Macrophages Are Alternatively Activated in Patients with Endometriosis and Required for Growth and Vascularization of Lesions in a Mouse Model of Disease

Monica Bacci,* Annalisa Capobianco,*
Antonella Monno,* Lucia Cottone,*
Francesca Di Puppo,[†] Barbara Camisa,[‡]
Margherita Mariani,[‡] Chiara Brignole,[§]
Mirco Ponzoni,[§] Stefano Ferrari,[†]
Paola Panina-Bordignon,[‡] Angelo A. Manfredi,*
and Patrizia Rovere-Querini*

From the Clinical Immunology and Innate Immunity and Tissue Remodelling Units,* Division of Regenerative Medicine, Stem Cells, and Gene Therapy, and the Department of Gynaecology and Obstetrics,[†] all from the H. San Raffaele Scientific Institute and Vita Salute San Raffaele University, Milano; Biozell Spa,[‡] Milano; and the Laboratory of Oncology,[§] G. Gaslini Children's Hospital, Genova, Italy

The mechanisms that sustain endometrial tissues at ectopic sites in patients with endometriosis are poorly understood. Various leukocytes, including macrophages, infiltrate endometriotic lesions. In this study, we depleted mouse macrophages by means of either clodronate liposomes or monoclonal antibodies before the injection of syngeneic endometrial tissue. In the absence of macrophages, tissue fragments adhered and implanted into the peritoneal wall, but endometriotic lesions failed to organize and develop. When we depleted macrophages after the establishment of endometriotic lesions, blood vessels failed to reach the inner layers of the lesions, which stopped growing. Macrophages from patients with endometriosis and experimental mice, but not nonendometriotic patients who underwent surgery for uterine leiomyomas or control mice, expressed markers of alternative activation. These markers included high levels of scavenger receptors, CD163 and CD206, which are involved in both the scavenging of hemoglobin with iron transfer into macrophages and the silent clearance of inflammatory molecules. Macrophages in both inflammatory liquid and ectopic lesions were equally polarized, suggesting a critical role of environmental cues in the peritoneal cavity.

Adoptively transferred, alternatively activated macrophages dramatically enhanced endometriotic lesion growth in mice. Inflammatory macrophages effectively protected mice from endometriosis. Therefore, endogenous macrophages involved in tissue remodeling appear as players in the natural history of endometriosis, required for effective vascularization and ectopic lesion growth. (Am J Pathol 2009, 175:547–556; DOI: 10.2353/ajpath.2009.081011)

Endometriosis is a common disease, characterized by the persistence and growth of vascularized endometrial tissue at ectopic sites, typically the pelvis, and associated with pelvic pain and infertility.^{1–3} Endometriotic lesions possibly originate from shed endometrial tissue that reaches the abdominal cavity via the Fallopian tubes during menstruation.⁴ This process, referred to as retrograde menstruation, occurs in most women during the fertile years. In women who develop endometriosis, endometrial tissue attaches to the peritoneal wall. The following steps include invasion of the underlying basement membrane and recruitment of novel vessels from the peritoneal vasculature.^{5,6}

The events underlying the susceptibility of menstruating women to endometriosis are obscure. The ability of shed endometrium, to attach and to infiltrate the peritoneum, to survive, and to recruit vessels are possibly limiting steps. Neo-angiogenesis physiologically takes place during wound healing as a consequence of macrophage activation.^{7,8} Macrophages represent a major source of neo-angiogenic signals in solid tumors, which depend on newly formed vessels for their growth and spreading. In solid tumors, macrophages also deliver

Supported by the AIRC, by the MIUR, and by the Ministero della Salute.

Accepted for publication April 28, 2009.

Address reprint requests to Patrizia Rovere-Querini, Istituto Scientifico Ospedale San Raffaele, DIBIT, 3A1, via Olgettina 58, 20132, Milano Italy. E-mail: rovere.patrizia@hsr.it.

signals that limit apoptosis under hypoxic conditions and facilitate local infiltration and metastatic spreading.⁹

Recombinant cytokines or microbial products elicit the *in vitro* differentiation of macrophage precursors toward such a reparative phenotype (also called alternative or M2 activation) or toward inflammatory M1 cells, better suited to fight invading pathogens. The signals that commit precursors in living tissues to polarize toward M1 or M2 macrophages, in conditions of infection, sterile injury, or neoplastic transformation, are only beginning to be elucidated. Interestingly, a heterogeneity in response to innate signals among the population has been demonstrated, suggesting that environmental signals result in diverse patterns of macrophage activation in different subjects.^{10,11}

Differences in macrophage activation in the peritoneal cavity could therefore be involved in the susceptibility/resistance to endometriosis: a “permissive” macrophage activation would facilitate the survival of shed endometrial tissue via the production of trophic and anti-apoptotic signals, facilitate invasion of the mesothelial layer via interference with the balance between metalloproteinase and their inhibitors,¹² and could be later on required for neo-angiogenesis. In the current study, using an experimental mouse model, we verified the hypothesis that macrophage activation is involved in the establishment, survival, and spreading of ectopic lesions from shed endometrium. We finely characterized macrophages in the peritoneal fluid and in established endometriotic lesions, in experimental mice and human, and found that they share evident features of alternative, disease-permissive, activation.

Materials and Methods

Mice

All procedures were performed in the animal facility of H San Raffaele Scientific Institute (Italy) in accordance with European Union guidelines and with the approval of the Institutional Animal Care and Use Committee of our Institution. Eight-week-old female Balb/C mice were purchased from Charles River (Calco, Italy). We used 10 animals per experimental group for each independent experiment.

