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Macrophage immune memory controls endometriosis in mice and humans

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SUMMARY

Endometriosis is a frequent chronic inflammatory gynecological disease characterized by the presence of ectopic endometrial tissue causing pain and infertility. Macrophages play a central role in lesion establishment and maintenance by driving chronic inflammation and tissue remodeling. Macrophages can be reprogrammed to acquire memory-like characteristics following antigenic challenge to reinforce or inhibit a subsequent immune response; a phenomenon termed ‘trained immunity’. Here, whereas BCG-training enhances the lesion growth in a mice model of endometriosis, tolerization with repeated low doses of lipopolysaccharide (LPS^{low}) or adoptive transfer of LPS^{low}-tolerized macrophages elicits a suppressor effect. LPS^{low}-tolerized human macrophages mitigates the fibro-inflammatory phenotype of endometriotic cells in an IL-10-dependent manner. A history of severe GRAM-infection is associated with reduced infertility duration and alleviated symptoms, in opposition to patients with GRAM+ infections history. Thus, the manipulation of innate immune memory may be effective to dampen hyper-inflammatory conditions, opening the way to promising therapeutic approaches.

Introduction

Endometriosis is a chronic estrogen-dependent inflammatory gynecological disease that affects about 5-15% of women in reproductive age (Chapron et al., 2019). The disease is characterized by the presence and growth of endometrial glands and stroma outside the uterine cavity (Giudice and Kao, 2004) and the inclusion of fibrosis in the definition has been proposed (Vigano et al., 2018). The current theory on endometriosis pathogenesis is based on the “retrograde menstruation phenomenon” defined as the reflux of endometrial fragments regurgitated through the fallopian tubes during menstruation with subsequent implantation on the peritoneum, or other organs of the peritoneal cavity (Burney and Giudice, 2012). However, menstrual regurgitation occurs in most women and only 5 to 15% suffer from endometriosis, indicating that additional factors favor the development of the disease. Indeed, molecular factors like inflammation, altered steroid biosynthesis and receptor response and increased invasiveness are involved in endometriosis development. Numerous studies have emphasized the role of chronic inflammation and aberrant immune response as major mechanisms for endometriosis development (Riccio et al., 2018). Abnormalities of both innate and adaptive immune responses such as decreased T cell cytotoxicity, polyclonal activation of B cells and altered activation of peritoneal macrophages (M ϕ s) with modifications in inflammatory mediators (Riccio et al., 2018) have been reported.

M ϕ s play a central role in the establishment of the chronic inflammatory process occurring in the pelvic cavity and promoting ectopic endometriotic tissue growth. In endometriosis, the frequency of M ϕ s, their activation and cytokine production are increased (Capobianco and Rovere-Querini, 2013; Králíčková and Vetvicka, 2015). Their enhanced ability to produce pro-inflammatory mediators maintains pelvic inflammation and promotes other immune cells’ recruitment, endometriotic cells’ proliferation and angiogenesis (Yang et al., 2017). It has been demonstrated that in certain models of bacterial and viral infections, protection from reinfection occur independently of T and B cells and involve classical innate immune cells, such as monocytes/M ϕ s (Netea et al., 2020). This paradigm, called “innate immune memory” or “trained immunity” is based on metabolic changes and epigenetic reprogramming modifying the innate immune cells’ capacity to respond to a second stimulation (Gardiner and Mills, 2016; Netea et al., 2016, 2019). Interestingly, innate immune memory results either in an enhanced immune response upon reinfection with some microbial challenge such as the *Bacille Calmette Guérin* (BCG) and *Candida albicans* (Kleinnijenhuis et al., 2012; Quintin et al., 2012) or, at the opposite, in an immunoparalysis with a decreased immune response upon a second infection induced by repeated low doses of lipopolysaccharide (LPS) (Cheng et al., 2016; Hotchkiss et al., 2013). Based on the experimental evidence that M ϕ s can be trained with long-lasting effects, and the well-established role of M ϕ s in the pathogenesis of endometriosis, we propose to analyze the immunomodulatory effects of memory M ϕ s on endometriotic cells’ implantation and to apply these properties as promising therapeutic approaches.

Results

***In vivo* immune training modulates macrophages' phenotype and cytokine production**

We have first analyzed the expression of surface molecules on peritoneal M ϕ s to better characterize the phenotypic changes upon *in vivo* immune training with LPS^{low} or BCG (Figure 1A). Peritoneal M ϕ s from BCG-mice showed an enhanced expression of CD43, CD206, CCR2, CXCR4, CD80, TLR2 and Ly6C compared to PBS-mice (Figure 1B and Figure S1A-C). LPS^{low} mice showed an overlapping profile with an increased expression of CD206 and CD43 as observed in BCG-mice but a downregulation of the expression of CCR2, CXCR4, CD80 and Ly6C on their peritoneal M ϕ s compared to those of PBS-mice. MHC II expression on peritoneal M ϕ s did not change in any of the *in vivo* training protocols (Figure S1C). These phenotypic modifications are challenging the dualistic concept of classic (M1) or alternative (M2) M ϕ polarization, as a mixed expression of those markers is observed rather than a skewing toward a specific M ϕ subpopulation.

Then, we assessed the cytokine production capacity of peritoneal M ϕ s from *in vivo* trained mice upon an *ex vivo* inflammatory challenge. Inflammatory stimulation of M ϕ s from PBS-mice increased the release of IL-6, TNF- α and IL-10 when compared to basal cytokine production in non-stimulated M ϕ s. BCG training was accompanied by a higher capacity to produce pro-inflammatory cytokines (TNF- α and IL-6) upon an inflammatory stimulation compared to the stimulated PBS-M ϕ s and to a lesser extent CCL2 level ($p=0.15$) but did not affect IL-10 production (Figure 1C). By contrast, *ex vivo* stimulated M ϕ s from LPS^{low}-mice showed a significant decrease in the production of TNF- α , IL-6 and CCL2 but an important increase in IL-10 production.

Innate immune memory has been shown to modify the cellular metabolism as reflected by a shift towards an increased glycolysis with either an elevated lactate production or a defect in the energy metabolism (Arts et al., 2016; Cheng et al., 2016). We measured the lactate production in the supernatant of trained M ϕ s upon inflammatory LPS stimulation. BCG training induced a significant increase of lactate production compared to PBS-mice while no difference was detected with LPS^{low} stimulation (Figure 1D). Chromatin reorganization is a hallmark of innate immune memory and is responsible for epigenetic regulation of gene expression. We assessed the enrichment of H3K4me3 (associated with an increased gene expression) and showed that spleen-derived M ϕ s from BCG-mice had an increased H3K4me3 on the promoters of IL-6 and TNF- α while LPS^{low} stimulation significantly upregulated H3K4me3 enrichment on the promoter of IL-10 (Figure S2).

Altogether, these results highlight the important phenotypic and functional effects that *in vivo* training may exert on peritoneal M ϕ s according to the nature of antigenic challenge.

***In vivo* training of macrophages affects endometriosis development in mice and are accompanied by specific immunological changes**

Mice were subjected to *in vivo* immune training prior to the endometriosis surgery (Figure S3A). Endometriotic lesions are believed to generate signals that drive the activation of peritoneal M ϕ s, and thus act in our model as a second antigenic stimulation on the trained M ϕ s. In LPS^{low}-EDT mice, endometriotic lesions were smaller and less active (no fresh blood, less vascularization and fewer glands) compared to the EDT-PBS mice (referred as control in this experiment), whereas BCG training resulted in larger and more active endometriotic fragments at the macroscopically level (Figure 2A-a). Microscopically, the implants from EDT-BCG mice showed an enhanced cellular infiltration and collagen deposition as reflected by Sirius Red staining, while EDT-LPS^{low} had less pronounced cellular activity and fibrosis mark compared to control (Figure 2A-c and Figure 2A-d).

Implant volume evaluated by ultrasonography (Figure 2A-b) at day 0 and day 22 was significantly decreased in the EDT-LPS^{low} group when compared to control. This variation was evidenced by a reduced kinetic of lesions' growth in the EDT-LPS^{low} group compared to control while lesions from EDT-BCG animals showed an accelerated increase (Figure 2B). Furthermore, implant weight from EDT-LPS^{low} was significantly reduced at day 23 compared to control. Activating M ϕ s with BCG injections induced a slight but significant increase in implants' volume and weight when compared to control (p=0.04) (Figures 2B-C).

