Tamoxifen, Flaxseed, and the Lignan Enterolactone Increase Stroma- and Cancer Cell–Derived IL-1Ra and Decrease Tumor Angiogenesis in Estrogen-Dependent Breast Cancer

Gabriel Lindahl[1], Niina Saarinen[1,2], Annelie Abrahamsson[1], and Charlotta Dabrosin[1]

Abstract

The proinflammatory cytokines IL-1α and IL-1β promote tumor angiogenesis that might be counteracted by the IL-1 receptor antagonist (IL-1Ra), anakinra, a clinically approved agent. A diet with high amounts of phytoestrogens, such as flaxseed (Flax), genistein (GEN), and the mammalian lignan enterolactone (ENL), may affect breast cancer progression in a similar fashion as the antiestrogen tamoxifen. Both cancer cells and tumor stroma may be targets for cancer therapy. By using microdialysis in a model of human breast cancers in nude mice, we could perform species-specific analyses of released proteins in the microenvironment. We show that tumors treated with tamoxifen and fed Flax or ENL exhibited decreased *in vivo* release of IL-1β derived from the murine stroma and decreased microvessel density whereas dietary GEN had no effects. Cancer cell–released IL-1Ra were approximately 5 times higher than stroma-derived IL-1Ra. Tamoxifen, Flax, and ENL increased IL-1Ra levels significantly whereas GEN did not. The tumor stroma contained macrophages, which expressed the estrogen receptor. *In vitro*, estradiol decreased IL-1Ra released from breast cancer cells and from cultured macrophages. IL-1Ra decreased endothelial cell proliferation significantly *in vitro* whereas breast cancer cell proliferation was unaffected in presence of estradiol. Finally, IL-1Ra therapy of tumor-bearing mice opposed estrogen-dependent breast cancer growth and decreased angiogenesis. We conclude that the release of IL-1s both by cancer cells and the stroma, where macrophages are a key component, may offer feasible targets for antiestrogen therapy and dietary interventions against breast cancer. *Cancer Res; 71(1); 51–60. ©2011 AACR.*

Introduction

The inflammatory cytokines interleukin-1 (IL-1) α and IL-1β may contribute to angiogenesis, tumor proliferation, and local invasion of breast cancer (1, 2). It has been suggested that IL-1β is important for the initiation and completion of angiogenesis and IL-1 α or IL-1 β knockout mice have been shown to exhibit dramatically impaired ability of tumor development and angiogenesis (3). Treatment with the naturally occurring IL-1 receptor antagonist (IL-1Ra), which antagonizes the activities of IL-1s without any agonist effect, may lead to decreased inflammatory response and inhibited tumor development in mice (4, 5).

Estrogens play an important role in the development and progression of breast cancer and approximately two thirds of all breast cancers are estrogen receptor (ER) positive. Blocking the action of estrogens is a corner stone in the medical treatment of breast cancer today (6, 7).

Phytoestrogens, plant compounds structurally similar to estrogen, may have both agonistic and antagonistic actions in breast cancer dependent on the type and dose (8, 9). In Eastern societies, with a diet containing large amounts of phytoestrogens, the incidence of breast cancer is low and migrant studies support that lifestyle factors rather than genetics affect breast cancer risk (10, 11). There are 2 major classes of phytoestrogens: isoflavones, found in high concentrations in soy with genistein (GEN) being the main compound, and lignans, found in high amounts in flaxseed (Flax), with enterolactone (ENL) as a main potent active metabolite (12, 13).

Recent data suggest that the effects of Tamoxifen (Tam), the most widely used drug against ER$^+$ breast cancer, are potentiated if both the stroma and cancer cells are affected (14). We have previously found inhibition of angiogenesis of ENL by a reduction of VEGF secretion both by breast cancer cells and the tumor stroma (15). Whether tamoxifen and/or various phytoestrogens affect IL-1s released by tumor cells and/or stroma is unknown.

In this study, human breast cancer tumors were established in nude mice creating a model with species-specific components in the tumor microenvironment. By using microdialysis, which we previously have validated for use in tumor tissue (16–23), proteins in the tumor microenvironment were sampled.

Authors' Affiliations: [1]Linkoping University, Clinical and Experimental Medicine, Division of Oncology, Linkoping, Sweden; and [2]Functional Foods Forum, University of Turku, Turku, Finland

G. Lindahl and N. Saarinen contributed equally to this work.

Corresponding Author: Charlotta Dabrosin, Division of Oncology, University Hospital, SE-581 85, Linkoping, Sweden. Phone: 46-10-103-85-95; Fax: 46-10-103-30-90. E-mail: charlotta.dabrosin@liu.se

We show for the first time that Flax, ENL, and Tam decreased IL-1β released by stroma and increased the levels of both cancer cell- and stroma-derived IL-1Ra. A diet of GEN did not affect the release of the IL-1s. *In vitro*, IL-1Ra decreased cell proliferation of human umbilical vein endothelial cells (HUVEC) whereas the proliferation of cancer cells was unaffected. Moreover, treatment of tumor-bearing mice with recombinant IL-1Ra decreased tumor growth and angiogenesis.

Materials and Methods

Cells culture

Reagents were obtained from Gibco BRL unless otherwise stated. MCF-7 (HTB-22) human breast cancer cells, characterized by The American Type Culture Collection, by receptor analysis, growth pattern, and morphology, were maintained in phenol red–free Dulbecco's minimum essential medium (DMEM) supplemented with 10% FBS, 50 IU/mL penicillin, 50 µg/mL of streptomycin, and 2×10^{-3} mol/L of L-glutamine at $37°$C and 5% CO_2. Trypsinized (0.05% trypsin and 0.02% EDTA) cells were seeded, 20,000 cells/cm^2, into petri dishes (CoStar), and exposed to 10^{-9} mol/L of E2 (17β-E2; Apoteket), 10^{-6} mol/L of Tam (Sigma), 10^{-6} mol/L of ENL, 10^{-6} mol/L of GEN, or their combinations. ENL (purity > 98%) was obtained from VTT. GEN (purity = 98%) was a generous gift from Professor William Helferich (University of Illinois). Hormones were added in serum-free DMEM/F12 (1:1) medium without phenol red supplemented with 10 µg/mL of transferrin (Sigma Chemicals), 1µg/mL of insulin (Sigma), and 0.2 mg/mL of BSA (bovine serum albumin; Merck). The medium was changed everyday.

