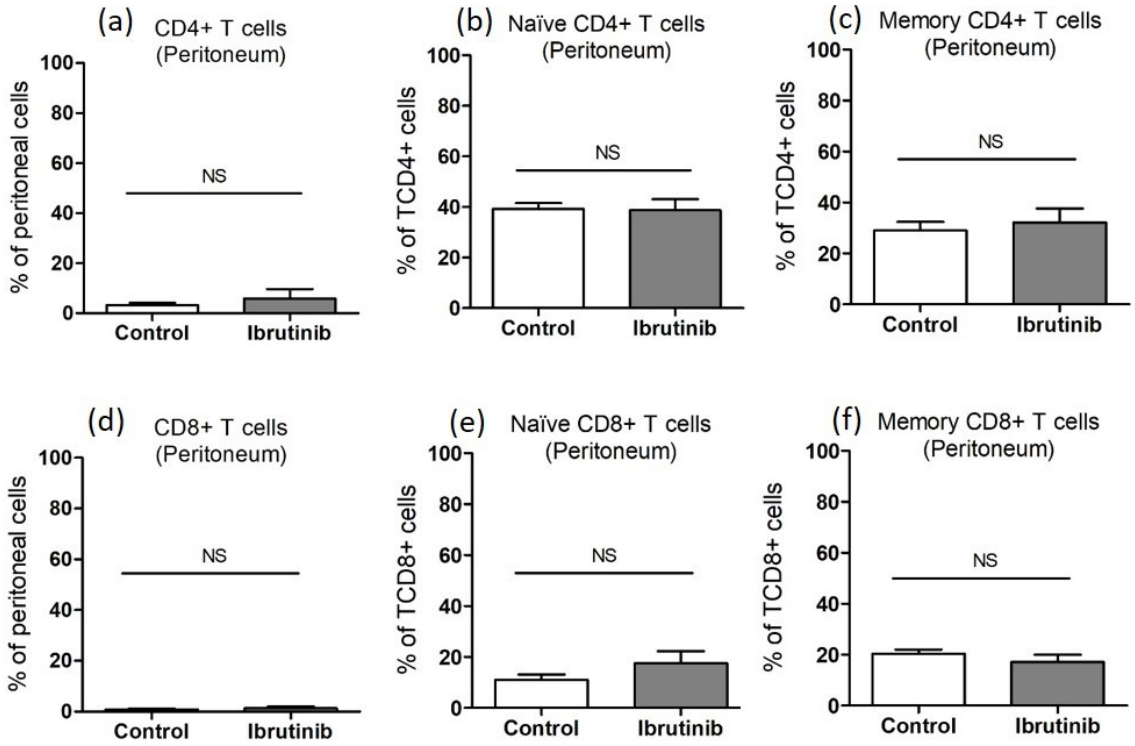


Figure 12 – Effects of Ibrutinib on peritoneal T lymphocytes. Frequency of peritoneal T lymphocytes subsets TCD4⁺ **(a)** and TCD8⁺ **(d)**: Naïve (CD62L^{high} CD44^{low}) **(b;e)** and memory (CD62L^{low} CD44^{high}) **(c;f)**. Data represent mean ± SEM. Each group had n=10 mice. The Mann Whitney test was used to detect significant differences. NS: Non-significant; *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001

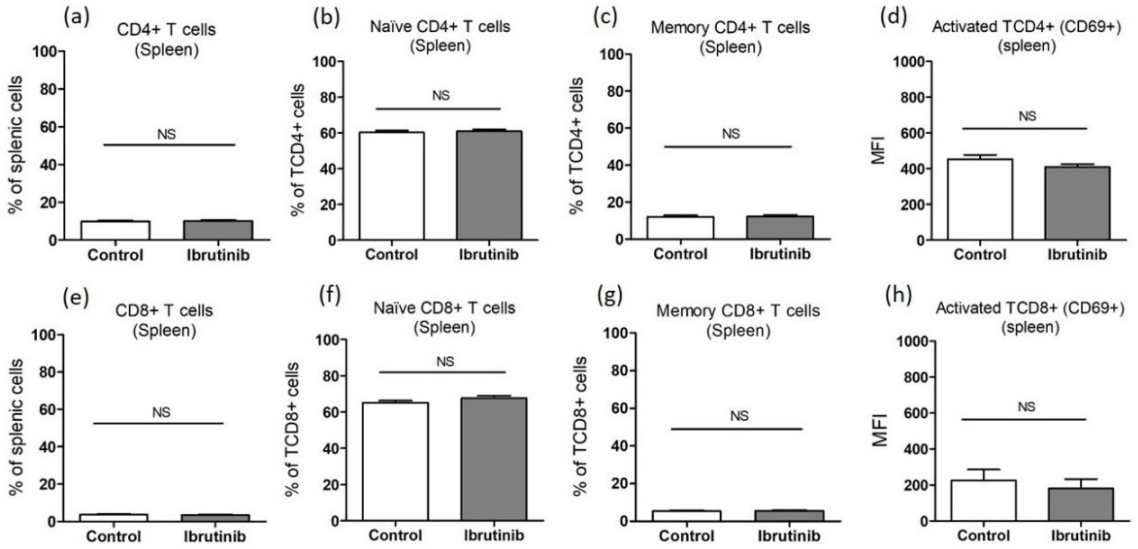


Figure 13 – Effects of Ibrutinib on splenic T lymphocytes. Frequency of splenic T lymphocytes subsets TCD4⁺ **(a)** and TCD8⁺ **(e)**: Naïve (CD62L^{high} CD44^{low}) **(b;f)** and memory (CD62L^{low} CD44^{high}) **(c;g)**. Data represent mean ± SEM. Activated CD4⁺ T cells (CD69 mean fluorescence intensity) among either total CD4⁺ **(d)** or CD8⁺ **(h)** T cells. Each group had n=10 mice. The Mann Whitney test was used to detect significant differences. NS: Non-significant; *p≤0.05; **p≤0.01; ***p≤0.001

5.6 Effects of Ibrutinib on cytokine balance

Cytokine concentration was measured in the serum (Figure 14) and peritoneal fluid (Figure 15) of endometriotic mice. In the serum, Ibrutinib treatment decreased TNF-α (188.2 ± 25.24 *vs* 245.3 ± 26.13 pg/mL, p=0.0014, Figure 14a) and IL-6 concentrations (36.87 ± 1.18 *vs* 66.98 ± 4.19 pg/mL, p<0.0001, Figure 14b) and increased IL-10 levels compared to Control Group (273.1 ± 24.23 *vs* 175.5 ± 9.48 pg/mL, p=0.0015, Figure 14c); no significant difference was observed for IL-13 levels (p=0.6784, Figure 14d).

In the peritoneal fluid, Ibrutinib treatment increased IFN-γ concentration (3231 ± 656.2 *vs* 1951 ± 1229 pg/mL, p=0.0133, Figure 15d) and decreased IL-13 (35.23 ± 29.84 *vs* 145.2 ± 73.29 pg/mL, p=0.0006, Figure 15e) and IL-4 concentrations (9.371 ± 19.79 *vs* 30.03 ± 13.10, p=0.0220, Figure 15f), when compared to controls. No differences in peritoneal concentrations of TNF-α (p=1.000, Figure 15a), IL-6 (p=0.4470, Figure 15b), IL-10 (p=0.4470, Figure 15c) or IL-1β (p=0.3070, Figure 15g) were observed with Ibrutinib treatment compared to controls.

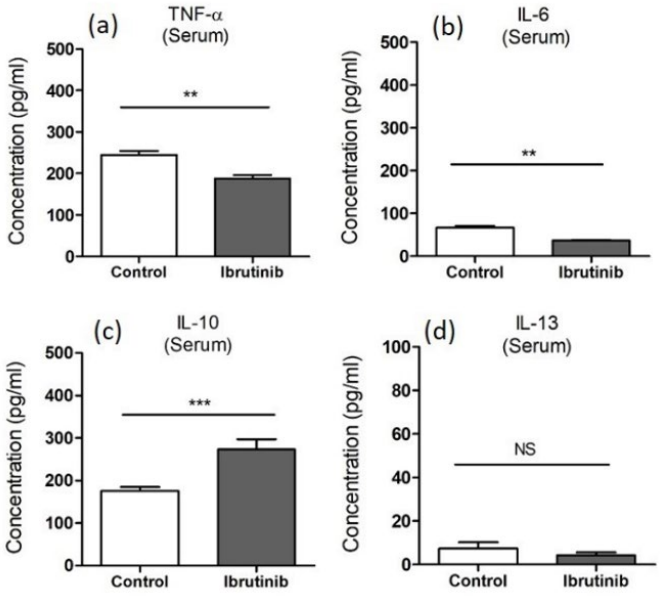


Figure 14 – Effects of Ibrutinib on systemic cytokines of endometriotic mice. **(a)** TNF-α; **(b)** IL-6; **(c)** IL-10 and **(d)** IL-13 concentrations in the sera of mice measured by ELISA. Data represent mean ± SEM. Each group had n=10 mice. The Mann–Whitney test was used to detect significant differences. NS, non-significant; *p ≤ 0.05; **p ≤ 0.01; *** p ≤ 0.001

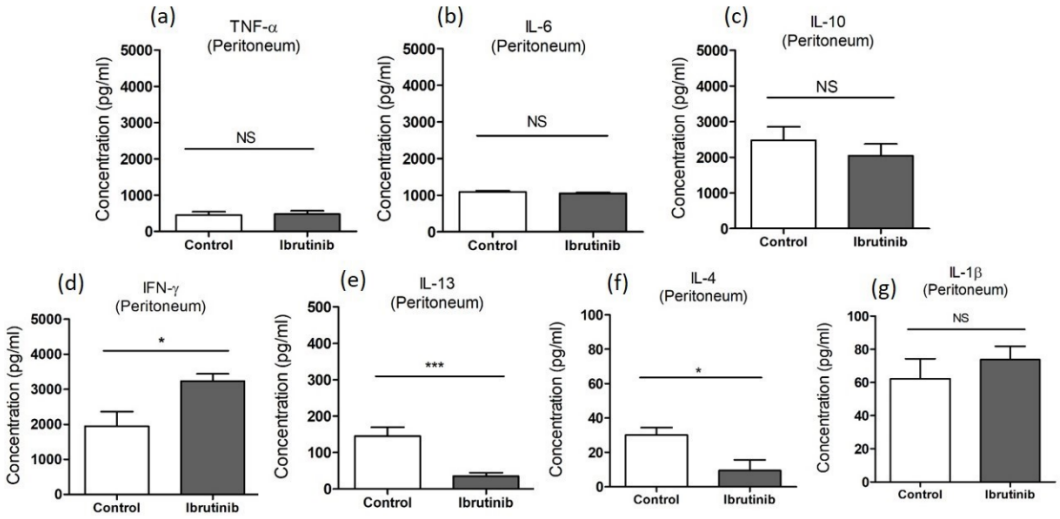
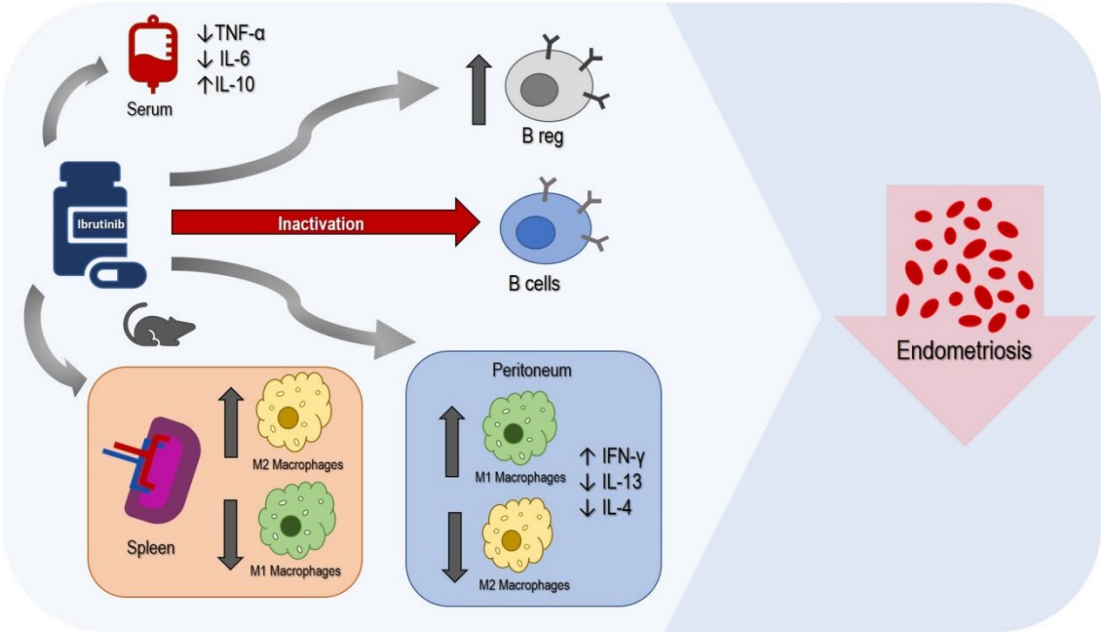


Figure 15 – Effects of Ibrutinib on peritoneal cytokines of endometriotic mice. **(a)** TNF-α; **(b)** IL-6; **(c)** IL-10; **(d)** IFN-γ; **(e)** IL-13; **(f)** IL-4 and **(g)** IL-1β concentrations in peritoneal fluid of mice measured by ELISA. Data represent mean ± SEM. Each group had n=10 mice. The Mann–Whitney test was used to detect significant differences. NS, non-significant; *p ≤ 0.05; **p ≤ 0.01; *** p ≤ 0.001

Figure 16 summarizes Ibrutinib effects that limited endometriosis development in mice.



