Animal Models

Soluble Human IL-1 Receptor Type 2 Inhibits Ectopic Endometrial Tissue Implantation and Growth

Identification of a Novel Potential Target for Endometriosis Treatment

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Endometriosis is often associated with a chronic pelvic immuno-inflammatory process, which is closely related to disease pathogenesis and major symptoms. Our studies led to the detection of a marked imbalance between IL-1 and its natural inhibitor IL-1 receptor type 2 (IL1R2) in women with endometriosis. This points to a deficiency in the local control of IL-1 that, in view of the cytokine's elevated levels and potent proinflammatory, angiogenic, and growth-promoting effects, may contribute to endometriosis development. Using an *in vivo* model in which human endometrial tissue was inoculated into nude mice and left to establish before any further treatment, our data showed that sIL1R2 interferes with the capability of endometrial tissue to invade, grow, disseminate, and stimulate angiogenesis into the host tissue. sIL1R2 significantly down-regulated the expression of major cell adhesion receptors (α v and β 3 integrins), matrix metalloproteinases (MMP-2 and -9), and vascular endothelial cell growth factor. Interestingly, treatment with sILR2 (5 μ g/kg) led to a concomitant upregulation of matrix metalloproteinases natural inhibitors (TIMP1 and TIMP2) and down-regulation of BclII, a potent anti-apoptotic protein. This creates an imbalance between pro- and anti-proteolytic and apoptotic factors and may further contribute to IL1R2 growth-inhibitory effects. This study pro-

vides evidence that sIL1R2 alters ectopic endometrial tissue growth, remodeling, and survival *in vivo* and may represent an interesting potential therapeutic tool. (Am J Pathol 2012, 181:1197–1205; <http://dx.doi.org/10.1016/j.ajpath.2012.06.022>)

Endometriosis is a serious gynecological pathologic condition and a major public health concern in view of its physical, psychological, and socioeconomic impacts. This disease affects 6% to 10% of reproductive-aged women, 50% to 60% of teenage girls with pelvic pain, and up to 50% of women with infertility.^{1,2} Endometriosis is estrogen dependent; however, several risk factors are thought to contribute to the pathogenesis of the disease. Abundant menstrual reflux and early menarche,³ genetic and environmental factors,^{4–7} hormonal dysfunctions,⁸ and impaired immune response have been suggested.^{9,10} The most predominant hypothesis defines endometriosis as an abnormal implantation of endometrial tissue outside the uterus, mainly in the peritoneal cavity.¹¹ This is supported by a growing body of evidence corroborating our first data and showing the intrinsic capability of endometrial tissue to invade, implant, and grow into the host tissue, and a significant contribution of immune factors to the development of endometriosis and its major clinical symptoms.^{10,12} Endometriosis is often associated with a chronic pelvic immunoinflammatory reaction.¹ Autoantibodies nonspecific or specific to endometrial antigens have been found in the peritoneal fluid of patients along with an increased recruitment of proinflam-

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matory cells, especially activated macrophages, and higher levels of reactive oxygen species, proteolysis enzymes, prostaglandins, and proinflammatory cytokines.^{10,13–17}

IL-1 is a major proinflammatory cytokine that exerts its biological effects via the IL-1 receptor type 1 (IL1R1), the signaling receptor. IL-1 is regulated by IL-1 receptor antagonist (IL1RA) by competing for binding to IL1R1. This control system is consolidated by another potent, specific, and natural IL-1 inhibitor, IL-1 receptor type 2 (IL1R2). This decoy receptor has no signaling properties. It instead counterregulates IL-1 signaling by sequestering active and inactive IL-1, thereby restricting the cytokine's bioavailability and effects. Interestingly, both the membrane-bound receptor and the soluble form (sIL1R2), which results from a proteolytic cleavage form IL1R2 extracellular domain, can bind to and capture IL-1.^{18–20}

Our and other recent studies have led to the detection of an imbalance between IL-1 and sIL1R2 in the peritoneal fluid of patients with endometriosis, where a significant decrease in the levels of IL1R2 and a concomitant increase in those of IL-1 were noted.^{13,21} This was quite obvious in initial endometriosis stages and is consistent with the marked down-regulation of IL1R2 protein and mRNA expression in the earliest and most active endometriotic lesions.^{22,23} These findings point to endometriosis-associated major defects in local IL-1 counterregulatory mechanisms, which, considering IL-1 proinflammatory, remodeling, angiogenic, and growth-promoting effects, may play an important role in the establishment and development of endometriosis.