HUVEC were prepared from umbilical cords donated anonymously according to national ethical legislation. Cells were isolated by digestion at $37°$C using M199 medium with 0.1% collagenase (Roche Diagnostics GmbH) and 2% FBS for 20 minutes. Cells were cultured in M199 culture medium containing 20% FBS, 20 mmol/L of HEPES, 1% nonessential amino acids, 50 IU/mL penicillin, 50 µg/mL of streptomycin, 20 µg/mL of endothelial cell growth factor (Roche Diagnostics GmbH), and 1 mmol/L of sodium pyruvate in 1% gelatin-coated bottles at $37°$C with 5% CO_2 until 70% to 90% confluent. Cell passages 3 to 5 were used.

Proliferation of MCF-7 cells and HUVEC

MCF-7 cells were seeded in 96-well plates (3,000 cells per well). After 24 hours, E2 and/or recombinant IL-1Ra (Kineret, Biovitrum) were added in serum-free D-MEM/F12. HUVEC were seeded (5,000 cells per well) in 1% gelatin-coated, 96-well plates. After 24 hours, E2 and/or recombinant human IL-1Ra were added in M199 medium with 1% FBS and 0.1% BSA. After 3 days, the proliferation was analyzed with MTS kit (Promega Biotech AB) according to the manufacturer's instructions.

Macrophages preparation for cell culture and ERα staining

According to the Swedish ethical legislation and after informed consent, human venous blood was collected in heparin tubes, diluted with RPMI medium (Invitrogen), and separated by Ficoll-paque Plus (GE Healthcare) density gra-

dient centrifugation. Mononuclear cells were washed twice, stained with Turk's solution (Merck), and counted. Monocytes, 0.5×10^6 per well, were seeded in 24-well plates and incubated for 2 hours at $37°$C, 5% CO_2 to allow macrophage adhesion. Nonattached cells were removed by washing. The cells were exposed to 10^{-9} mol/L of E2, plus 10^{-6} mol/L of Tam, 10^{-6} mol/L of ENL, or 10^{-6} mol/L of GEN. After 24 hours, medium was collected, centrifuged, and stored at $-20°$C.

For ERα analysis, 5×10^4 mononuclear cells were cytocentrifuged onto glass slides, air dried, and stored at $-20°$C. For staining, mouse anti-human ERα (Dako) and MACH4 (Histolab) was used.

IL-1α, IL-1β, and IL-1Ra analyses

Cell culture media and microdialysis samples were analyzed for Il-1α, Il-1β, and IL-1Ra using human and murine immunoassay kits (R&D Systems). Total protein content of cell lysates was determined using Bio-Rad DC Protein Assay (Bio-Rad).

Animals housing conditions and study protocol

Linkoping University animal ethics research board approved the study. Athymic female mice (Balb/cA nu/nu, 6–7 weeks old, Taconic) were housed in pathogen-free isolation facility with a 12-hour light/dark cycle, administered sterilized diet (Special Diet Services) and water *ad libitum*. The animals were oophorectomized under intraperitoneal ketamine/xylazine anesthesia and implanted with E2 pellets, 0.18 mg/90-day release providing physiologic E2 concentrations (Innovative Research of America). Four days after surgery, MCF-7 cells (5×10^6 cells) were injected subcutaneously (s.c.) on the flanks. Tumor area was calculated using [(length $\times$ width)/2] $\times$ π.

In a first experiment, at the time of cancer cell injection, mice were fed phytoestrogen-free AIN-93G basal diet (BD), where soy oil was replaced with corn oil and sterilized by irradiation. At similar tumor sizes, mice were divided into 4 dietary treatment groups: BD (E2 + BD), BD + 100 mg/kg of GEN (E2 + GEN), BD + 100 mg/kg of ENL (E2 + ENL), and BD + 10% ground Flax (BD + Flax). Body weights were similar in all groups and food intake and body weights were monitored weekly. Administration of diets resulted in exposure of 10.0 to 16.8 mg/kg of body weight per day of GEN and ENL resulting in serum concentrations of approximately 0.5 µmol/L (Warri et al., 2009, unpublished results), which is in the range seen in women consuming soy products (24, 25) or Flax (26).

In the second experiment, mice with similar tumor sizes were divided; 1 group continued with the E2 treatment only, whereas in the other group Tam (1 mg for every 2 days s.c.) was added.

In the third experiment, mice with similar tumor sizes were divided and 1 group received daily s.c. injections (5 mg per mouse) of IL-Ra (Kineret, Biovitrum) whereas the control group was treated with daily injections of the solvent (PBS).