SOURCE: Riccio et al., 2019

Figure 16 – Summary of Ibrutinib effects that limited endometriosis development in mice

6 DISCUSSION

6 DISCUSSION

Many studies have attempted to clarify the involvement of the immune system in endometriosis and various abnormalities have been detected, including increased B lymphocyte number and activation with excessive production of autoantibodies. Although there is evidence of an abnormal B-cell compartment in patients with endometriosis, its role in the development of the disease is not well understood. To evaluate the role of B cells on endometriosis a dual strategy was applied in the present study: a complete depletion of B cells using anti-CD20 treatment or an immunomodulatory strategy using a Btk inhibitor that blocks B cell activation.

It was observed that treatment with Ibrutinib in a mouse model of endometriosis 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 is an enzyme involved in the conversion of arachidonic acid to prostaglandins and has been associated with the inflammatory response and with lesion activity and growth in endometriosis (Cho et al., 2010; Santulli et al., 2016). Moreover, increased expression of type I collagen and αSMA, marking myofibroblast differentiation, has also been associated with severe forms of endometriosis (González-Foruria et al., 2015; Vigano et al., 2018).

However, complete elimination of B cells using 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 three 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 as well as autoimmune and systemic inflammatory diseases, such as rheumatoid arthritis and vasculitis (Edwards et al., 2004; Coiffier et al., 2009; Harrison, 2012; Guillevin et al., 2014).

Once B lymphocyte inactivation by Ibrutinib was effective in limiting endometriosis development in mice, but complete B cells depletion had no effects,

we have hypothesized that regulatory B cells (Breg) might play a role in controlling endometriosis. These immunosuppressor cells are increased in both number and suppressive ability in response to inflammation (Rosser and Mauri, 2015) and they can also be depleted by anti-CD20 (Lee-Chang et al., 2019).

6.1 Ibrutinib

Ibrutinib is an irreversible inhibitor of Btk, a non-receptor kinase essential for B cells development and function of mature B cells. Btk was initially shown to be defective in the primary immunodeficiency X-linked agammaglobulinemia (XLA) and shortly after its discovery, it 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 activity of Ibrutinib against Btk-restricted targets (Honigberg et al., 2010; Woyach et al., 2012).

Btk has a crucial function in oncogenic signaling that is critical for proliferation and survival of leukemic cells in many B cell malignancies. Inhibitors of Btk have shown anti-tumor activity, first in animal models and subsequently in the clinics, with durable remissions against a variety of B-cell malignancies, including mantle cell lymphoma, follicular lymphoma, and chronic lymphocytic leukemia (Harrison, 2012; Byrd et al., 2013; Jain et al., 2018).

In normal cells, Btk controls B cell signaling and has been shown to be important for B cell tolerance. Indeed, transgenic mice overexpressing Btk develop a systemic lupus erythematosus like syndrome, while inhibiting Btk by genetic or pharmacologic manipulation in mice and humans can prevent autoimmune proinflammatory disorders such as systemic lupus, rheumatoid arthritis, or type 1 diabetes (Honigberg et al., 2010; Chang et al., 2011; Hutcheson et al., 2012; Manda et al., 2015).

Btk-deficient patients and mice suffer from humoral immunodeficiency, as their B cells fail to progress beyond the bone marrow (Rawlings et al., 1993; Thomas et al., 1993). They have reduced follicular compartments with expanded transitional populations and a severe decrease in B-1 B cells, serum IgM and

IgG3 levels and defective responses to various B cell activators (Khan et al., 1995). However, Btk excision in fully developed, mature peripheral B cells, using a *Btk*^{flox}*/Cre-ER*^{T2} mouse model did not reduce follicular B cells nor B-1 cells, contrasting their near absence in global Btk-deficient mice. However, if B-1-related natural IgM levels remained normal in *Btk*^{flox}*/Cre-ER*^{T2} mouse, B cell proliferation and activation especially against T-independent immunization were still blunted (Nyhoff et al., 2018). These observations are in line with the normal frequency of B cells and the decrease in B cell activation upon treatment of endometriotic mice with the Btk inhibitor (Barnes et al., 2018; Tang et al., 2018; Paydas, 2019).

6.2 Regulatory B cells

Regulatory B cells (Breg) secrete IL-10, controlling effector immune responses and autoimmune diseases, such as encephalomyelitis (Fillatreau et al., 2002), chronic intestinal inflammatory condition (Mizoguchi et al., 2002), and arthritis (Mauri et al., 2003). It has been described that defective Breg function and development result in chronic inflammation (Rosser and Mauri, 2015).

The current main theory about the origin of Breg supports that the primary requisite for their cell differentiation is the environment in which a B cell finds itself (Rosser and Mauri, 2015). It is known that Breg cells increase in number during the inflammatory phase of several autoimmune disorders (Mizoguchi et al., 2002; Evans et al., 2007) and their differentiation is also induced by pro-inflammatory cytokines (Rosser and Mauri, 2015). Studies have shown that chronic inflammatory diseases are exacerbated in the absence of Breg (Fillatreau et al., 2002; Carter et al., 2011, 2012).

Despite splenic populations of B cells had been most frequently studied, Breg have also been found in the lymph node draining the site of inflammation (Mizoguchi et al., 2002). It has been demonstrated that Breg can develop and acquire their suppressive abilities outside the spleen, as splenectomy had no effect on their generation (Matsumoto et al., 2014). These findings support that

the induction of Breg differentiation is driven by an inflammatory environment (Rosser and Mauri, 2015).

In the present study, we have identified the presence of Breg in the spleen and peritoneal fluid of mice with endometriosis (in both Control and Ibrutinib groups), and they were completely depleted in Anti-CD20 group. We have also observed that Ibrutinib treatment led to an increased frequency of Breg in the spleen, when compared to controls, and this finding may contribute to the drug effects observed in endometriosis development.

The role of Btk in the development of Breg is unclear (Rosser and Mauri 2015). However, in mice lacking B-cell linker (BLNK), a Btk adaptor molecule also implicated in B cell signaling, the percentages of $CD1d^{high}CD5^{+}$ regulatory B cells were markedly increased (Jin et al., 2013), as observed in the present study in Ibrutinib-treated endometriotic mice. Additionally, the frequencies of marginal zone B cells and transitional stage 2 marginal zone precursor B cells, which have been reported to contain IL-10–producing Breg, were also increased in $BLNK^{-/-}$ mice.

Until the present, the role of Breg in endometriosis has not been studied. However, our findings and the previous information concerning other chronic inflammatory diseases may lead us to consider that these immunosuppressive cells may play a role in controlling endometriosis development. Further studies in this field could help better understanding the role of regulatory B cells in endometriosis.

6.3 Effects of Ibrutinib on macrophages

The effects 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 (Fiorcari et al., 2016; 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 (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.

MAC-1 can be expressed by both M1 and M2 macrophages. Regarding the origins of peritoneal macrophages, two macrophage subsets coexist in the peritoneal cavity (PerC) in adult mice. One, called the large peritoneal macrophage (LPM), contains approximately 90% of the PerC macrophages, originates from the yolk-sac and appears to be maintained by self-renewal and independent of hematopoiesis. The second population, called small peritoneal macrophage (SPM), derives from blood monocytes that rapidly enter the PerC after antigen stimulation and differentiate to mature SPM within two to four days. Both macrophage subsets express MAC-1 but to a lesser extend for SPM macrophages.

Therefore, if a systemic event may possibly impact macrophages differentiation, the polarization of macrophages may largely depend on the peritoneal microenvironment. Indeed, an increased M1/M2 ratio in the peritoneal cavity was shown in the present study, consistent with the increased levels of IFN-γ and the decrease in the IL-4 and IL-13 levels in the peritoneal cavity of Ibrutinib-treated animals compared to controls (Gabhann et al., 2014).

These results are also consistent with the increase in the M1/M2 ratio in endometriotic lesions from Ibrutinib-treated mice as reflected by the highest

CD86 and iNOS mRNA levels and the lowest Fizz mRNA levels in the endometriotic lesions from ibrutinib-treated animals compared to controls.

6.4 Effects of Ibrutinib on T lymphocytes

T lymphocytes play an important role in the development of endometriosis (Riccio et al., 2018). Ibrutinib impacts mainly B cell through interaction with Btk, but investigators (Cheng et al., 2014; Kokhaei et al., 2016; Long et al., 2017) have described Ibrutinib also as an Itk inhibitor, subverting Th2 immunity and potentializing Th1 based immune responses (Dubovsky et al., 2013).

The Itk inhibition by Ibrutinib lead to increased number and function of T lymphocytes in chronic lymphocytic leukemia patients (Long et al., 2017; Parry et al., 2019; Solman et al., 2020) However, affinity of Ibrutinib for Btk is 20 times higher than the one for Itk (Honigberg et al., 2010), and the double Btk-Itk inhibition in mice was achieved with a 25 mg/kg/day dose (Dubovsky et al., 2013), much higher than the one used in this study.

It is also known that Btk is present in T lymphocytes (Tomlinson et al., 2004), although its role in these cells is not completely clarified, probably due to its much lower expression, about 0.1–1% of that in B lymphocytes (Smith et al., 1994; Xia et al., 2020).

In the present study, Ibrutinib had no effect on peritoneal or splenic T lymphocytes number or activation through flow cytometry evaluation, and there was no difference in CD3 gene expression in the endometriotic implants from mice treated or not with Ibrutinib. Thus, the findings of effective control of endometriosis development by Ibrutinib seem to be due to its Btk inhibition pathway in B lymphocytes, to its effects on regulatory B cells and on M1/M2 macrophages distribution, 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 development.

6.5 Effects of Ibrutinib on cytokines

B cells overexpressing wild-type Btk were selectively hyper responsive to B cell receptor (BcR) stimulation and showed enhanced Ca^{2+} influx, NF-κB 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 TNF-α induced by Ibrutinib in the present study.

The role of Btk in IL-10 production is more complex as $Btk^{-/-}$ 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). Such systemic modulation of cytokines by Ibrutinib has already been observed in other models of inflammatory diseases, like rheumatoid arthritis (Chang et al., 2011).

In addition, increased Breg population in Ibrutinib-treated mice contributed to the higher IL-10 concentration in this group, as they secrete this immunomodulatory cytokine (Fillatreau et al., 2002). In endometriosis, decreased levels of IL-6 and increased IL-10 have been associated with an amelioration of the diseases (Schwager et al., 2011), as observed in this study.

The cytokine distribution observed with Ibrutinib treatment in the present study was characterized by a shift into a systemic Th2-like immunoregulatory profile, with decreased pro-inflammatory cytokines TNF-α and IL-6 and increased anti-inflammatory IL-10; and a local Th1-like inflammatory profile in the peritoneum, with increased IFN-γ and decreased IL-4 and IL-13.

6.6 Murine model of endometriosis

In the present study we have used an animal model of endometriosis: a syngeneic murine surgically induced model. It is a low-cost, easily reproducible

method, with rapid development of the disease. However, considering that the endometrium is sutured onto the peritoneum, it leads to the question if this model is representative of all three phenotypes of the disease or if it only represents peritoneal endometriosis.

However, after three weeks of the implantation, it was possible to identify pelvic organs adhesions (including bowel), large complex solid-cystic peritoneal lesions, and cysts with hemorrhagic fluid inside. These findings were consistently similar to human's deep infiltrating endometriosis lesions and ovarian endometriomas, so it can therefore be concluded that the model was appropriately representative of all three phenotypes of endometriosis.

The method we used to induce endometriosis in mice includes the administration of 56 μg/kg/day of 17β-estradiol in two steps of the protocol. First, it is necessary to give estradiol to donors to increase the volume of the uterus, so it becomes technically viable to perform hysterectomy, open the horns longitudinally and prepare the grafts. Then, all operated mice receive a post-operative oral gavage of 17β-estradiol for three days after implantation, to ensure endometrial grafting to the peritoneum and further development of endometriotic lesions, as they are estrogen-dependent.

The effects of estrogen in the immune system in humans (Bouman et al., 2005; Kovats, 2015; Khan and Ansar Ahmed, 2016) and mice (Verthelyi, 2001) have already been described. Women have a higher incidence of autoimmune diseases, increased cellular and humoral immune responses and are more resistant to certain infections, compared to men (Bouman et al., 2005). Estrogen can interfere on most immune cell phenotypes, through mechanisms dependent or independent of estrogen receptors (Khan and Ansar Ahmed, 2016).

However, all three groups (Ibrutinib, Anti-CD20 and Control) equally received 17β-estradiol as part of the protocol of surgically induced endometriosis in mice. In addition, the effects of three-day treatment with 17β-estradiol fade out and probably did not interfere on immune cells phenotype after endometriosis is stablished nor in its analysis 21 days later. So, we did not consider that the estrogen used in the endometriosis protocol had influence on the differences observed between the groups in this study.