Model of Endometriosis

Mice were initially treated subcutaneously with estradiol benzoate (3 µg/mouse, Intervet s.r.l., Milan, Italy). Seven days later mice were sacrificed (one donor mouse for every two to be challenged with endometrium), uteri were removed, seeded in a Petri dish containing warmed saline, and split longitudinally with a pair of scissors. Each uterine horn was identically processed, including isolation of endometrial tissue and its careful mechanical disruption in small cell aggregate suspension, of a maximal diameter that was consistently lower than 1 mm, before intraperitoneal injection of an experimental/control mice pair.^{13,14} By this approach each mouse received the

suspension derived from a half uterus. Twelve days after endometrial tissue injection, mice were euthanized individually by cervical dislocation. The abdominal cavity was immediately opened and lesions excised and processed for disease assessment or immunohistochemistry evaluation. The extent of endometriosis was evaluated by assessing the dry weight of all lesions from each mice, as described.^{13,14}

In Vivo Macrophage Depletion

To verify the role of macrophages in the establishment and growth of ectopic endometrial lesions, mice were treated i.p. with the depleting anti-F4/80 Ab (0.80 µg/g/ mice clone Cl: A3-1, SeroTec, Oxford, UK) or PBS, or with liposomes containing either clodronate (Sigma Aldrich, St Louis, MO) or PBS, prepared as described.¹⁵ When indicated, depleting agents were injected intraperitoneally. The anti-F4/80 depleting mAb was injected at day -2, the treatment was repeated at day 0, +2, +4, +6, +8, +10 after uterus transfer. Liposomes containing PBS or clodronate were injected at days -1, 0, +4, and +8 after endometrial tissue transfer. The final clodronate liposome suspension contained 5 mg of clodronate/ml. Both treatments kill monocytes and macrophages, while are not per se otherwise toxic. Control mice were injected i.p. with PBS. To assess the effect of macrophage depletion on ectopic endometrial tissue early survival and adhesion to the peritoneal cavity, depletion was performed only at day 0. To assess the effect of macrophage depletion on already established lesions, the treatment was performed exclusively at days +4, +8, ie, after lesion engraftment. To verify the efficacy of the treatment, blood or peritoneal fluid was retrieved at various times and analyzed by flow cytometry. Samples (30 µl) were incubated for 10 minutes at room temperature with PBS containing 10% fetal calf serum. Phycoerythrin-conjugated anti-F4/80 mAb (clone Cl: A3-1, SeroTec) and/or allophycocyanin-conjugated anti-CD11b mAb (clone M1/70, BD Biosciences) (2 µl/sample) were added for 20 minutes. Red blood cell lysis buffer (0.15 M/L NH₄Cl, 1 mmol/L KHCO₃, and 0.1 mmol/L Na₂EDTA) was added for 10 minutes at room temperature before analysis (FACS Calibur flow cytometer and CellQuest software, BD Biosciences).

Propagation of Polarized Macrophages From Mouse Bone Marrow Precursors

Bone marrow precursors from Balb/C female mice were retrieved and cultured for 7 days in α-MEM (GIBCO, Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Lonza, Basel, Switzerland) and recombinant murine (rm) macrophage-colony-stimulating factor (100 ng/ml), yielding non polarized macrophages (M0). Cells were then further cultured for 48 hours in the presence of rm interferon-γ (50 ng/ml) (PeproTech, Rocky Hill, NJ) to obtain inflammatory/M1 macrophages or with rm macrophage-colony-stimulating factor (10 ng/ml) + rm interleukin (IL)-10 (10 ng/ml) for 96 hours to obtain alternatively activated M2 cells. Polarization was verified by flow

cytometry as described.¹⁶ The secretion of prototypical M1 and M2 cytokines, such as tumor necrosis factor (TNF)- α and IL-10 respectively, was verified by enzyme-linked immunosorbent assay (R&D Systems).

In Vivo Delivery of Polarized Macrophages

In selected experiments undifferentiated (M0), M1, and M2 macrophages generated *in vitro* as above, were injected in the peritoneal cavity of recipients mice at days -2, 0, and +2. At day 0, mice received the intraperitoneal injection of endometrial tissue and were sacrificed at day +12. Lesions were excised and processed for disease assessment or immunohistochemistry evaluation as above.

Macrophages Assessment in the Mouse Peritoneal Liquid

Peritoneal cells were retrieved by peritoneal lavage of treated and untreated animals, and resuspended in cold PBS containing 2 mmol/L EDTA. Cell viability, verified by trypan blue exclusion, was typically >98%. Cells were stained with allophycocyanin-conjugated anti-CD11b Ab (BD, Pharmingen), phycoerythrin-conjugated anti-F4/80 Ab (R&D), or with purified unlabeled rat anti-mouse anti-CD206 Ab and purified antibody rabbit anti-mouse anti-CD163 (SeroTec). Phycoerythrin-conjugated goat anti-rat or fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit Ab were used as second step reagents before analysis by 2- and 4-parametric flow cytometry.

Immunohistochemistry of Murine Lesions

Endometriotic lesions were frozen in liquid N₂-cooled isopentane. Serial 6-mm thick sections were fixed with 4% paraformaldehyde (10 minutes at room temperature) and successively treated with 0.3% H₂O₂ (10 minutes at room temperature) to quench endogenous peroxidase activity. To evaluate macrophage infiltration, tissue sections were incubated in PBS + 5% bovine serum albumin for 1 hour at room temperature and then overnight at 4°C with rat anti-mouse CD68 mAb (2 mg/ml, clone FA-11, SeroTec), CD163 (1 mg/ml, clone M-96, Santa Cruz Biotechnology, Inc, Santa Cruz, CA), and CD206 (1 mg/ml, clone MR5D3, SeroTec). Endothelial cells were identified by staining with anti-mouse CD31 mAb (SeroTec). Primary Abs were revealed using biotin conjugated anti-rat polyclonal IgG (1.5 mg/ml, eBiosciences, San Diego, CA) and R.T.U horseradish peroxidase streptavidin (Vector Laboratories, Burlingame, CA), which was detected using Vector NovaRED substrate kit (Vector Laboratories). Slides were counterstained with hematoxylin and examined under a Nikon Eclipse 55i microscope (Nikon, Tokyo, Japan). Images were captured with Digital Sight DS-5 M digital camera (Nikon) using Lucia G software (Laboratory Imaging, Prague, CZ). Parallel slides in which primary Abs had been omitted were identically processed and used as negative controls.