In endometriosis, the inflammatory peritoneal environment influences the behavior of endometrial ectopic cells. As M ϕ s play an important role in driving inflammation in the peritoneal cavity, we have assessed the immunological changes associated with the *in vivo* immune training. As shown in Figure 2D, endometriosis itself induced phenotypic modifications of peritoneal M ϕ s consisting in an upregulation of CCR2, CXCR4, CD206, CD43 and CD80 expression compared to the sham group. Interestingly, when compared to control, LPS^{low} stimulation prior to the endometriosis surgery induced a significant reduction in the expression of CCR2, CXCR4, CD80, CD43 and CD206 on peritoneal M ϕ s while BCG training enhanced their expression compared to PBS-mice (Figure 2D).

We then analyzed the ability of peritoneal M ϕ s to secrete soluble factors (Figure 2E-F) and showed that endometriosis itself was associated with an increased production of IL-6, TNF- α , IL-10, IL-18, CCL2, CCL3, CCL5 and CXCL1 by peritoneal M ϕ s compared to sham mice. LPS^{low}-EDT mice had a reduced IL-6, TNF- α , CCL2, CCL3, CCL5 and CXCL1 production while IL-10 release was increased compared to control. The EDT-BCG group had a significant increase in IL-6, TNF- α , CCL2 and CCL5 compared to the control.

At the systemic level, endometriosis in EDT-PBS mice was associated with an increase in the sera of IL-13 and TGF- β compared to sham mice. LPS^{low} *in vivo* stimulation reduced the systemic levels of these two cytokines. EDT-BCG mice had an only increased seric TGF- β level (Figure 2G).

Lesions in PBS-EDT mice were associated with an 8-fold and 4-fold increase of *colla1* and *cox-2* mRNA expression reflecting an enhanced fibrosis and active inflammation. LPS^{low}

training induced a significant reduction of *coll1a1* and *cox-2* mRNA expression in the implants' samples compared to the control while BCG training had no effect (Figure 2H).

Thus, *in vivo* training of mice with LPS^{low} prior to the surgical implants of the uterine horns hampered the peritoneal inflammation associated with the endometriotic lesions and reduced their growth and activity while BCG training worsened them. These data support the modulatory effect of *in vivo* immune training on endometriosis development through the modifications of peritoneal Mφs' phenotype, local and systemic cytokine production.

***In vitro* adoptive transfer of LPS^{low}-macrophages reduces endometriotic lesions' growth**

To ascertain the role of Mφs training in the course of endometriotic lesions development, we have assessed the effects of weekly *in vitro* trained BMDM injections in EDT-mice (Figure 3A). As shown in Figure 3B-C, peritoneal injections of PBS-Mφs did not affect the implant's volume and weight as well as the kinetic of implants' growth in EDT-mice (control group) (Figure 3D). Interestingly, weekly injections of LPS^{low}-Mφs reduced the volume (Figure 3C), the weight (Figure 3B) and the slope of lesion growth of the implants (Figure 3D). Analysis of the peritoneal Mφs and their cytokine production capacity showed that LPS^{low}-Mφs injection decreased CCR2, CXCR4 and CD206 expression and diminished IL-6 and TNF-α release in contrast to IL-10 production that was enhanced (Figure 3E-F). Of note, treatment with PBS-Mφs had no effect on neither the assessed Mφ markers nor cytokine production. These results further confirm that LPS^{low}-Mφs are effective to alleviate the endometriotic lesions' growth with a notable impact on the peritoneal inflammatory environment during endometriosis.

Co-culture of trained macrophages to human endometriotic (EDT) cells

To characterize the relevance of our findings in human endometriosis for further therapeutic purposes, we evaluated the impact of Mφs training on inflammation and fibrosis in a co-culture model using different cell-to-cell combinations. Ectopic EDT cells have spontaneously increased IL-6 and CCL2 secretion when compared to eutopic endometrial cells from non-EDT controls, while IL-10 level in the supernatant remained unchanged (Figure 4A-C). Addition of PBS-Mφs did not affect the levels of the assessed cytokines. Interestingly, addition of LPS^{low}-Mφs decreased IL-6 and CCL2 concentration in the supernatant while BCG Mφs increased their levels (Figure 4A-B). IL-10 secretion was strikingly elevated in the co-culture of EDT cells and LPS^{low}-Mφs (Figure 4C) showing that EDT cells acted as a trigger on LPS^{low}-Mφs and induced an important release of IL-10 in the supernatant. Of note, basal cytokine secretion of non-stimulated trained Mφs cultured alone did not show any difference for all assessed markers (Figure S4A).

EDT cells had a higher mRNA expression of genes associated with fibrosis (*Coll1a1*), cellular adhesion (*cd44*, *icam1*, *cd29*) and inflammation (*cox2*) compared to eutopic endometrial cells (Figure 4D). Co-culture of LPS^{low}-Mφs with EDT cells was effective in reducing the mRNA expression of all these assessed genes. BCG-Mφs only increased the *in vitro* level of *cd44* and *Icam-1* (16-fold change). Human eutopic endometrial cells from HC co-cultured with trained Mφs did not show any differences in *Cox-2*, *Coll1a1*, *Cd29* and *Cd44* genes except for

Icam-1 that was upregulated in the BCG-M ϕ s–eutopic endometrial cells co-culture but to a lesser extent than that observed in the co-culture with the EDT cells (7 fold change).

Taken together, these data suggest that adding LPS^{low}-M ϕ s could dampen the fibro-inflammatory phenotype of diseased endometriotic cells *in vitro* paving the way for the use of trained M ϕ s-based cellular therapy for this condition in humans.

LPS^{low}-tolerized macrophages modulate endometriotic cells' phenotype in an IL-10 dependent manner

The LPS^{low} training *in vivo* and *in vitro* enhances the capacity of M ϕ s to produce the immunoregulatory IL-10 cytokine. In addition, it is established that endometrial cells express constitutively the IL-10 receptor (Vigano et al. 2002). We thus focused on this cytokine to get further into the mechanistic aspect of the immunomodulatory effects of LPS^{low} stimulation *in vivo* and the LPS^{low}-M ϕ s in co-culture with endometriotic cells. We first analyzed the expression of the target genes of IL-10 in the endometrial implants of mice and observed a significant increase of *socs3* and *bcl3* mRNA expression in the implants from EDT-LPS^{low} compared EDT-PBS mice, while BCG training had no effect (Figure 5A). Then, to ascertain the immunomodulatory role of IL-10, we performed a co-culture model using the endometrial RL95 cell line that exhibits enhanced adhesiveness, migration and epithelial to mesenchymal transition properties (Yao et al., 2019). This cell line constitute an interesting *in vitro* tool to study the endometriosis pathophysiology (Au et al., 2015; Jh et al., 2013). The stimulation of the RL95 cells with the recombinant human IL-10 (rh-IL10) cytokine upregulated the phosphorylation of STAT3 (Figure 5B) and significantly increased the *socs3* mRNA expression, while the expression of genes associated with migration (*cxcr4*), epithelial to mesenchymal transition (*oct4*, *snail*, *n-cadherin*) and extracellular matrix deposition (*colla1*) were significantly downregulated (Figure 5C). Similarly, LPS^{low}-M ϕ s co-cultured with the endometrial RL95 increased the STAT3 phosphorylation (Figure 5D) and were efficient to downregulate the assessed gene markers (Figure 5E). Importantly, mRNA IL-10 silencing of the LPS^{low} M ϕ s co-cultured with RL95 cells, as well as the inhibition of pSTAT3, abrogated the effects observed with wild-type LPS^{low} M ϕ s on RL95 genes expression (Figure 5D-E and Figure S5).

Collectively, these data provide an additional mechanistic insight into the immunomodulatory effects of the LPS^{low} M ϕ s on endometriotic cells and highlight the role of IL-10 in this process.