6.7 Strengths and limitations

Infertility is one of the main issues of endometriosis, however most of non-surgical treatment options available are contraceptive, leaving women affected by the disease with the difficult choice between controlling the pain or 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 a drug approved by Food and Drug Administration (FDA) and by *Agência Nacional de Vigilância Sanitária* (ANVISA) that targets immune cells was shown to be effective in controlling endometriosis development in mice. This study has the limitations of using an animal model and perhaps not completely clarifying all the mechanisms and pathways of the drug efficacy observed.

There is still a long path between animal studies results and the use of Ibrutinib to treat endometriosis in humans. Further studies are necessary to confirm the results and the efficacy of the drug in humans. Even if we consider the hypothetical success of future research, there are still other issues: the current estimated monthly average wholesale price of Ibrutinib is USD 13 324 (Barnes et al., 2018), and endometriosis is a chronic disease that affects young women that may need treatment for several years. Therefore, it is not a currently viable option in terms of cost-effectiveness. In addition, Ibrutinib is a drug with several collateral effects including some rare but severe, such as bleeding and atrial fibrillation (Tang et al., 2018; Paydas, 2019), and this fact may lead to questioning if its use is worthy to treat a benign disease.

However, the efficacy of Ibrutinib in controlling endometriosis development in mice observed in the present study can open the door to further studies and to the development of new immunoregulatory therapeutic strategies for endometriosis.

6.8 Final considerations

Btk inhibitor Ibrutinib controlled endometriosis development in mice, while total B cell depletion using an anti-CD20 antibody had no effect on the course of the disease. In addition, Breg were depleted by anti-CD20 antibody and preserved by Ibrutinib, suggesting that regulatory B cells might help blocking the development of endometriotic lesions. Btk Inhibitor Ibrutinib was effective probably due to its effects on multiple immune cells: B lymphocytes, regulatory B cells, M1 and M2 macrophages. The use of Ibrutinib to skew activated B cells towards regulatory B cells and increase the M1/M2 ratio into the peritoneal cavity opens new perspectives in both understanding and treating endometriosis. Further studies are necessary to better understand these pathways and to stablish if this drug could by a therapeutic option to treat endometriosis in humans.

7 CONCLUSIONS

7 CONCLUSIONS

- B lymphocytes inactivation by Ibrutinib limited endometriosis development in mice and B lymphocytes total depletion with anti-CD20 antibody did not have impact in the disease's development. These findings lead us to hypothesize if regulatory B cells may play a role in controlling endometriosis, as they were depleted by Anti-CD20 and increased by Ibrutinib.
- B cell blockade impacted the proportion of M1 and M2 macrophage subsets: Ibrutinib treatment induced a decreased M1/M2 ratio in the spleen and increased M1/M2 ratio in the peritoneal fluid of mice. These changes in the distribution of macrophages subsets probably contributed to Ibrutinib's effects in limiting endometriosis development.
- Ibrutinib is a double Btk/Itk inhibitor, but there were no significant differences in T lymphocytes subsets number or activation. This may be due to the use of a dose under the required to achieve double inhibition.
- The cytokine distribution observed with Ibrutinib treatment was characterized by a shift into a systemic Th2-like immunoregulatory profile, with decreased pro-inflammatory cytokines TNF-α and IL-6 and increased anti-inflammatory IL-10; and a local Th1-like inflammatory profile in the peritoneum, with increased IFN-γ and decreased IL-4 and IL-13. These findings were consistent with the effects of Ibrutinib on B cells, Breg and M1/M2 macrophages.

8 REFERENCES

8 REFERENCES

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APPENDIXES

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Immunology of endometriosis

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A B S T R A C T

The pathophysiology of endometriosis is not completely understood, but an aberrant immune response in the peritoneal environment seems to be crucial for the proliferation of ectopic endometrial cells – as those cells escape apoptosis and peritoneal cavity immunosurveillance. The growth of endometrial implants leads to the recruitment of a large number and diversity of immune cells and intense inflammation with increased pro-inflammatory cytokines, growth factors, and angiogenesis. There is substantial evidence of aberrant function of almost all types of immune cells in women with endometriosis: decreased T cell reactivity and NK cytotoxicity, polyclonal activation of B cells and increased antibody production, increased number and activation of peritoneal macrophages, and changes in inflammatory mediators. New clinical treatments for endometriosis are an urgent need, especially nonhormonal drugs. The study of immunology may

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clarify its role in the pathogenesis of endometriosis and contribute to the development of new therapeutic strategies.

Endometriosis is a hormono-dependent inflammatory gynecological disease whose pathophysiology is not completely understood. A peritoneal environment that allows the proliferation of ectopic endometrial cells associated with an aberrant immune response seems to contribute to the development of the disease. Although several immunological abnormalities have already been reported, the role of the immune system in endometriosis is not well established [1].

Disturbances in immune homeostasis are associated with increase in implantation, proliferation, and angiogenesis of the ectopic endometrial tissue [2]. However, it is not clear whether the modifications of the immune response lead to the development of the disease or if they are consequences of the ectopic endometrial growth [3].

The study of immunological dysfunctions in the context of endometriosis may help in understanding its role in the pathogenesis of the disease and could contribute to the development of new therapeutic strategies in the future.

Immunosurveillance: importance of immunological disorders in the survival and proliferation of ectopic endometrial cells

One of the main theories of the pathogenesis of endometriosis is the retrograde menstruation – the dissemination of endometrial cells through the uterine tubes – first described by Sampson (1927) [4]. However, it is known that this phenomenon occurs in most women of reproductive age, but not all of them develop the disease. Once they reach the peritoneal cavity, the endometrial cells in healthy women do not implant and are eliminated by an "immunosurveillance" system through apoptosis [3].

It was proposed that, in women with endometriosis, changes in cell-mediated and humoral immunity may contribute to the development of the disease [5]. These changes probably prevent the clearance of the endometrial cells that reach the peritoneal cavity and allow their implantation and development [3].

The exact mechanisms of immunosurveillance evasion by ectopic endometrial cells remain unclear, and some hypotheses have been formulated to explain this phenomenon. The production of proteins by the implants – such as the soluble form of the ICAM (intercellular adhesion molecule)-1, the sICAM-1 – could interfere in their recognition by the leukocytes. It has been described that the expression of ICAM-1 mRNA and the secretion of sICAM-1 are increased in endometriotic stromal cells compared to those in stromal cells from eutopic endometrium. The circulating sICAM-1 binds to leukocyte function antigen-1 (LFA-1) and makes leukocytes less available to recognize the aberrant endometrial cells through their cell surface ICAM-1 [6].

Dysfunctional cells are eliminated by apoptosis in the normal endometrium as part of a tissue repair mechanism during each menstrual cycle. This normal mechanism of programmed cell death does not occur in ectopic endometrial cells that reach the peritoneal cavity. The overexpression of antiapoptotic factors and decreased expression of proapoptotic factors [7] may interfere in peritoneal homeostasis and contribute to the development of the disease.

The Fas-FasL and TNF-α apoptosis pathways seem to play a key role in the immunosurveillance of the peritoneal microenvironment [8]. It was shown that the peritoneal environment in endometriosis induces FasL expression in stromal cells, leading to a Fas-mediated apoptosis of activated immune cells that express Fas (T cells and NK cells), as a mechanism of immunosurveillance escape [9]. The regulation of apoptosis can be a target for the treatment of endometriosis. It has been shown that the use of GnRH analogs increases the expression of the proapoptotic protein Bax and decreases the expression of the antiapoptotic protein Bcl-2 [10].

It has been suggested that endometrial stromal cells are involved in cellular adhesion to the intraperitoneal surface, whereas glandular epithelial cells play a role in invasion and growth of the lesion [11]. Anomalous expressions of various matrix metalloproteinases seem to be responsible for an

increased proteolytic capacity in endometriosis [11]. Genetic polymorphisms of matrix metalloproteinase were associated with disease progression, as the combined polymorphisms of genes 12 and 13 seem to protect from deep infiltrating endometriosis [12].

The growth of the ectopic endometrial implants leads to the recruitment of immune cells and an intense inflammatory response, with increased proinflammatory cytokines, growth factors, and angiogenesis. In addition, a mechanism of mobilization of fibroblasts and proliferation of connective tissue is activated to attempt to heal the injury [3].

Endometriosis is a chronic inflammatory disease, and inflammation plays a key role through mitogen-activated protein kinase (MAPK) signaling pathways [13]. Increased cyclooxygenase-2 (COX-2), interleukins, and oxidative stress act through the MAPK pathways [13]. MAPK are dysregulated in endometriotic lesions, and it was shown that their inhibitors can control the disease progression both in vitro and in animal models [14]. However, the use of MAPK inhibitors in the treatment of endometriosis is still limited owing to their teratogenicity and specific adverse side effects [13].

The MAPK pathway can increase inflammation and endometriosis clinic repercussion by recruitment of immune cells and amplification of the inflammatory response [15], generation of an anti-apoptotic signal [16], increased growth factor expression leading to angiogenesis [17], playing a role in the development of pain and hypersensitivity to pain [18] or acting as intracellular and extracellular signal transducers in endometriotic cells [13].

Functional changes in the immunological components of the peritoneal fluid of women with endometriosis have been described, such as in monocytes/macrophages, natural killer (NK) cells, T lymphocytes, B cells, and cytokines [19]. However, the role of these alterations in the development of the disease has not been clarified [20].

Innate immunity: the role of macrophages and NK cells

Macrophages

In the peritoneal fluid, macrophages are the most prevalent type of immune cells [19], and their number and activation are increased in endometriosis [21], as well as their production of cytokines [22,23].

The activated macrophages can regulate the peritoneal environment by phagocytosis that removes red blood cells, damaged tissue fragments, and cellular debris [22] or by the production of soluble mediators like cytokines, prostaglandins, complement components, and enzymes. Through the secretion of these immune mediators, the macrophages can induce inflammation, tissue repair, and neovascularization and may favor the recruitment of fibroblasts and endothelial cells [21,24]. The macrophage-derived cytokines stimulate the activation of other immune cells such as T and B lymphocytes.

Despite the increased activation, the phagocytic activity of the macrophages is reduced in endometriosis [22], as they fail to eliminate the ectopic endometrial cells that reach the cavity through retrograde menstruation. The phagocytosis is regulated through expression of CD36 receptor and activation of matrix metalloproteinases, and both mechanisms are suppressed by prostaglandin E2, which are overexpressed in patients with endometriosis [25].

The scavenger function of the peritoneal macrophages depends on their attachment to extracellular matrix components. Increased nonadherent macrophages have been described in the peritoneal fluid of women with endometriosis, suggesting a defective scavenger function that could lead to the survival of ectopic endometrial cells [23].

In addition to the reduction of phagocytosis ability of macrophages, the amount of regurgitated endometrial cells in the peritoneal cavity may be higher than the capacity of the macrophages to remove them. This factor could contribute to the adhesion and proliferation of these cells and development of the disease [2].

Macrophages exhibit a phenotypic plasticity in their various microenvironments and are classified as two main groups, with different functions [26]: The M1 macrophages, which produce high quantities of inflammatory cytokines and are specialized in the elimination of microorganisms and defective cells, and the M2 macrophages, which modulate adaptive immune response, promote angiogenesis and tissue repair, and scavenge cellular debris.

An imbalance in M1 macrophages was shown in the eutopic endometrium of women with endometriosis [27]. However, M2 $CD163^{+}$/$CD206^{+}$ macrophages are significantly upregulated in the peritoneum and lesions of women [28] and rhesus macaques with the disease [29]. Experiments with macrophage depletion further demonstrated the key role of M2 macrophages in endometriotic grafting, development, and persistence [28,30]. In addition, selective adoptive transfer of M2 macrophages indicated that they promote endometriosis progression [28].

A recent study [31] evaluated the imbalance in macrophage subtypes in a murine model of endometriosis, considering the classification in large peritoneal macrophages (LPMs) and small peritoneal macrophages (SPMs). The authors have shown an increased proportion of SPMs and an opposite trend for the LPMs. They proposed that this new classification of macrophages should be included in further studies in endometriosis field.

To summarize, the macrophages play a key role in the development of endometriosis once they fail to eliminate the ectopic endometrial cells that reach the peritoneal cavity by retrograde menstruation. In addition, production of inflammatory mediators by macrophages contributes to the implantation and proliferation of endometrial cells, resulting in the development of endometriotic lesions [32].

NK cells

NK cells are lymphocytes of the innate immune system that can kill an array of target cells and secrete cytokines that participate in the shaping of the adaptive immune response and tissue repair. A feature of NK cells resides in their capacity to distinguish stressed cells that have undergone some degree of injuries from normal cells.

The NK cell detection system includes a variety of cell surface activating (KAR) and inhibitory (KIR) receptors, the engagement of which regulates NK cell activities. Among the cell surface activating receptors, two main receptors can be distinguished: NKG2D and CD16 (FcγRIIIa). The latter has the ability to bind and destroy immunoglobulin G (IgG)-coated stressed cells by a mechanism called antibody-dependent cell-mediated cytotoxicity. In addition, the cytotoxic activity of the NK cells can be increased by cytokines such as interleukin-2 (IL-2) [3].