Based on the above-reported evidence, we undertook an *in vivo* study to investigate the effects of an intraperitoneal administration of sIL1R2 on endometriosis progression using a murine model of endometriosis.

Materials and Methods

Subjects and Tissue Handling

Women who were included in this study provided informed consent for a research protocol approved by Saint-François d'Assise Hospital ethics committee. Endometrial tissue was collected using a Pipelle de Cornier (Prodimed, Neuilly-en-Thelle, France) during the proliferative phase (day 2 to day 13) of the menstrual cycle from 10 healthy, normally cycling women (mean $\pm$ SD age, 36.2 ± 5.2 years) who were undergoing laparoscopy for benign conditions, did not have endometriosis, and were not receiving anti-inflammatory or hormonal medication for at least 3 months before surgery. A sample of each tissue was kept at -80°C for histological confirmation of the menstrual phase.²⁴ The remaining tissue, destined for the *in vivo* studies, was immediately washed in PBS, dissected into small (1 mm^3) cubes, and labeled with a fluorescent tracker, carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE) (8.10^{-6} M in PBS) (Invitrogen, Burlington, ON, Canada).²⁵ Tissue labeling was confirmed by fluorescence stereomicroscopy.

Establishment of Endometrial Tissue Implants in Nude Mice

The protocol was approved by the committee of animal protection of Laval University. Five- to 7-week-old female athymic Nude-Foxn1nu mice (Harlan Laboratories, Indianapolis, IN) were used after a 1-week acclimatization period. Mice were housed under laminar-flow filtered hoods at 28°C with a 12:12-hour light-dark cycle. Housing materials, food, and water were sterilized before use. Animals were given buprenorphine (1.68 g per mouse) by intradermal injection for analgesia, then anesthetized with a mixture of oxygen (1.5:1) and isoflurane (3% to 4%) (Abbot Laboratories, Saint-Laurent, Quebec, Canada). A small (1 cm) cutaneous and peritoneal incision was made in a sterile environment, and 0.1 mL of PBS containing 13 CFDA-labeled endometrial tissue fragments (Figure 1, A and B) was injected into the peritoneal cavity using a micropipette. The incision was closed with Coated NB (polyglactin 910) sutures (Ethicon Johnson & Johnson, Markham, ON, Canada) for the peritoneal tissue and MikRon autoclip 9 mm (Clay Adam Brand, Sparks, MD) for the cutaneous tissue. Implanted mice were monitored daily for comfort, survival, and weight. Ten biopsy samples from 10 different patients were used. Each sample was used to inoculate one control mouse (treated with PBS) and one mouse treated with one of the sIL1R2 doses.

Treatment of Mice with sIL1R2 and Quantification of Lesions

Twelve days after tissue injection, animals received a daily intraperitoneal injection of 100 μL of sterile PBS solution (control group, $n = 10$) or containing 5 or 25 $\mu\text{g/kg}$ sIL1R2 ($n = 5$ for each group) (R&D Systems, Inc., Minneapolis, MN) and were monitored for survival and weight. Two weeks later, mice were anesthetized with isoflurane/ O_2 mixture, then euthanized in an atmosphere saturated by CO_2 (10 L/min), and the abdominal cavity was examined under a fluorescence stereomicroscope connected to a color CCD HD camera (DAGE-MTI, Michigan City, IN). A detectable signal depends on the brightness of the fluorophore and tissue penetration of both the excitation and emission light. Fluorescence of CFDA SE was detected at $\chi_{\text{ex}465}/\chi_{\text{em}535}$.²⁶ Using these excitation/emission wavelengths and fluorescence filters, labeled tissue was detected without noticeable tissue autofluorescence or background noise. Lesions (Figure 1, C and D) were numbered, measured, and photographed *in situ* before being collected and stored at -80°C for histological studies using H&E staining, CD10, and cytokeratin immunostaining, and quantitative real-time (qPCR) analyses.