Patients and Controls

Venous blood, peritoneal liquid, and tissue samples (lesions and disease-free peritoneum) were obtained between September 2006 and June 2007 from 23 patients with patho-

Figure 1. Peritoneal macrophages are required for endometriosis establishment. **A:** Estradiol benzoate-treated female mice were sacrificed and uteri removed and split. Endometrial tissue was isolated and mechanically disrupted before intraperitoneal injection of an experimental/control mice pair. Control mice (open squares) and depleted mice (filled squares) were treated i.p. at day 0, 4, and 8 with liposomes containing PBS (PBS-L) or clodronate (Clo-L) respectively. Lesions were evaluated 12 days after endometrial tissue injection. **B:** The percentage of peritoneal F4/80⁺ and of CD11b⁺ macrophages was evaluated by flow cytometry at different time points (left panels). Right panels represent the quantitative expression of the results obtained at day 12, expressed as percentage of positive cells in mice treated with PBS-L (open columns) or Clo-L (filled columns) respectively. **C:** The dry weight of endometriotic lesions from mice treated with PBS-L or with Clo-L was assessed at day 12. Results are expressed as mean of total weights of lesions from six independent experimental/control mouse pair $\pm$ SEM. **D:** Estradiol benzoate-treated female mice were sacrificed and uteri removed and split. Endometrial tissue was isolated and mechanically disrupted before intraperitoneal injection of experimental/control mice pairs. Mice were treated with PBS (open squares) or F4/80 mAb mice (filled squares) i.p. 2 days before injection of syngeneic endometrial tissue (day -2) and every other day since then (days 0, 2, 4, 6, 8, 10, and 12). Lesions were evaluated at day 12, when mice were sacrificed. **E:** The dry weight of endometriotic lesions from mice treated with PBS (open columns) or with F4/80 mAb (filled columns) was assessed at day 12. Results are expressed as mean of total weights of lesions from six independent experimental/control mouse pair $\pm$ SEM. **F:** The dry weight of endometriotic lesions from mice treated with PBS or with F4/80 mAb was assessed at day 12. Results from each independent experimental/control mouse pair, in which endometrial tissue from a single donor was split and injected in a mouse previously injected with PBS (open columns) or with F4/80 mAb (filled columns). Significantly different from control, * $P < 0.05$ and ** $P < 0.01$.

logically proven endometriosis and 13 matched controls undergoing surgery for uterine leiomyomas, all followed in the Department of Obstetrics & Gynaecology of the H San Raffaele Scientific Institute (Italy). The San Raffaele Ethical Committee approved the study protocol and all subjects provided written informed consent. Patients and controls were menstruating women between 20 and 45 years that did not receive hormonal and/or anti-inflammatory medications for at least 3 months before sample collection. The stage of disease was established according to the revised American Fertility Society (AFS) classification.¹⁷ In our analysis, we considered three stages, mild (AFS stages I–II; $n = 5$), moderate (AFS stage III; $n = 8$), and severe (AFS stage IV; $n = 8$).

Monocyte/Macrophage Assessment in Human Samples

Peripheral blood mononuclear cells were obtained by centrifugation over a ficoll density gradient (Ge Health

Care, Piscataway NJ) and inflammatory cells within the peritoneal fluid were obtained by centrifugation after lysis of red blood cells with ammonium chloride (0.15 M/L NH_4Cl , 1 mmol/L KHCO_3 , 0.1 mmol/L Na_2EDTA). Viable cells, identified by trypan blue exclusion, were consistently $>90\%$. 1 to 1.5×10^5 cells/sample were stained with: Percp-conjugated anti-CD45 and anti-CD14, phycoerythrin-conjugated anti-CD16, and anti-CD163, FITC-conjugated anti-CD206 anti-CD36, anti-CD86, anti-HLA-ABC, anti-HLA-DR mAbs, all from Becton Dickinson (BD Pharmingen, California). When indicated, cells were stained with purified anti-receptor for advanced glycation end products (rabbit polyclonal IgG, Santa Cruz Biotechnology) or with biotinylated anti- $\alpha\text{V}\beta 3$ mAbs (Chemicon International). FITC-conjugated anti-rabbit Ab or FITC-conjugated streptavidin were used as second step reagents. Cells were analyzed by flow cytometry (FACS Calibur, Becton Dickinson California). Analysis was performed using a FACS express version 3 software.

Figure 2. Peritoneal macrophages sustain the growth of established endometriotic lesions. Estradiol benzoate-treated female mice were sacrificed and uteri removed and split. Endometrial tissue was isolated and mechanically disrupted before i.p. injection of an experimental/control mouse pair. Control mice (open squares) and depleted mice (filled squares) were treated i.p. with liposomes containing PBS (PBS-L) or clonazepam (Clo-L) 24 hours before (A) or 4 and 8 days after (B) i.p. injection of syngeneic endometrial tissue. Lesions were evaluated at day 12 day after endometrium injection. The dry weight of endometriotic lesions from mice treated with PBS-L (open columns) or with Clo-L (filled columns) 24 hours before (C) or 4 and 8 days after (E) i.p. injection of syngeneic endometrial tissue was assessed at day 12. Results are expressed as mean of total weights of lesions from six independent experimental/control mouse pair $\pm$ SEM. F4/80⁺ and CD11b⁺ macrophages were identified by flow cytometry in animals treated with PBS-L or Clo-L 24 hours before (D) or 4 and 8 days after (F) i.p. injection of syngeneic endometrial tissue at sacrifice at day 12. Panels report the results expressed as percentage of peritoneal positive cells in mice treated with PBS-L (open columns) or Clo-L (filled columns). **G:** Macrophages (CD68⁺ cells) and endothelial cells (CD31⁺ cells) in the endometriotic lesions obtained from mice treated with PBS-L or with Clo-L were evaluated by immunohistochemistry at day 12. **H:** The treatment effect on vascularization was also quantitatively assessed by counting the number of CD31⁺ cells per field of vision. Results represent the mean $\pm$ SEM of five independent field of vision, as assessed by two independent blinded expert pathologists. Significantly different from control, * $P < 0.05$ and *** $P < 0.001$.