Bacterial Gram-negative infection hampers endometriosis severity in humans

To support our *in vitro* and *in vivo* findings, we explored whether a clinical history of severe gynecological infection requiring hospitalization, depending on the type of germs, can have an impact on the clinical features of endometriosis. As shown in Table S1, the patients from the three groups were matched for age, weight and body mass index. As expected, infected patients (either with Gram-positive –GP- or Gram-negative –GN- bacteria) had a lower level of hemoglobin and elevated levels of white blood cells and CRP. Strikingly, patients with history of severe gynecological GN bacterial infections had a shortened duration of infertility compared to

uninfected patients. Patients from the GP infection group reported more pelvic pain compared to the “No infection” and GN groups, while GN-infected patients tended to show lower pelvic pain compared to the “No infection” group, though not significant. Importantly, compared to the “No infection” group, the frequency of ovarian endometrioma were significantly higher in the GP group while it was decreased in the GN group. Furthermore, the number of endometriotic lesions (including pelvis, digestive and urinary tracts) was significantly lower in GN-infected patients compared to uninfected patients and higher in the GP group. These results indicate that acute GN sepsis in patients with endometriosis is associated with an alleviated gynecological symptomatology and less severe endometriotic lesions while GP infection may be associated with more severe endometriotic symptomatology.

Discussion

Endometriosis is an inflammatory disorder with a dysregulated innate and adaptive immune response that inappropriately reacts with ectopic endometrial debris (Ahn et al., 2015). Here, the concept of innate immune memory using two opposite methods of M ϕ training (LPS^{low} and BCG) has been tested in a mice model of endometriosis and in a human cellular co-culture model to evaluate its immunomodulatory properties on the outcome of the disease.

The main finding of this study was to show the immunomodulatory effects of innate immune memory on endometriotic lesions growth consistent with obvious changes of peritoneal M ϕ s' phenotype and cytokine production. M ϕ s are involved in endometriosis as they drive inflammation, angiogenesis and cellular growth (Symons et al., 2018). Increased frequency of M ϕ s in the endometriotic tissue and peritoneal fluid from women with endometriosis has been reported (Berbic et al., 2009) and this invasion is correlated with the early onset and the disease activity (Khan et al., 2004). The genesis of endometriotic lesions is associated with an altered peritoneal immune environment characterized by a heightened inflammation and subsequent fibrogenesis in severe forms of endometriosis (Vigano et al., 2018). In addition, peritoneal immune cells and M ϕ s produce higher levels of pro-inflammatory cytokines in women with endometriosis such as TNF- α , IL-6 and chemokines such as CCL2, CCL3 and CCL5 that favors the macrophages recruitment and angiogenesis (Gazvani and Templeton, 2002; Jørgensen et al., 2017; Wang et al., 2010). Ectopic lesions in the peritoneal cavity are a potent trigger of the immune system and induce a subsequent inflammation that ultimately tend to support the survival and growth of ectopic endometrial tissue fragments (Lebovic et al., 2001). Here, peritoneal M ϕ s from EDT-LPS^{low} mice show a hyporesponsive phenotype but an increased release of the immunosuppressive IL-10 cytokine. This anergic status of the M ϕ s at the very early stage of the disease may have broken down the pathophysiological process leading to a less severe clinical presentation of endometriosis in our model. On the contrary, *in vivo* BCG training induced a heightened inflammation in the peritoneal cavity and accelerated growth of the endometriotic lesions.

Endometriotic tissue exhibits an aberrant expression of the aromatase enzyme (Kyama et al., 2008), which is responsible for an upregulation of estrogen synthesis that exerts an autocrine

effect and promotes the growth and invasiveness of the ectopic lesions. The aromatase expression is upregulated by some proinflammatory cytokines such as IL-6 (Che et al., 2014) and TNF- α (Zhao et al., 1996) while IL-10 has been shown to suppress it (Martínez-Chacón et al., 2018). Importantly, it has been shown that sex hormones do not influence BCG-induced trained immunity (de Bree et al., 2018). However, given the potential interaction between the sex hormones and the cytokine production, further studies should explore the role of estrogen in the context of endometriosis and innate immune memory.

In endometriosis, the role of M ϕ M1/M2 polarization (Italiani and Boraschi, 2014) is highly discussed. In some studies, M2 M ϕ s are found in elevated numbers in endometriotic lesions and peritoneal fluid from women with endometriosis and have been shown to promote lesion growth (Bacci et al., 2009; Nie et al., 2018), while others did not show any difference (Itoh et al., 2013). Conversely, a study showed that the M1 M ϕ subtype was predominant in the eutopic endometrium of endometriosis women compared to healthy controls (Takebayashi et al., 2015). The dualistic concept of M ϕ polarization has been recently challenged, as it may not represent the complex *in vivo* immune environment, and different subsets of M2 M ϕ s with contradictory functions have been described (Martinez and Gordon, 2014). Moreover, the emergence of the concept of innate immune memory drastically changed our understanding of M ϕ s' physiology as memory M ϕ s have been shown to express a "mixed" M1 and M2 phenotype which contests a simple shift of M ϕ differentiation (Quintin et al., 2012).

Intraperitoneal injections of *ex vivo* LPS^{low}-M ϕ s alleviated the endometriotic lesions' growth and affected the immune peritoneal environment by reducing the CXCR4, CCR2 and CD206 expression. The CXCR4-CXCL12 axis has been shown to play a role in the pathogenesis of endometriosis (Leconte et al., 2014). The CCL2 chemokine is increased in the peritoneal fluid of women with endometriosis (Kalu et al., 2007), correlated with disease severity (Arici et al., 1997) and is involved in the survival and the invasiveness of endometrial stromal cells through the Akt and MAPK/Erk1/2 signal pathways (Li et al., 2012). So far, only one study has performed an intraperitoneal injection of M ϕ s in an experimental model of endometriosis, showing that the adoptive transfer of M2 M ϕ s enhanced endometriotic growth in mice while M1 M ϕ s protected mice from endometriosis (Bacci et al., 2009). This model using the overinterpreted M1/M2 paradigm is in contrast with our findings. Injection of LPS^{low}-M ϕ s, reduce the lesions growth, and decrease the IL-6 and TNF- α production in the peritoneum. In line with our results, endometrial and peritoneal TNF- α expression is reported to be increased in women with endometriosis with a positive correlation between its level and lesion size in early stages of the disease, highlighting its role when the lesions are establishing (Cheong et al., 2002; Harada et al., 1997).

To confirm the immunomodulatory properties of memory M ϕ s in human, we showed that *ex vivo* LPS^{low} human M ϕ s modify the fibro-inflammatory phenotype of diseased EDT cells *in vitro* by downregulating the gene expression of the inflammation marker *cox2*, the adhesion molecules *cd29*, *cd44*, *icam-1* as well as the fibrotic marker *colla1*. Cox-2 is overexpressed in ectopic endometrial tissue and plays a role in maintaining lesion activity and growth (Santulli et

al., 2014). The pro-fibrotic nature of endometriosis has been clearly established (Vigano et al., 2018) with the presence in high frequency of *Coll1a1* producing myofibroblasts in peritoneal lesions and deep infiltrating endometriosis (van Kaam et al., 2008). An elevation of CD29 expression in monocytes is correlated with disease severity in EDT patients (Gebel et al., 1995). The hyaluronic receptor CD44 molecule plays an important role in cellular adhesion and T cells activation (Senbanjo and Chellaiah, 2017). Its increased expression in eutopic endometrium is correlated with a higher risk of EDT development (Pazhohan et al., 2018). *Cd44*^{-/-} mice were shown to have an impaired development of early endometriotic lesions (Knudtson et al., 2016). The intercellular adhesion molecule-1 (ICAM1) that favors cellular adhesion and leukocyte migration is also involved in the pathogenesis of EDT (Pabalan et al., 2015). Thus, the *in vitro* immunomodulatory effects of the memory Mφs on the expression of molecules associated with endometriosis progression and severity consolidates the possibility of designing therapeutic tools that could be effective to reduce the severity of lesions in patients.

In our study, we show an enhanced capacity of IL-10 production by peritoneal Mφs in LPS^{low} mice, upon adoptive transfer of LPS^{low}-Mφs and in the supernatant of EDT cells co-cultured with LPS^{low} Mφs. In endometriosis, the role of IL-10 is highly debated. IL-10 is mainly secreted by Mφs, dendritic cells, T cells and has immunosuppressive properties as it inhibits the production of pro-inflammatory cytokines and the antigen presentation (Moore et al., 2001). Controversial data exist on IL-10 in endometriosis patients: an increased level of IL-10 has been described in the peritoneum of women with endometriosis compared to controls (Suen et al., 2014) while other authors have concluded that the “low producer of IL-10” ACC/ACC genotype was associated with a higher rate of endometriosis (Riiskjaer et al., 2011). Here, we show that LPS^{low}-Mφs, modulate the *in vivo* growth of the endometriotic implants as well as the endometriotic cells’ phenotype in an IL-10-dependent manner by increasing STAT3 phosphorylation and decreasing genes involved in cellular adhesion and migration, epithelial to mesenchymal transition and fibrosis (Reitamo et al., 1994; Yamamoto et al., 2001; Yamamoto-Tabata et al., 2004).