Vimentin and Cytokeratin Immunostaining

Tissue cryosections ($5\text{ }\mu\text{m}$) were rinsed in cold PBS, treated with 3% hydrogen peroxide to block endogenous peroxidase activity, and incubated for 1 hour at room temperature with rabbit polyclonal anti-human cytokeratin

Figure 1. Staining of biopsy samples with CFDA-SE on day 0 (A and B), fluorescent lesion after 26 days postinoculation (C and D), and effect of endometrial tissue inoculation and treatment with shIL1R2 on the survival rate (E and G) and body weight (F and H) of animals. Figure summarizes results obtained from 10 control mice and five mice treated with 5 or 25 μ g/kg shIL1R2. * $P < 0.05$ as compared with control group.

tin 7 antibody (GeneTex, Irvine, CA) [1:100 dilution in PBS/bovine serum albumin (BSA) 3%] and anti-human vimentin (Novus Biologicals, Littleton, CO) (1:100 dilution in PBS/BSA 3%). Sections were then incubated with a peroxidase-conjugated goat anti-rabbit antibody (1:500 dilution in PBS/BSA 3%) (Jackson ImmunoResearch Laboratories, Mississauga, ON, Canada) before being incubated with the peroxidase substrate 3,3'-diaminobenzidine for 5 minutes, rinsed in PBS, counterstained with hematoxylin, and mounted in Mowiol.

RNA Extraction and qPCR

RNA was extracted with Trizol (Invitrogen), and qPCR was performed using an ABI 7000 Thermal Cycler (Applied Biosystems, Foster City, CA). Each PCR reaction contained 2 μ L of reverse transcriptase product, 0.5 μ L of primer (final concentration, 0.1 mM), 12.5 μ L of SYBR Green PCR Master Mix (Invitrogen) containing TaqDNA polymerase buffer, deoxynucleotide triphosphate mix, SYBR green I, $MgCl_2$, and TaqDNA polymerase. Primers were designed with Primer Premier 5 software to cross intron-exon boundaries and specificity to human tissue was verified with Basic Local Alignment Search Tool (BLAST) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Table 1). Samples were tested in duplicate, and, for each reaction, negative controls without RNA or reverse transcriptase, RNA from mouse tissue (negative control), and RNA from endometrial tissue (positive control) were added.

Statistical Analysis

Data related to the number and volume of lesions followed a nonparametric distribution and were analyzed using the *U*-test. Data related to the weight of mice and qPCR followed a Gaussian distribution and were analyzed using the Student's *t*-test (GraphPad Software, San Diego, CA). Differences were considered as statistically significant for $P < 0.05$.

Results

Survival and Body Weight Recovery After Tissue Inoculation and Treatment with shIL1R2

To first ascertain the effect of tissue inoculation before any treatment and that of shIL1R2 on animal health, we monitored animal body weight and survival rate. As shown in Figure 1, E and F, all inoculated animals survived, but had a higher weight during the first 7 days after inoculation ($P < 0.05$). However, no weight difference between inoculated and control mice was then observed until day 12, corresponding to the start of treatment. Similarly, all animals survived after daily injection of shIL1R2 (25 μ g/kg) for 2 weeks or an equal volume of a vehicle solution (PBS) (Figure 1G). However, animals in both groups showed a slight weight loss during the first 3 days, which, in control mice, reached $91.3\% \pm 0.5\%$ at the end of treatment but was fully recovered ($99.4\% \pm 0.8\%$) in shIL1R2-treated mice (Figure 1H).

Endometrial Implants Preserve Typical Endometrial Histomorphology

Endometrial tissue was allowed to implant and establish before starting treatment. Histological assessment of implants showed a well-preserved typical endometrial histomorphology comparable to initial endometrial tissue, with stroma surrounding glandular structures and distinguishable from the underneath murine host tissue (Figure 2, A and B).

