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Proangiogenic Tie2⁺ Macrophages Infiltrate Human and Murine Endometriotic Lesions and Dictate Their Growth in a Mouse Model of the Disease

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Endometriosis affects women of reproductive age, causing infertility and pain. Although immune cells are recruited in endometriotic lesions, their role is unclear. Tie2-expressing macrophages (TEMs) have nonredundant functions in promoting angiogenesis and growth of experimental tumors. Here we show that human TEMs infiltrate areas surrounding newly formed endometriotic blood vessels. We set up an *ad hoc* mouse model in which TEMs, and not Tie2-expressing endothelial cells, are targeted. We transplanted in wild-type recipients bone marrow cells expressing a suicide gene (Herpes simplex virus type 1 thymidine kinase) under the Tie2 promoter/enhancer. TEMs infiltrated endometriotic lesions. TEM depletion by ganciclovir administration arrested the growth of established lesions, without toxicity. Lesion architecture was disrupted, with: i) loss of glandular organization, ii) reduced neovascularization, and iii) activation of caspase 3 in CD31⁺ endothelial cells. Thus, TEMs are important for maintaining the viability of newly formed vessels and represent a potential therapeutic target in endometriosis. (Am J Pathol 2011; 179:2651–2659; DOI: 10.1016/j.ajpath.2011.07.029)

Endometriosis is a gynecological disease affecting 10% to 15% of women during their reproductive years. It is characterized by the persistence and growth of vascu-

larized endometrial tissue at ectopic sites, typically the pelvis, and it is associated with pelvic pain and infertility.^{1–3} Initially, shed endometrial tissue attaches to the peritoneal wall. Subsequent steps include invasion of the underlying basement membrane and recruitment of novel vessels from the peritoneal vasculature.^{4–6}

The causes of endometriosis and the molecular events underlying susceptibility to developing the disease are poorly understood. The disease originates when shed endometrium invades the peritoneal layer and initiates an inflammatory circuit that leads to an angiogenic response.^{5–8} The events responsible for neoangiogenesis have been extensively studied in solid tumors, where their participation is essential. Tumor-associated macrophages (TAMs) represent a source of trophic and proangiogenic signals.^{9–13} TAMs comprise cell populations endowed with diverse functions, including promotion of tumor cell intra- and extravasation, survival, and subsequent growth.¹⁴ Tumor infiltrating cells derived from Tie2-expressing macrophages (TEMs) represent a fraction of TAMs with high proangiogenic activity.^{15–19} Their selective elimination in mouse tumor models by a suicide gene strategy that spares other tumor infiltrating inflammatory leukocytes, such as macrophages and granulocytes, is sufficient to inhibit tumor angiogenesis.²⁰

In the present study we demonstrate, using an experimental mouse model of endometriosis, that TEMs play a nonredundant role in the persistence and growth of ectopic endometrial lesions, preventing caspase-3 activation and apoptosis of endothelial cells in neoformed blood vessels.

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Table 1. Endometriosis Patient Characteristics

Patient	Age (years)	Sterility	Hormonal treatment	Cycle phase	Stage*
E1	30	Yes	Yes	Proliferative	IV
E2	30	Yes	NA	Proliferative	III
E3	32	Yes	Yes	Secretive	III
E4	30	No	Yes	Secretive	III
E5	38	Yes	Yes	Secretive	I
E6	39	Yes	Yes	Proliferative	IV
E7	32	Yes	Yes	Proliferative	IV
E8	25	Yes	Yes	Proliferative	IV
E9	33	Yes	Yes	Secretive	III
E10	28	Yes	Yes	Secretive	IV
E11	42	Yes	Yes	Secretive	IV
E12	28	No	Yes	Proliferative	III
E13	31	Yes	Yes	Secretive	III
E14	35	Yes	Yes	Proliferative	III
E15	27	Yes	Yes	Proliferative	III

*According to criteria of the revised ASRM classification.²¹

Materials and Methods

Patients

We analyzed surgical specimens derived from 15 women affected by endometriosis (age range, 25 to 42 years) and from 10 patients with leiomyoma (age range, 23 to 47 years). Selected characteristics for the endometriosis patients are given in Table 1. All patients were followed in the Department of Obstetrics and Gynecology of the San Raffaele Scientific Institute, Milan, Italy. The Institutional Ethical Committee approved the study.