Microdialysis of tumor tissue

Microdialysis probes (CMA/20: diameter = 0.5 mm, length = 10 mm, 100 kDa cutoff PES membrane; CMA Microdialysis AB) were inserted into tumor tissue and perfused at 0.6 µL/min with 0.9% saline containing 40 mg/mL of dextran (Meda) as previously described (21). After a 30-minute equilibration
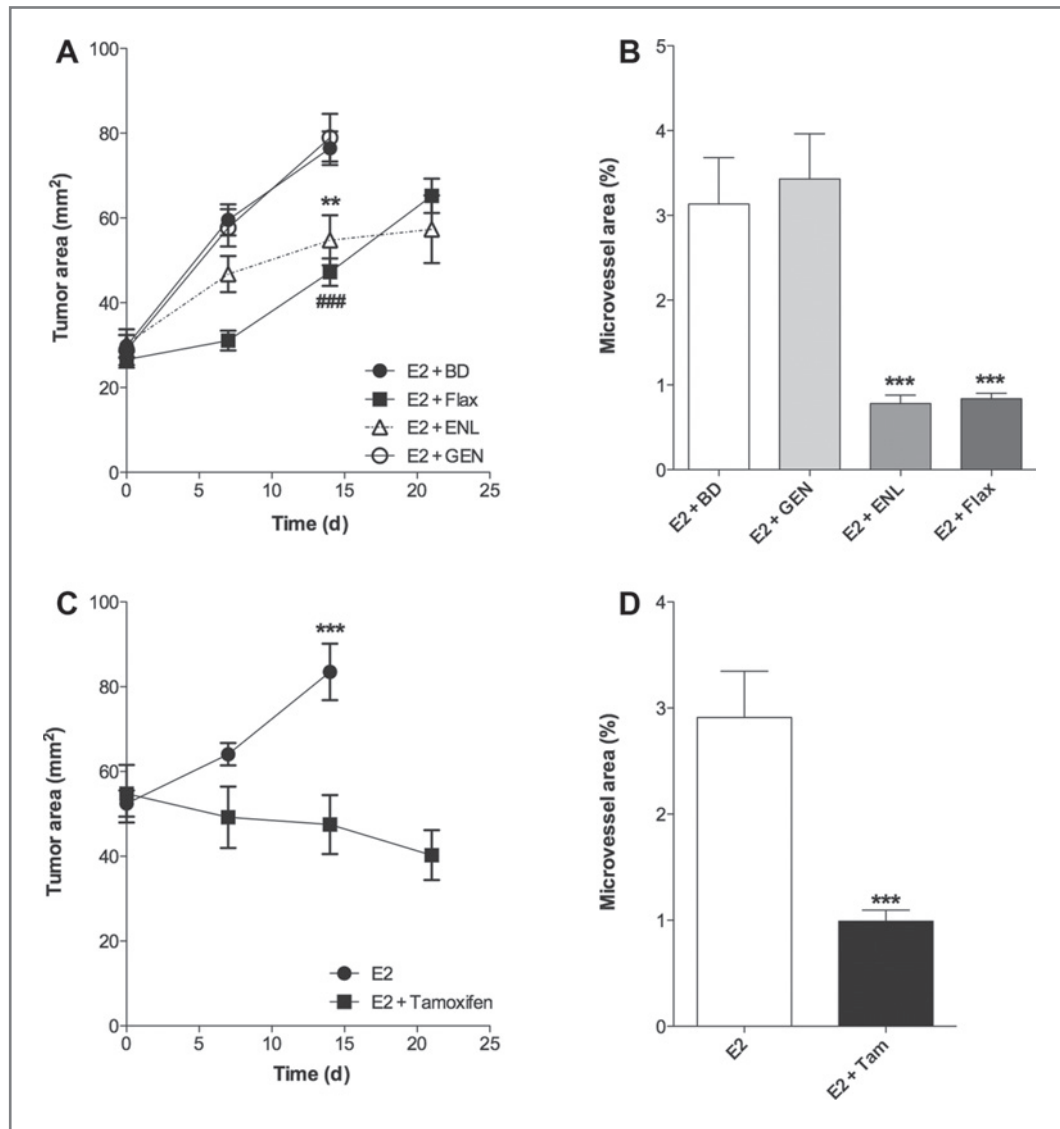
Figure 1. Tumor growth and tumor angiogenesis *in vivo*. Oophorectomized nude mice were supplemented with physiologic levels of E2 and injected s.c. with MCF-7 cells on the flanks. *A* and *B*, at similar tumor sizes, mice were allocated into 4 diet groups; controls fed with AIN-93G diet (E2 + BD) or BD containing 100 mg/kg of E2 + GEN, 100 mg/kg of E2 + ENL, or BD + 10% E2 + Flax. A, tumor growth over time. **, $P < 0.01$ compared with E2 + BD and E2 + GEN; ###, $P < 0.0001$ compared with E2 + BD and E2 + GEN; n = 14 to 23 tumors in each group; dots, mean; and bars, SE. B, sections from tumors were stained for microvessels with anti-Von Willebrand's factor and percentage of stained area was digitally quantified. ***, $P < 0.0001$ compared with E2 + BD and E2 + GEN; n = 9 in each group; columns, mean; and bars, SE. Cand D, at similar tumor sizes, 1 group continued with E2 alone and in 1 group, tamoxifen (1 mg s.c.) every second day was added (E2 + Tam). C, tumor growth over time. ***, $P < 0.0001$; n = 8 to 9 in each group; Dots, mean; and bars, SE. D, microvessel area (percent) of tumors stained with anti-von Willebrand's factor and digitally quantified. ***, $P < 0.0001$; n = 9 in each group; Columns, mean; and bars, SE.

period, microdialysates were collected on ice and stored at −70°C. The microdialysis samples were analyzed undiluted and results are given as raw data.

Immunohistochemistry of tumor sections

Formalin-fixed, paraffin-embedded tumors were cut in 3-μm sections, deparaffinized, and subjected to immunohistochemistry, IL-1α and IL-1β (mouse anti-human, 5 μg/mL; R&D Systems), stroma ERα (rabbit anti-mouse ERα; dilution 1:2,000; Lifespan Bioscience) and the macrophage marker F4/80 (rat anti-mouse F4/80, 0.67 μg/mL clone CI:A3–1;

Abcam) with Envision detection (DAKO) or Rat on Mouse HRP Polymer Kit (Bio Care Medical). For microvessel staining, anti-von Willebrand's factor was used (rabbit anti-human, which cross-reacts with mouse protein, dilution 1:1,000: Dako A/S). Sections were counterstained with Mayer's hematoxylin. Negative controls did not show staining. In a blinded manner, high-power fields (200×) were examined from tumors in each group. The IL-1 staining was scored as weakly or strongly positive. Vessel quantification (% area) of tumor sections was conducted using images acquired on an Olympus BX41 microscope at 200× magnification. The images were digitally

analyzed and percentage of area stained positively for von Willebrand's factor was quantified using ImageJ software version 1.39u (NIH).

Statistical analyses

The values represent mean $\pm$ SE. Statistical analyses were performed using Student's t test, Fishers's exact test, and ANOVA with Tukey's *post hoc* test where appropriate (Graph-Pad Prism 5.0).

Results

### ENL and Flax, but not GEN, reduced tumor growth and tumor angiogenesis *in vivo*

In this model, E2 is required for tumor take and continued tumor growth of MCF-7 in nude mice. Therefore, no untreated control group could be included. Instead, the various diets and treatments were added with a stable background of estradiol (E2) at physiologic levels. As previously shown (15), ENL and Flax decreased tumor growth whereas mice fed with GEN exhibited similar tumor growth as mice fed BD (Fig. 1A). After 14 days of diet, tumors in the E2 + ENL group were significantly smaller compared with the E2 + BD and E2 + GEN groups ($P <$ 0.01; Fig. 1A). Tumors of mice in the E2 + Flax group did also exhibit significantly smaller tumors compared with E2 + BD and E2 + GEN mice ($P < 0.0001$; Fig. 1A). The microvessel areas from E2 + ENL-exposed and E2 + Flax-exposed animals were decreased compared with E2 + BD and E2 + GEN tumors (Fig. 1B; $P < 0.0001$). E2 + GEN did not affect tumor growth or microvessel area compared with E2 + BD. In the Tam, ENL, and Flax group, no mice succumbed heavy tumor burden or other illnesses before the end of the experiment.