Table 1. List of PCR Primers

Gene	Primers	Tm (°C)	GenBank accession no.
<i>Bax</i>	F: 5'-TCAACTGGGGCCGGTGTGTC-3' R: 5'-CCTGGTCTTGGATCCAGCCCAAC-3'	60	NM_004324.3
<i>Bcl2</i>	F: 5'-GGCACACGCCCCATCCAGCC-3' R: 5'-GCCGGGGGCGCCGGGTCT-3'	60	NM_000657.2
<i>GAPDH</i>	F: 5'-CAGGGCTGCTTTTAACCTCTGG-3' R: 5'-TGGGTGAATCATATTGGAACA-3'	60	NM_002046.3
<i>Integrin αv</i>	F: 5'-GGAGCAATTCGACGAGCACT-3' R: 5'-TTCATCCCGCAGATACGCTA-3'	60	NM_001144999.1
<i>Integrin β3</i>	F: 5'-TGACGAAAATACCTGCAACCG-3' R: 5'-GCATCCTTGCCAGTGCTCTTAA-3'	60	NM_000212.2
<i>MMP2</i>	F: 5'-TTGACGGTAAGGACGGACTC-3' R: 5'-ACTTGCACTACTCCCATCG-3'	60	NM_001127891.1
<i>MMP9</i>	F: 5'-TTGACAGCGACAAGAAGTGG-3' R: 5'-CCCTCAGTGAAGCGGTACAT-3'	60	NM_004994.2
<i>TIMP1</i>	F: 5'-AAGGCTCTGAAAAGGGCTTC-3' R: 5'-GAAAGATGGGAGTGGGAACA-3'	60	NM_003254.2
<i>TIMP2</i>	F: 5'-GAGAAGGAAGTGGACTCTGGAAC-3' R: 5'-AAACTCTATATCCTTCTCAGGCC-3'	56	NM_003255.4
<i>TIMP3</i>	F: 5'-ACATGCTCGCCAGCCAC-3' R: 5'-TGCACATGGGCATCTTGG-3'	60	NM_000362.4
<i>VEGF</i>	F: 5'-GCTCTACCTCCACCATGCCA-3' R: 5'-CACCACTTCGTGATGATTCTGC-3'	60	NM_001171630.1

F, forward; R, reverse.

Treatment with shIL1R2 Reduces the Number and Volume of Endometrial Implants

Using this *in vivo* endometriosis model with established endometrial implants, we next studied the impact of shIL1R2 treatment using two different doses (5 and 25 μ g/kg). For each shIL1R2 dose, five mice versus five vehicle-treated mice (controls) were used. In other terms, there were 10 vehicle-treated animals and 5 mice per treatment. It is noteworthy that no significant difference between the control groups was observed, either in the

number (mean $\pm$ SD, 8.0 ± 4.84 and 8.2 ± 2.58) or in the volume (mean $\pm$ SD, 1.89 ± 1.49 and 1.31 ± 0.82) of endometriotic lesions ($P = 0.84$ and $P = 0.11$, respectively). Stereomicroscopic identification of fluorescence-labeled endometrial implants showed a recovery rate of $71.0\% \pm 0.2\%$. Lesions in shIL1R2-treated mice exhibited an obvious decrease in size as compared with control mice (Figure 3A). Statistical analysis showed that shIL1R2 (5 and 25 μ g/kg) exerts a significant inhibitory effect on the development of endometrial implants by decreasing their number ($P < 0.05$) and total volume (6.6- and 4.8-fold reduction, respectively) ($P < 0.001$) (Figure 3, B and C). Histological evaluation further showed disrupted structures in lesions from shIL1R2-treated mice, as compared with lesions from vehicle-treated control mice showing well-defined endometrial glands and stroma surrounded by host mouse tissue (Figure 4, A and B). Immunostaining of vimentin and cytokeratins confirmed the presence of endometrial stromal and glandular cells, respectively (Figure 4, C and E).

shIL1R2 Limits Endometrial Tissue Invasiveness and Dissemination

Analysis of harvested lesions according to the implantation site showed several invaded organs and tissues in control mice, mainly the intestines, the peritoneum, the liver, and fat tissue, whereas shILR2 treatment appeared to increase the resistance of many organs to invasion by human endometrial implants (Figure 5, A–C). Statistical analysis showed that in mice treated with shIL1R2 (5 and 25 μ g/kg), lesion size and number were significantly reduced in these organs and tissues. Furthermore, lesions fully disappeared in the uterus and ovaries (Figure 5, D–I).

Figure 2. Histological examination of human endometrial implants in nude mice 2 weeks after tissue injection. Note the preserved stromal structure surrounding glands in endometrial implants (B), which is comparable to initial endometrial tissue (A) and distinguishable from murine tissue. H&E staining. EG, endometrial gland; MT, mouse tissue. Scale bar = 20 μ m.

