Retinoic acid suppresses growth of lesions, inhibits peritoneal cytokine secretion, and promotes macrophage differentiation in an immunocompetent mouse model of endometriosis

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Abstract

Objective—To determine the effects of retinoic acid (RA) on establishment and growth of endometrial lesions, peritoneal IL-6 and MCP-1 concentrations, and CD38, CD11b, F4/80 expression on peritoneal macrophages in an immunocompetent mouse model of endometriosis.

Design—Experimental transplantation study using mice.

Setting—Academic medical center.

Animals—C57BL/6 recipient mice and syngeneic Green Fluorescent Protein transgenic (GFP+) mice.

Intervention(s)—Recipient mice were inoculated with GFP+ minced uterine tissue to induce endometriosis and treated with RA (400 nmol/day) or vehicle for 17 days (3 days before to 14 days after tissue injection).

Main Outcome Measure(s)—Total number of GFP+ implants in recipient mice, number of implants showing visible blood vessels, total volume of established lesions per mouse, concentrations of IL-6 and MCP-1 in peritoneal fluid, expression of CD11b, F4/80 and CD38 on peritoneal macrophages.

Results—17 days of RA treatment reduced the number of implants versus controls and decreased the frequency of lesions with vessels. Peritoneal washings in RA-treated animals had lower IL-6 and MCP-1 than controls 3 days after endometrial inoculation and lower levels of IL-6 on day 14 after inoculation. Concomitant with these effects on day 14, CD38, CD11b, and F4/80 were higher on macrophages from RA-treated mice vs. controls.

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Conclusions—RA inhibits the development of endometriotic implants. This effect may be caused, at least in part, by reduced IL-6 and MCP-1 production and enhanced differentiation of peritoneal macrophages.

Keywords

endometriosis; retinoic acid; cytokines; GFP-transgenic mice

INTRODUCTION

Endometriosis is a chronic inflammatory syndrome, commonly associated with ectopic peritoneal implants of endometrial tissue (1). Its etiology appears to involve genetic and environmental factors, including aspects of diet and exercise (1–4). The gene-environment interaction contributes to local inflammatory responses in the uterine and peritoneal cavity, which are thought to play a key role in the establishment and maintenance of endometriosis (5). To this end, the pathogenesis and progression of the disease are promoted by the collective effects of cytokines, chemokines, proteases, and angiogenic factors in the peritoneal cavity (5). These mediators are derived from endometriotic implants, mesothelial cells, and particularly activated peritoneal macrophages (5). Studies have suggested that defects in macrophage activation/differentiation programs lead to chronic immune activation with accompanying reduction in scavenging (6–8). Both of these consequences are thought to contribute to the establishment, growth, and maintenance of endometriotic lesions (9, 10).

All-trans-retinoic acid (RA), the active metabolite of vitamin A, has many biologic effects, including a variety of immunomodulatory and anti-inflammatory activities (11, 12). RA has been shown to modulate inflammation in autoimmune disease via its ability to enhance regulatory T cell (T-reg) suppression of pro-inflammatory cells (13, 14). In model systems involving activated monocytes/macrophages, RA decreased pro-inflammatory cytokines while increasing anti-inflammatory proteins such as IL-10 (15). The role of retinoids in the female reproductive tract and in particular in the endometrium has been investigated in our laboratory and others' (7, 16–19). Some studies suggest the possibility that the retinoid pathway is involved in the pathophysiology of endometriosis (7, 16, 17, 20).

Preclinical rodent models of endometriosis have been used to investigate the link between certain hormones (e.g. estrogen and progesterone) and the growth of endometriotic implants (21–23), and to test the efficacy of potential therapeutic agents (24, 25). In this context, immune-compromised mice (e.g., "nude" mice), have been extensively used as models for xenografting, whereas in rats, surgical implants of autologous endometrial tissue has been utilized (26–28). Unfortunately, immune deficient models are limited for investigating immune system-disease interactions, while surgical auto-transplantation models bypass the attachment/implantation phases of the disease. As a result, investigators have refined an immunocompetent mouse model whereby endometrial fragments from syngeneic donors are injected into the peritoneal cavity of estradiol-primed recipients to generate endometriotic lesions (29, 30). This non-surgical approach closely mimics the establishment of endometriosis in affected women via retrograde menses (31) and can be used to longitudinally study inflammatory signals associated with endometriotic lesion growth (32).

In this study, we examined the effects of RA on the growth and angiogenesis of endometriotic lesions in immunocompetent mice. We also evaluated the effects of RA on peritoneal macrophage activation/differentiation markers CD38, CD11b, and F4/80, and on pelvic washing concentrations of interleukin-6 (IL-6) and macrophage chemotactic factor-1 (MCP-1), cytokines known to be elevated in the peritoneal fluid of women with endometriosis (33–35).