MCF-7 tumors express cellular IL-1α and IL-1β

Immunohistochemical staining confirmed expression of IL-1α and IL-1β in MCF-7 cells. In 10 randomly selected areas of 3 different tumors in each treatment group, the intensity of staining was scored. There were no differences in intensity of the staining between the treatments, IL-1α stained strongly positive in 27 of 30, 26 of 30, 26 of 30, 28 of 30, 27 of 30 areas in E2, E2 + Tam, E2 + ENL, E2 + Flax, and E2 + GEN, respectively (Fig. 2A). For IL-1β, 29 of 30, 30 of 30, 28 of 30, 30 of 30, 29 of 30 areas were scored as weakly positive in E2, E2 + Tam, E2 + ENL, E2 + Flax, and E2 + GEN tumor sections (Fig. 2B).

ENL and Flax, but not GEN, decreased extracellular stroma-derived IL-1β and increased extracellular stroma- and cancer cell–derived IL-1Ra

Microdialysis was performed on size-matched tumors from the different diet groups. As the tumors consisted of human cancer cells surrounded by a murine stroma, it was possible to distinguish from which compartment in the tumor microenvironment, the cytokines were released. As hypoxia may affect several factors important in angiogenesis regulation, the tumors were size matched and exhibited no necrotic areas confirmed by hematoxylin and eosin (H&E) staining. IL-1α (human and murine) and human IL-1β were not detectable in the microdialysates. Stroma-derived murine IL-1β was
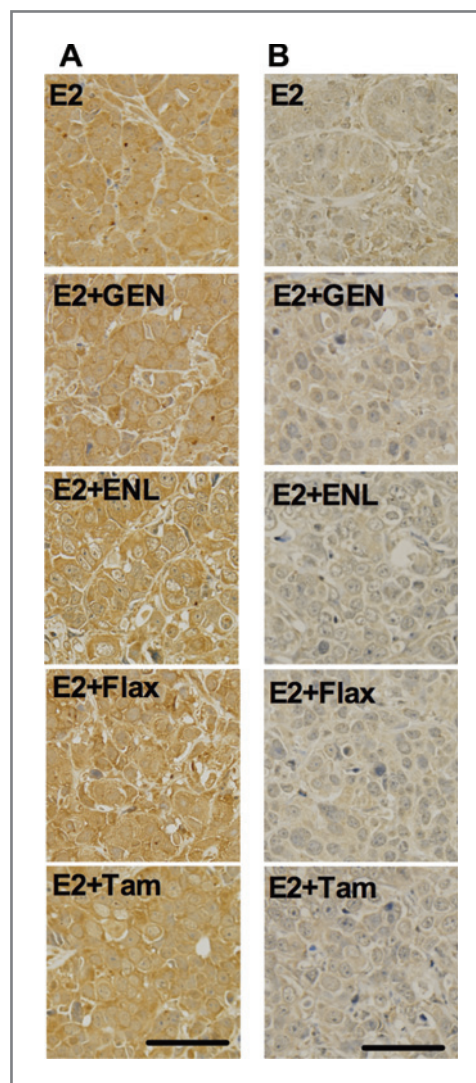
Figure 2. Staining for IL-1α and IL-1β of MCF-7 tumors. Oophorectomized nude mice were supplemented with physiologic levels of E2 and injected s. c. with MCF-7 cells on the flanks. At similar tumor sizes, mice were allocated into 4 diet groups; controls fed with AIN-93G diet (E2 + BD) or BD containing 100 mg/kg of E2 + GEN, 100 mg/kg of E2 + ENL, or BD + 10% E2 + Flax or tamoxifen (1 mg s.c.) every second day was added (E2 + Tam). No detectable differences were revealed between the different treatment groups. Scale bar, 100 μm. A, representative staining of IL-1α. B, representative staining of IL-1β.

significantly decreased in tumors grown in mice in the E2 + ENL and E2 + Flax exposed groups (Fig. 3A, E2 + ENL vs. E2 + BD; $P < 0.05$, and E2 + Flax vs. E2 + BD; $P < 0.01$). There were approximately 4 times higher levels of cancer cell–derived human IL-1Ra compared with stroma-derived murine IL-1Ra in the tumors from E2 + BD group, 82 ± 5.3 pg/mL of human IL-1Ra compared with 18 ± 2.5 pg/mL of murine IL-1Ra ($P < 0.0001$). Both cancer cell- and stroma-derived IL-1Ra were significantly increased in tumors of E2 + ENL-exposed and E2 + Flax-exposed animals compared with E2 + BD fed mice (Fig. 3B and C; $P < 0.001$ and $P < 0.0001$, respectively). E2+GEN did not affect IL-1s compared with E2 + BD.
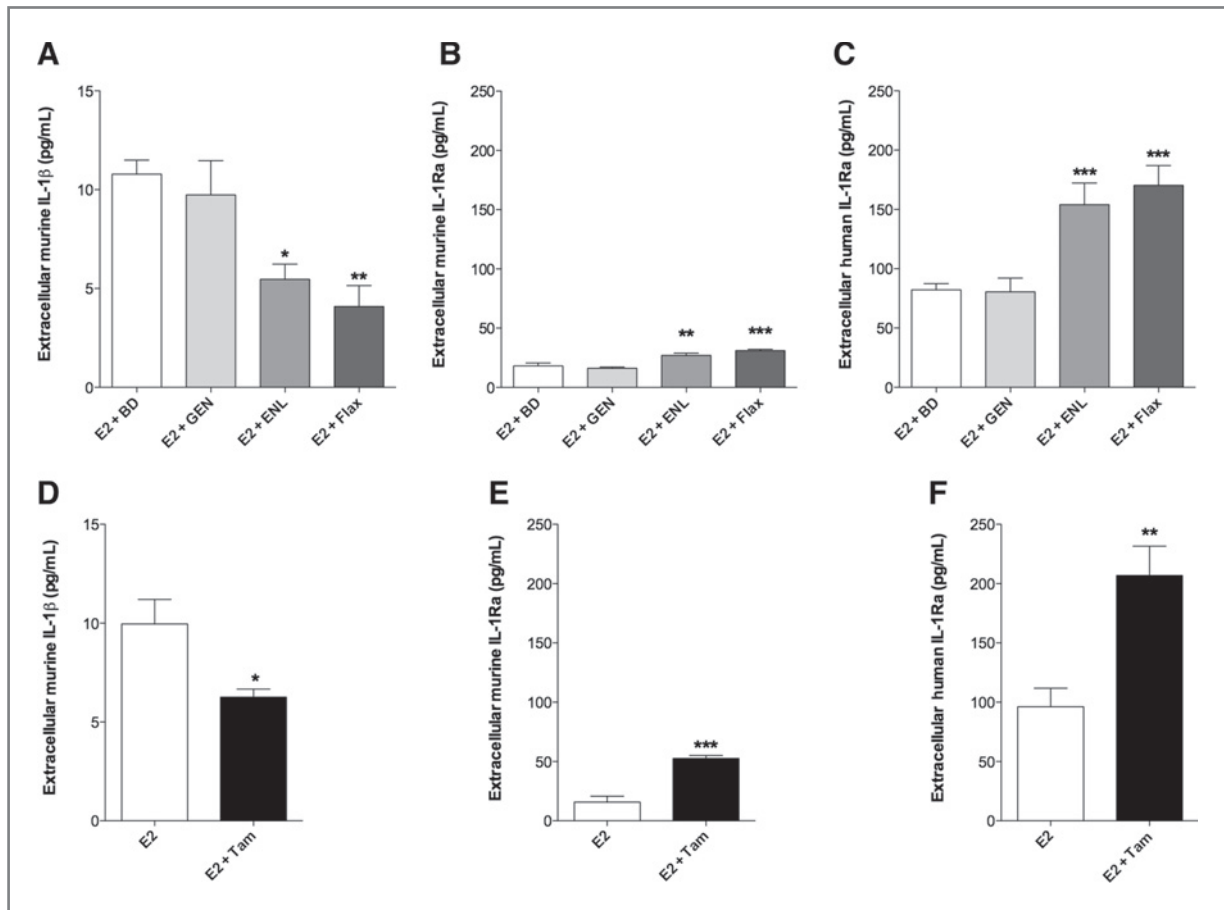
Figure 3. Microdialysis for *in vivo* sampling of extracellular stroma- and cancer cell–derived IL-1s in tumor tissue. Oophorectomized nude mice were supplemented with physiologic levels of E2 and injected s.c. with MCF-7 cells on the flanks. Microdialysis was performed for sampling of extracellular proteins *in vivo*. IL-1β derived from cancer cells was not detectable in the microdialysates. A–C, at similar tumor sizes, mice were allocated into 4 diet groups; controls fed with AIN-93G diet (E2 + BD) or BD containing 100 mg/kg of E2 + GEN, 100 mg/kg of E2 + ENL, or BD + 10% of E2 + Flax. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$ compared with E2 + BD and E2 + GEN; $n = 5$ to 7 in each group; Columns, mean; bars, SE D–F, at similar tumor sizes, 1 group continued with E2 alone and in 1 group, tamoxifen (1 mg s.c.) every second day was added (E2 + Tam). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$; $n = 5$ to 10 in each group; Columns, mean and bars, SE.