Immunohistochemistry of Human Lesions

Samples were immediately frozen in optimal cutting temperature compound at -80°C . Five- to six-micron thick sections were fixed with 4% paraformaldehyde for 10 minutes at room temperature, before staining with purified mouse anti-human-CD68, anti-CD163 (Dako), anti-CD206 (BD Pharmingen) overnight at room temperature. Biotinylated rat-anti-mouse antibodies were used as second-step reagents. Samples were then processed as described¹⁸ and examined under a Nikon Eclipse 55i microscope (Nikon, Tokyo, Japan). Images were captured with Digital Sight DS-5 M digital camera (Nikon) using Lucia G software (Laboratory Imaging, Prague, CZ). Parallel slides in which primary Ab had been omitted were identically processed and used as negative controls. Infiltration score was calculated independently by two blinded investigators, using grades from 1 to 4: 1, no infiltrating macrophages expressing the given marker; 2, sparse infiltrates, ≤ 5 infiltrating macrophages expressing the given marker per 0.6-mm lesion core; 3, moderate infiltrates, for >5 but ≤ 25 infiltrating macrophages expressing the given marker per 0.6-mm lesion core, and 4, dense infiltrates, for >25 infiltrating macrophages expressing the given marker per 0.6-mm lesion core.

Statistical Analysis

Statistical analysis was performed using bivariate correlation and linear regression analysis for continuous variable and two-tailed Student's *t*-test for unpaired samples with unequal variance to analyze differences between groups. Analysis was performed with SPSS (11.0 Mac OS X Version).

Results

Ectopic Endometrial Lesions Depend on Peritoneal Macrophages for Their Growth and Vascularization

We compared the establishment of ectopic endometrial lesions in Balb/C mice treated with macrophage-depleting liposomes containing clodronate or with control sham liposomes containing PBS. To minimize the risk of artifacts, mice cohorts were divided in pairs (control and experimental), each receiving identical amounts of endometrial cell suspension from a single syngeneic donor (Figure 1, A). At sacrifice, 12 days after intraperitoneal injection of syngeneic endometrial tissue, lesions had developed in all mice. Depletion was effective, since the fraction of F4/80⁺ and CD11b⁺ macrophages abated in the peritoneal cavity of mice treated with liposomes containing clodronate, but not in those treated with liposomes containing PBS (Figure 1B). Endometriotic lesions from macrophage-depleted mice were significantly smaller as reflected by their total weight (Figure 1C). To verify that the result of the treatment was due to macrophage depletion, we injected animals 48 hours before

intraperitoneal injection of endometrial tissue with a different macrophage-depleting agent, ie, the F4/80 mAb. mAb injection was then repeated every 48 hours (Figure 1D). Significantly smaller ectopic lesions developed in mice treated with macrophage-depleting mAb than in animals injected with PBS (Figure 1, E–F).

We then assessed whether macrophages were required for the implant of endometrial tissue on the peritoneal membrane and for the ensuing growth of established ectopic lesions. We depleted macrophages with liposomes containing clodronate 24 hours before injection of endometrial tissue (Figure 2A). Peritoneal F4/80⁺ and CD11b⁺ macrophages had returned to almost normal levels at the time of sacrifice (day 12), indicating that the effect of the treatment was indeed transient (Figure 2D). Lesions number, weight and microscopic architecture were not apparently affected in depleted animals (Figure 2C).

We also injected syngeneic endometrium at day 0 and treated mice with liposomes containing clodronate or PBS at days 4 and 8 (Figure 2B). Only small numbers of F4/80⁺ and CD11b⁺ macrophages were detectable in the peritoneal fluid at day 12, when animals were sacrificed, indicating that the depletion was maintained (Figure 2F). The number of the implanted endometriotic lesions did not differ in treated animals ($4.83 \pm 0.3/\text{mouse}$ and $3.33 \pm 0.71/\text{mouse}$ in control and macrophage-de-

Figure 3. Peritoneal macrophages of mice with established endometriotic lesion express markers of alternative activation. **A:** Peritoneal cells were retrieved at day 12 from mice injected either with PBS (control mice, open symbols) or from mice 12 days after injection of syngeneic endometrial tissue (endometriotic mice, filled symbols), as described in *Materials and Methods*. **Left** plots: CD11b⁺ cells (y axis, red fluorescence) were analyzed by flow cytometry for the expression of markers of alternative activation, such as the CD163 and the CD206 antigens (x axis, green fluorescence). **Right** panels: histograms represent the fraction of peritoneal cells expressing each marker in control (open columns) or endometriotic (filled columns) mice. Results represent the mean $\pm$ SEM of three independent experiments. **B:** Endometriotic lesions were characterized by immunohistochemistry for the expression of CD68, CD163, and CD206 in infiltrating macrophages.

Figure 4. Peritoneal macrophages of endometriotic patients express markers of alternative activation. **A:** Peritoneal cells from endometriotic patients (filled columns) and relevant controls (open columns) were analyzed for the expression of macrophage lineage, activation, and differentiation markers by flow cytometry. The cluster analyzed was defined based on the expression of the CD45 common leukocyte antigen (y axis, red fluorescence) by cells with appropriate side scatter characteristics (x axis, green fluorescence) as illustrated. **B:** % of peritoneal macrophages expressing the indicated markers in endometriotic patients (filled columns) and relevant controls (open columns). Results are expressed as the mean $\pm$ SEM of 23 endometriotic patients and 13 matched controls. **C:** Expression of indicated markers by peritoneal macrophages from endometriotic patients (filled columns) and relevant controls (open columns) as assessed by the associated relative fluorescence intensity (y axis) calculated as described in *Materials and Methods*. Results are expressed as the mean $\pm$ SEM of 23 endometriotic patients and 13 matched controls. Significantly different from control, * $P < 0.05$ and ** $P < 0.01$.

pleted animals respectively), but the total weight of the lesions was significantly lower (Figure 2E). At the microscopic levels, lesions in control mice consisted of dilated glands with columnar or cuboid epithelial endometrial cells, surrounded by narrow stroma layers, with newly formed vessels characterized by CD31⁺ endothelial cells (Figure 2G). CD68⁺ macrophages infiltrated endometriotic lesions (Figure 2G). In contrast, in macrophage-depleted animals, the glandular and stromal architecture was disrupted (Figure 2G), with severely reduced vascularization, as assessed by staining of endothelia with the anti-CD31 mAb (Figure 2, G and H).