MATERIAL AND METHODS

Chemicals and reagents

All-trans-retinoic acid (RA) was purchased from Sigma Chemical Co. (St. Louis, MO) and diluted in corn oil to the indicated concentrations for the experiments. IL-6 and MCP-1 ELISA kits were obtained from RayBiotech, Inc (Norcross, GA). Antibodies against CD38 and F4/80, and against CD11b were purchased from eBbioscience, Inc. (San Diego, CA) and from BD Pharmingen (Franklin Lakes, NJ), respectively. The reagents were equilibrated at room temperature for 0.5 hours before use.

Mice

This study was approved by the Emory Institutional Animal Care and Use Committee. All the procedures were performed according to the NIH Guidelines for Care and Use of Laboratory Animals. Wild-type C57BL/6 mice and transgenic C57BL/6-Tg(UBC-GFP) 30Scha/J donor mice (Tg-GFP) were purchased from Jackson Laboratory (Bar Harbor, ME). The Tg-GFP mice express enhanced Green Fluorescent Protein (GFP) under the direction of the human ubiquitin C promoter (36). Studies have demonstrated that C57BL/6 mice show little, if any, anti-GFP immune responses when transplanted with tissue from their Tg-GFP counterparts (37). Tg-GFP mice are viable, fertile, equivalent in size and do not display gross physical or behavioral differences. Food (standard pellet diet) and water were provided ad libitum. The mice were housed in a light/dark cycle of 12 h/12 h under standard conditions and allowed to acclimatize to these conditions for at least 1 week prior to inoculation of endometrial fragments.

Experimental Model

The mouse endometriosis model was established as previously described (30) with minor modifications using transplantation of Tg-GFP into syngeneic recipients, which yielded permanent expression of GFP in endometriotic tissue. This model provides the benefit of increased sensitivity in the identification of endometriotic lesions and more accurate quantification of lesion size and growth rates. Tg-GFP donor mice were treated subcutaneously (s.c.) with 100 μg/kg estradiol valerate (dissolved in corn oil) one week before sacrifice to stimulate proliferation of their endometrial tissue for transplantation. All procedures were conducted under aseptic conditions. Uteri were dissected, finely minced with iris scissors, and further fragmented by serial tituration through an 18-gauge adjuvant-mixing needle (Popper and Sons, Inc., New Hyde Park, NY) in 0.5 ml of sterile PBS. Each recipient mouse received an equal quantity of tissue (equivalent to one uterine horn [~35 mg]) via intraperitoneal (i.p.) injection with an 18-gauge needle through the abdominal wall just below the umbilicus. Recipient mice received 100 ug/kg/week estradiol valerate s.c. in corn oil each week, starting one week before i.p. inoculation of endometrial tissue, in order to synchronize their estrus cycles.

Experimental design and drug treatment

In the treatment groups, retinoic acid was administered by oral gavage. The dose of RA (0.1 ml of 4 mM/day) was previously shown to achieve therapeutic serum concentrations in anti-tumor studies without adverse side effects (38). Mice were treated daily with RA or vehicle control (corn oil) starting three days before inoculation with endometrial fragments (day-3) until sacrifice. Three - 14 days after endometrial fragment inoculation, we sacrificed the recipients and examined their peritoneum under 488 nm light (excitation wavelength peak of GFP) using interference filter eyeglasses to observe GFP+ endometrial implants (emission wavelength peak: 502 nm). Based on our experience, at 5 days after challenge endometriotic lesions are visible in all animals. The animals were killed by cervical dislocation 24 hours

after the last administration of drug or vehicle. 3 ml of PBS was injected intraperitoneally (see below), and the abdominal skin and peritoneum were opened to harvest peritoneal washings and macrophages, examine the visceral organs and evaluate the presence of endometriotic implants and vascularization. The sizes of all the lesions were measured in situ with calipers. We confirmed the authenticity of the implants by verifying the histopathological criteria for endometriosis, including formation of endometriotic cysts with endometrial glands, stroma, vessels and hemorrhage (Fig. 1D).