Our retrospective clinical study of the relationship between severe gynecological infections and endometriosis severity consolidates our *in vitro* model as it shows that a history of severe GN infection is associated an alleviated EDT symptomatology, in opposition to patients with GP infections history. Indeed, mice simulation with LPS derived from the GN *E.Coli* induced an immunoparalysis, dampened EDT severity while training mice with the GP BCG was immunostimulatory, and aggravated EDT. This dichotomy can be explained at a molecular level. The TLR4-CD14 complex is the main receptor on Mφs that recognize the LPS from GN bacteria (Elson et al., 2007) while TLR2-deficient Mφs are hyporesponsive to GP bacterial infections (Takeuchi et al., 1999). Repeated or chronic exposure of Mφs to endotoxin through TLR4 has been shown to be the dominant pathway to induce innate tolerance compared to the stimulations of alternative TLRs (Butcher et al., 2018). By contrast, innate immune reprogramming of Mφs through TLR2 induces a hyper-inflammatory response as reflected by an enhanced resistance to

Leptospirosis (Santecchia et al., 2019), an increased production of type I interferon (Perkins et al., 2013) and a M1-like phenotype switch (Silva et al., 2017).

So far, innate immune memory was mainly used to explore therapeutic strategies in the field of infection diseases and vaccine development. Our findings indicate that targeting immune dysfunction through the manipulation of innate immune memory can constitute a serious therapeutic alternative in endometriosis, where curative treatment is lacking. Moreover, it appears from our clinical observation that bacterial components can train M ϕ s to modulate a chronic inflammatory disease highlighting the role that infections may exert in triggering/worsening or preventing/improving endometriosis and more generally non-infectious inflammatory disorders.

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Figure titles and legends

Figure 1. *In vivo* immune training modulates peritoneal macrophages' phenotype and cytokine production.

(A) Experimental design

(B) Flow cytometry analysis of peritoneal macrophage phenotype on day 7 after training. Data represent the mean fluorescence index (MFI) and SEM from n=6 mice.

(C) Cytokine/chemokine (TNF- α , IL-6, CCL2 and IL-10) production measurement by ELISA in culture supernatant.

(D) Lactate production (mmol/l) of trained peritoneal macrophages pre-incubated with LPS (100 ng/ml).

Each box represents mean \pm SEM of triplicates obtained with cell culture from a single mouse, and results are representative of two separate experiments ANOVA test with Bonferroni correction. M ϕ : Macrophages. NS: Non-significant; *p \leq 0.05; **p \leq 0.01; ***p \leq 0.001. See also Figures S1 and S2.

Figure 2. *In vivo* training of macrophages affects endometriosis development in mice and is accompanied by specific immunological changes.

(A) (a) Macroscopic view of the implants on day 23. (b) Ultrasonography images of peritoneal implants in mice on day 22. (c) Staining with Hematoxylin & Eosin of implants on day 23. (d) Coloration with Sirius Red showing the collagen fibrils deposition in the extracellular matrix of implants at day 23.

(B) Implant weight on day 23 (mg).

(C) Volume evolution of the implants between day 0 (day of the surgery) and day 22 evaluated by ultrasound (mm³).

(D) Flow cytometry analysis of peritoneal macrophage phenotype on day 23 after training.

(E-F) ELISA assessment of cytokine/chemokine production of peritoneal macrophages (pg/ml).

(G) Assessment of IL-13 and TGF- β in sera of mice on day 23. Each box represents mean \pm SEM of duplicates obtained with cell culture from a single mouse (N=8 mice per group at least). ANOVA test with Bonferroni correction.

(H) *cox-2* and *colla1* mRNA expression in the implant lesions of mice on day 23. Unpaired T-test.

M ϕ : Macrophages. NS: Non-significant; *p \leq 0.05; **p \leq 0.01; ***p \leq 0.001. Data represent the mean fluorescence index (MFI) and SEM from at least n=8 mice. See also Figure S3.

Figure 3. *In vitro* adoptive transfer of LPS^{low}-macrophages reduces endometriotic lesions' growth.

(A) Experimental design of the adoptive transfer of trained macrophages in EDT-mice (n=10 in each group).

(B) Implants' weight at day 25 (mg).

(C) Implants' volume at day 25 (mm³).

(D) Volume implant evolution between day 0 and day 22 (mm³) evaluated by ultrasound. Data are mean ± SEM.

(E) Flow cytometry analysis of peritoneal macrophage (CD11b+ F4/80+) phenotype on day 25 after the surgery and the intraperitoneal macrophage injection.

(F) Assessment of cytokine production of peritoneal macrophages.

Data represent the mean fluorescence index (MFI) and SEM obtained from at least n=8 mice.

Figure 4. Co-culture of trained macrophages to human endometriotic cells influences the inflammatory and fibrotic profile of the co-cultured cells.

(A-C) ELISA measurement of IL-6, CCL2 and IL-10 in co-culture supernatant (pg/ml). Data are mean ± SEM of triplicates of each co-culture condition. ANOVA test with Bonferroni correction.

(D) RT-qPCR assessment of *cox2*, *colla1*, *cd29*, *cd44* and *icam1* mRNA expression in cells. Unpaired T-test. See also Figure S4B.

Figure 5. LPS^{low}-tolerized macrophages modulate endometriotic cells' phenotype in an IL-10 dependent manner.

(A) RT-qPCR assessment of *socs3* and *bcl3* mRNA expression in the implants. Unpaired T-test.

(B-C) Endometrial RL-95 cells were treated with 10μM of pSTAT3 Inhibitor V or vehicle (PBS + 1% DMSO) for about 30 minutes prior to human recombinant IL-10 exposure (100 ng/ml). (B) Western blotting analysis of STAT3, pSTAT3 and β-actin expression. (C) RT-qPCR assessment of *cxcr4*, *colla1*, *oct4*, *snail*, *n-cadherin* and *socs3* mRNA expression. Representative experiment of two performed.

(D-E) Differentiated macrophages from the HL-60 cell line were stimulated by either PBS or tolerized by LPS^{low} and subjected to mRNA silencing of IL-10 cytokine with small interfering RNA. Cells were then co-cultured with the RL95 endometrial cells in six-plicates. After 48h of incubation at 37°C 5% CO₂, cells were analyzed by western blotting for the expression of STAT3, pSTAT3 and β-actin (D) and RT-qPCR assessment of *cxcr4*, *colla1*, *oct4*, *snail* and *n-cadherin* mRNA expression (E). Representative experiment of two performed. See also Figure S5.

STAR METHODS

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Frédéric Batteux (Frederic.batteux@aphp.fr).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

The entire original and source data generated in this study paper is available online in Mendeley dataset at this address: Mendeley Data, V2, <http://dx.doi.org/10.17632/htzhskjy78.2>

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

Eight-week-old female BALB/c mice, weighing 15-17g, were purchased from Janvier Laboratory (Le Genest Saint Isle, France). All mice were housed in ventilated cages with free access to food and water and maintained under standard 12-h photoperiod. They were given humane care in compliance with institutional guidelines and all experimental procedures and animal care were approved by the Ethics Committee, Paris Descartes University (CEEA 34 : *Comité d’Ethique en matière d’Expérimentation animale Paris Descartes*: PROJET N° 2016040716219897 – V6 –APAFiS # 7283).

Patients

We performed an observational cross-sectional cohort study from July 2015 to May 2018 using data from a prospective database. For each patient, personal history data were obtained during face-to-face interviews conducted by the doctor, using a highly structured and previously published questionnaire (Chapron et al., 2010). The database consists of clinical and biological data from non-pregnant patients less than 42 years of age who underwent assisted reproductive technology at our institution. Women who did not consent to participate in the study were excluded from this population. Signed informed written consent was obtained from all included patients. All data were fully anonymized before use. The study protocol was approved by the ethics committee of our institution (*Comité Consultatif de Protection des Personnes dans la Recherche Biomedicale de Paris – Cochin* reference no. 05- 2006). The patients’ characteristics can be found in Table S1 and the inclusion strategy is shown in Flow chart S1.