Mice

Eight-week-old female BALB/c and male FVB/N mice were purchased from Charles River Laboratories International (Wilmington, MA). FVB/N transgenic (Tg) male mice (Tie2-HSV-Tk) (which express the Herpes simplex virus type 1 thymidine kinase under the control of the Tie2 promoter and have been described previously¹⁵) were maintained in specific pathogen-free conditions. We used 10 animals per experimental group for each independent experiment. Eight-week-old female BALB/c mice were treated with estrogens, as described previously⁷ (see further below). 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.

Bone Marrow-Derived Stem Cell Transplantation

Eight-week-old female FVB/BALB/c WT (wild type) and FVB/BALB/c Tg mice, F1 generation, were sacrificed. Bone marrow (BM) cells were collected by flushing femurs and tibias and were injected into the tail vein (1×10^7 cells/mouse) of 8-week-old female BALB/c

Figure 1. TEMs are recruited into human endometriotic lesions. **A:** TEMs, identified as CD163⁺TIE2⁺VWF⁻ mononuclear cells, were significantly more abundant in samples from endometriotic patients ($n = 20$) than in control tissues obtained from patients with leiomyoma ($n = 15$). Results represent the mean $\pm$ SEM of five independent fields of view (FOV), as assessed by two independent blinded expert pathologists. ** $P < 0.01$ versus control. **B:** Confocal images were obtained from endometriotic lesions and tissues from patients with leiomyoma. Tissues were analyzed for TIE2, CD163, and Von Willebrand factor expression. Confocal planes are shown after merging (top row) or individually. TEMs (arrows) are found preferentially in the perivascular areas of endometriotic lesions only. Scale bar = 15 μ m.

mice that had been lethally irradiated to allow full engraftment of the transplanted hematopoietic stem cells (7 Gy). We transplanted pooled BM cells derived from five donors into 10 recipient animals for each condition.

Model of Endometriosis and Treatment

Mice were treated subcutaneously with estradiol benzoate (3 mg/mouse; Intervet, Milan, Italy). Seven days later, they were sacrificed. Uteri were removed, seeded in Petri dishes containing warmed saline, and split longitudinally with a pair of scissors. Each uterus horn was processed identically: endometrial tissue was isolated and carefully disrupted mechanically, yielding small-cell aggregate suspensions with a maximal diameter consistently <1 mm. One suspension was then used for intraperitoneal injection of an experimental/control mice pair.^{22,23} Each mouse of a pair received the cell suspension derived from a half uterus; all injected animals developed peritoneal endometriosis (overall take, 100%). At 12 days after endometrial tissue injection, mice were euthanized individually by cervical dislocation. The abdominal cavity was immediately opened and lesions were excised and processed for disease assessment or immunohistochemical evaluation. The overall extent of endometriosis was evaluated by assessing the dry weight of all lesions from each mouse, as described previously.⁷ When indicated, endometriotic transplanted mice were treated with ganciclovir (GCV; 100 mg/g per mouse) or PBS daily for 8 days, starting from 4 days after endometrium injection.