Specimen Collection and flow cytometry

Peritoneal fluid washings were harvested for assessment of IL-6 and MCP-1 levels by ELISA, and peritoneal leukocytes were evaluated by flow cytometry for quantitation of macrophage markers (CD11b and F4/80) and the differentiation/activation marker CD38. Resident peritoneal cells were obtained as described (39). Briefly, after sacrifice, 3 ml of ice cold PBS was injected i.p. using an 18 g needle with 5 ml syringe. After gentle massage, the abdominal wall was carefully incised and peritoneal fluid-containing cells was harvested and centrifuged at 1000 rpm for 10 min at 4°C. The lavage fluid was concentrated 6-fold using Amicon Ultra-15 Centrifugal Filter Tubes (Millipore, Billerica, MA) and the concentrate then stored at −80°C for furthe r analysis of IL-6 and MCP-1 levels. The cell pellet was reconstituted with washing media (PBS with 2% FBS). After counting, 1×10^6 cells were suspended in 100 μl washing media and incubated at 4°C for 1 hour with appropriate dilutions of the following antibodies: FITC-conjugated rat anti-mouse CD11b (BD Pharmingen), PE-Cy5 conjugated rat anti-mouse CD38 (eBioscience), APC-conjugated rat anti-mouse F4/80 Antigen (eBioscience). Isotype control antibodies were used at the same dilution as their counterparts. The cells were washed and analyzed on a LSRII flow cytometer (Becton Dickinson, San Jose, CA) within 4 hours or fixed with 1% paraformaldehyde and run within 4 days after storage in dark/cold. For each experiment, 10,000 events were collected and analyzed using the Flowjo 7.6 program. The data were expressed as mean fluorescence intensity (MFI; arbitrary units).

Statistical analysis

Statistical analysis was performed using SPSS (release 19.0 SPSS, Inc., Chicago, IL, USA). Data are presented as mean ± SEM. Differences between treatment groups were analyzed by *t* test (two tailed) where $p < 0.05$ was considered statistically significant.

RESULTS

Effects of RA on the establishment and growth of endometriotic lesions

In a previous study of the effects of RA on tumor growth in a mouse model (38), we optimized an RA treatment protocol to achieve a mean peak serum concentration of 1 μM over extended periods (up to 30 days). We have utilized this protocol in the present study since this concentration of RA was shown to be optimal for inducing a variety of alterations in macrophages and endometrial cells that might be beneficial for reducing the invasive phenotype of ectopic endometrial tissue (7, 20, 40, 41). In pilot experiments, we determined that 100% of the animals had detectable peritoneal lesions by 5 days after challenge with uterine fragments. By 14 days, mature, GFP+ implants were prominent and many contained well-formed blood vessels. The presence of the latter tended to correlate with the invasive quality of the implant (depth of invasion into host tissue) (Fig. 1A-C). Therefore, our evaluation of lesion formation delineated both total number of invading lesions and those showing visible vessels in animals where RA or vehicle treatment began 3 days before intraperitoneal inoculation of endometrial cells and continued for 14 days until sacrifice. As shown in Fig. 2, RA-treated animals showed a reduction in the mean number of total implants (2.00 ± 0.26) vs. vehicle-treated controls (3.14 ± 0.40, P<0.03) and a decrease in

the mean number of lesions with visible vessels (RA, 0.55 ± 0.17; controls, 1.36 ± 0.28, P<0.02). In addition, the average volume of all endometriotic lesions per mouse in the RA group (7.2 ± 1.6 mm^3) was significantly less than those in the control group (18.4 ± 4.4 mm^3, P<0.03). There was no significant difference between the mean weights of the control versus RA-treated animals at the time of sacrifice (control, 18.3 ± 0.3 g; RA-treated, 18.4 ± 0.3 g).

Suppressive effects of RA treatment on cytokine secretion

The proinflammatory cytokines IL-6 and MCP-1 are known to be elevated in the peritoneal fluid of women with endometriosis (33–35). Studies in mice have demonstrated that secretion of these cytokines peak within the first few days after i.p. injection of endometrial cells and subsequently decrease to near background levels within a week (42). Thus, after concentrating the peritoneal lavage fluid, we measured IL-6 and MCP-1 levels in short-term experiments before lesions were apparent (3 days after endometrium injection) as well as at the 14-day time point when the endometriotic lesions were quantified. Fig. 3 shows that the concentrations of IL-6 diminished over the 11-day interval, and were significantly lower in the RA-treated groups compared with the control groups. On day 3, MCP-1 was significantly lower in the in the peritoneal washings of RA-treated mice compared to controls, but had fallen below detectable concentrations by day 14.

Effects of RA treatment on expression of macrophage activation/differentiation markers CD38, CD11b, and F4/80

Mouse macrophages are typically characterized by surface F4/80 glycoprotein and CD11b expression, but these markers are also expressed to lesser degrees by eosinophils and neutrophils (Fig. 4, top) (43–45). Peritoneal cells were harvested from washings after 14 days. As a means of discriminating the macrophage population from eosinophils and neutrophils in flow cytometric analyses, we gated on the CD11b^{high}F4/80^{high} populations (Fig. 4, top). There were no significant differences in the subpopulation profiles (i.e. relative number of macrophages, eosinophils, and neutrophils) between the control and RA-treated animals. Relative changes in differentiation between control and RA treatment groups were assessed by quantitation of CD38, CD11b, and F4/80 expression as mean fluorescence intensity (MFI). Fig. 4, bottom shows that in macrophages, all three surface markers were significantly increased in the RA-treated versus control mice.