LPS and BCG

LPS was from *E. coli* serotype 0127: B8 (Sigma Aldrich), the BCG vaccine was purchased from Sanofi Pasteur and composed of 0.5 mg of the Brazilian strain (BCG Biomed-Lublin Laboratory).

Cell lines

Human promyelocytic leukemia cell line HL-60 (ATCC® CCL-240) were differentiated into M ϕ s. Endometrial carcinoma RL-95 (ATCC® CRL-1671™) cell line was used to mimic the endometriotic cells behavior *in vitro*.

METHODS DETAILS

***In vivo* innate immune training**

The *in vivo* training experiment was performed with 6 eight-week-old female BALB/c mice per group, weighing 15-17g, as shown in Figure 1A. On day 1 and day 5, the BCG group received a peritoneal injection of BCG (37.5 μ g/kg corresponding to 6x10⁵ CFU per mice per injection; Brazilian strain BCG Biomed-Lublin Laboratory) to induce an enhanced M ϕ response as described previously (Kleinnijenhuis et al., 2012). LPS training consisted of five peritoneal injections from day 1 to day 5 of low doses (0.1 mg/kg) of LPS (Sigma Aldrich) to achieve M ϕ s immunoparalysis as previously described (Zhang et al., 2014). The PBS group (considered as control for the first experiment) was treated with peritoneal injections of vehicle PBS (Gibco, Life technologies) from day 1 to day 5. To determine induction of innate immune memory responses after BCG and LPS stimulation, animals were sacrificed on day 7, after which splenic and peritoneal M ϕ s were isolated for *ex vivo* experiments.

Surgically induced endometriosis in innate immune trained mice

Endometriosis was surgically induced in mice by syngeneic transplantation of uterine tissue as previously described (Marcellin et al., 2017). Fifteen eight-week-old female BALB/c donor mice, weighing 15-17g, provided uterine horns to generate endometriosis-like lesions. The donors received 56 μ g/kg/day 17 β -estradiol (Provames®, Sanofi-Aventis, France) by oral gavage daily for 3 days before sacrifice. Donor mice were sacrificed by cervical dislocation and uterine horns were surgically extracted and transferred into a Petri dish containing 37 °C-warm sterile PBS. The uterine horns were opened longitudinally with microscissors and cut in half, so each uterus provided 4 samples that were prepared for grafting in the peritoneal cavity of recipient mice. 30 eight-week-old female BALB/c recipient mice, weighing 15-17g, were anesthetized with isoflurane and mechanically ventilated. An incision was made on the ventral midline, and half horn fragment was sutured onto the parietal peritoneum with two 7/0 polypropylene stitches (Prolen®, Ethicon, Somerville, NJ), one sample on the right side and another on the left side. In all mice, tissue samples were sutured at identical positions of the abdominal wall to ensure that host tissue sites exhibited a comparable vascularization. The cut was sutured with a 6/0 nylon thread. A postoperative oral gavage of all recipient mice with 56

$\mu\text{g/kg/day}$ 17 β -estradiol (Provames®, Sanofi-Aventis, France) was performed for 3 days after implantation. For more details, see also Figure S3.

For the endometriosis (EDT)-trained immunity model, the operated mice were divided into three groups, with 10 mice each: (1) EDT-PBS group (controls), (2) EDT-LPS^{low} group and EDT-BCG group, as shown in Figure S3. The three groups were subjected to *in vivo* immune training as described in the first experiment prior to surgical induction of endometriosis.

Implants size evaluation by ultrasonography

The endometriotic implants were measured at day 0 (day of the surgery) and day 22 after the surgery through serial ultrasonography as previously described (Santulli et al., 2016) with the Vevo 2100 high-frequency ultrasound imaging system (VisualSonics; Toronto, Canada). The probe has a 40-MHz center frequency (MS550) and an adaptable focal depth. The spatial resolution at the focus is $40 \times 80 \times 80 \mu\text{m}^3$. 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, and an image sequence with two-dimensional axial views of the endometriotic implant was acquired, as the probe was swept from the upper to the lower abdominal wall of the mouse. The implant volume was calculated as described: $\text{TV (mm}^3) = (\text{L} \times \text{W}^2)/2$ (Tomayko and Reynolds, 1989). 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.

Endometriotic implants’ collection

Twenty-three days after implantation, animals were sacrificed by cervical dislocation. Endometriotic implants were surgically removed, weighed and measured using a rule caliper. The right-side implant of each mouse was fixed with 10% formaldehyde for further histological analysis. The left-side implant was frozen in liquid nitrogen for further RNA extraction followed by Reverse Transcription - Quantitative real-time PCR analysis.

Histology

All the right-side mice implants were embedded in paraffin. Multiple 5- μm paraffin sections were prepared and stained with hematoxylin and eosin (H & E) and Sirius Red prior to histological examination with light microscopy. Stained tissue sections were examined histologically by pathologists experienced in endometriosis and blinded to the experiment groups (P.S. and F.B.).

Isolation and stimulation of murine peritoneal M ϕ s

Isolation of peritoneal murine M ϕ s was performed as described previously (Zhang et al., 2008). Briefly, peritoneal cells were retrieved by peritoneal lavage with 10ml of cold PBS containing 2 mmol/L of ethylenediaminetetraacetic acid (EDTA). Monolayers of peritoneal M ϕ s were obtained by seeding $4\text{--}6 \times 10^6$ total peritoneal cells/ml in complete RPMI supplemented with 10% FBS, 2 mM L-glutamine, 1% streptomycin-penicillin, 10 mM HEPES (all from Gibco

Life Technologies) to 6-well tissue culture plates. After incubation of 4 hours at 37°C, non-adherent cells were removed by gently washing twice with warm PBS. Cellular purity was verified by flow cytometry and was typically >90 % of Mφs. To explore the cytokine production of memory Mφs, 10⁶ adherent peritoneal Mφs were stimulated in triplicates for each mouse with 100 ng/ml of LPS for 24h. Non-stimulated peritoneal Mφs obtained from PBS-trained mice were considered to evaluate the basal cytokine production. Supernatant was collected and stored at -80°C for further enzyme-linked immunosorbent assay (ELISA) assessment.

Flow cytometry analysis of peritoneal Mφs

Peritoneal Mφs were selected as described above. Cells suspensions were incubated for 20 min with 10 µg/mL anti-CD16/CD32 antibody (clone 93, BioLegend) for Fc receptor saturation. Cells were then incubated with the appropriate labeled antibody at 4°C for 30 min in the dark in PBS with 2% normal fetal bovine serum (FBS). Flow cytometry was performed using a fluorescence-activated cell sorting (FACS) Fortessa II flow cytometer (BD Biosciences), according to standard techniques. The following monoclonal antibodies were used : F4/80-BV711 (BM8, Catalog#123147, dilution 1/200), Zombie UV (Catalog#423107), CD45-APC-Cy7 (30-F11, Catalog#103115), CD11b-APC/Cy7 (M1/70, Catalog#101226, dilution 1/200), CXCR4-BV421 (L276F12, Catalog#146511, dilution 1/200), CD282-PE Toll-Like receptor 2 (TLR-2) (CB225, Catalog#148603, dilution 1/200), Ly6-C-PerCP/Cy5.5 (HK1.4, Catalog#128011, dilution 1/200), CCR2-PE (SA203G11, Catalog#150609, dilution 1/200) and CD80-APC (16-10A1, Catalog#104714, dilution 1/200) from BioLegend (Ozyme France, 78180 Montigny-le-Bretonneux) and CD11b-BV510 (M1/70, Catalog#562950, dilution 1/200), CD43-BV421 (S7, Catalog#562958, dilution 1/200), CD206-Alexa Fluor 647 (MR5D3, Catalog#565250, dilution 1/200), IA-IE-FITC (MHC-II) (2G9, Catalog#553623, dilution 1/100) from eBiosciences (Thermo Fisher Scientific, Villebon-Sur-Yvette, France). Data were analyzed with FlowJo software (Tree Star, Ashland, OR). Gating strategy for identification of peritoneal macrophages by flow cytometry is shown in Figure S1D.

RNA extraction and reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR)

Total mRNA was extracted from crushed samples or cells with TRIzol reagent (Invitrogen). The RT-qPCR was performed with a QuantiTect SYBR® Green RT-PCR Kit on a LightCycler 480 II instrument (Roche Applied Science, France). The relative fold-change of each target gene (normalized to the reference gene: β-actin for murine samples and GAPDH for human samples) was calculated to the reference group using the formula $-2^{\Delta\Delta C_t}$. Primers used for PCR are listed in Table S2.