Ectopic endometrial cells that reach the peritoneal cavity achieve to escape the clearance and are not targeted or removed by NK cells in a not completely understood mechanism called “immunoescaping” [8].

A decreased NK cell cytotoxic activity against endometrial cells in women with endometriosis was first described by Oosterlynck et al. (1991) [33] and has been well established since then [34], and it is correlated to the advanced stages of the disease [35]. It is more evident for NK cells from the peritoneal cavity [35,36], of women with endometriosis but is also observed for NK cells in the peripheral blood [35].

Despite the decreased NK cell function in endometriosis, the mechanisms of this suppression are not clear. There is also no consensus regarding the percentage or number of NK cells in endometriosis both in the blood and in the peritoneal cavity [33,37]. Qualitatively, an increased expression of KIR on peritoneal NK cells from women with endometriosis was reported, which could explain the decreased peritoneal NK cell activity in these patients [38].

A decreased cytotoxic function of NK cells could explain the immunoescaping mechanism of endometrial cells, leading to their adhesion and proliferation and resulting in endometriotic lesions. However, it is also possible that this aberrant NK cell function is a consequence of the chronic inflammatory environment provided by the disease [39,40].

González-Foruria et al. (2015) [41] evaluated ligands for NKG2D (a NK cell receptor that triggers a cytotoxic response that activates NK cells) in the peritoneal fluid of women with endometriosis. The authors demonstrated a significant increase in soluble NKG2D ligands, which means a lower expression of these ligands in ectopic endometrial cell surface, and as these soluble NKG2D ligands act as decoy receptors heading toward greater evasion from NK cell recognition.

Macrophage-derived factors such as prostaglandins and cytokines produced in this environment may also modulate NK activity. This hypothesis is corroborated by studies showing the suppression of NK cytotoxic activities by serum and peritoneal fluids of women with endometriosis compared to fluids from control patients [35].

It has been shown that NK cells are important for the interface between innate and adaptive immune response and that they have different subtypes. The NK T cells represent 15–20% of these cells

and express T-cell receptor (TCR)—CD3 membrane complex, in addition to classical CD 16 expression. They can both kill target cells and secrete cytokines such as IL-4 and IL-10 — which are important in the control of autoimmunity [42].

NK cells contribute to the balance of immune self-tolerance by targeting cells that present self-antigens. Therefore, their reduced activity in endometriosis could explain the increased autoimmune reactivity observed in the disease [2].

Adaptive immunity: T and B lymphocytes

Cell-mediated immunity: T lymphocytes

The B and T lymphocytes are essential subsets for adaptive immunity, which play an essential role in the survival and proliferation of endometrial cells. Indeed, endometriosis is characterized by the reduced activity of cytotoxic T cells, the modulation of cytokine secretion by T helper cells, and autoantibody production by B lymphocytes [22,43].

T lymphocytes are derived from stem cells in the bone marrow and in the fetal liver, and they migrate to the thymus to complete their development. They are classified as several subtypes. The two main groups are those that express the glycoproteins CD4 and CD8, which function as co-receptors for MHC class II and class I molecules, respectively [3,44].

The CD4 T cells can be classified in Th1 and Th2, with different functions: Th1 cells promote the differentiation of the CD8 cells and facilitate cell-mediated immunity by activating monocytes and macrophages; Th2 cells lead to the differentiation of B cells into plasma cells that secrete antibodies. The CD8 T cells can activate macrophages and kill cells that are infected by virus or intracellular pathogens [3,44]. The two groups of lymphocytes secrete different cytokines: Th1: IL-2, IL-12, interferon (IFN)-γ, tumor necrosis factor (TNF)-α and TNF-β; Th2: IL-4, IL-5, IL-6, IL-10, and IL-13 [20].

Studies that have evaluated T lymphocytes in patients with endometriosis showed higher CD4/CD8 ratio and increased concentration of each subset in the peritoneal fluid of the patients, but with a relative reduction in Th1 cells [36]. The endometriotic lesions showed higher concentration of T lymphocytes when compared to that in the eutopic endometrium, but with a similar CD4/CD8 ratio. There were no changes in the peripheral blood [3,44]. Endometriotic lesions also showed higher Th17 lymphocyte fraction when compared to eutopic endometrium [45].

The mechanism of implantation of the ectopic endometrial cells in the peritoneal cavity depends on altered macrophages. These cells also produce inflammatory cytokines that recruit and activate Th1 and Th2 T cells [36].

Another important subset of the T lymphocytes is the regulatory T cells (Treg). They are potent suppressors of inflammatory immune responses and are responsible for maintaining antigen-specific T-cell tolerance and immune homeostasis. A recent systematic review [46] evaluated the role of Treg in endometriosis. The authors concluded that in the peritoneal fluid and in the endometriotic lesions of women with endometriosis, there is a higher concentration of Treg cells and/or their expression markers, when compared with those in controls. However, there is no consensus about the concentration of Treg cells in the eutopic endometrium and peripheral blood of these patients.

Humoral immunity: B lymphocytes and antibodies

Even though immunosurveillance seems to have a defect in endometriosis, some aspects of the immune system are upregulated, such as the widespread polyclonal activation of B cells [47]. B lymphocytes produce antibodies against antigens, and they seem to contribute to the pathogenesis of endometriosis through secretion of autoantibodies [48].

Startseva (1980) [44] first described an increased reactivity of B cells in endometriosis. The same year, another study demonstrated IgG and complement deposits in the endometrium and decreased serum complement, suggesting an autoimmune response with complement consumption by the antigen-antibody complex in patients with endometriosis [49].

A few years later, the presence of antiendometrial antibodies in the serum of women with endometriosis was described [50]. Immunohistochemistry demonstrated that these antibodies could bind

to topic and ectopic endometrial tissues [51]. Bohler et al. (2007) [52] evaluated the presence and reactivity of IgG in the serum of women with endometriosis against antigens (derived from the membrane, nucleus, and cytosol) from endometrial and ovarian cells. There was a significantly higher level of autoantibodies in patients with endometriosis, when compared to that in controls, and the intensity of the reaction increased with disease progression.

Chishima et al. (2000) [53] proposed possible common alterations between endometriosis and autoimmune diseases: increased B-1 B cells in the peritoneal fluid and B cell production of ANA in the serum of women with endometriosis. However, despite the similarities, it is not possible to consider endometriosis as an autoimmune disease yet. Even if there is a genetic component in endometriosis, a specific association with HLA alleles has not been demonstrated so far [54], nor the specific activation of complement in the endometrium of women with endometriosis [55].

Moreover, polyclonal B cell activation, B-1 cell proliferation, and autoantibodies production may be associated with infertility in these patients [53]. An increased number of B lymphocytes was also observed in the follicular fluid of infertile patients with endometriosis, and it was suggested that this could contribute to endometriosis-related infertility [56].

Some authors have described increased B cells in patients with endometriosis with an inversed correlation with the severity of the disease, suggesting that mild endometriosis (Stages I and II) may be immunologically more active than severe endometriosis (Stages III and IV) [57].

High levels of cytokines that activate B cells were observed in endometriotic lesions, such as BLyS (B lymphocyte stimulator) [58]. This molecule is produced by macrophages and stimulates the development and differentiation of B lymphocytes into plasma cells [59]. Increased BLyS was also described in patients with autoimmune diseases and could be a target for therapeutic strategies for diseases with B cell defects [60]. Interestingly, heterozygosity for the BLyS 817C/T polymorphism was associated with reduced risk of deep infiltrating endometriosis [61].

A recent review [62] evaluated 22 studies concerning the role of B lymphocytes in endometriosis, and almost all of them reported increased number and activation and/or production of antibodies by B cells. It seems that B cells play a role in the pathogenesis of endometriosis; however, further studies are necessary to better understand this association.

Inflammatory mediators: cytokines, chemokines, and growth factors

Increased soluble factors such as autoantibodies, cytokines, growth factors, adhesion molecules, enzymes, hormones, prostaglandins, and reactive oxygen species [16,21,63–66], have been described in the blood, peritoneal fluid, and lesions of patients with endometriosis. This fact is probably a consequence of the high number of leukocytes, macrophages, and other immune cells in the peritoneal cavity of these patients.

These proteins work as mediators of the immune system [67], regulating the proliferation and the differentiation of immune cells, the release of enzymes and acute phase proteins, immunoglobulin secretion, and the cytotoxic activities of immune cells [20].

Studies have shown that the higher concentration of inflammatory mediators in the peritoneal fluid in endometriosis has toxic effects on oocyte pick up by the fimbria, sperm–oocyte interaction, and embryo implantation, leading to an aberrant reproductive function in these women. These effects were reversed during hormonal treatment [3].

Many cytokines – IL-1 [68,69], IL-4 [70], IL-6 [71], IL-8 [72,73], IL-10 [36], IL-33 [74], and TNFα [75] – and growth factors – transforming growth factor (TGF-β) [71], insulin-like growth factor (IGF-1) [76,77], hepatocyte growth factor (HGF) [78], epidermal growth factor (EGF) [79], platelet-derived growth factor (PDGF) [80,81], and vascular endothelial growth factor (VEGF) [24,82]– are significantly increased in endometriosis [83]. In addition, studies have shown that there are changes in the Th1/Th2 balance toward Th2 in endometriosis [22,43].

In endometriotic lesions, VEGF induces angiogenesis and its immunostaining was observed in the epithelium of endometriotic implants [84], particularly in hemorrhagic red implants [85]. VEGF is also increased in the peritoneal fluid of women with endometriosis [47]. However, is not yet clarified whether it is produced by endometriotic lesions [84,86], or by activated peritoneal macrophages [24].

Interleukin (IL)-6 is one of the main cytokines in the inflammatory cascade in endometriosis. It is elevated in the peritoneal cavity and blood of these patients, and it is correlated with disease activity [64,87]. IL-10 is also a potent modulator of inflammatory responses and immune cell function – as B cells and macrophages – so it is likely that both IL-6 and IL-10 are partially responsible for the aberrant immune regulation observed in endometriosis [20].

IL-6 can inhibit the proliferation of endometrial stromal cells [88], but it has been shown that in endometriotic lesions, these cells are resistant to IL-6, showing no inhibitory response [89,90]. This cytokine induces T cell activation and differentiation of B lymphocytes into antibody-producing plasma cells, and it can lead to polyclonal B cell stimulation in autoimmune diseases [3]. IL-1 is another cytokine that affects B cells and production of antibodies in addition to increasing prostaglandins, collagen, and tissue repair [5,19].

IL-1 and TNF-α usually initiate the cascade of cytokines and inflammatory response. TNF-α is increased in the peritoneal fluid of women with endometriosis, and it has higher concentrations in the later stages of the disease [91]. It has been suggested that it may contribute to the adhesion of endometrial cells to the peritoneal cavity [92].

IL-8 is also increased in endometriosis [72,73]. IL-8 contributes to cell adhesion [65] and is a potent angiogenic factor [3]. IL-8 stimulates the growth of topic and ectopic endometrial cells [73], probably through TNF-α activation [93]. It is produced by the mesothelium as a response to proinflammatory cytokine stimuli. IL-8 levels can be correlated to the severity of the disease [72].

Concerning the IL-10 family, it was demonstrated that IL-19 and IL-22 are both significantly decreased in the sera of women with ovarian endometrioma without deep infiltrating endometriosis [94]. In addition, there was a reverse correlation between levels of these cytokines and the occurrence of deep dyspareunia in those patients. The authors concluded that these anti-inflammatory cytokines exert immunosuppressive effects favorable to the development of ovarian endometrioma.

IL-13 is another anti-inflammatory cytokine that was shown to be decreased in endometriosis. It is a potent regulator of macrophage activation; hence, its reduction in the peritoneal fluid of women with endometriosis could contribute to the pathogenesis of the disease [95].

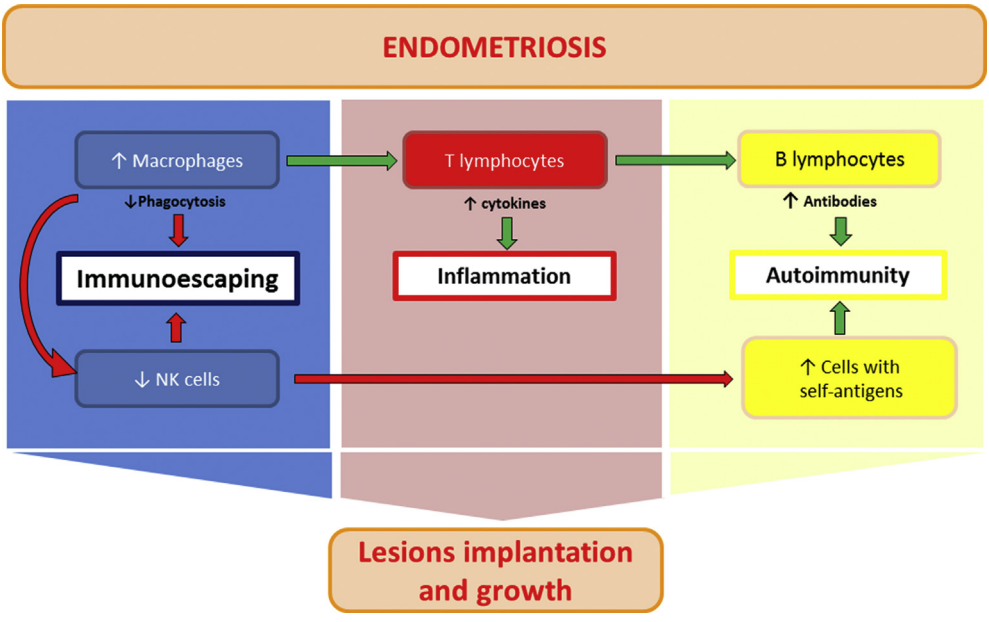

Fig. 1. Immune response in endometriosis.