DISCUSSION

This study demonstrated that *in vivo* RA treatment suppressed the establishment and growth of endometriotic lesions in an immunocompetent mouse model of endometriosis. RA also decreased the peritoneal fluid levels of IL-6 and MCP-1, two inflammatory cytokines that are elevated in the peritoneal fluid of women with endometriosis and have been implicated in its pathogenesis (5, 33, 35). In addition, RA treatment modulated the "differentiated state" of peritoneal macrophages as reflected by increased expression of CD38, CD11b, and F4/80. On a quantitative bases, both F4/80 and CD11b increase with differentiation of monocytes to macrophages and with inflammatory reactions that are associated with maturity and increased macrophage function including phagocytosis (44–47). CD38 is a type II transmembrane glycoprotein widely used as a marker of lymphocyte and macrophage activation and differentiation (48). RA has been shown to upregulate CD38 on immune cells via an RA response element located in the first intron rather than the 5'-flanking region of the CD38 gene (49). In macrophages, RA induction of CD38 is associated with an increase in differentiated functions including antigen presentation and cell adhesion (48, 50). These findings reveal intergrated anti-inflammatory and immunomodulatory effects of RA, which we propose contribute to the suppression of endometriotic lesions, and suggest the

therapeutic potential of agents that target the retinoic acid pathway for the treatment of clinical endometriosis.

Numerous studies provide evidence of the anti-proliferative effects of RA on cancer cells both *in vitro* and *in vivo* (51–53). Our results indicate that RA inhibited the growth of endometriotic lesions as reflected by decreases in the average number and volume of established implants in the retinoid-treated group. In contrast, *in vitro* studies of RA on primary endometrial cell cultures have not demonstrated direct antiproliferative activity (unpublished). This fact suggests that growth inhibition by RA on endometriotic implants is indirect, perhaps mediated by changes in macrophage function or altered production of cytokines involved in the survival of ectopic endometrial cells. To this end, aberrant regulation of IL-6 responses is thought to play a role in the development of endometriosis (54, 55). The mechanism of action of IL-6 on endometriotic cell growth is unknown, but may be mediated directly or through its ability to co-activate macrophages and lymphocytes, resulting in the sustained production of other cytokines involved in chemotaxis and cellular growth (7). Concomitant reduction of both IL-6 and MCP-1 levels in the peritoneal fluid of RA-treated mice is consistent with this latter hypothesis. In addition, both IL-6 and MCP-1 have been shown to be potent pro-angiogenic cytokines which can stimulate endothelial cell proliferation and migration (56, 57), and thereby promote the de novo vasculature necessary for the sustained growth of endometriotic lesions. To this end, the reduced number of lesions with vessels that formed in RA-treated animals is consistent with the reduced levels of these cytokines in their peritoneal fluid. Results of our *in vivo* studies support previous work showing that RA inhibited the expression and secretion of IL-6 from endometrial cells *in vitro* and suppressed MCP-1 expression in spleen cells from mice with collagen induced arthritis (20, 58).

Macrophages play an essential role in the establishment of endometriosis and we have suggested that pharmacologic manipulation of macrophage function may provide a novel mechanism for treating this disease (7). Peritoneal fluid macrophages are involved in the removal of red cells and endometrial tissue products arising via retrograde menstruation. In endometriosis, peritoneal macrophages have diminished capacity to scavenge cellular debris, which may lead to the persistence of endometriotic lesions (6). In addition, secretion of inflammatory cytokines from peritoneal macrophages is increased, leading to a positive feedback loop and the further propagation of a peritoneal inflammatory environment. As an example, we have shown that the expression of CCR1, a high affinity receptor for RANTES (Regulated upon Activation, Normal T cell Expressed and Secreted), is upregulated in peritoneal fluid macrophages from women with endometriosis (59).

RA treatment resulted in a more differentiated phenotype of peritoneal fluid macrophages as shown by upregulation of CD38, CD11b, and F4/80. These findings are consistent with *in vitro* studies showing that RA can upregulate CD38 and CD14 on human macrophages (60, 61). Additional promising anti-inflammatory actions of RA include induction of T-reg cells with resulting inhibition of IL17-producing helper cells (14), and stimulation of immunosuppressive CD8+ T cells (62) and myeloid-derived suppressor cells (63). Further studies are needed to delineate the salutary RA effects in our mouse model.