Tamoxifen decreased extracellular stroma-derived IL-1β and increased extracellular stroma- and cancer cell–derived IL-1Ra

To elucidate if Tam induced similar effects as ENL and Flax on the secretion of IL-1s, another set of animals were set up. As expected, Tam significantly reduced tumor growth as shown in Figure 1C. This was associated with reduced angiogenesis, confirming our previous data (Fig 1D). As Figure 3D shows, Tam decreased IL-1β in a similar fashion as ENL and Flax, E2 + Tam versus E2; $P < 0.05$. There were also similar effects on IL-1Ra as with ENL and Flax. There was a pronounced upregulation of stroma-derived IL-1Ra after Tam exposure ($P < 0.0001$; Figs. 3 and 4A and B). Also, the cancer cell–derived IL-1Ra was increased after Tam treatment ($P < 0.01$; Fig. 3E).

Macrophages and ERα-expressing cells in the tumor stroma

By using an antibody raised against murine ERα without cross-reactivity to the human receptor, we show that some of the stroma cells expressed ERα (Fig. 4A). Thereafter, we stained the sections for immune cells and found very few/ no NK (natural killer) cells and neutrophils in the tumors (data not shown). However, the stroma contained large areas of macrophages (Fig. 4B). In line with previous data (27, 28) showing that macrophages express ERα we found that the areas staining for macrophages also were stained for ERα (Fig. 4A and B). To confirm this, we isolated mononuclear cells from venous blood and found that macrophages expressed ERα (Fig. 4D). To further analyze if macrophages responded to our treatments, we cultured isolated macrophages *in vitro* and exposed them to E2, Tam, ENL, and GEN. In line with previous data (29), E2 decreased the IL-1Ra levels significantly compared with controls ($P < 0.01$; Fig. 4E). This decrease was counteracted with all treatments (Fig. 4D). The macrophages also secreted low levels of IL-1α; 34 ± 3.4 pg/10^6 cells to 44 ± 5.0 pg/10^6 cells without any differences between the various treatment groups. The levels of IL-1β were between 522 ± 52 pg/10^6 cells and 852 ± 81 pg/10^6 cells in the different treatment groups with a significantly higher level in the E2 + GEN group compared with E2 alone; $P < 0.05$.
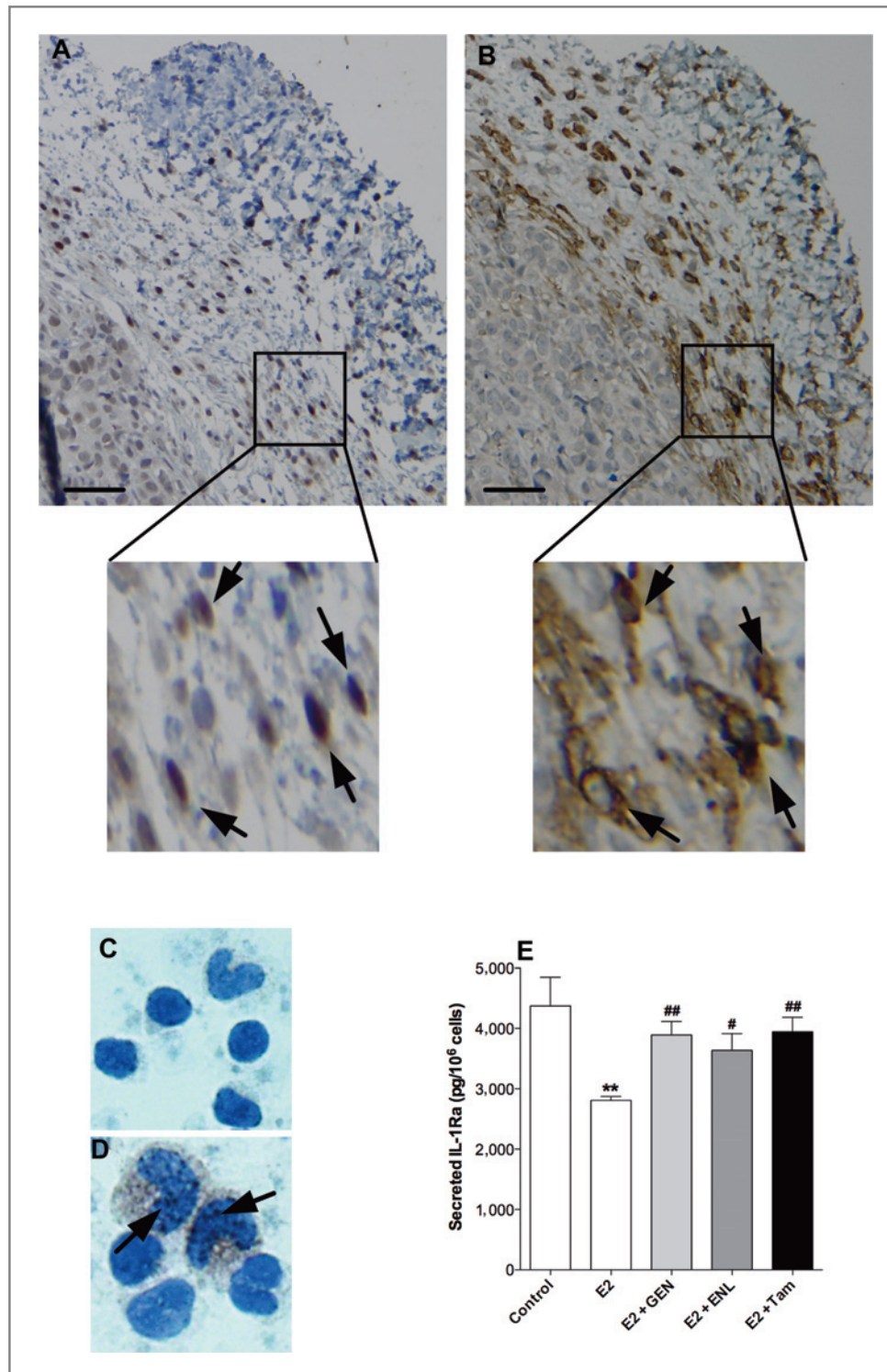
Figure 4. Macrophages, ERα expression, and secretion of IL-1Ra from cultured macrophages *in vitro*. A and B, oophorectomized nude mice were supplemented with physiologic levels of E2 and injected s.c. with MCF-7 cells on the flanks to form tumors. Serial tumor sections were stained for ERα and the macrophage marker F4/80. Scale bar, 100 μm. A, ERα-stained tumor section. B, F4/80-stained tumor section. A and B, magnification. ERα-stained cells also stained for F4/80 in serial sections. C and D, mononuclear cells were isolated from venous blood, cytocentrifuged onto glass slides and stained for ERα. C, negative control. D, ERα-stained macrophages. E, mononuclear cells were isolated from venous blood and macrophages were cultured for 24 hours in presence of vehicle (control), 10^{-9} mol/L of E2, 10^{-6} mol/L of E2 + GEN, 10^{-6} mol/L of E2 + ENL, or 10^{-6} mol/L of E2+Tam. **, $P < 0.01$ compared with Control; #, $P < 0.05$; and ##, $P < 0.01$ compared with E2; $n = 4$ in each group; Columns, mean; and bars, SE.