Histology and Immunohistochemistry

Endometriotic human lesions were frozen in isopentane-liquid nitrogen and embedded in optimum cutting temperature compound (Bio-Optica, Milan, Italy). For immunofluorescence, sections (10 μ m thick) were fixed in paraformaldehyde (4%) and incubated with mouse monoclonal antibodies anti-human CD163 (BD Bioscience, San Jose, CA) at a final concentration of 0.5 μ g/mL, rabbit antibodies anti-human Von Willebrand factor (Abcam, Cambridge UK) at a final concentration of 7 μ g/mL, and goat antibodies anti-human Tie2 (R&D Systems, Minneapolis, MN) at a final concentration of 5 μ g/mL for 20 hours at 4°C. AlexaFluor 488-conjugated anti-mouse, AlexaFluor 633-conjugated anti-rabbit, and AlexaFluor 594-conjugated anti-goat antibodies were used as second-step reagents at a final concentration of 4 μ g/mL (Invitrogen, Milan, Italy). Samples were then washed and mounted with a coverslip in glycerol. Sections were analyzed using a Perkin Elmer (Waltham, MA) confocal UltraVIEW imaging system. Four randomly selected areas at $\times 400$ magnification were analyzed. TEMs were identified and quantified by the simultaneous expression of the Tie2 and the CD163 markers. Explanted endometrium or endometriotic lesions were immediately fixed in paraformaldehyde (4%) and sucrose gradient 10-20-30%, then in optimum cutting temperature compound-embedded tissue blocks. Sections (5 μ m thick) were

stained with H&E or with rat anti-mouse CD31 antibody (BD Biosciences, San Jose, CA) at a final concentration of 5 μ g/mL and rat anti-mouse CD68 monoclonal antibody (AbD Serotec, Oxford, UK) at a final concentration of 1 μ g/mL for immunohistochemistry. Immunofluorescence analysis was performed on thicker specimens (10 μ m) using a biotinylated anti-mouse Tie2 antibody (eBioscience, San Diego, CA) at a final con-

Figure 2. Effective and selective depletion of TEMs infiltrating endometriotic lesions in Tie2-HSV-Tk/BM mice. **A:** Schematic representation of the experimental group and of the relevant controls. **B:** Endometriotic lesions from Tie2-HSV-Tk/BM mice, treated with saline (PBS) or GCV were analyzed for expression of the CD163, CD31, and Tie2 markers. Confocal planes are shown after merging (**top row**) and individually. Perivascular CD163⁺CD31⁺Tie2⁺ TEMs were evident in the perivascular areas of endometriotic lesions of PBS-treated mice (**arrows**) but were depleted by treatment with GCV. Scale bar = 15 μ m.

Figure 3. Disruption of endometriotic lesion growth and architecture in the absence of TEMs. **A:** The number of endometriotic lesions for each experimental group was assessed at necropsy, 12 days after intraperitoneal injection of endometrial tissue. Data are expressed as means $\pm$ SEM of results from three independent experimental and control mouse pairs. **B:** The weight of endometriotic lesions in each experimental group was assessed at day 12. Results are expressed as means $\pm$ SEM of total weights of lesions from three independent experimental and control mouse pairs. ** $P < 0.01$ versus control. **C:** Representative endometriotic lesions obtained from experimental mice and control groups (H&E stain). The architecture of lesions from Tie2-HSV-Tk/BM mice treated with GCV is severely disrupted.

centration of 10 $\mu\text{g/mL}$, a rabbit anti-mouse cleaved caspase 3 antibody (Cell Signaling Technology, Danvers, MA) at a final concentration of 0.25 $\mu\text{g/mL}$, or a rabbit anti-mouse CD163 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a final concentration of 1 $\mu\text{g/mL}$.

TUNEL Assay

For detection and quantification of apoptosis, we verified DNA degradation by terminal deoxynucleotidyl transferase dUTP nick end-labeling (in situ cell death detection kit; Roche Diagnostics-Roche Applied Science, Mannheim, Germany), according to the manufacturer's instructions.

Statistical Analysis

Statistical analysis was performed using a two-tailed Student's *t*-test for unpaired samples with unequal variance

to analyze differences between groups. Analysis was performed with SPSS software (version 11.0 Mac OS X; SPSS, Chicago, IL).