In conclusion, our results indicate that RA inhibits the development of endometriotic implants *in vivo*. We postulate that this effect is due, at least in part, to suppression of IL-6 and MCP-1 production, and to promotion of peritoneal macrophage differentiation. These preclinical *in vivo* findings emphasize the potential use of retinoids to treat women with endometriosis.

Acknowledgments

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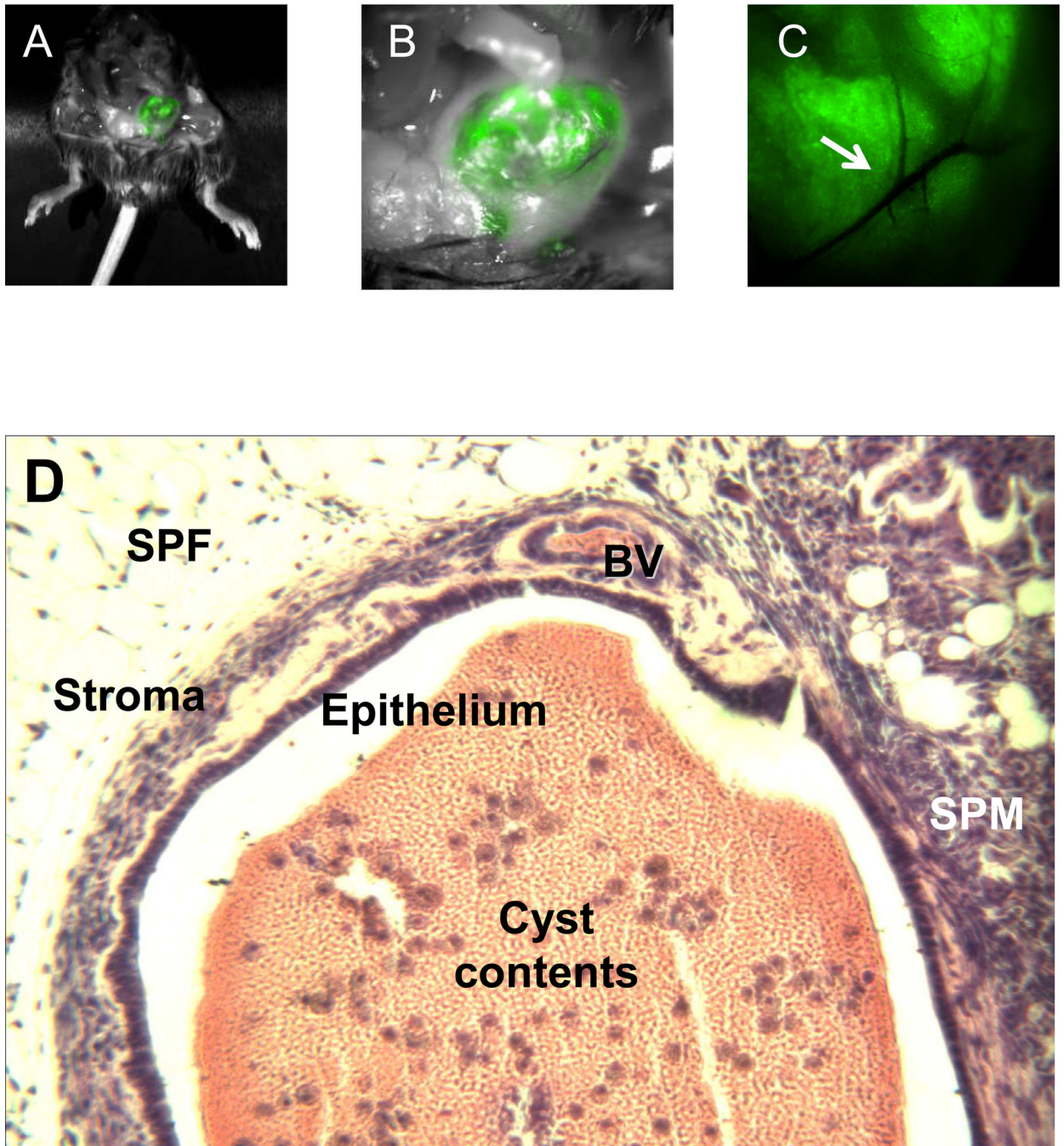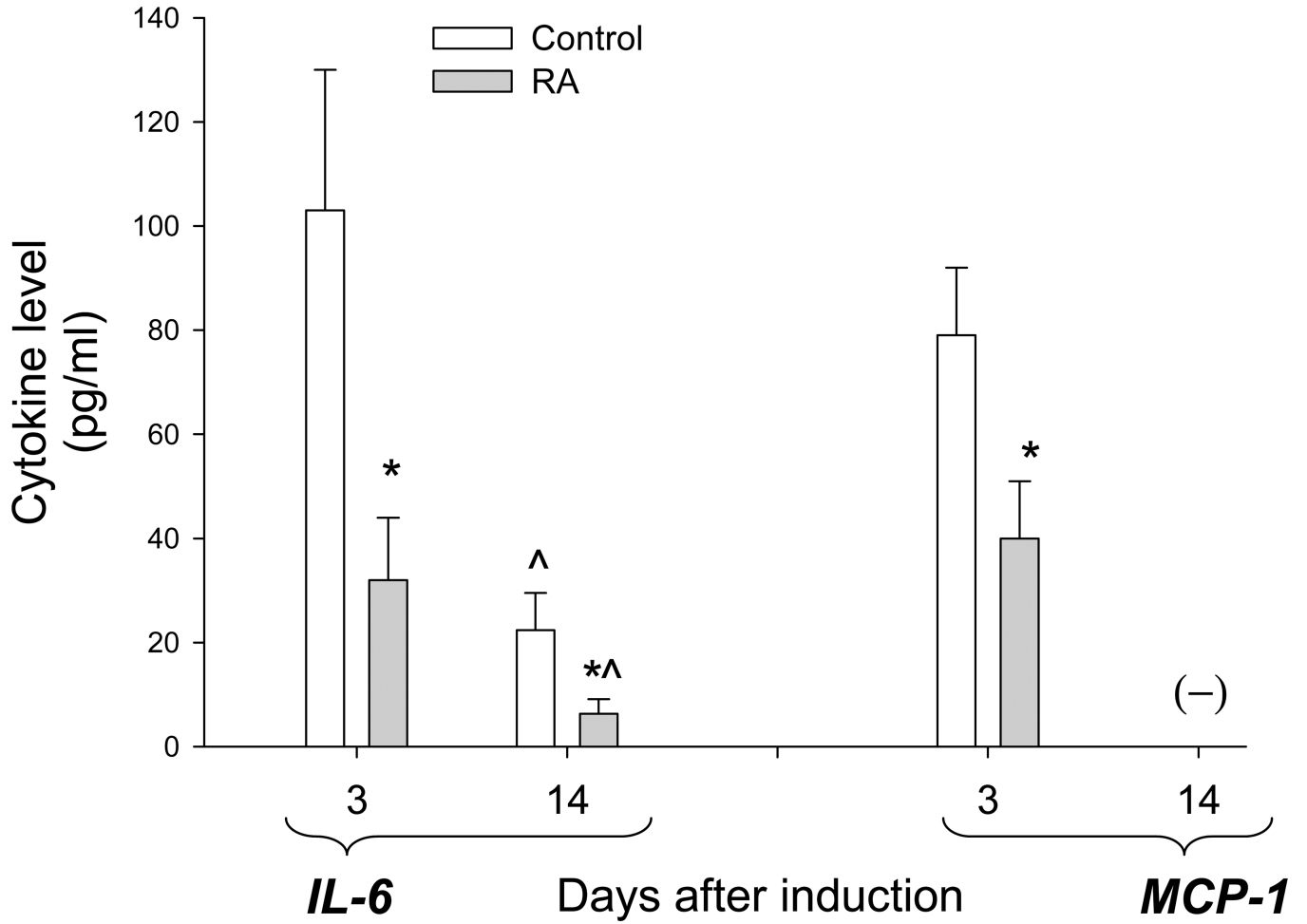
Figure 1.
Endometriotic lesions in recipient mice, 14 d after inoculation. **A.** GFP+ lesions were visible in situ using interference filter eyeglasses (excitation wavelength, 488 nm; emission wavelength, 502 nm). **B.** Higher power (5.6x) view shows large cystic and two small satellite GFP+ lesions invading anterior parietal peritoneum and preperitoneal fat in the left lower pelvis. **C.** High magnification (12x) view of GFP+ lesion shows vascular arcade of established GFP+ implant with arrow indicating vessel branchpoint. Pictures were taken with a Hamamatsu Multiplier CCD Camera (Bridgewater, NJ). **D.** H&E histology of murine endometriotic lesions was nearly identical to that observed in the human counterpart. This