Figure 3. Effect of shIL1R2 treatment on number and volume of endometrial lesions. Endometrial implants were observed at sacrifice by fluorescence stereomicroscopy (A) in mice treated with vehicle (left) or shIL1R2 (5 µg/kg) (right). Implants as observed by optic light (upper) or epi-fluorescence (middle). Merged images are shown (lower). Number (B) and volume (C) of implants were determined *in situ*. Figure summarizes results obtained from control ($n = 10$) and shIL1R2-treated mice ($n = 5$ for each shIL1R2 dose). Bars represent mean $\pm$ SD. * $P < 0.05$ and *** $P < 0.001$, as compared with control group.

Impact of shIL1R2 Treatment on Adhesion, Survival, Invasion, and Angiogenesis Factors

The development of endometriosis requires an intricate network of biological processes, including cell adhesion, invasion and angiogenesis, which enable endometrial tissue to implant and grow within the host tissue, as well as the deployment of anti-apoptotic mechanisms that favor its ectopic survival. To investigate whether any significant changes in these biological processes may underlie the shIL1R2-induced diminution in the number, size, and dis-

semination of endometrial implants, the expression of main tissue adhesion, apoptosis, proteolysis, and angiogenesis mediators found to be significantly dysregulated in human endometriotic lesions was assessed.

As shown in Figure 6, A and B, treatment with shIL1R2 (5 and 25 µg/kg) led to a significant and dose-dependent down-regulation of the adhesion receptors integrins αv ($P < 0.05$ and $P < 0.001$, respectively) and $\beta 3$ ($P < 0.05$) in endometrial implants. We then investigated the expression of Bax and Bcl-2, which are two main regulatory proteins of cell apoptosis and survival, respectively. For Bax expression, no statistically significant difference between control lesions and those collected from mice treated with 5 µg/kg shIL1R2 was noted. However, at a higher shIL1R2 dose (25 µg/kg), Bax expression was significantly decreased ($P < 0.01$) (Figure 6C). As for Bcl2, the inhibitory effect of shIL1R2 was dose dependent and statistically significant at both shIL1R2 doses ($P < 0.001$) (Figure 6D).

Matrix metalloproteinases play a critical role in the invasive capacity of endometrial implants and the deployment of a new vessel network. Therefore, we analyzed the expression level of MMP-2 and MMP-9, two MMPs found to be up-regulated in active endometriotic lesions and involved in endometriosis development.^{27–30} The results showed a significant decrease in MMP-2 mRNA levels in mice treated with 5 ($P < 0.01$) and 25 µg/kg ($P < 0.001$) of shIL1R2, compared with control mice (Figure 7A). As for MMP-9 mRNA levels, we noted a significant diminution in endometriotic lesions collected from mice

Figure 4. Histological evaluation of endometrial implants from mice treated with vehicle (A) or shIL1R2 (B) at sacrifice (magnification, $\times 10$). Endometrial stromal and glandular cells were identified using vimentin (C) and cytokeratin (E) immunostaining. Sections incubated without the primary antibody were used as negative control for immunostaining (D) (original magnification, $\times 20$). ck+, cytokeratin-positive immunostaining; EG, endometrial gland; MT, mouse tissue; vm+, vimentin-positive immunostaining.

Figure 5. Effect of shIL1R2 on endometrial tissue invasiveness and dissemination. Endometrial implants were localized using fluorescence stereomicroscopy, then counted and measured *in situ*. Figure summarizes number (A–D, F, and H) and volume (E, G, and I) of lesions per mouse according the implantation site after treatment with vehicle (control) or with 5 or 25 µg/kg shIL1R2. Results are from 10 control mice and five mice for each concentration of shIL1R2. Data are mean ± SD. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, as compared with control group.