Results

TEMs Selectively Infiltrate Areas Surrounding Neovessels within Human Endometriotic Lesions

We analyzed ectopic endometrial tissues from 15 consecutive endometriotic patients. Columnar or cuboidal epithelial endometrial cells surrounded by narrow stroma layers characterize the ectopic endometrial tissue. Infiltrating macrophages are roughly distributed throughout the tissue. To identify TEMs in the lesions, we traced TIE2⁺ cells that also express the hemoglobin/haptoglobin scavenger receptor CD163, a marker

Figure 4. Impaired neoangiogenesis of endometriotic lesions in the absence of TEMs. **A:** Immunohistochemical analysis of CD31⁺ endothelial cells in endometriotic lesions obtained from experimental mice and control groups. Scale bar = 30 μm . **B:** The mean vascular area was calculated by digital image analysis and the data are expressed as means $\pm$ SEM of results obtained in three independent experimental and control mouse pairs. ** $P < 0.01$ versus control.

of alternative activation that is broadly expressed by macrophages within endometriotic lesions.⁷ We also analyzed the expression of von Willebrand factor (vWF), which identifies endothelial cells. Similar to human solid tumors,¹⁷ substantial infiltration by CD163⁺vWF⁺TIE2⁺ cells was detectable in all endometriotic samples (Figure 1A). TEMs were found preferentially in the perivascular areas of newly formed vessels (Figure 1B). We used surgical specimens derived from 10 patients with leiomyoma, a benign con-

dition characterized by limited neovascularization and extensive fibrosis that only partially reproduces the features of endometrial tissue. TEMs were significantly less abundant in leiomyomas and were not detectable in areas surrounding vessels (Figure 1A). Moreover, TEMs were not detectable in eutopic endometrial tissues from estrogen-treated healthy mice, even if CD68⁺ and CD163⁺ resident macrophages were easily detected (see Supplemental Figure S1 at <http://ajp.amjpathol.org>).

Figure 5. Selective caspase-3 activation in endothelial cells of endometriotic lesions in the absence of TEMs. **A:** Endometriotic lesions from experimental mice and their respective controls were analyzed by immunofluorescence for the expression of Tie2, cleaved activated caspase-3, and the CD31 endothelial cell marker. Confocal planes are shown after merging (top row) and individually. Tie2⁺caspase-3⁺CD31⁺ endothelial cells and Tie2⁺caspase-3⁺CD31⁻ TEMs were present only in GCV-treated Tie2-HSV-Tk/BM mice. Scale bar = 15 μ m. **B:** The number of Tie2⁺caspase-3⁺CD31⁺ endothelial cells/FOV was compared in experimental mice and relevant controls. * $P < 0.05$ versus control.

Table 2. Caspase Activation in TEM-Depleted Mice

	CD31 ⁺ cells/FOV (no. $\pm$ SEM)	Caspase3 ⁺ CD31 ⁺ cells/FOV (no. $\pm$ SEM)
Tie2-HSV-Tk/BM PBS	24 $\pm$ 4.16	6.33 $\pm$ 1.67
Tie2-HSV-Tk/BM GCV	46.83 $\pm$ 2.5*	26.83 $\pm$ 1.83*
WT/BM PBS	26 $\pm$ 1.87	7.2 $\pm$ 0.72
WT/BM GCV	24.2 $\pm$ 2.1	5.6 $\pm$ 1.03

* $P < 0.005$ versus controls not treated with GCV.

BM, bone marrow; FOV, field of view; GCV, ganciclovir; TEM, Tie2-expressing macrophage; WT, wild type.