E2 decreased whereas ENL and Tamoxifen increased secreted levels of IL-1Ra released by MCF-7 cells *in vitro*

MCF-7 cells were cultured to explore if the *in vivo* data on IL-1Ra could be verified. Secreted IL-1α and IL-1β were below detection levels of the ELISA. E2 significantly decreased IL-1Ra compared with controls without hormones ($P < 0.0001$; Fig. 5). The addition of GEN did not significantly affect the IL-1Ra compared with E2 alone. ENL reversed the E2-induced decreased IL-1Ra levels back to control levels, E2 versus E2 + ENL; $P < 0.0001$. The addition of Tam did not only reverse the decreased levels by E2 but increased the levels significantly also compared with the control group ($P < 0.0001$; Fig. 4). When used alone without E2, ENL and GEN increased the levels of IL-1Ra to some extent compared with the control cells whereas Tam increased the levels significantly, approximately 3 times above the control levels.

In presence of E2, MCF-7 proliferation was unaffected by IL-1Ra

At high concentrations of IL-1Ra, a decrease of MCF-7 cell proliferation was detected ($P < 0.01$; Fig. 6A). However, in presence of E2, similar to the *in vivo* situation IL-1Ra, at any concentrations, did not affect proliferation compared to E2 alone (Fig. 6B).

IL-1Ra inhibited cell proliferation of HUVEC *in vitro*

As we detected decreased angiogenesis in the tumor sections, we set up HUVEC cells for the proliferation assay. As shown in Figure 6C and D, recombinant IL-Ra did significantly inhibit the proliferation of HUVEC in a dose–response fashion, both with and without E2; $P < 0.0001$.

IL-1Ra inhibited E2-induced tumor growth and angiogenesis *in vivo*

In a final experiment, we wanted to confirm if IL-1Ra had the ability to reduce tumor growth and/or angiogenesis in estrogen-dependent breast cancer. At similar tumor sizes, 1 group of animals was treated with daily injections of IL-1Ra and the other group received daily injections of the solvent. Tumors of mice treated with IL-1Ra exhibited tumor stasis/tumor regression whereas the tumors in the control group continued to grow. After 3 weeks of treatment, there was a significant difference in tumors sizes between E2 and E2 + IL-1Ra ($P < 0.0001$; Fig. 7A). The tumors in the IL-1Ra did also exhibit decreased microvessel density ($P < 0.0001$; Fig. 7B and C).

Discussion

Here, we show that effects on IL-1s, released both by cancer cells and the stroma, are involved in the therapeutic effects of Tam in estrogen-dependent breast cancer. As previously shown, GEN did not inhibit or enhance estrogen-stimulated breast cancer growth whereas both Flax and ENL alone counteracted tumor growth in a similar manner (15, 30). Tumors of mice treated with Tam, fed Flax or ENL exhibited decreased *in vivo* release of IL-1β derived from the murine stroma and these tumors also demonstrated significantly lower microvessel density whereas dietary GEN had no effects. IL-1α and IL-1β
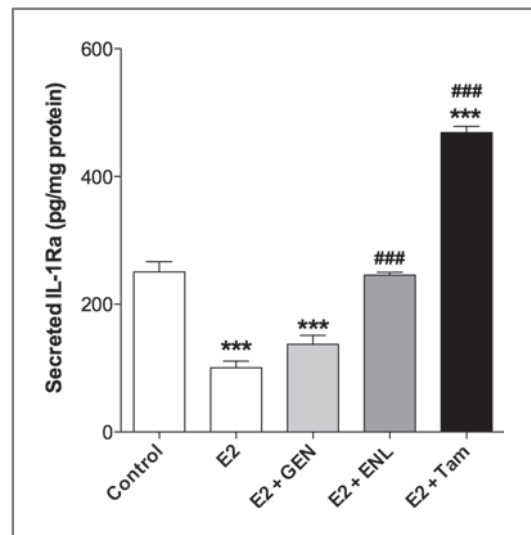