Mouse Macrophages in the Peritoneal Fluid and Infiltrating Endometriotic Lesions Express Markers of Alternative Activation

We derived and analyzed by flow cytometry peritoneal macrophages from control and endometriotic mice. The fraction of peritoneal CD11b⁺ macrophages did not differ in the two groups of mice. In mice with endometriosis, we observed a substantial and significant increase in the fraction of peritoneal macrophages that expressed the F4/80 antigen ($96.35 \pm 0.6\%$ of CD11b⁺ cells; $P = 0.002$ vs controls). CD11b⁺ macrophages from the peritoneal liquid of endometriotic mice co-expressed markers of alternative activation, the hemoglobin scavenger receptor, CD163 and the mannose receptor, CD206, which were virtually absent in CD11b⁺ macrophages of control mice (58.41 ± 8.4 of CD163⁺, 56.8 ± 13.8 of CD206⁺ cells; $P = 0.01$ and 0.03 respectively versus controls) (Figure 3, A and B). CD68⁺ macrophages infiltrating endometriotic lesions expressed the CD163 and the CD206 receptors (Figure 3B), while those associated to

the peritoneal tissue of control mice were next to negative (*not shown*).

Macrophages in the Peritoneal Fluid of Endometriotic Patients or Infiltrating Lesions Express Markers of Alternative Activation

To verify the relevance of the results obtained in the mouse model for the human disease, we analyzed peritoneal cells and tissues from 21 consecutive endometriotic patients and 12 matched controls. To unambiguously identify macrophages in the peritoneal fluid, flow cytometric analysis has been performed on the cluster depicted in Figure 4A, in which events with congruent side scatter physical characteristics were selected among cells expressing the CD45 common leukocyte antigen. Cells were then analyzed for the expression of lineage and activation markers, as depicted in Figure 4A for the CD14 receptor. While CD14⁺ peritoneal macrophages were similar in patients and controls, CD16⁺ cells were 50.04 ± 8.6 of macrophages in endometriotic patients and 5.68 ± 3.2 in control subjects ($P < 0.01$); macrophages expressing the CD163 hemoglobin scavenger receptor were 19.04 ± 5.4 in patients and 1.75 ± 0.56 in controls ($P < 0.01$); macrophages expressing the CD206 mannose receptor were 68.82 ± 5.6 in patients and 33.68 ± 7.6 in controls ($P < 0.01$) (Figure 4B). The fraction of peritoneal macrophages from endometriotic patients and control subjects that express the CD86 receptor (Figure 4B) or the MHC class I and II, $\alpha\beta$ 3, CD40, and CD36 molecules was similar (*not shown*), while the difference in the fraction of anti-receptor for advanced glycation end products-expressing macrophages, although significant, was modest (Figure 4B). The level of

expression of each molecule, as assessed by the associated relative fluorescence intensity, was similar in CD14⁺ peritoneal macrophages from patients and controls, with the exception of CD16, whose expression was significantly higher in patients (relative fluorescence intensity was 6.89 ± 2.3 in patients and 2.37 ± 0.66 in controls, $P < 0.05$; Figure 4C). The differences were specifically associated to the peritoneal environment, because we failed to detect any significant difference between the expression of these markers in circulating leukocytes from patients and controls (*not shown*). We did not observe any correlation between the expression of markers of macrophage alternative activation and the clinical stage of the disease.¹⁹ However, only five patients with mild endometriosis (stages I–II) were studied. Data on a larger cohort of patients will be necessary to draw conclusions on the influence of endometriosis stage on this parameter.

The immunohistochemical study confirmed that CD68⁺ macrophages preferentially infiltrate endometriotic lesions, even if they can be detected also in the disease-free peritoneum of patients and controls and in uterine leiomyomas. Endometriotic macrophages only expressed the CD163 and CD206 receptors, which were virtually absent in control tissues (Figure 5A). The difference was statistically significant ($P < 0.05$; Figure 5B).

Opposite Effect of M1 and M2 Macrophages on Endometriotic Lesions Growth

To directly address the role of macrophage activation and polarization *in vivo* we propagated from syngeneic bone marrow precursors either undifferentiated M0 macrophages or cells polarized toward an M1 inflammatory or M2 alternatively activated macrophages (Figure 6A). M0 cells did not secrete significant amounts of cytokines. In contrast M1 macrophages produced significant amounts of TNF- α and negligible amount of IL-10. The opposite was true for M2 macrophages, which secreted IL-10 but not TNF- α (Figure 6B). M0, M1, and M2 macrophages were transferred in the peritoneum of recipient mice 2 days before and at the time of the injection of endometrium. Macrophage transfer was repeated 2 days after. Non differentiated M0 macrophages did not influence the establishment and the growth of endometrial lesions, as assessed at the time of animal sacrifice. In contrast lesions growth was significantly reduced in the presence of M1 macrophages (Figure 6C) and significantly enhanced in the presence of M2 macrophages (Figure 6C). Lesions in mice injected with M1 macrophages were barely detectable and exhibited a severely disrupted architecture (Figure 6D).