Endometriotic Lesions Persist in Vivo Only in the Presence of TEMs

To verify whether TEMs have a role in the natural history of endometriotic lesions, we used a mouse model of the disease.^{7,22,23} To selectively deplete TEMs from mice carrying endometriotic lesions, we devised a bone marrow (BM) transplantation strategy (Figure 2A). We first generated Tie2-HSV-Tk transgenic mice on a mixed FVB/BALB/c background by crossing FVB/Tie2-HSV-Tk with BALB/c mice and using the F1 generation (FVB:BALB/c/Tie2-HSV-Tk mice). We then transplanted BM cells from these mice into lethally irradiated wild-type (WT) BALB/c female mice, to obtain Tie2-HSV-Tk/BM chimeric mice. On hematopoietic reconstitution, Tie2-expressing cells can be depleted by administration of GCV. With this approach, GCV selectively kills TEMs but not endothelial cells (which are not BM-derived). To obtain control mice, we transplanted BM cells derived from FVB $\times$ BALB/c F1 female mice (FVB:BALB/c mice) into lethally irradiated WT BALB/c female mice, thus obtaining WT/BM chimeric mice.

Eight weeks after BM transplant, we intraperitoneally injected endometrial tissue from syngeneic mice. We allowed endometriotic lesions to establish for 4 days, and then administered either GCV (100 mg/g per mouse) or saline (PBS) daily for 8 days to deplete TEMs. At sacrifice (12 days after intraperitoneal injection of endometrial tissue), the number of implanted endometriotic lesions did not differ between GCV- and PBS-injected Tie2-HSV-Tk/BM mice (3.43 ± 1.28 for GCV versus 3.22 ± 0.66 for PBS) or between GCV- and PBS-injected WT/BM mice (4.4 ± 0.75 for GCV versus 3 ± 0.71 for PBS) (Figure 3A). CD163⁺CD31⁺Tie2⁺ cells were present in the endometriotic lesions of PBS-injected mice, but were virtually absent in GCV-treated Tie2-HSV-Tk/BM mice, indicating that TEMs were effectively depleted (Figure 2B). As expected, GCV did not alter CD163⁺CD31⁺Tie2⁺ cell infiltration or distribution in the endometriotic lesions of WT/BM mice.

In TEM-depleted mice, the dry weight of the lesions was strikingly lower (Figure 3B), and their glandular and stromal architecture was disrupted (Figure 3C), indicating that established lesions depend on TEMs for their integrity and growth. By contrast, endometriotic lesions developed normally in control PBS-injected Tie2-HSV-Tk/BM mice (Figure 3) and in WT/BM mice treated with

GCV or PBS. These data exclude unwanted effects on the endometriotic lesions related to transplant or transgene procedures.

In the absence of TEMs, the neovasculature associated with endometriotic lesions is disorganized and is reduced overall (Figure 4A). Moreover, endothelial cells had a reduced size and an elongated aspect, with a striking reduction of vessel lumen dimensions. The actual vascular area was also significantly reduced, specifically in GCV-treated Tie2-HSV-Tk/BM mice (Figure 4B). Notably, endometriotic lesions in these mice were characterized by a high number of CD31⁺ endothelial cells; however, these CD31⁺ cells were mostly isolated and not organized in vascular structures, which were instead clearly evident in the three control groups (Figure 4A). A substantial fraction (>50%) of the CD31⁺ endothelial cells expressed the cleaved active form of caspase 3 (the key enzyme of the apoptotic machinery), specifically in GCV-treated Tie2-HSV-Tk/BM mice (Figure 5 and Table 2). Moreover, selective DNA degradation occurs in endothelial cells of GCV-treated mice, as assessed by the TUNEL assay (Figure 6B). These data strongly suggest that TEMs are required to maintain

Figure 6. Selective apoptosis induction in endothelial cells of endometriotic lesions in the absence of TEMs. Endometriotic lesions from experimental mice and relevant controls were analyzed by immunofluorescence for the expression of CD31 and for apoptosis induction, as assessed by TUNEL staining. Apoptotic cells were present as expected throughout the ectopic endometrial tissues of Tie2-HSV-Tk/BM mice treated with saline (A) and after depletion of TEMs with GCV (B). However, apoptotic endothelial cells were substantially more frequent in TEM-depleted mice (arrows, B). Scale bar = 50 μ m.

the viability of endothelial cells recruited to endometriotic lesions, allowing angiogenic spots to develop into actual neovessels.