Figure 5. Extracellular IL-1Ra of MCF-7 cells in culture. MCF-7 cells were cultured for 7 days and exposed to vehicle (control), 10^{-9} mol/L of E2, 10^{-6} mol/L of E2 + GEN, 10^{-6} mol/L of E2 + ENL, or 10^{-6} mol/L of E2 + Tam. Cell culture supernatants were analyzed for IL-1Ra using quantitative ELISA. ***, $P < 0.0001$ compared with Control; ###, $P < 0.0001$ as compared with E2; $n = 5$ to 9 in each group; Columns, mean; and bars, SE.

derived from the cancer cells were not detectable extracellularly. This is most likely due to IL-1α being an intracellular protein and very low levels of secretion of human IL-1β by the cancer cells. However, immunohistochemistry confirmed expression of these cytokines in our model. IL-1Ra in the tumor microenvironment was released both by the stroma and the cancer cells with approximately 5 times higher levels from the cancer cells than the stroma-derived IL-1Ra. Tam, Flax, and ENL increased these levels significantly whereas GEN did not. *In vitro*, E2 decreased IL-1Ra and this could to some extent be counteracted by GEN whereas ENL reversed this decrease back to the control levels. Tam not only counteracted the reduced IL-1Ra levels but also further increased levels above the control cells. We detected a slight but significant decrease of the cancer cell proliferation by adding IL-1Ra to the cells but this was abolished in presence of E2 in the cell culture. Contrary to cancer cells, HUVEC proliferation was significantly decreased by IL-1Ra in a dose-dependent manner both with and without E2 in the culture. This suggests that the therapeutic effect of IL-1Ra mainly depends on decreased angiogenesis without significant effects on cancer cell proliferation. IL-1Ra therapy to tumor-bearing mice resulted in tumor stasis/regression and this suggests IL-1Ra to be a key mediator of estrogen-dependent breast cancer progression mainly by effects on the tumor stroma. These findings are consistent with other studies showing no effect on cancer cell proliferation *in vitro* by adding IL-1Ra but a significant tumor growth inhibition *in vivo* (31). As previously shown, macrophages expressed ERα, although not all ERα stained cells in the tumor stroma were macrophages and not all tumor macrophages stained for ERα (27, 28). *In vitro* culturing of macrophages also showed that our treatments affected IL-1Ra with a downregulation by E2 and higher levels
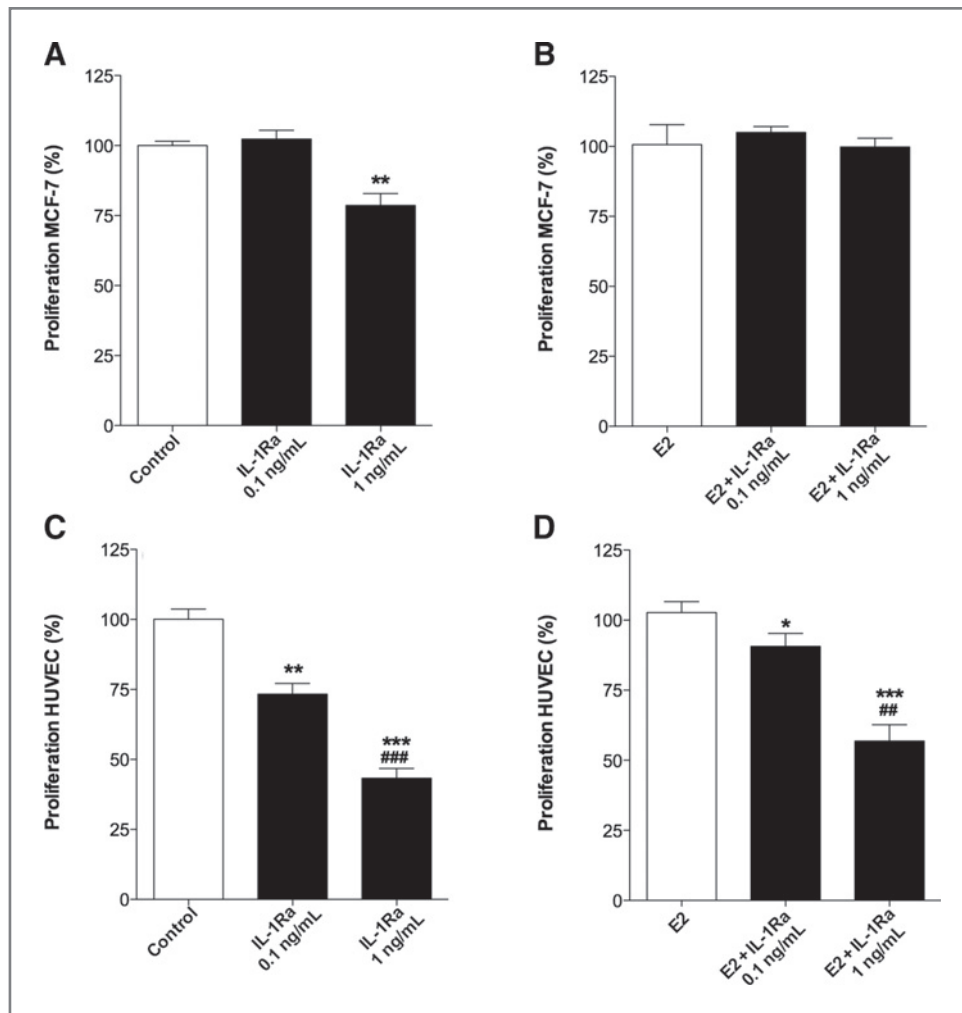
Figure 6. Proliferation of HUVEC and MCF-7 cells after IL-1Ra treatment MCF-7 cells and freshly isolated HUVEC were cultured *in vitro* and exposed to IL-1Ra for 3 days at different concentrations in presence or absence of 10^{-9} mol/L of E2. Proliferation was analyzed using MTS assay. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$ as compared with control cells; ##, $P < 0.01$; and ###, $P < 0.0001$ as compared with IL-1Ra (0.1 ng/mL); $n = 5$ in each group; Columns, mean; and bars, SE.

in the treatment groups. This suggests that macrophages may be 1 component involved in stroma-derived release of IL-1 in tumor tissue and a target for cancer therapies.