The cytokine production in the immune system works in a cascade mode, the biosynthesis of one type of cytokine activates the production of a whole group of inflammatory mediators. In addition, each cytokine has different target tissues and biologic effects, which makes more difficult to clarify the role of a specific mediator in the development of endometriosis. It has also been shown that they can be produced by endometriotic cells, mesothelium, and other resident cells in the peritoneal cavity [87,96]. Cytokines are also deregulated in the peripheral blood of women with endometriosis, suggesting a systemic effect of the disease [3,94,97].

Summary

Even if the pathophysiology of endometriosis is not completely understood, it is well established that the immune system plays a key role in this disease. There is substantial evidence of aberrant function of almost all types of immune cells in women with endometriosis with decreased T cell reactivity and NK cytotoxicity, polyclonal B cells activation and increased antibody production, increased number and activation of peritoneal macrophages, and changes in inflammatory mediators (Fig. 1). In addition, some alterations are similar to those observed in autoimmune diseases [23].

New clinical treatments for endometriosis are an urgent need, especially nonhormonal drugs. Most of the current therapies are contraceptive, and women with endometriosis may have to choose between managing the pain and trying to conceive [62].

The ability of ectopic endometrial cells to escape apoptosis and cell-mediated destruction to later achieve peritoneal adhesion and invasion may be a target to new nonhormonal therapies for endometriosis. However, these mechanisms should be more deeply understood to make possible the design therapeutic strategies [3].

Practice points

- The physiopathology of endometriosis is not completely understood. There seems to be a peritoneal environment that allows the proliferation of ectopic endometrial cells associated with an aberrant immune response.
- There is substantial evidence of aberrant function of almost all types of immune cells in women with endometriosis: decreased T cell reactivity and NK cytotoxicity; polyclonal activation of B cells and increased antibody production; increased number and activation of peritoneal macrophages; and changes in inflammatory mediators.
- New clinical treatments for endometriosis are an urgent need, especially nonhormonal drugs. The study of immunology may clarify its role in the pathogenesis of endometriosis and contribute to the development of new therapeutic strategies.

Research agenda

- Further studies of the immunology of endometriosis could help clarifying the pathophysiology of the disease and lead to the development of new treatments.
- The ability of ectopic endometrial cells to escape apoptosis and cell-mediated destruction to later achieve peritoneal adhesion and invasion may be a target to new nonhormonal therapies for endometriosis. However, these mechanisms should be more deeply understood to make possible the design of therapeutic strategies.

Conflicts of interest

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or nonfinancial interest in the subject matter or materials discussed in this manuscript.

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Review article

The role of the B lymphocytes in endometriosis: A systematic review

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ABSTRACT

The physiopathology of endometriosis is not completely understood and its progression is associated with a local and systemic inflammatory reaction. It is important to clarify the potential role of the immune system to better understand its implication in the pathogenesis of endometriosis, which includes the study of the role of B cells and antibodies. The aim of this study was to review the literature about the role of B lymphocytes in endometriosis. A search for "*endometriosis*", "*B cells*" and "*B lymphocytes*" in databases resulted in 140 citations; after applying inclusion and exclusion criteria, a total of 22 studies were assessed. The analyzed samples in the studies varied and different markers and techniques were used by the authors to evaluate the direct or indirect role of B lymphocytes in endometriosis. Most studies demonstrated increased number and/or activation of B cells while seven studies found no difference and two studies showed decreased number of B cells. Increased B lymphocytes and excessive production of autoantibodies in endometriosis have been described in the literature, but their role in the development of the disease is not well understood. Moreover, the association of these factors with clinical symptoms, location and severity of the disease has not been investigated. Further studies are necessary to clarify the role of B cells in the development of endometriosis and propose new therapeutic strategies such as the use of drugs that target these cells.

1. Introduction

Endometriosis is a benign gynecologic disease characterized by the growth of endometrial glands and stroma outside the uterine cavity (Giudice and Kao, 2004; Tosti et al., 2015). It affects 5–15% of women in reproductive age (Missmer and Cramer, 2003).

The physiopathology of endometriosis is not completely understood. There are many theories about its pathogenesis, including: a) retrograde menstruation (Sampson, 1927); b) blood and lymphatic dissemination of endometrial cells (Abrão et al., 2006); c) celomic metaplasia (Bulun, 2009); d) endometrium-derived stem cells migrating to ectopic sites (Hufnagel et al., 2015); e) epigenetic changes leading to a pro-inflammatory microenvironment (Laganà et al., 2017); f) environmental toxicants acting as endocrine disrupters on the female reproductive system (Sofo et al., 2015). However, none of these theories can explain the disease in all its aspects.

Many important factors may contribute to the development of endometriosis, such as a welcoming environment for the proliferation of endometrial cells associated with an aberrant immune response. Many authors have attempted to clarify the role of the immune system in endometriosis and several abnormalities have been reported (Christodoulakos et al., 2007).

Disturbances in immune homeostasis can allow implantation, proliferation and angiogenesis of the ectopic endometrial tissue (Matarese et al., 2003). Despite the fact that there is probably a defect in the immunosurveillance, some aspects of the immune response are increased in endometriosis. A widespread polyclonal activation of B cells has been identified (Lebovic et al., 2001), as well as the presence of anti-endometrial autoantibodies (Fernandez-Shaw et al., 1993; Wild and Shivers, 1985). The aim of this study was to review the literature on the role of B lymphocytes in endometriosis to better understand their impact in pathogenesis of the disease, which could lead to the future development of new therapeutic strategies.

2. Material and methods

This review was conducted according to PRISMA statement (Liberati et al., 2009) at the Department of Obstetrics and Gynecology,

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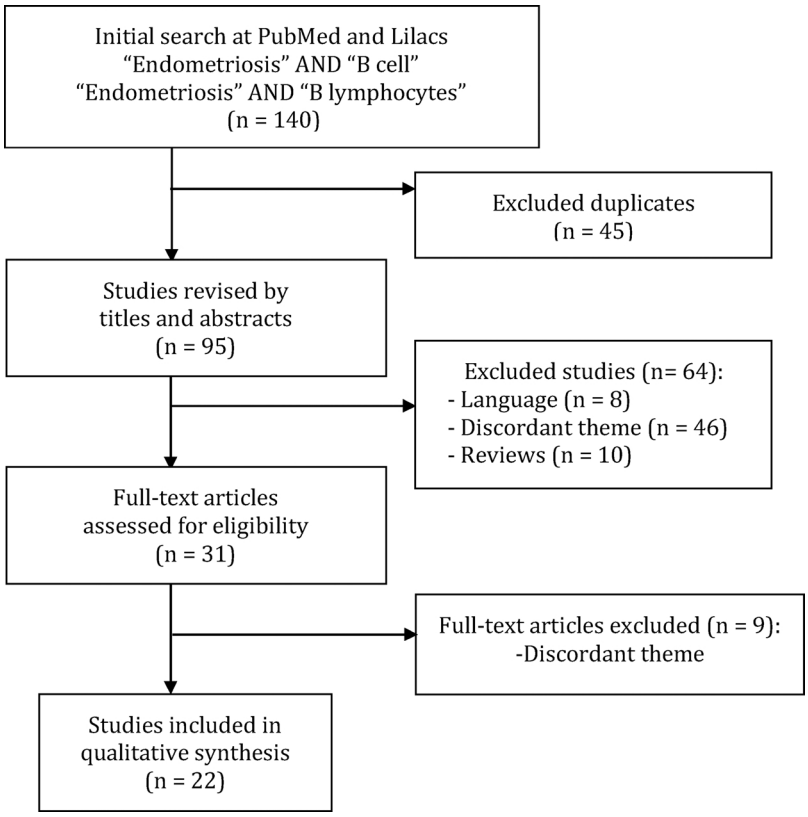

Fig. 1. Results of the search on the databases, 2017.

School of Medicine, University of São Paulo, Brazil and at INSERM U1016-Batteux, Institut Cochin, France. Studies were identified through systematic searches in PubMed/MEDLINE and Lilacs, considering the keywords: "*endometriosis*"; "*B cells*" and "*B lymphocytes*". Articles written in English; Portuguese; French or Spanish were included. No limits of date of publication were imposed and reviews were excluded. The selection of the 22 studies assessed in this review is shown in Fig. 1. There is no PROSPERO registration number.

3. Results

We summarized the content of 22 selected studies in Table 1, organized by type of sample evaluated. Different markers and samples were assessed by the authors to analyze the direct or indirect role of B lymphocytes in endometriosis.

Most studies demonstrated increased number and/or activation of B cells (Antsiferova et al., 2005; Badawy et al., 1987, 1989; Berbic et al., 2013; Chishima et al., 2000; Gleicher et al., 1987; Hever et al., 2007; Lachapelle et al., 1996; Odukoya et al., 1995, 1996a,b; Scheerer et al., 2016), seven studies found no difference (Christofolini et al., 2011; Gebel et al., 1993; Klentzeris et al., 1995; Nava Loya et al., 1996; Nomiyama et al., 1997; Witz et al., 1994; Yeol et al., 2015) and two studies showed decreased number of B cells (Oosterlynck et al., 1993; Gagne et al., 2003).

4. Discussion

4.1. Differences among the evaluated studies

Since the literature included in this review was published over a long period of time – between 1987 (Badawy et al., 1987) and 2016 (Scheerer et al., 2016) – it should be considered that the methods have improved in meantime. The authors used: Avidin-biotin immunoperoxidase technique (ABS), Immunobead rosette technique (IBT), Enzyme-linked immunosorbent assay (ELISA), Immunofluorescence (IF), Flow cytometry, Immunohistochemistry (IHC) and Protein chain reaction (PCR).

Some techniques, such as IBT and ABS, were more frequently used in 1980s and 1990s; the markers and antibodies used in ELISA and IHC have since improved and new target genes and primers have been developed for the PCR evaluations.

Since the most recent publication that evaluated B cells in endometriosis through flow cytometry (Antsiferova et al., 2005), several new phenotype markers have been used to study these cells in other diseases (Kaminski et al., 2012).

B cells phenotypes have been analyzed through flow cytometry in chronic autoimmune diseases such as systemic lupus erythematosus (Bertsias et al., 2010); Sjögreńs syndrome (Abdulahad et al., 2011) and rheumatoid arthritis (Leandro et al., 2006). The B cells subsets include transitional, mature-naïve, memory and antibody-secreting cells. The current knowledge on these subsets offers different possibilities of profiling that could help optimize diagnostic and therapeutic protocols for many diseases in the future (Kaminski et al., 2012).

Studies have also evaluated different types of samples from endometriosis patients and the results can represent different aspects of the disease: findings in blood/serum are systemic; the ectopic endometrium represents the endometriotic lesions directly; pelvic lymph nodes may account for the influence of a lymphatic spread of the endometrial cells; peritoneal fluid represents the peritoneal cavity environment; and the follicular fluid could enable the assessment of infertility in the context of the disease. It is also important to study the eutopic endometrium since its alteration in patients with endometriosis has already been described.

To summarize, there are many differences between the studies included in this review, especially in the methods and samples, and therefore it can be difficult to compare their results. However, it is possible to consider their findings complementary in describing different aspects of the disease.

Table 1
Studies included on the role of B lymphocytes in endometriosis.