Discussion

Macrophages play a critical role, via their ability to guide tissue regeneration, in the growth of tumors and in diseases characterized by persistent tissue remodeling.^{9,20–25} Several lines of evidence suggest that macro-

Figure 5. Macrophages infiltrating endometriotic lesions and apparently healthy peritoneum express markers of alternative activation. **A:** Macrophages in endometriotic lesions, leiomyomas, and apparently nonaffected peritoneum from patients with endometriosis and controls were characterized by immunohistochemistry for the expression of a lineage marker (CD68) and for markers of alternative activation (CD163 and CD206). **B:** Infiltrating macrophages expressing CD68, CD163, and CD206 were quantitatively assessed by the infiltration score, performed as described in *Materials and Methods*. * $P < 0.05$, significantly different from control.

phages are locally activated and not only “trapped” in ectopic endometrial lesions.^{1,26–31} For example, the nuclear factor- κ B transcription factor is activated in macrophages from endometriotic patients²⁷ and responsive genes that determine macrophages functions undergo transactivation, supporting angiogenesis and tissue remodeling.^{32,33} Accordingly, the *in vivo* inhibition of nuclear factor- κ B interferes with the growth of experimental lesions.³⁴ The environmental cues that activate peritoneal macrophages are still uncertain: local hypoxia apparently plays a major role.^{35,36} Aging erythrocytes, accumulating and dying into the pelvic cavity because of retrograde menstruation, cause iron overload.³⁷ Iron overload is greater in peritoneal macrophages, which are in charge of erythrocytes phagocytic clearance.^{20,38} Iron overload could as well influence macrophage activation.

Our goal was to verify whether macrophage activation is an epiphenomenon or is causally related to endometriosis. We deleted cells before and at different times after intraperitoneal injection of syngeneic endometrium in ex-

Figure 6. Endometriotic lesions growth is dependent on macrophages activation. **A:** Estradiol benzoate-treated female mice were sacrificed and uteri were removed and split. Endometrial tissue was isolated and mechanically disrupted before i.p. injection of four experimental/control mice pair per each treatment group. Control mice were treated with PBS. Experimental animals were injected i.p. at day -2, 0, and +2 with M0, M1, and M2 macrophages (1.5×10^6 cells/mouse/100 μ l). Animals were sacrificed 12 days after endometrial tissue injection. **B:** TNF- α and IL-10 secretion by *in vitro* polarized M0, M1, and M2 macrophages was assessed by enzyme-linked immunosorbent assay. Results are expressed as the mean $\pm$ SEM of at least three independent experiments. **C:** The dry weight of endometriotic lesions was assessed in mice treated with PBS or injected with M0, M1, or M2 macrophages. Results are expressed as mean of total weights of lesions from at least four mice $\pm$ SEM. * P < 0.05, significantly different from control group. **D:** Representative H&E staining and immunohistochemical analysis for CD68+ cells in endometriotic lesions obtained from mice treated with M1 and M2 macrophages.

perimental animals. Results in Figures 1–2 indicate that endogenous peritoneal macrophages are required for the experimental model of the human disease. Accordingly, a recent study has shown that the depletion of macrophages in the rat peritoneum by local injection of liposomal alendronate reduces both the initiation and the growth of endometriotic lesions.³⁹

The establishment of endometriotic lesions is characterized by infiltration of the underlying tissue and attraction of novel vessels, with development of an organized ectopic glandular and of stromal architecture. The latter events are disrupted in the absence of macrophages (Figures 1–2). Macrophages deliver trophic and anti-apoptotic signals, in particular during the very first days after endometrium injection,⁴⁰ before the formation of novel vessels,^{36,41,42} which possibly facilitate the survival of ectopic cells in a relatively hypoxic environment.⁴⁰

Endometriotic macrophages are actively involved in both *i)* the phagocytic clearance of aged red cells^{20,38} and endometrial cell debris,⁴³ and *ii)* the secretion of trophic and neo-angiogenic molecules. These features are typical of macrophages that have acquired an alternatively activated phenotype.^{8,44} We addressed this possibility characterizing membrane receptors expressed by macrophages in the peritoneal fluid and in the tissues of endometriosis patients and of experimental mice. We focused on two markers of M2-polarized macrophages, the CD206 man-

nose receptor and the CD163 hemoglobin scavenger receptor.⁴⁵ CD206 belongs to the C-type lectin superfamily, behaves as a pattern recognition receptor and has been involved in the silent clearance of inflammatory signals⁴⁶ CD163 physiologically scavenges haptoglobin by inducing endocytosis of haptoglobin–hemoglobin complexes, with degradation and consequent rescue of heme-iron components for erythropoiesis.⁴⁷ Its up-regulation reveals vascular defect and haptoglobin-hemoglobin complex stimulation *in vivo*.⁴⁸

Peritoneal macrophages express high levels of both markers both in patients and mice (Figures 3–4). Both macrophages in the peritoneal fluid and macrophages actively infiltrating ectopic lesions display features of alternative activation (Figures 3–4). This suggests that signals in the peritoneal cavity of endometriotic subjects and restricted to the ectopic tissue, switch the differentiation program of endogenous blood-derived precursors toward an alternative reparative phenotype. In turn, alternatively activated macrophages are responsible for lesions vascularization and growth.

We directly addressed this model by adoptive transfer of inflammatory/M1 and alternatively activated/M2 macrophages. The treatment resulted in a dramatically impaired or enhanced establishment of endometriotic lesions respectively (Figure 6). This effect probably depends on several features of polarized macrophages including

their ability to secrete different arrays of soluble factors (Figure 6). In support to this model, the peritoneal fluid of patients with endometriosis interferes with *in vitro* differentiation of precursors challenged with recombinant cytokines, favoring their differentiation toward macrophages⁹ and immature dendritic cells injected into endometriotic mice intraperitoneally actively migrate and infiltrate ectopic endometrial lesions, where they contribute to lesions further development.⁴⁹

In conclusion, our findings indicate that the peritoneal environment controls the differentiation of macrophage precursors, committing them toward an alternatively activated, reparatory phenotype. In turn, alternatively activated macrophages are necessary for ectopic lesions to vascularize and grow. These results are important for several reasons. First, they suggest that the alternative activation of macrophages is a clue to the heterogeneity in the susceptibility to endometriosis among menstruating women, especially in light of human data showing how environmental stimuli have shaped the genetic variations of our innate response, possibly originating complex individual responses to infectious agents¹¹ but also endogenous alarm signals. Second, they suggest that it may be possible to treat human endometriosis through local inhibition of macrophage reparative actions.