Studies have shown that IL-1β is required for tumor angiogenesis and that this effect can be inhibited by IL-1Ra and that IL-1β knockout mice exhibit impaired angiogenesis (3, 4, 32). We have previously shown a proangiogenic effect of E2 in breast cancer with a potent effect on VEGF and IL-8 secretion (18–20, 33). Here, we demonstrate that altering the levels of IL-1s may be another mechanism of the proangiogenic effect of E2. The tumor stroma is also a target for cancer therapeutics and it has been shown that a reactive stroma may predict the response to chemotherapy in ER-negative breast cancer (34). Moreover, Tam has been shown to induce a microenvironment, which is suppressive to breast cancer cells (14). In line with these data, we show in this study that the stroma cells in the tumors express ERα. Hence, the tumor stroma may be a target for antiestrogen treatment as we demonstrate that Tam increased stroma-derived IL-1Ra and decreased stroma-derived IL-1β. Several studies have reported that high IL-1β concentration within the tumor microenvironment is associated with a more virulent tumor phenotype and correlates

with a higher rate of recurrence (2, 35). Moreover in breast cancer patients, it has been shown that high levels of IL-1Ra and low levels of IL-1 at the tumor site is associated with a better prognosis (36) emphasizing the clinical relevance of our results. The cancer cells in our model did produce low levels of extracellular IL-1β. However, significant amounts of IL-1β derived from the stroma were present and a target for IL-1Ra therapy as we show that daily s.c. injections with IL-1Ra resulted in decreased tumor growth. This supports the concept of blocking IL-1 as a successful therapy against cancer as recently reviewed by Dinarello (37).

The majority of breast cancers are ER^+ and antiestrogen therapy is a corner stone in the medical treatment of this disease (6). Despite antiestrogen therapy, some patients will develop resistance and eventually die of metastatic disease and the antiestrogen therapies, such as Tam, and aromatase inhibitors may induce severe side effects. Hence, it is important to study the mechanisms involved in estrogen and antiestrogens action in breast cancer to develop novel therapeutic strategies against this disease. Components in our diet, such as phytoestrogens, may attenuate the incidence of breast cancer and progression of established disease (8, 9, 38, 39). We have

Figure 7. Growth and angiogenesis of MCF-7 tumors during IL-1Ra treatment. Oophorectomized nude mice were supplemented with physiologic levels of E2 and injected s.c. with MCF-7 cells on the flanks to form tumors. At similar tumor sizes, 1 group received daily injections of IL-1Ra (5 mg) whereas the other group received the vehicle. A, tumor growth was followed. ***, $P < 0.0001$; $n = 6$ to 7 in each group; Dots, mean; and bars, SE. B, microvessel area of tumors stained with anti-von Willebrand's factor and digitally quantified. ***, $P < 0.0001$; $n = 6$ in each group; Columns, mean; and bars, SE. C, representative tumors sections of. Scale bar, 100 μm.
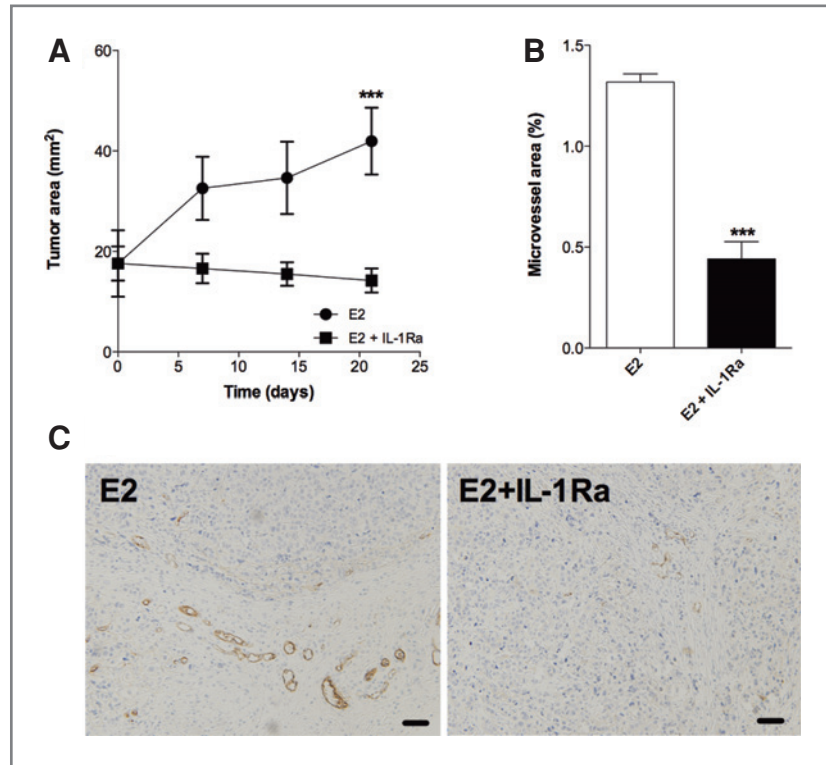
previously shown that ENL in absence or presence of E2 inhibited breast cancer growth by enhanced apoptosis and decreased VEGF secretion without any estrogenic effects on uteri and that GEN similar to E2 increased MCF-7 cell proliferation whereas ENL did not (15, 30, 40, 41). Both GEN and ENL can interact with ERs with different affinity and with tissue specificity depending on dose and presence/absence of E2, suggesting an ER-related mechanism of action by these compounds (42, 43). Our present results demonstrate that phytoestrogens may also affect IL-1s. Ingestion of Flax or ENL exerted similar effects of those seen in mice treated with Tam although Tam induced a more potent effect. GEN did not affect the IL-1s demonstrating that various phytoestrogen may have very different effects on tumor biology. Our data support previous studies showing an attenuation of the inflammatory response by tamoxifen and phytoestrogens (44, 45).

We conclude that IL-1Ra may be involved in estrogen-dependent breast cancer growth and that treatment with IL-1Ra to tumor-bearing mice decreased estrogen-dependent breast cancer growth. Tam, Flax, and ENL affected IL-1s released by the tumor stroma and we show that, at least in part, this may be attributable to macrophages, which expressed the ERα. *In vitro*, IL-1Ra did not have any direct effect on the tumor cells but significantly decreased endothelial cell proliferation. This suggests that the antitumor effects of IL-1Ra were mainly mediated through a tumor–stroma interaction. Our results demonstrate previously unrecognized mechanisms of E2, Tam, and dietary factors on breast cancer growth.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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