Chromatin Immunoprecipitation and quantitative PCR

Adherent splenic Mφs were isolated as previously described (Wang et al., 2013), fixed in 37% methanol-free formaldehyde and the pellet was stored at -80°C. The chromatin preparation and immunoprecipitation were performed as described in the Diagenode user manual (reference

C01010059). Briefly, 300 μ L of chromatin was obtained from 10^6 frozen fixed cells and sonicated with a Bioruptor® Pico, for 10 cycles (30 seconds ON, 30 seconds OFF). The immunoprecipitation was performed with 100 μ L of chromatin with the H3K4me3 antibody and IgG as negative control. A qPCR (using SensiAST™ SYBR® No-ROX Kit from Bioline) and the appropriate primers (chosen in the H3K4me3 mouse ChIP seq peaks from data of Quintin et al (Quintin et al., 2012) was performed on DNA obtained from the decrosslinking of the immunoprecipitate. The primers are listed in Table S2. Concentrations were calculated from a standard curve according to the manufacturer's protocol.

Extraction and immune training of bone marrow derived M ϕ s (BMDM)

Bone marrow cells were collected from femurs and tibias of eight-week-old female BALB/c mice, weighing 15-17g, and differentiated into BMDM using BMDM medium as previously described (Weischenfeldt and Porse, 2008). BMDM medium contains RPMI 1640 medium (from Sigma-Aldrich, Ref #8758) with 20% L929-DMEM conditioned medium cell supernatant (L929 produce GM-CSF required for M ϕ differentiation), 10% heat inactivated FBS, 1% antibiotic/antimycotic, 1% HEPES, and 1% sodium pyruvate (Eurobio Ingen. Les Ulis, France). Floating cells were removed and discarded 48h after the plating. At day 7, cells were washed with warm Ca²⁺/Mg²⁺-free PBS and collected by brief trypsin-EDTA (Gibco) treatment. Cells were spun at 1200 rpm for 7 minutes to form a pellet and then resuspended in complete RPMI (supplemented with 10% FBS, 2 mM L-glutamine, 1% streptomycin-penicillin, 10 mM HEPES, all from Gibco Life Technologies). LPS training was achieved by adding one daily dose of 10 ng/mL (referred as low dose) of LPS from *E. Coli* serotype 0127: B8 (Sigma Aldrich), from day 1 to day 3 with daily changes of culture medium. Control M ϕ s were obtained by daily treatment with 100 μ L of sterile PBS. All cells were incubated at +37°C in an atmosphere containing 5% CO₂. At day 14, cells were collected by brief trypsin-EDTA (Gibco) treatment, counted and used for intraperitoneal injection.

ELISA assays

Assessment of IL-6 (Mouse Ref # 88-7064-88 , Human Ref # 88-7066-88), IL-10 (Mouse Ref # 88-7105-88 , Human Ref # 88-7106-88), IL-13 (Mouse Ref # 88-7137-88), TNF- α (Mouse Ref # 88-7324-88), CCL2 (Mouse Ref # 88-7391-88 , Human Ref # 88-7399-88) and TGF- β (Mouse Ref # 88-8350-88), CCL5 “RANTES” (Mouse Ref # BMS6009INST), IL-18 (Mouse Ref # BMS618-3), CCL3 “MIP-1 α ” (Mouse Ref # 88-56013-22) was performed by specific ELISA kits from eBiosciences (Invitrogen) (Thermo Fisher Scientific, Villebon-Sur-Yvette, France). Assessment of mouse CXCL1/KC (Mouse Ref # DY453-05) was performed by specific ELISA from R&D Systems (Bio-Techne Brands, Rennes France). Concentrations were calculated from a standard curve according to the manufacturer's protocol.

Lactate measurement

Lactate production by memory peritoneal M ϕ s stimulated with inflammatory dose of LPS was quantified using the Cobas® 8000 modular analyzer series (Diagnostics Roche, France).

Intraperitoneal injection of *in vitro*-trained bone marrow-derived Mφs in EDT-mice

A total of 30 eight-week-old female BALB/c mice, weighing 15-17g, underwent surgery-induced endometriosis (day 0) as described above. Mice were then separated into three groups containing 10 mice each and subjected to a total of four intraperitoneal injections at day 1, day 7, day 14 and day 21 of either 200 µl of sterile PBS (Control mice), 2.10⁶ PBS-treated Mφs in 200µl of sterile PBS (EDT+Mφ PBS mice) or 2.10⁶ LPS-treated Mφs in 200 µl of sterile PBS (EDT + MφLPS^{low} mice). An ultrasound was performed at day 0 (day of surgery) and at day 22 of the experiment and mice were sacrificed at day 25 for further analysis.

Human Endometrial cells-Mφs co-culture experiments

Blood samples for Mφ isolation were obtained from healthy human female volunteers in their luteal phase. After PBMC isolation by Ficoll density gradient, cells were washed twice, adjusted to 10⁶ cells per well in a 6-well plate and incubated in complete RPMI medium for 12h at 37°C in 5% CO₂. Adherent monocytes were selected by washing out non-adherent cells with warm PBS. Purity of cells was checked by FACS and was around 94 % (defined as CD14 positive cells). Cells were then counted and trained with PBS, LPS, BCG as described above for murine Mφs. Human ectopic endometriotic cells were obtained from patients with painful endometriosis who underwent surgery for complete removal of the endometriotic lesions. The isolated cells are stromal cells purified from biopsies as described previously (Ngô et al., 2009). The purification of stromal and epithelial cells was confirmed by staining with anti-cytokeratin and anti-vimentin antibodies. Stromal cells are cytokeratin negative and vimentin positive. Eutopic endometrial cells were collected from endometriosis-free patients who underwent benign gynecological surgery (considered as healthy control). Patients and control subjects gave their informed consent for all the biopsies. Samples were immediately diced with scalpels in Collagenase (Sigma Aldrich, Saint Quentin France) and Dispase (Gibco) for 2 hours at 37°C. Cells were rinsed, filtered, and cultured in complete DMEM at 37°C in 5% CO₂. Endometriotic and control endometrial cells were expanded for 3 to 5 days and then seeded in 6-well plates (5x10⁴ cells) with the memory Mφs at a 1:1 ratio. All the co-cultures conditions were performed in triplicates. Cells were incubated for 48h at 37°C in 5% CO₂. Supernatants were collected and stored at -80°C for further cytokines measurement. Cellular pellet was used to perform mRNA extraction for RT-qPCR analysis. We used triplicates each time for the PCR and cytokine assessments. Co-culture experiments were performed and reproduced twice with two different female healthy controls donors for circulating monocytes and two different donors for endometriotic stromal cells.

RL95 cells-HL-60 differentiated Mφs co-culture experiments

The human promyelocytic leukemia cell line HL-60 (ATCC® CCL-240) were differentiated into Mφs as described by Fleck et al (Fleck et al., 2005). Briefly, HL-60 cells were cultured in RPMI medium supplemented with 10% FBS (Gibco), 1% streptomycin-penicillin (Gibco) and 0.8% of N,N-Dimethylformamide (Sigma Aldrich) and incubated at 37°C 5% CO₂ for 6 days in order to achieve complete differentiation into Mφs. The endometrial carcinoma RL-

95 (ATCC® CRL-1671™) cell line was used to mimic the endometriotic cells behavior *in vitro*, as they exhibit potent proliferative, migratory, fibrotic and epithelial to mesenchymal transition properties (Au et al., 2015; Jh et al., 2013). LPS^{low} innate immune memory was induced in the differentiated HL-60 Mφs (either wild type or IL-10 KO) as described above. For co-culture experiments, differentiated HL-60 Mφs (either LPS^{low} wild type, IL-10 KO-LPS^{low} or PBS stimulated Mφs) were seeded at a density of 1.10^6 cells and cultured with 1.10^6 RL-95 cells in 2ml of complete RPMI medium supplemented with 10% FBS, 1% HEPES (Eurobio Scientific, les Ulis, France) and 0.005 mg/ml of insulin (Novorapid 1000UI/10ml, Novo Nordisk France). After 48h of incubation at 37°C 5% CO₂, the supernatant and the cellular pellet were collected and stored at -80°C for further analysis. The different considered conditions were performed in three-plicates or six-plicates according to the condition, and the experiments were carried out twice.