Samples	Study design	Population	Methods	Markers	B Lymphocytes in EDT	References
Blood/serum	Case-control	EDT x controls	IBT	Monoclonal antibodies	Increased B cells	Badawy et al. (1987)
	Descriptive	59 EDT	ELISA	FAN; IgG; IgM lupus anticoagulant	Abnormal polyclonal B cells activation	Gleicher et al. (1987)
	Case-control	19 EDT x 26 infertile	IBT; ELISA	B cells; IgA; IgG.	Increased B cells and IgG	Badawy et al. (1989)
	Case-control	42 EDT x 20 infertile x 22 controls	In vitro stimulation with polyclonal B-cell activators	IgG1; IgG2; IgG3	No difference in B cells. ↓ polyclonal IgG2 production in stages III and IV EDT.	Gebel et al. (1993)
	Case-control	21 EDT x 18 controls	Chemiluminescent ELISA	sCD23	Activation of B cells	Odukoya et al. (1995)
	Case-control	25 EDT and idiopathic infertility	Flow cytometry	CD 19	No difference	Nava Loya et al. (1996)
	Case-control	57 EDT x 40 controls	ELISA	sCD23; IgG	Increased amount and activation of B cells	Odukoya et al. (1996a)
	Case-control	31 EDT x 14 controls	Flow cytometry; IF	CD5; ANA	B cells are related to ANA production.	Chishima et al. (2000)
	Case-control	175 EDT x 131 controls	Flow cytometry	CD 20	Decreased B cells	Gagné et al. (2003)
	Case-control	15 EDT x 20 controls	Flow cytometry	CD 20; CD5	No difference	Antsiferova et al. (2005)
	Case-control	10 OMA x 10 adenomyosis x 10 leiomyoma	IHC; PCR; ELISA	BLyS; Plasma cells	Increased BLyS	Hever et al. (2007)
	Case-control	87 EDT x 33 adenomyosis x 205 controls	PCR	BLyS 817C/T polimorphism	Heterozygosity ↓ risk of DIE; BLyS may play a role in the pathogenesis.	de Graaff et al. (2010)
	Case-control	165 infertile EDT x 83 idiopathic infertility x 145 control	PCR	BLyS −817C/T polymorphism	No difference	Christofolini et al. (2011)
Peritoneal fluid	Case-control	EDT x controls	IBT	Monoclonal antibodies	Increased B cells	Badawy et al. (1987)
	Case-control	19 EDT x 26 infertile	IBT; ELISA	B cells; IgA; IgG.	Increased B cells, IgA and IgG	Badawy et al. (1989)
	Case-control	25 EDT and idiopathic infertility	Flow cytometry	CD 19	No difference	Nava Loya et al. (1996)
	Case- control	47 EDT x 35 controls	Chemiluminescent ELISA	sCD23	↑ B cell activation; higher in stages I and II	Odukoya et al. (1996b)
	Case-control	31 EDT x 14 controls	Flow cytometry; IF	CD5; ANA	Increased B-1 cells	Chishima et al. (2000)
	Case-control	46 EDT x 52 controls	ELISA; PCR	IgG; IgA; Bcl-6; Blimp-1	↓ Bcl-6 and ↑ Blimp-1 No difference in Ig	Yeol et al. (2015)
Eutopic and ectopic endometrium	Descriptive		IHC	CD 22	No difference	Witz et al. (1994)
	Case-control	30 infertile EDT x 10 controls	IHC	IgG	No difference	Nomiyama et al. 1997
	Case-control	15 EDT x 20 controls	Flow cytometry	CD 20; CD5	↑ amount in eutopic endometrium; ↑ amount and activation in ectopic endometrium	Antsiferova et al. (2005)
	Case-control	10 OMA x 10 adenomyosis x 10 leiomyoma	IHC; PCR; ELISA	BLyS; Plasma cells	Increased BLyS and plasma cells.	Hever et al. (2007)
	Case-control	48 EDT X 24 adenomyosis X 12 controls	IHC	CD 20	↑ amount in EDT lesions, adenomyosis and endometrium	Scheerer et al. (2016)
	Descriptive	15 EDT	ABC; IHC	anti-leu-12	Very few B cells in the lesions	Oosterlynck et al. (1993)
	Case-control	87 EDT x 33 adenomyosis x 205 controls	PCR	BLyS 817C/T polimorphism	Heterozygosity ↓ risk of DIE; BLyS may play a role in the pathogenesis.	de Graaff et al. (2010)
	Case-control	21 infertile EDT x 18 controls	IHC	CD 22	No difference in eutopic endometrium	Klentzeris et al. (1995)
Follicular fluid	Case-control	12 infertile EDT x 35 tubal factor x 13 idiopathic	Flow cytometry	CD3; CD4; CD8; CD14; CD20; CD45; CD56	Increased B cells	Lachapelle et al. (1996)
Pelvic lymph nodes	Case-control	7 EDT x 9 controls	IHC	CD 20; CD79; plasma cells	↑ in lymph nodes during proliferative phase	Berbic et al. (2013)

ABC: avidin-biotin immunoperoxidase technique; ANA: antinuclear antibodies; Bcl-6: B cell leukemia lymphoma-6; Blimp-1: B lymphocyte inducer of maturation program-1; BLyS: B lymphocyte stimulator; DIE: deep infiltrating endometriosis; EDT: Endometriosis; ELISA: enzyme-linked immunosorbent assay; IBT: Immunobead rosette technique; IF: Immunofluorescence; IHC: Immunohistochemistry; OMA: ovarian endometrioma; PCR: protein chain reaction.

4.2. Importance of immunological disorders in survival and proliferation of ectopic endometrial cells

Studies have demonstrated the importance of the immune system in the pathogenesis of endometriosis. In this context, inefficient peritoneal immunosurveillance and persistence of ectopic endometrial cells remain a crucial issue. This complex process could explain why although retrograde menstruation is a common phenomenon among women of reproductive age, not all of them develop endometriosis.

The role of apoptosis in normal endometrium is to eliminate the dysfunctional cells – a tissue repair mechanism during each menstrual cycle. In endometriosis, the cells regurgitated into the peritoneal cavity do not exhibit normal mechanisms of programmed cell death.

The Fas-Fas-L and TNF-α apoptosis pathways play a key role in modulating the peritoneal microenvironment (Vetvicka et al., 2016). The overexpression of anti-apoptotic factors and decreased expression of pro-apoptotic factors (Tosti et al., 2015) may interfere in peritoneal homeostasis and contribute to the development of the disease. Therefore, the regulation of apoptosis can be a target for the treatment of endometriosis. It has been shown that the use of GnRH analogues increases the expression of the pro-apoptotic proteins Bax and FasL and decreases the expression of the anti-apoptotic protein Bcl-2 (Bilotas et al., 2007).

In addition, endometrial cells in the peritoneal cavity are not targeted and removed by phagocytes and NK cells, thereby escaping the clearance and surviving to invade the peritoneum, through a mechanism called “immunoescaping” (Vetvicka et al., 2016). It is important to study the role of these immune cells as well as their recently described subsets, such as the Invariant Natural Killer T cells (iNKT) in the pathogenesis of endometriosis (Laganà et al., 2016).

The adhesion of the fragments of endometrium to the intraperitoneal surface probably involves the expression of extracellular membrane molecules and their co-receptors (Lebovic et al., 2001). Current knowledge suggests that endometrial stromal cells are involved in cellular adhesion, whereas glandular epithelial cells play a role in invasion and growth of the lesion (Ahn et al., 2015).

In endometriosis, the endometrium presents an increased proteolytic capacity. Anomalous expressions of plasminogen activator system proteins as well as various matrix metalloproteinases seem to be responsible for this phenomenon (Ahn et al., 2015; Gilabert-Estellés et al., 2003).

Once established, endometriotic lesions secrete various pro-inflammatory molecules. There is evidence of peritoneal macrophages activation with increased production of cytokines, though the phagocytic activity is reduced (Králíčková and Vetvicka, 2015).

4.3. The role of B lymphocytes and immunoglobulins in the pathogenesis of endometriosis

Many lymphocytes were identified in endometriotic implants, and they may contribute to the progression of the disease. The immune cells of lymphoid lineage play an essential role in the survival and proliferation of endometrial cells (Klein et al., 1992). There is an aberrant functioning of these immune cells in endometriosis: reduced activity of cytotoxic T cells and NK cells; secretion of cytokines by T helper cells and autoantibody production by B lymphocytes (Králíčková and Vetvicka, 2015; Osuga et al., 2011).

B lymphocytes are responsible for the humoral immune response through the production of antibodies against antigens. In the pathogenesis of endometriosis, these cells seem to contribute to the occurrence of the disease by autoantibody secretion (Straub, 2007).

Endometriosis is an estrogen-dependent disease, and the role of estrogens in immunomodulation has been described as a paradox: they can act both as anti-inflammatory and pro-inflammatory substances. This may explain why women have a higher inflammatory response and an increased incidence of autoimmune diseases compared to men. Estrogen can stimulate antibody production by B cells, probably by inhibiting T cell suppression of these cells. In contrast, high concentrations of estrogens may lead to a suppression of B lymphocyte lineage precursors (Straub, 2007).

An increase in the reactivity of B lymphocytes in endometriosis was first suggested in 1980 (Startseva, 1980). The same year, another study demonstrated IgG and complement deposits in the endometrium and decreased serum complement, suggesting an autoimmune response with complement consumption by the antigen-antibody complex (Weed and Arquembourg, 1980).

B lymphocytes seem to contribute to the pathogenesis of endometriosis by producing antibodies anti-endometrium and also anti-DNA, antiphospholipid and antinuclear antibodies (ANA), usually observed in autoimmune diseases (Osuga et al., 2011). These changes may be related to specific genetic variants in autoimmune-related genes (Bianco et al., 2012).

Wild and Shivers (1985) first described by indirect immunofluorescence the presence of anti-endometrial antibodies in the serum of women with endometriosis. Immunohistochemical analysis revealed that these anti-endometrial antibodies bind to endometrial glands and also to the ectopic tissue (Fernandez-Shaw et al., 1993). A subsequent western blot analysis demonstrated that autoantibodies react with membrane proteins of the endometrial cells and that the immunoreactivity increases with disease progression (Bohler et al., 2007).

Although many studies have demonstrated an aberrant production of autoantibodies in endometriosis, there is no consensus about the concentration of B lymphocytes and their role in this disease. Badawy et al. (1987) reported increased B cells in blood and peritoneal fluid from patients with endometriosis. The same group later described increased IgG and IgA in the peritoneal cell cultures and also an increased number of T cells, B cells, and increased ratio of CD4/CD8 lymphocytes in blood and peritoneal fluid. These findings suggest that immunoglobulin production by the activated B cells may be regulated by the increased presence of T cells, and specifically helper cells (CD4) (Badawy et al., 1989).

Some studies have suggested that endometriosis has an autoimmune etiology, presenting changes in both humoral and cellular immunity (Nothnick, 2001) that lead to inflammatory reactions and proliferation of endometriotic cells (Osuga et al., 2011). Nothnick (2001) lists common characteristics between endometriosis and autoimmune diseases: tissue injury, polyclonal activation of B cells, abnormalities of B and T lymphocytes, changes in apoptosis, association with other autoimmune disorders, multiple organ involvement, familial occurrence and possible environmental and genetic factors associated.

Possible common backgrounds of immune dysfunctions between autoimmune diseases and endometriosis were also proposed by Chishima et al. (2000). They reported that B cells are related to ANA production in the blood of patients with endometriosis and also found increased B-1 cells in peritoneal exudate cells of these women. It is speculated that the infertility associated endometriosis is partly due to autoantibody abnormalities regarded as the result of polyclonal B-cell activation associated with B-l-cell proliferation.

Hever et al. (2007) analyzed significantly upregulated genes in endometriosis versus control endometrium and concluded that 53 genes associated with immune responses had altered expression.

While evaluating the role of B cells through soluble CD23 and IgG autoantibodies, Odukoya et al. (1995, 1996a,b) demonstrated increased amount and activation of B cells in the blood and peritoneal fluid of women with endometriosis. They also described higher concentration of soluble CD23 in patients with stage I and II endometriosis, suggesting that mild endometriosis may be immunologically more active than severe endometriosis. Gebel et al. (1993) findings also agree with this statement as they have reported reduced polyclonal IgG2 production in stage III and IV endometriosis.

Through the analysis of CD22+, Klentzeris et al. (1995) and Witz et al. (1994) did not find any difference in B lymphocytes of eutopic and

ectopic endometrium of patients with endometriosis compared to controls. However, Klentzeris et al. (1995) concluded that functional differences between leukocytes could not be excluded and, therefore, the analysis of cytokines secreted by these cells would be helpful.

There was also no difference in CD29+ cells analyzed by flow cytometry in the blood and peritoneal fluid of women with endometriosis compared to those with idiopathic infertility (Nava Loya et al., 1996). Oosterlynck et al. (1993), through the IHC analysis of anti-leu-12, reported very few B cells in the ectopic endometrium of 15 women with endometriosis.

Nomiyama et al. (1997) analyzed the concentration of immunoglobulins in eutopic and ectopic endometrium of infertile women with endometriosis and found no difference with controls. They concluded that the difference in local immune response in endometrial implants did not affect systemic immunity.

Lymphocyte subsets were evaluated in the blood of 306 patients and CD20+ B cells were decreased, as well as subsets of CD20 cells co-expressing either HLADR or high level of CD44 molecules in women with endometriosis (Gagné et al., 2003). The authors concluded that although some specific B-cell clones are activated to produce autoantibodies, the relative number of total B lymphocytes expressing either HLADR or CD44 molecules is downregulated in the blood of patients with endometriosis.

Higher IgG and IgA concentrations in the peritoneal fluid of patients with endometriosis were reported by Yeol et al. (2015), but the differences were not statistically significant. They also evaluated the transcriptional factors that play a key role in B cells function: *B lymphocyte inducer of maturation program* (Blimp)-1 − a crucial regulator of plasma cell differentiation − and its antagonist *B cell leukemia lymphoma* (Bcl)-6. Blc-6 mRNA level was significantly lower and Blimp-1 mRNA level was significantly higher in the endometriosis group, with significant correlations among transcriptional factors, immunoglobulins and cytokines.

Increased B cells were described in the follicular fluid of infertile patients with endometriosis (Lachapelle et al., 1996), suggesting that this could be one of the factors impairing their fertility.