example of a cystic lesion was lined by dense epithelium and surrounded by endometrial stroma. The lesion invaded subperitoneal fat (SPF) and subperitoneal muscle (SPM). The cyst wall contained blood vessels (BV) and the cyst contents included erythrocytes (pink) and leukocytes (purple). Magnification = 40x.
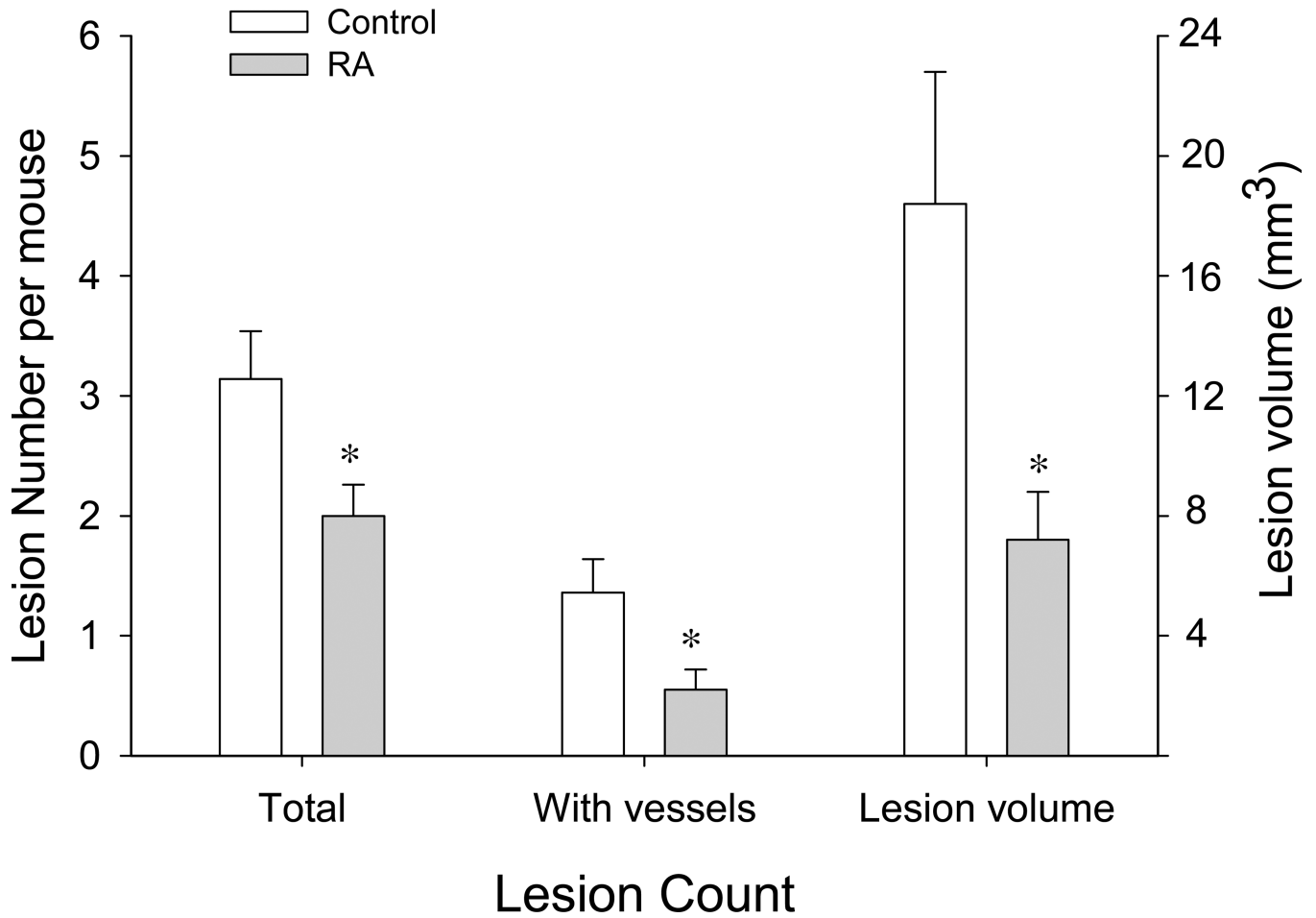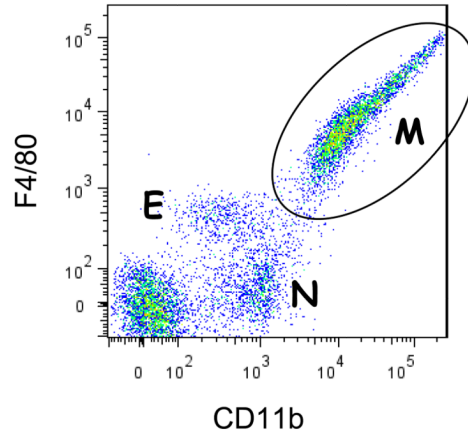
Figure 2.
Inhibition of lesion growth by retinoic acid. RA or vehicle control treatment began 3 days before intraperitoneal inoculation of endometrial cells and continued for 14 days until sacrifice. RA treatment significantly reduced the mean number of total lesions and number of vascularized lesions vs. vehicle-treated controls (n=22 for both groups). In addition, the average volume of established endometriotic lesions in the RA group was significantly less than those in the control group. Values represent the mean ± SEM of the indicated lesion count parameter. *, Significant decrease of the indicated lesion parameter compared with control-treated mice (p< 0.03).
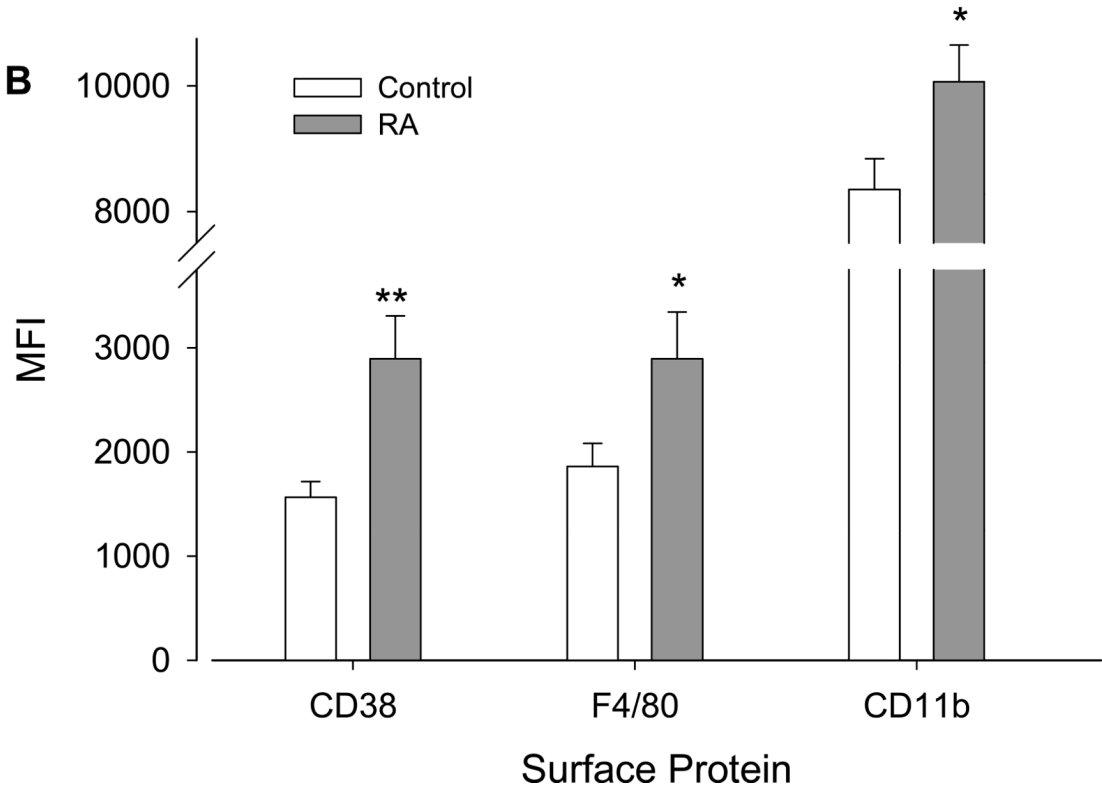
Figure 3.
Suppressive effects of retinoic acid treatment on peritoneal fluid IL-6 and MCP-1 levels.
Values represent the mean ± SEM of cytokine levels found in control and RA-treated
animals 3 days (n=10, both groups) and 14 days (n=22, both groups) after induction of
endometriosis. (-) indicates below the level of detection. *, Significant decrease in levels of
the indicated cytokine compared with control-treated mice (p< 0.05) ^, Significant reduction
in 14-day control and RA-treated samples compared with those from correspondingly
treated groups at day 3 (p<0.01).

A

B

Figure 4.
A. Representative dot plot showing CD11b and F4/80 fluorescent intensity of peritoneal cells from a control mouse 14 days after induction of endometriosis. Subpopulations of viable eosinophils (E, CD11b^{medium}F4/80^{medium}), neutrophils (N, CD11b^{medium}F4/80^{negative}), and macrophages (M, CD11b^{high}F4/80^{high}) are indicated. Dot blot analysis of RA-treated mice showed similar population profiles. **B.** Cells gated on the macrophage (M) population were analyzed for CD11b, F4/80, and CD38 expression. Upregulation by RA of the macrophage markers (CD11b and F4/80) and the differentiation/

activation marker CD38 was observed. Values represent the MFI ± SEM of the surface proteins as indicated in control (n=22) and RA-treated (n=22) mice. *, p< 0.03; **, p<0.002.