Mφs IL-10 knock out (IL-10 KO) with small interfering RNA (siRNA-IL10)

Differentiated Mφs from HL-60 cells (ATCC® CCL-240) were plated at 1.10^5 cells per well in a 6-well plate in 2ml of antibiotic free normal growth medium supplemented with 10% of FBS (Gibco) for 24 h. The siRNA IL-10 (sc-39634) and control (Scrambled sequence) siRNA (sc-37007) obtained from Santa Cruz Biotechnology were used for to obtain IL-10 KO Mφs by using siRNA transfection medium (sc-36868) and siRNA transfection reagent (sc-29528) (Santa Cruz Biotechnology) according to the manufacturer's instructions. The impact of siRNA IL-10 interference on IL-10 mRNA was evaluated by ELISA and Western Blotting.

***In vitro* stimulation with human recombinant IL-10 and STAT3 inhibition**

The specific STAT3 Inhibitor V, Stattic sc-202818 (Santa Cruz Biotechnology, Catalog # 19983-44-9) was used as described before (Cevey et al., 2019). Stattic solution was resuspended in DMSO to generate a 50mM stock solution and a 1/100 dilution in PBS was performed to obtain a working solution of 500μM. The RL-95 cells were treated with 10μM of the STAT3 Inhibitor V in 2 ml of complete DMEM medium supplemented with FBS 10%, HEPES 1% (Eurobio Scientific, les Ulis, France) and 0.005mg/ml of insulin (Novorapid 1000UI/10ml, Novo Nordisk France) 0.005mg/ml and incubated for 30 minutes at 37°C 5% CO₂. A control vehicle condition was considered with untreated RL-95 cells cultured in complete DMEM medium with PBS/DMSO (Final concentration of DMSO 1%). Then, RL-95 cells were stimulated with the human recombinant IL-10 from BioLegend (Ref #571002, Ozyme France, 78180 Montigny-le-Bretonneux) at a concentration of 100 ng/ml and incubated for 24h at 37°C 5% CO₂. The different conditions were performed in six-plicates and the experiments were carried out twice. Cells were then collected to perform further analysis (Western Blot and RT-qPCR).

Western blotting of Cell Lysates

Cells were lysed in ice-cold RIPA buffer (10 mmol/L Tris-HCl, pH 7.5; NaCl, 150 mmol/L; 1% Triton X-100; 0.1% sodium dodecyl sulfate) supplemented with 25 mmol/L sodium fluoride, anti-protease 1%, and 0.5 mmol/L sodium orthovanadate. Equal amounts of protein (30

µg) were loaded and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Biorad Marnes-la-Coquette, France). After transfer and blocking with 5% of fat-free milk and 0.1% Tween in PBS, nitrocellulose membrane was incubated overnight at 4°C with a 1:100 dilution of a rabbit anti-human STAT3 IgG (Sigma Aldrich, Saint Quentin France), a 1:200 dilution of a mouse anti-human pSTAT3 IgG2b, a 1:200 dilution of a mouse anti-human IL-10 IgG2b (sc-8438 Santa Cruz Biotechnology, Santa Cruz, CA) and a 1/200 mouse anti-β-Actin–Peroxidase antibody (Sigma Aldrich, Saint Quentin France). Specific unconjugated proteins were detected using a 1:10000 dilution of a horseradish peroxidase-conjugated goat anti-rabbit IgG (Life Technologies, Catalog #31490) and visualized by an enhanced chemiluminescence system (Advansta, Diagnostics, France).

Clinical study for the relationship between gynecological infections and endometriosis

For the purpose of this study, among the 267 patients in the database, only 255 women who have given their informed consent and presenting endometriosis were retained. Diagnosis and staging of endometriosis were based on previously published imaging criteria using transvaginal ultrasound (TVUS) (Abrao et al., 2007; Guerriero et al., 2016), magnetic resonance imaging (MRI) (Guerriero et al., 2018; Medeiros et al., 2015; Millischer et al., 2015), or transrectal ultrasonography (Chapron et al., 2004) or, for patients with a history of prior surgery, the diagnosis was also confirmed by histological proof of endometriosis. Data related to the presence of a previous infection were retrospectively collected (i.e. details on the microbiological characterization of the infection).

In this study, three groups were compared: the “patients with Gram-positive (GP) bacterial infections”, the “patients with Gram-negative (GN) bacterial infections” and the “patients without history of gynecological infection (No infection)”. Of note, women with fungal infection that are usually low or mild in severity and localized to the vagina were not reported in the clinical setting as only severe infections were considered.

QUANTIFICATION AND STATISTICAL ANALYSIS

All data were analyzed using GraphPad Prism 5 software (GraphPad Software Inc, California). A one-way analysis of variance (ANOVA) was conducted when more than two groups were compared. When group means were significantly different using the one-way ANOVA, pairwise comparisons were performed using Bonferroni’s post hoc test. Statistical details of experiments can be found in the figure legends. In the figures, the error bars represent the standard error of the mean. A p value of <0.05 was accepted as significant. P values are noted as follow: *p < 0.05, **p < 0.01, ***p < 0.001 and NS, not significant.

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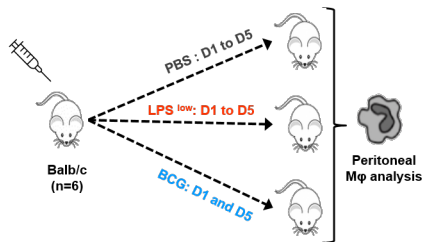
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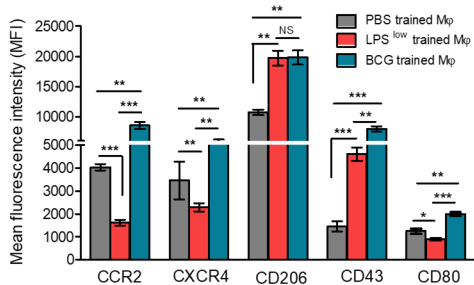
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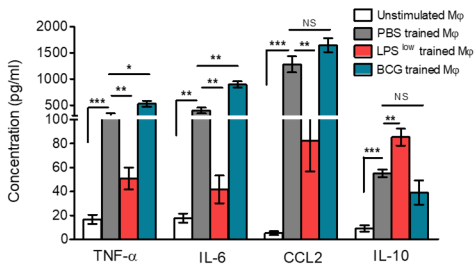
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A**B**

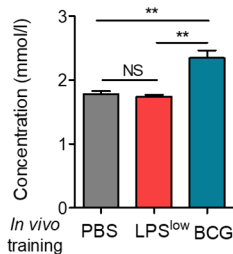
Peritoneal Mφ markers

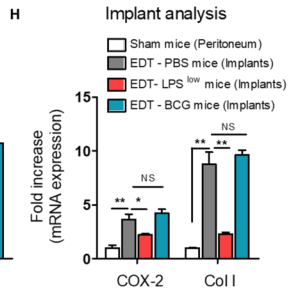
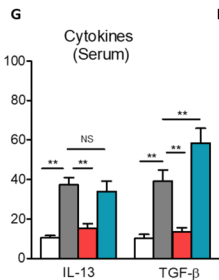
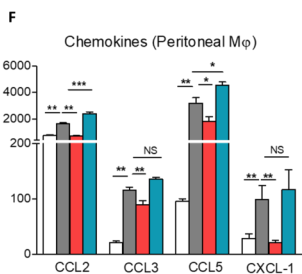
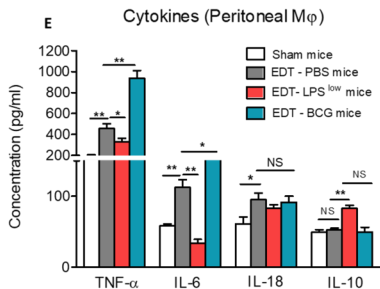
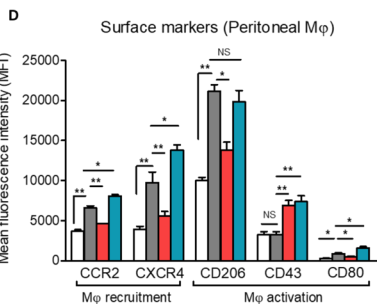
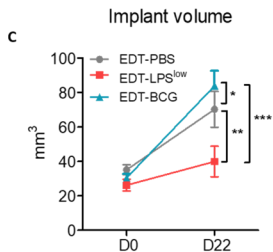
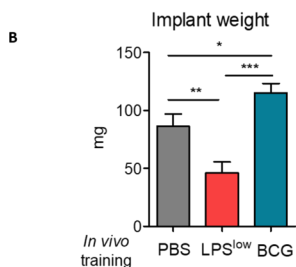
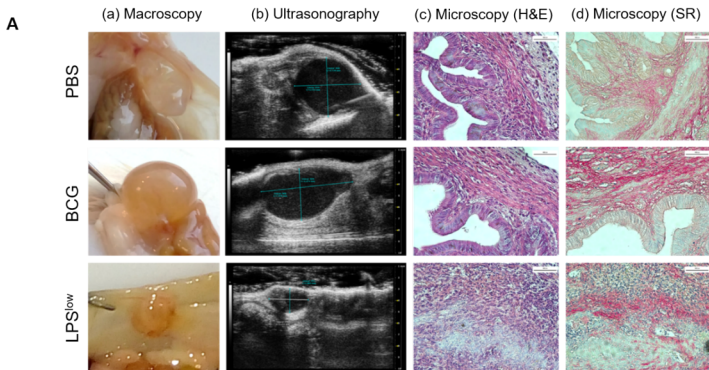
**C**