During the proliferative phase of the menstrual cycle, B lymphocytes were also increased in pelvic lymph nodes of women with endometriosis. This finding may support the theory of a lymphatic spread of endometriosis (Berbic et al., 2013).

Immune cells infiltrates of lesions, myometrium and endometrium of women with endometriosis and adenomyosis showed increased CD20+ B cells in IHC (Scheerer et al., 2016).

Endometriotic lesions show high levels of cytokines that activate B cells, such as BLyS (B lymphocyte stimulator) (Hever et al., 2007). This cytokine is produced by macrophages and plays an important role in the normal development of B cells and their differentiation into plasma cells (Schiemann et al., 2001). High levels of BLyS were also identified in the plasma of patients with autoimmune diseases, and it is a potential target in the treatment of diseases with B cell defects (Krivosikova et al., 2009).

Another study (de Graaff et al., 2010) evaluated the BLyS 817C/T polymorphism in women with endometriosis, adenomyosis and controls. They observed a reduced risk of deep infiltrating endometriosis associated with heterozygosity and concluded that BLyS may play a role in the pathogenesis of the disease. Christofolini et al. (2011) analyzed the same polymorphism in a specific group of infertile women with endometriosis and did not find any difference.

Antsferova et al. (2005) observed increased B cells in eutopic and ectopic endometrium and activated B cells in the lesions of patients with endometriosis in flow cytometry. They concluded that the development of peritoneal lesions is associated with the activation of systemic and local humoral reactions due to an increase in the amount of Th2 lymphocytes.

The importance of the immune system in the pathogenesis of endometriosis supports the idea of using therapeutic strategies involving drugs that modulate specifically the functions of immune cells (Osuga et al., 2011). There is an urgent need for new approaches to the medical treatment of endometriosis, especially non-hormonal therapies since most of the drugs currently used are contraceptives and women may have to choose between managing painful symptoms and trying to conceive. An increased understanding of the immune aspects of endometriosis would be beneficial in the search for novel treatment strategies.

5. Conclusion

The pathogenesis of endometriosis is multifactorial. Many studies have explored the role of genetics, environmental factors and the immune system in the development of the disease. Several authors have attempted to clarify the role of the immune system in endometriosis and various abnormalities have been detected in this association, including increased B lymphocytes and excessive production of autoantibodies. Although there is evidence of abnormal production of autoantibodies in patients with endometriosis, their role in the development of the disease and the concentration of B cells in this context are not well understood.

The use of flow cytometry techniques with new subset markers could be a valuable tool to evaluate different B cells phenotypes in endometriosis. Moreover, the association of these factors with clinical symptoms, location and severity of the disease has not been investigated. Further studies are necessary to clarify the role of B cells and antibodies in the development of endometriosis and propose new therapeutic strategies such as the use of drugs that target these cells.

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ORIGINAL ARTICLE ***Gynaecology***

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.

STUDY FUNDING/COMPETING INTEREST(S): This research was supported by a grant of Institut National de la Santé et de la Recherche Médicale and Paris Descartes University. None of the authors has any conflict of interest to disclose.

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; Vigano *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 $CD4^+$ 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_2O 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: $TV\ (mm^3) = (L \times W^2)/2$, 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 1 (Fizz-1)—and one reference gene, Beta-actin (B-actin) as internal control, were analyzed by RT-qPCR (primers are listed in Supplementary Table SI).

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). Concentrations 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

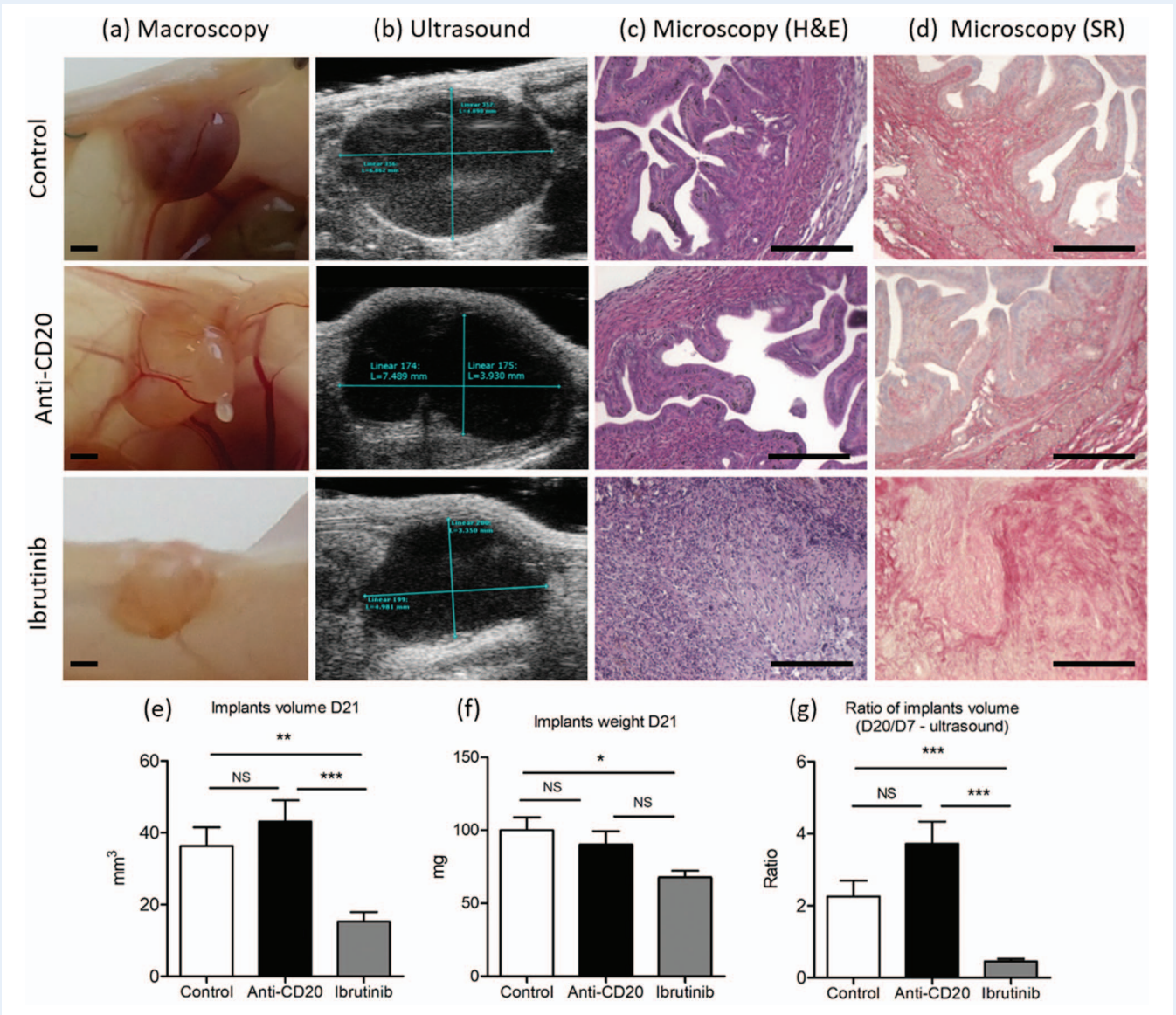

Figure 1 Effects of B cell modulating treatment on endometriotic implants development in mice. (**a**) Macroscopic view of the implants. (**b**) Ultrasonography images of peritoneal implants in mice on Day 20. (**c**) Coloration with H&E of implants at Day 21. (**d**) Coloration with SR of implants at 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 µm.

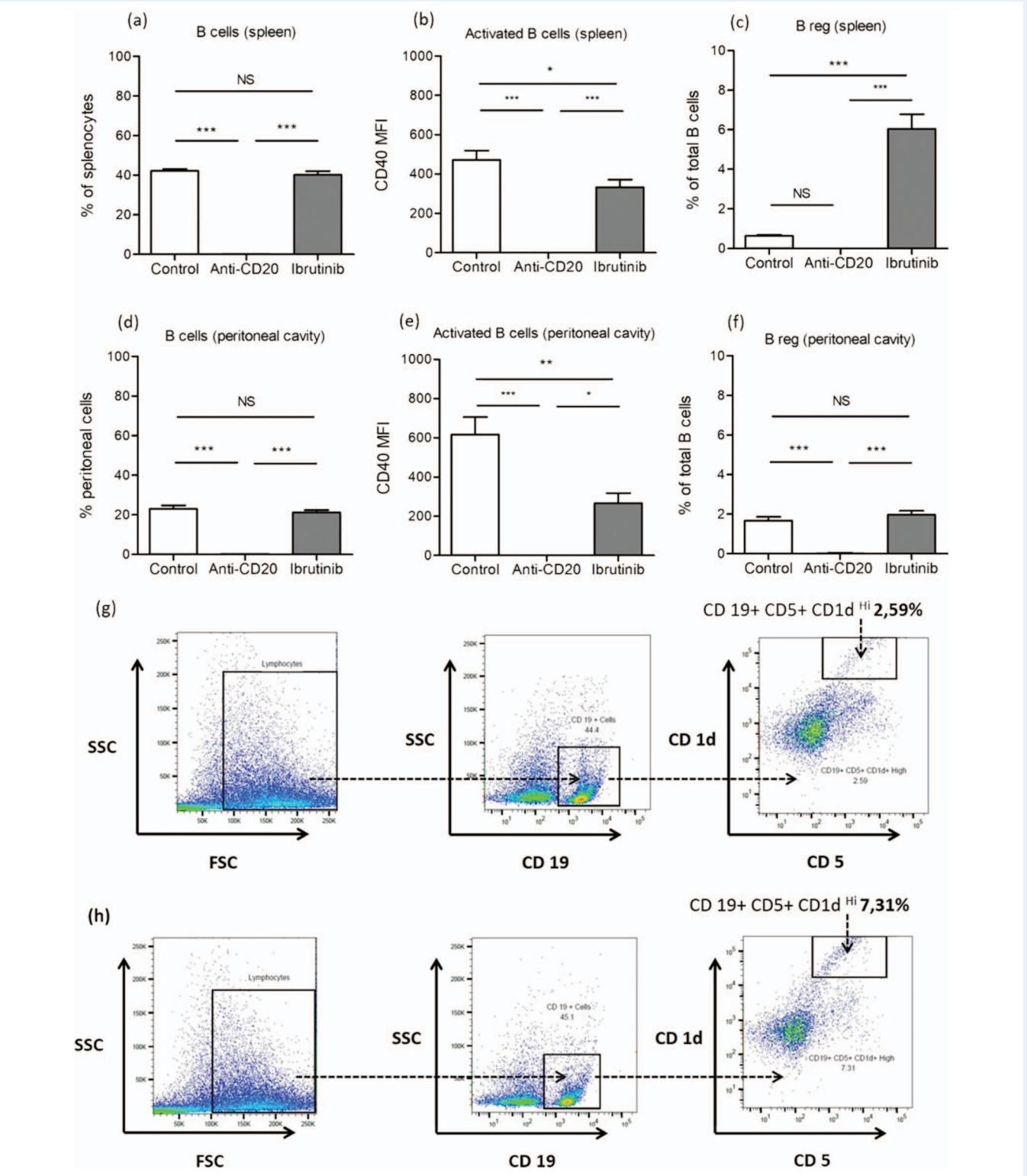

Figure 2 B cell phenotype analysis in spleen and peritoneal cavity of endometriotic mice. Frequency of B cells ($B220^+CD19^+$) in spleen (**a**) and peritoneal cavity (**d**) of mice. Data represent mean ± SEM. Surface CD40 expression in B cells (activated B cells) in spleen (**b**) and peritoneal cavity (**e**). Data represent the MFI of CD40 expression ± SEM. Frequency of Breg ($B220^+CD19^+CD5^+CD1d^{high}$) in spleen (**c**) and peritoneal cavity (**f**). Data represent mean ± 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 $B220^{+}CD19^{+}$) 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 $CD19^{+}CD5^{+}CD1d^{high}$ 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