References

- Lebovic DI, Mueller MD, Taylor RN: Immunobiology of endometriosis. *Fertil Steril* 2001, 75:1–10
- Giudice LC, Kao LC: Endometriosis. *Lancet* 2004, 364:1789–1799
- Berkley KJ, Rapkin AJ, Papka RE: The pains of endometriosis. *Science* 2005, 308:1587–1589
- Sampson J: Peritoneal endometriosis due to menstrual dissemination of endometrial tissue into the peritoneal cavity. *Am J Obstet Gynecol* 1927, 14:422–469
- Dunselman GA, Groothuis PG: Etiology of endometriosis: hypotheses and facts. *Gynecol Obstet Invest* 2004, 57:42–43
- Becker CM, D'Amato RJ: Angiogenesis and antiangiogenic therapy in endometriosis. *Microvasc Res* 2007, 74:121–130
- van Amerongen MJ, Harmsen MC, van Rooijen N, Petersen AH, van Luyn MJ: Macrophage depletion impairs wound healing and increases left ventricular remodeling after myocardial injury in mice. *Am J Pathol* 2007, 170:818–829
- Martinez FO, Sica A, Mantovani A, Locati M: Macrophage activation and polarization. *Front Biosci* 2008, 13:453–461
- Allavena P, Sica A, Solinas G, Porta C, Mantovani A: The inflammatory micro-environment in tumor progression: the role of tumor-associated macrophages. *Crit Rev Oncol Hematol* 2008, 66:1–9
- Ferwerda B, McCall MB, Alonso S, Giamarellos-Bourboulis EJ, Mouktaroudi M, Izaguirre N, Syafruddin D, Kibiki G, Cristea T, Hijmans A, Hamann L, Israel S, ElGhazali G, Troye-Blomberg M, Kumpf O, Maiga B, Dolo A, Doumbo O, Hermesen CC, Stalenhoef AF, van Crevel R, Brunner HG, Oh DY, Schumann RR, de la Rua C, Sauerwein R, Kullberg BJ, van der Ven AJ, van der Meer JW, Netea MG: TLR4 polymorphisms, infectious diseases, and evolutionary pressure during migration of modern humans. *Proc Natl Acad Sci USA* 2007, 104:16645–16650
- Ferwerda B, McCall MB, Verheijen K, Kullberg BJ, van der Ven AJ, Van der Meer JW, Netea MG: Functional consequences of toll-like receptor 4 polymorphisms. *Mol Med* 2008, 14:346–352
- Wu MH, Shoji Y, Wu MC, Chuang PC, Lin CC, Huang MF, Tsai SJ: Suppression of matrix metalloproteinase-9 by prostaglandin E(2) in peritoneal macrophage is associated with severity of endometriosis. *Am J Pathol* 2005, 167:1061–1069
- Somigliana E, Vigano P, Rossi G, Carinelli S, Vignali M, Panina-Bordignon P: Endometrial ability to implant in ectopic sites can be prevented by interleukin-12 in a murine model of endometriosis. *Hum Reprod* 1999, 14:2944–2950
- Somigliana E, Vigano P, Filardo P, Candiani M, Vignali M, Panina-Bordignon P: Use of knockout transgenic mice in the study of endometriosis: insights from mice lacking beta(2)-microglobulin and interleukin-12p40. *Fertil Steril* 2001, 75:203–206
- Brignole C, Pastorino F, Marimpietri D, Pagnan G, Pistorio A, Allen TM, Pistoia V, Ponzone M: Immune cell-mediated antitumor activities of GD2-targeted liposomal c-myc antisense oligonucleotides containing CpG motifs. *J Natl Cancer Inst* 2004, 96:1171–1180
- Lolmede K, Campana L, Vezzoli M, Bosurgi L, Tonlorenzi R, Clementi E, Bianchi ME, Cossu G, Manfredi AA, Brunelli S, Rovere-Querini P: Inflammatory and alternatively activated human macrophages attract vessel-associated stem cells, relying on separate HMGB1- and MMP-9-dependent pathways. *J Leukoc Biol* 2009, 85:779–787
- Revised American Fertility Society classification of endometriosis: 1985. *Fertil Steril* 1985, 43:351–352
- Akoum A, Jolicoeur C, Kharfi A, Aube M: Decreased expression of the decoy interleukin-1 receptor type II in human endometriosis. *Am J Pathol* 2001, 158:481–489
- Revised American Society for Reproductive Medicine classification of endometriosis: 1996. *Fertil Steril* 1997, 67:817–821
- Brunelli S, Rovere-Querini P: The immune system and the repair of skeletal muscle. *Pharmacol Res* 2008, 58:117–121
- MacKinnon AC, Farnworth SL, Hodgkinson PS, Henderson NC, Atkinson KM, Leffler H, Nilsson UJ, Haslett C, Forbes SJ, Sethi T: Regulation of alternative macrophage activation by galectin-3. *J Immunol* 2008, 180:2650–2658
- Arnold L, Henry A, Poron F, Baba-Am Y, van Rooijen N, Plonquet A, Gherardi RK, Chazaud B: Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. *J Exp Med* 2007, 204:1057–1069
- Kamada N, Hisamatsu T, Okamoto S, Chinen H, Kobayashi T, Sato T, Sakuraba A, Kitazume MT, Sugita A, Koganei K, Akagawa KS, Hibi T: Unique CD14 intestinal macrophages contribute to the pathogenesis of Crohn disease via IL-23/IFN-gamma axis. *J Clin Invest* 2008, 118:2269–2280
- Kelly J, Ali Khan A, Yin J, Ferguson TA, Apte RS: Senescence regulates macrophage activation and angiogenic fate at sites of tissue injury in mice. *J Clin Invest* 2007, 117:3421–3426
- Lumeng CN, Bodzin JL, Saltiel AR: Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J Clin Invest* 2007, 117:175–184
- Lawson C, Al-Akoum M, Maheux R, Akoum A: Increased expression of interleukin-1 receptor type 1 in active endometriotic lesions. *Reproduction* 2007, 133:265–274
- Lousse JC, Van Langendonck A, Gonzalez-Ramos R, Defrere S, Renkin E, Donnez J: Increased activation of nuclear factor-kappa B (NF-kappaB) in isolated peritoneal macrophages of patients with endometriosis. *Fertil Steril* 2008, 90:217–220
- Minici F, Tiberi F, Tropea A, Fiorella M, Orlando M, Gangale MF, Romani F, Catino S, Campo S, Lanzone A, Apa R: Paracrine regulation of endometriotic tissue. *Gynecol Endocrinol* 2007, 23:574–580
- Zhang C, Maeda N, Izumiya C, Yamamoto Y, Kusume T, Oguri H, Yamashita C, Nishimori Y, Hayashi K, Luo J, Fukaya T: Killer immunoglobulin-like receptor and human leukocyte antigen expression as immunodiagnostic parameters for pelvic endometriosis. *Am J Reprod Immunol* 2006, 55:106–114
- Galleri L, Luisi S, Rotondi M, Romagnani P, Cobellis L, Serio M, Petraglia F: Low serum and peritoneal fluid concentration of interferon-gamma-induced protein-10 (CXCL10) in women with endometriosis. *Fertil Steril* 2008, 91:331–334
- Herrmann Lavoie C, Fraser D, Theriault MJ, Akoum A: Interleukin-1 stimulates macrophage migration inhibitory factor secretion in ectopic endometrial cells of women with endometriosis. *Am J Reprod Immunol* 2007, 58:505–513
- Timmer AM, Nizet V: IKKbeta/NF-kappaB and the miscreant macrophage. *J Exp Med* 2008, 205:1255–1259
- Hagemann T, Lawrence T, McNeish I, Charles KA, Kulbe H, Thompson RG, Robinson SC, Balkwill FR: "Re-educating" tumor-associated macrophages by targeting NF-kappaB. *J Exp Med* 2008, 205:1261–1268
- Gonzalez-Ramos R, Van Langendonck A, Defrere S, Lousse JC, Mettlen M, Guillet A, Donnez J: Agents blocking the nuclear factor-