treated with 5 ($P < 0.05$) and 25 µg/kg ($P < 0.001$) of shIL1R2 in comparison with control lesions (Figure 7B). Furthermore, as the invasive capacity of endometrial tissue depends on the balance between MMPs and their natural tissue inhibitor TIMPs, we further evaluated, in the same endometriotic lesions, the expression level of TIMPs 1, 2, and 3, known for inhibiting MMP-2 and MMP-9 activities.^{29,30} Data depicted in Figure 7, C–E, show that shIL1R2 had a significant dose-dependent impact on TIMP expression. Actually, treatment of mice with 5 µg/kg of shIL1R2 resulted in a significant up-regulation of TIMP1 ($P < 0.05$) and TIMP2 ($P < 0.05$) mRNA levels, as compared with vehicle-treated mice, whereas no statistically significant effect on TIMP3 mRNA levels was noted. However, treatment with 25 µg/kg of shIL1R2 rather had a down-regulatory effect on TIMPs 1, 2, and 3 expression ($P < 0.01$, $P < 0.01$ and $P < 0.001$, respectively). Furthermore, treatment with shIL1R2 either at 5 or 25 µg/kg significantly inhibited the expression of vascular endothelial cell growth factor (VEGF), a major angiogenic factor markedly expressed in active, early-stage, and highly vascularized endometriotic lesions³¹ ($P < 0.01$) (Figure 7F).

Discussion

This report, for the first time, provides evidence that shIL1R2 can inhibit ectopic endometrial tissue growth and endometriosis progression *in vivo*. Using a nude mouse model of endometriosis,^{32,33} in which human endometrial tissue was allowed to implant and to establish before any exogenous treatment, our data show that administration of shIL1R2 significantly reduced the number, volume, and dissemination of endometrial implants. Our data also show that shIL1R2 down-regulates the expression of integrins αv and $\beta 3$, MMPs 2 and 9, and VEGF and BclII, which are major mediators of adhesion, angiogenesis, tissue remodeling, and cell survival and known to be significantly involved in endometriosis.

These findings are of relevance for endometriosis pathophysiology. The development of endometriotic lesions requires critical steps including attachment of endometrial cells to and invasion of the peritoneal surface, recruitment of inflammatory cells, induction of angiogenesis in the surrounding host tissue, and endometrial cell survival and proliferation.

Integrin receptors play an important role in cell adhesion to the extracellular matrix (ECM). Both αv and $\beta 3$

Figure 6. Real-time PCR analysis of the expression of integrin αv (A), integrin $\beta 3$ (B), Bax (C), and BclII (D) in lesions harvested from mice treated with vehicle (control) or with 5 or 25 µg/kg shIL1R2. mRNA levels were normalized to that of the house-keeping gene GAPDH. Results were from 10 control mice and five mice for each concentration of shIL1R2. Data are mean ± SEM. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, as compared with control group.

Figure 7. Real-time PCR analysis of the expression of MMP-2 (A), MMP-9 (B), TIMP1 (C), TIMP2 (D), TIMP3 (E), and VEGF (F) in lesions harvested from mice treated with vehicle (control) or with 5 or 25 µg/kg shIL1R2. mRNA levels were normalized to that of the house-keeping gene GAPDH. Results were from 10 control mice and five mice for each concentration of shIL1R2. Data are mean ± SEM. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, respectively, as compared with control group.

integrins, which are also markers of active neovascularization,^{34,35} show an increased expression in endometriotic stromal cells.³⁶ Required for ECM degradation during tissue invasion^{10,37} and crucial for sprouting of capillaries from pre-existing vessels, MMPs were found to be overexpressed in active endometriotic lesions.^{27,29,30} This was also reported for VEGF, a key angiogenic factor, which, in addition, showed a high expression level in peritoneal macrophages of women with endometrio-

sis.^{31,38} Such an active tissue remodeling and angiogenesis is coupled with the abnormal ability of endometrial tissue to proliferate and to resist apoptosis. This is supported by the detection of numerous alterations in the apoptotic pathways, including a significant up-regulation of the anti-apoptotic molecule BclII and a significant down-regulation of the pro-apoptotic factor Bax.³⁹ In this study, we have selected some key genes that were previously reported as being involved in endometriosis. These genes are representative only of the intricate network of changes occurring during endometriosis development, leading to abnormal angiogenesis, tissue remodeling, survival, and growth. Indeed, further studies will be required to assess *in situ*, for instance, the impact of shIL1R2 treatment on these processes.