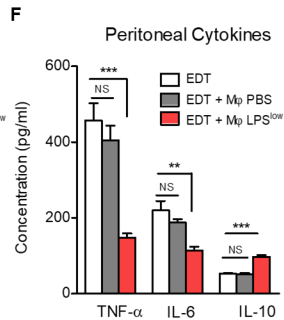
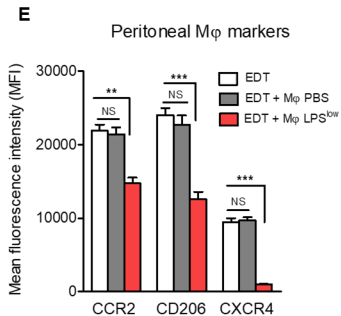
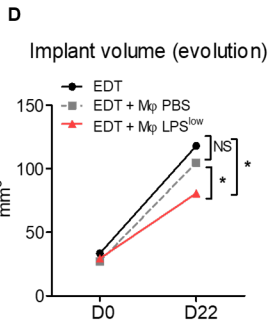
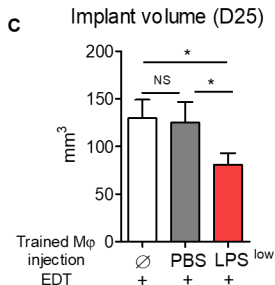
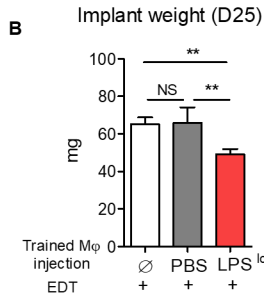
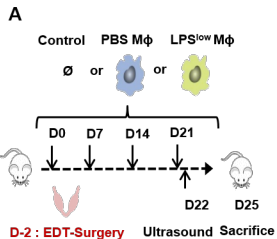
Cytokines produced by peritoneal Mφ

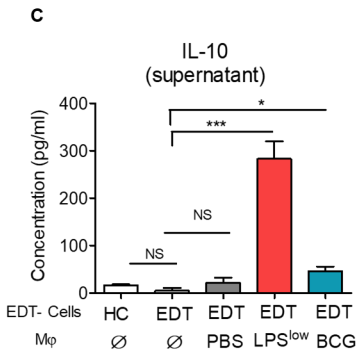
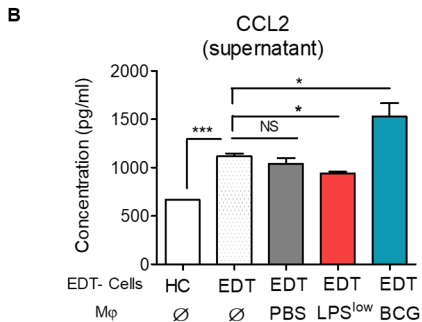
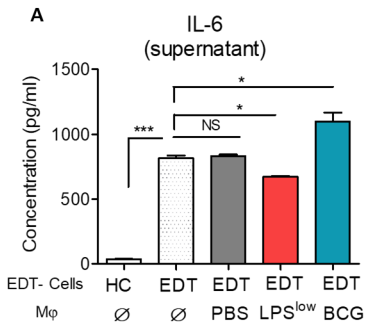
**D**

Lactates

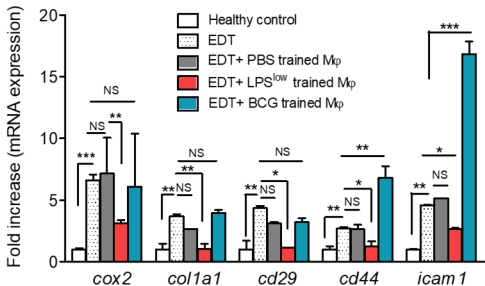




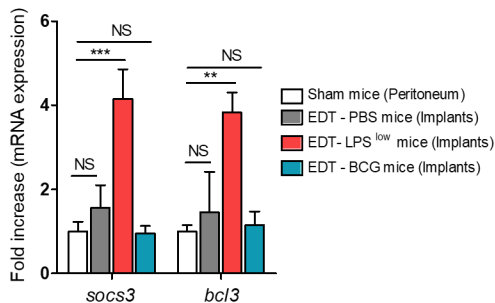




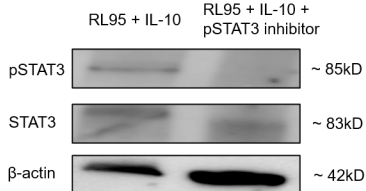
D Markers of human endometriotic cells - trained macrophages co-culture



A Analysis of IL-10 target genes in implants

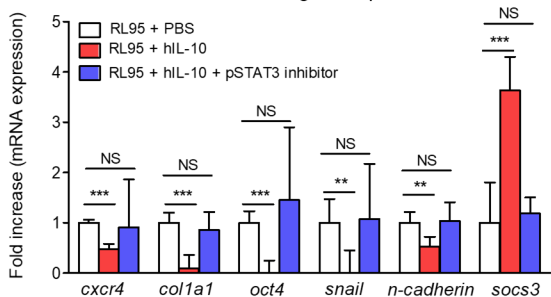


B

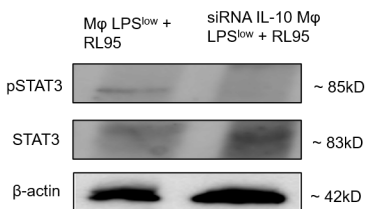


C

Assessment of RL95 endometrial cells gene expression

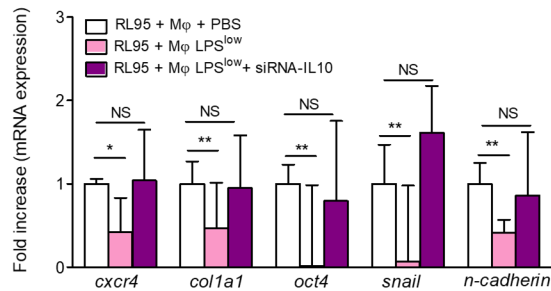


D



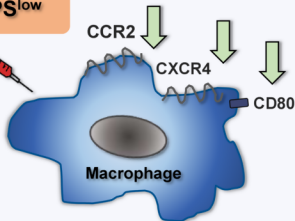
E

Assessment of RL95 - (un)trained Mφ



Innate immune memory

LPS^{low}

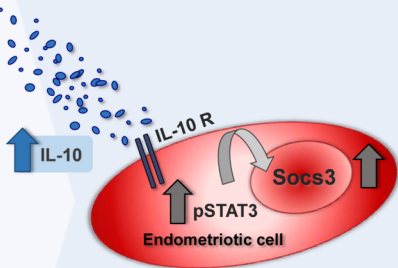


Macrophage

TNF- α CCL3
IL-6 CCL5
CCL2 CXCL10



Endometriosis



Inflammation

COX2

Adhesion

ICAM-1, CD29

Migration

CXCR4

Fibrosis

Type I Collagen

EMT

N-cadherin, oct4, snail