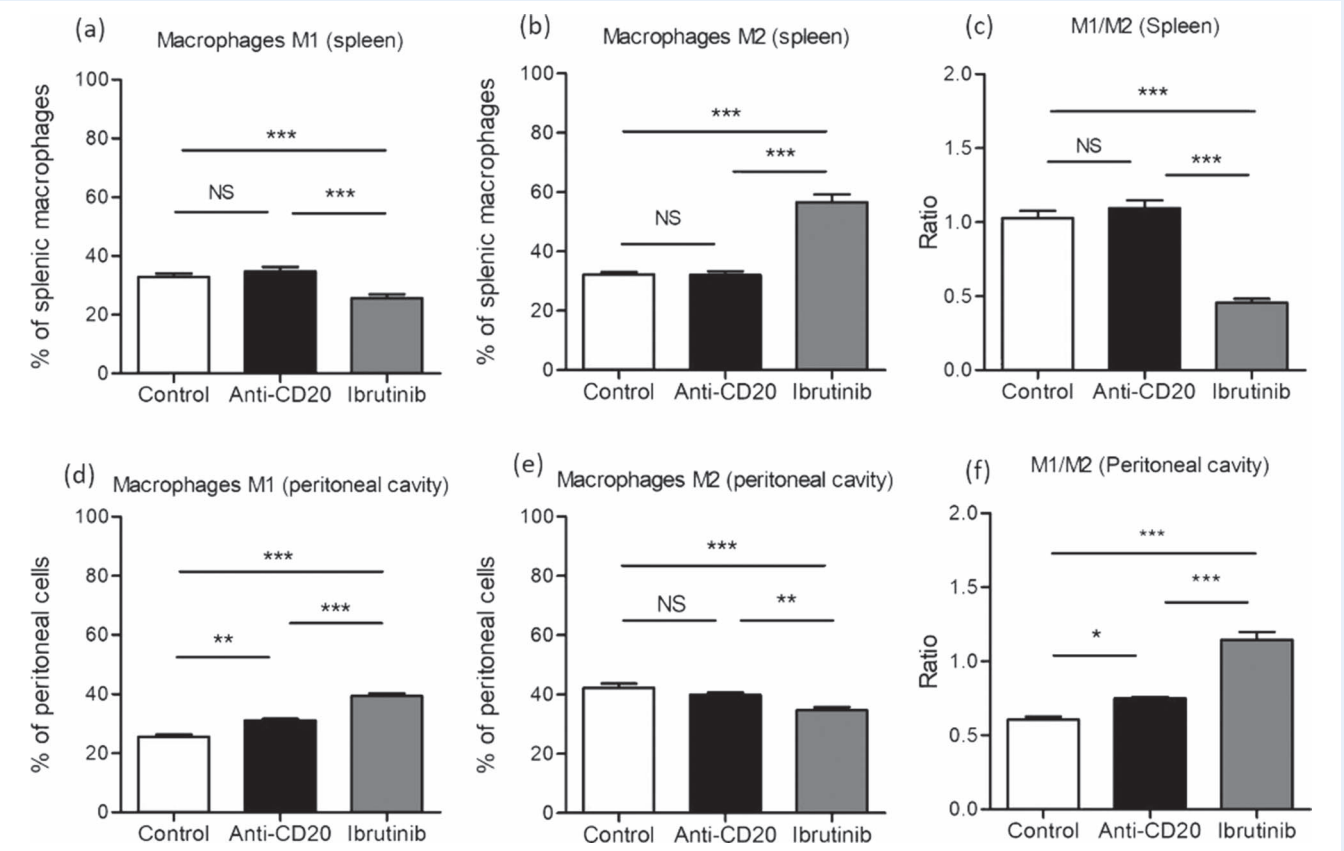

Figure 3 Macrophage M1/M2 distribution in spleen and peritoneal cavity in endometriotic mice. Macrophages were gated on $CD11b^{+}F4/80^{+}$ cells isolated from spleen (**a** and **b**) and from the peritoneal cavity (**d** and **e**). M1 macrophages (**a** and **d**) were further characterized as $CD43^{+}$ and $Ly6C^{high}$ and M2 macrophages (**b** and **e**) as $CD206^{+}$ and $Ly6C^{low}$. The ratio of M1/M2 population was calculated (**c** and **f**). Data represent 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; $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

(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.

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 $CD62L^{high}$ $CD44^{low}$) or memory ($CD62L^{low}$ $CD44^{high}$) $CD4^+$ and $CD8^+$ 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-

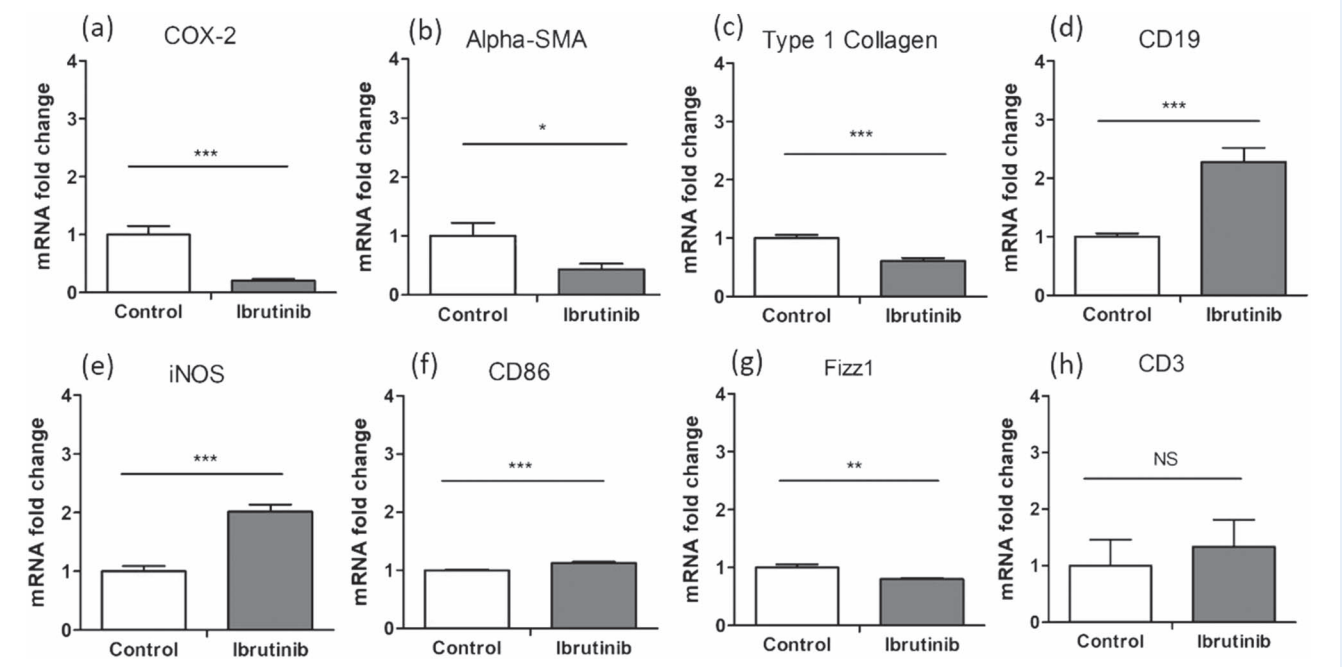

Figure 4 Effects of Ibrutinib in quantitative expression of genes in endometriotic implants of mice. (**a**) COX-2; (**b**) ASMA; (**c**) Type I collagen; (**d**) CD19 (for B lymphocytes); (**e**) iNOS (for M1 macrophages); (**f**) CD86 (for M1 macrophages); (**g**) Fizz-1 (for M2 macrophages) and (**h**) 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; $^*P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

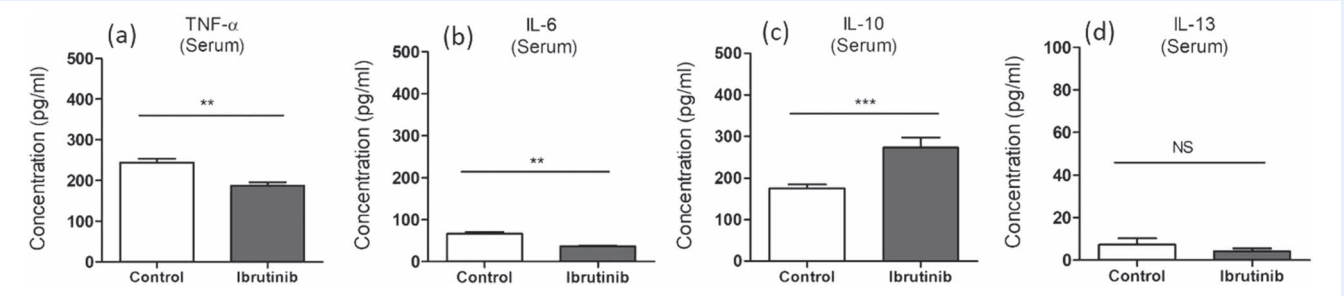

Figure 5 Effects of Ibrutinib on systemic cytokines of endometriotic mice. (**a**) TNFA; (**b**) IL-6; (**c**) IL-10 and (**d**) IL-13 concentrations in the sera of mice measured by ELISA. Data represent mean ± SEM. Each group had $n = 10$ mice. The Mann–Whitney test was used to detect significant differences.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

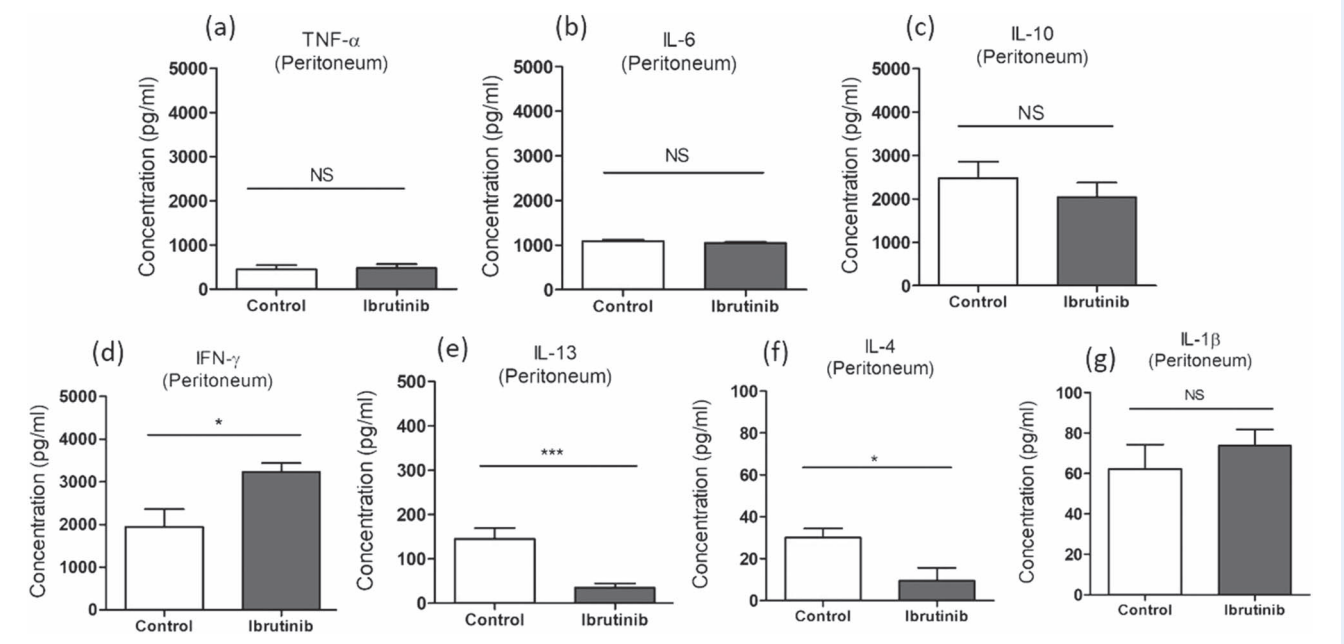

Figure 6 Effects of Ibrutinib on peritoneal cytokines of endometriotic mice. (**a**) TNFA; (**b**) IL-6; (**c**) IL-10; (**d**) IFNG; (**e**) IL-13; (**f**) IL-4 and (**g**) IL-1B concentrations in peritoneal fluid of mice measured by ELISA. Data represent mean ± SEM. Each group had $n = 10$ mice. The 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; Vigano *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

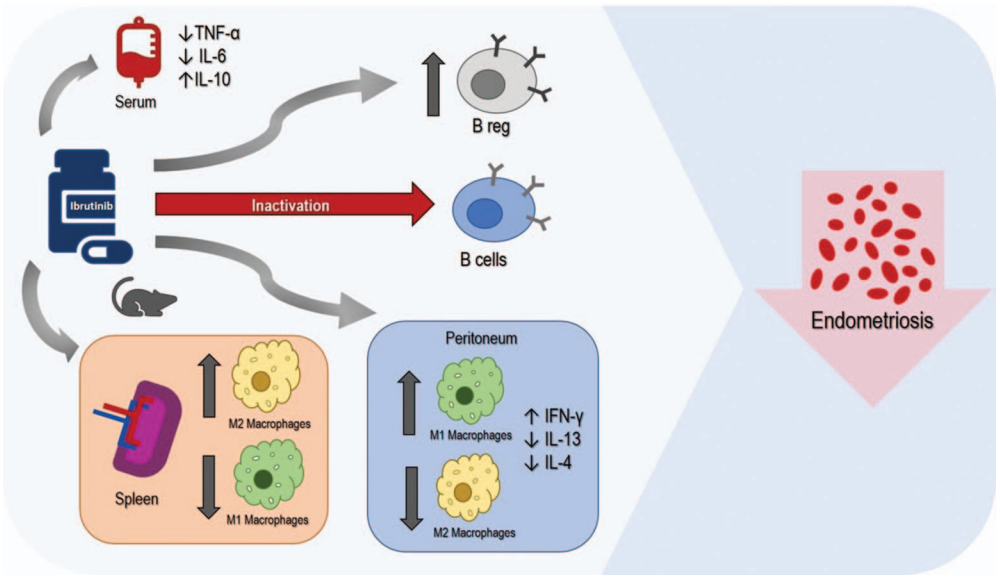

Figure 7 Summary of Ibrutinib effects that controlled endometriosis progression in mice.

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 $CD1d^{hi}CD5^{+}$ 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^{2+} influx, nuclear factor-κB 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 $Btk^{-/-}$ 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 (Ní 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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