The present study further showed that, although shIL1R2 treatment at 5 µg/kg had a down-regulatory effect on MMP-2 and MMP-9 expression levels, at the same time it displayed an up-regulatory effect on TIMP1 and TIMP2, MMP natural inhibitors.^{29,30} Furthermore, treatment with 5 µg/kg of shIL1R2 significantly down-regulated the expression of BclII, an anti-apoptotic molecule, but had no significant effect on Bax, which is endowed with anti-apoptotic properties. Indeed, assessment of apoptosis as well as MMP proteolytic activity will be required. In our model, endometrial implants were small, particularly those collected from mice treated with shIL1R2, and most of the lesions were used entirely for mRNA extraction. Tissue pooling will likely be necessary for the assessment of MMP proteolytic activity by zymography for instance and surgically induced endometriosis would provide a sufficient amount of tissue for quantitative analyses of expression and activity of proteins. However, our model is advantageous, as we introduced endometrial tissue into the peritoneal cavity of mice and allowed it to grow to mimic human peritoneal endometriosis development. Nevertheless, these results suggest that shIL1R2 treatment acts by altering the balance of pro- and anti-proteolysis molecules in endometriotic implants in the nude mice model and that of pro- and anti-apoptotic factors, which may contribute to the inhibition of endometrial tissue invasiveness, growth, and dissemination. Curiously, shIL1R2 effects were observed at 5 µg/kg, whereas a higher dose (25 µg/kg) instead decreased TIMP2, TIMP3, and Bax mRNA levels. This suggests that shIL1R2 exerts a dose-dependent action *in vivo*, and that the lowest dose used is more appropriate to achieve the expected beneficial effects of shIL1R2 treatment in this experimental model of endometriosis. Endometriosis is an estrogen-dependent disease. However, it is increasingly evident that immune-inflammatory changes occurring within the peritoneal cavity, where endometriosis is essentially found and diagnosed, play a significant role in the major symptoms of disease and the capability of endometrial tissue to implant and to develop endometriosis.^{10,40} IL-1, a major proinflammatory and growth-mediating factor, may represent a key element of the local inflammatory network that develops in the peritoneal implantation site. IL-1 expression levels are increased in peritoneal macrophages and endometriotic lesions, and concentrations are elevated in the peritoneal

fluid^{41–44} together with a concomitant diminution of the concentrations of its natural and potent inhibitor, sIL1R2.^{13,21,22} Such an imbalance in IL-1 counterregulatory mechanisms are also found in the eutopic endometrium and active endometriotic lesions.^{22,45} Mainly secreted by activated monocytes/macrophages, IL-1 plays an important role in the recruitment and activation of proinflammatory cells,⁴⁶ stimulates the adhesion of endometrial stromal cells,^{47–51} induces an angiogenic phenotype in ectopic endometrial cells,⁵² up-regulates MMP activity⁵³ and promotes cell survival.^{54–56} This seems to be due in part to a decrease in the expression of IL1R2^{22,55} and inhibition of apoptosis, particularly via the inhibition of Bax⁵⁷ and the induction of anti-apoptotic Bcl-2 family members.^{57,58}

Nude mice are deficient in T and B cells, but still have the capability to mount a partial immune response because of functional natural killer cells and macrophages⁵⁹ that may be activated by exogenous endometrial cells and that secrete IL-1. Previous studies showed that CD45-positive leukocytes are recruited into endometriotic lesions in nude mice and may be involved in ectopic endometrial tissue growth.⁶⁰ Therefore, one could hypothesize that inhibition of IL-1-mediated leukocyte recruitment and activation may represent one of the possible mechanisms underlying shIL1R2 effects observed in our study. Nevertheless, further studies will also evaluate shIL1R2 effects with endometrial tissue from women with endometriosis, as it differs from normal endometrium.

shIL1R2 has been subject to preclinical studies⁶¹ and evaluated as a potential treatment of degenerative osteoarthritis *in vivo*.⁶² The therapeutic interest of this molecule was confirmed by Jäger et al, who reported a significant decrease in inflammatory osteoclast and odontoclast cell recruitment in rats.⁶³ Therefore, our data demonstrate, for the first time, that shIL1R2 effectively interferes with the maintenance and growth of established endometriosis lesions and significantly reduces endometriotic lesion volume, size, and dissemination rate *in vivo*, and argues in favor of a potential therapeutic interest for this molecule. The current medical treatment of endometriosis, mainly based on the induction of a hypoestrogenic state, is associated with serious adverse effects and a high recurrence rate, and there is a crucial need for nonsteroidal therapeutic agents for more efficient and targeted treatment. Future pilot studies will be necessary to confirm these results in a nonhuman primate model for endometriosis, because of spontaneous endometriosis development,⁶⁴ and ultimately in humans.

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