- kappaB pathway are effective inhibitors of endometriosis in an in vivo experimental model. *Gynecol Obstet Invest* 2008, 65:174–186
35. Wu MH, Chen KF, Lin SC, Lgu CW, Tsai SJ: Aberrant expression of leptin in human endometriotic stromal cells is induced by elevated levels of hypoxia inducible factor-1alpha. *Am J Pathol* 2007, 170:590–598
 36. Becker CM, Rohwer N, Funakoshi T, Cramer T, Bernhardt W, Birsner A, Folkman J, D'Amato RJ: 2-methoxyestradiol inhibits hypoxia-inducible factor-1{alpha} and suppresses growth of lesions in a mouse model of endometriosis. *Am J Pathol* 2008, 172:534–544
 37. Defrere S, Lousse JC, Gonzalez-Ramos R, Colette S, Donnez J, Van Langendonck A: Potential involvement of iron in the pathogenesis of peritoneal endometriosis. *Mol Hum Reprod* 2008, 14:377–385
 38. Lousse JC, Defrere S, Van Langendonck A, Gras J, Gonzalez-Ramos R, Colette S, Donnez J: Iron storage is significantly increased in peritoneal macrophages of endometriosis patients and correlates with iron overload in peritoneal fluid. *Fertil Steril* 2008, 91:1668–1675
 39. Haber E, Danenberg HD, Koroukhov N, Ron-El R, Golomb G, Schachter M: Peritoneal macrophage depletion by liposomal bisphosphonate attenuates endometriosis in the rat model. *Hum Reprod* 2009, 24:398–407
 40. Lin YJ, Lai MD, Lei HY, Wing LY: Neutrophils and macrophages promote angiogenesis in the early stage of endometriosis in a mouse model. *Endocrinology* 2006, 147:1278–1286
 41. Grummer R: Animal models in endometriosis research. *Hum Reprod Update* 2006, 12:641–649
 42. Eggermont J, Donnez J, Casanas-Roux F, Scholtes H, Van Langendonck A: Time course of pelvic endometriotic lesion revascularization in a nude mouse model. *Fertil Steril* 2005, 84:492–499
 43. Harada T, Taniguchi F, Izawa M, Ohama Y, Takenaka Y, Tagashira Y, Ikeda A, Watanabe A, Iwabe T, Terakawa N: Apoptosis and endometriosis. *Front Biosci* 2007, 12:3140–3151
 44. Gordon S: The macrophage: past, present and future. *Eur J Immunol* 2007, 37 Suppl 1:S9–S17
 45. Verreck FA, de Boer T, Langenberg DM, van der Zanden L, Ottenhoff TH: Phenotypic and functional profiling of human proinflammatory type-1 and anti-inflammatory type-2 macrophages in response to microbial antigens and IFN-gamma- and CD40L-mediated costimulation. *J Leukoc Biol* 2006, 79:285–293
 46. Allavena P, Chieppa M, Monti P, Piemonti L: From pattern recognition receptor to regulator of homeostasis: the double-faced macrophage mannose receptor. *Crit Rev Immunol* 2004, 24:179–192
 47. Kristiansen M, Graversen JH, Jacobsen C, Sonne O, Hoffman HJ, Law SK, Moestrup SK: Identification of the haemoglobin scavenger receptor. *Nature* 2001, 409:198–201
 48. Borda JT, Alvarez X, Mohan M, Hasegawa A, Bernardino A, Jean S, Aye P, Lackner AA: CD163, a marker of perivascular macrophages, is up-regulated by microglia in simian immunodeficiency virus encephalitis after haptoglobin-hemoglobin complex stimulation and is suggestive of breakdown of the blood-brain barrier. *Am J Pathol* 2008, 172:725–737
 49. Fainaru O, Adini A, Benny O, Adini I, Short S, Bazinet L, Nakai K, Pravda E, Hornstein MD, D'Amato RJ, Folkman J: Dendritic cells support angiogenesis and promote lesion growth in a murine model of endometriosis. *FASEB J* 2008, 22:522–529