Discussion

Macrophages have the ability to secrete trophic factors and to support angiogenesis. They therefore contribute to the growth of neoplastic lesions and to the natural history of diseases characterized by sustained tissue remodeling.^{24–29} This applies to endometriosis as well. The lesions consistently contain infiltrating macrophages, which display clear features of being phagocytic cells, locally activated and not just passively entrapped in the ectopic tissues.^{3,30–37}

The depletion of all phagocytic cells^{7,38} and the interference with the NF- κ B transcription factor-dependent pathway,³⁹ which is activated in macrophages infiltrating endometriotic lesions,³³ attenuate the disease in models of endometriosis, further supporting the direct involvement of activated macrophages in the natural history of the disease.

Endometriotic lesions represent, from the immunological point of view, an autograft.³ The causes of macrophage activation and the nature of the signals they receive in the peritoneal cavity of otherwise healthy patients are elusive. Equally elusive are the signals that switch the differentiation program of endogenous blood-derived precursors, including monocytes and immature dendritic cells,⁴⁰ toward a reparative phenotype.^{7,41,42} Local hypoxia (which is well known to modulate Tie2 expression on TEMs and to influence their recruitment and functions in peripheral tissues^{43,44}) may contribute.^{6,45} Aging red blood cells that accumulate and die into the pelvis as a consequence of retrograde menstruation represent a source of iron.⁴⁶ Iron overload could also regulate innate cell activation.

The ability of macrophages to deal with iron changes with their functional polarization. Alternatively activated macrophages, which are the dominant population both in human and experimental endometriosis,⁷ effectively take up and spontaneously release iron at low concentrations and have limited storage ability. This macrophage population appears to be specifically involved in the recycling of the metal,^{47,48} and could find the peritoneal environment of endometriotic patients particularly advantageous.

The interaction between ectopic endometrial tissue and immune cells in the peritoneal environment is a critical area for the development of novel and more effective therapies for endometriosis, which represents an urgent medical need.⁴⁹ The identification of precise molecular targets is therefore a necessary step. Angiogenesis is a key event in the maintenance of endometrial lesions.^{50–54} Our data provide the first demonstration of the involvement of TEMs, which are a subset of macrophages that typically infiltrate tumors,^{16,19} in the angiogenic process in endometriosis, which is a non-neoplastic condition. When TEMs are specifically depleted, endothelial cells fail to organize effectively in neovessels and undergo programmed cell death, lead-

ing to disruption of the lesions. The preferential localization of TEM in perivascular areas¹⁶ and their relative proximity to endothelial cells may provide survival and growth signals to endothelial cells, and possibly also to endothelial progenitor cells.⁵⁵ Indeed, recent gene profiling studies of tumor-derived TEMs and other TAM subsets showed that TEMs express several proangiogenic and survival factors, including insulin-like growth factor 1.²⁰

Previous studies have indicated that macrophage alternative activation is critical for endometriosis.⁷ Alternatively activated macrophages comprise various cell populations endowed with diverse functions.⁵⁶ The gene expression profile of tumor-infiltrating TEMs is similar to that of alternatively activated TAMs and differs from that of inflammatory TAMs.^{20,29} TEMs account only for a small fraction of both alternatively activated TAMs¹⁷ and of macrophages that infiltrate endometriotic lesions. The selective expression by TEMs of proangiogenic genes encoding for molecules that bind hyaluronic acid (HA) (ie, *Lyve1*, the hyaluronan receptor-1 expressed on lymphatic endothelial cells, and *Stab1*, stabilin-1, a hyaluronan receptor) or its low molecular weight fragmentation products (ie, *Tlr4*, a Toll-like receptor that recognize exogenous and endogenous inflammatory signals)²⁰ is probably critical for their ability to support endometriotic neovasculation and underlies the finding that their selective ablation jeopardizes growth and spreading of the lesions, despite persistence in the tissue of Tie2[–] alternatively activated macrophages.

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