Infiltrating Macrophages Induce ERα Expression through an IL17A-mediated Epigenetic Mechanism to Sensitize Endometrial Cancer Cells to Estrogen

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Abstract

Persistent unopposed estrogen stimulation is a central oncogenic mechanism driving the formation of type I endometrial cancer. Recent epidemiologic and clinical studies of endometrial cancer have also revealed a role for insulin resistance, clinically manifested by chronic inflammation. However, the role of inflammation in estrogen-driven endometrial cancer is not well characterized. In this study, we investigated the association between infiltrating macrophages and estrogen sensitivity in endometrial cancer. Evaluating tissue samples and serum from patients with precancerous lesions or endometrial cancer, we found that tissue macrophage infiltration, but not serum estradiol levels, correlated positively with endometrial cancer development. Furthermore, IL4/IL13-induced CD681 CD1631 macrophages enhanced the proliferative effects of estradiol in endometrial cancer cells by upregulating estrogen receptor alpha (ERα), but not ERβ. Mechanistic investigations revealed that CD681 CD1631 macrophages secreted cytokines, such as IL17A, that upregulated ERα expression through TET1-mediated epigenetic modulation of the ERα gene. Overall, our findings show how cytokines produced by infiltrating macrophages in the endometrial microenvironment can induce epigenetic upregulation of ERα expression, which in turn sensitizes endometrial cells to estrogen stimulation. The concept that inflammation-induced estrogen sensitivity in the endometrium acts as a driver of type I endometrial cancer has implications for infiltrating macrophages as a prognostic biomarker of progression in this disease setting.


Introduction

Unopposed estrogen exposure has long been regarded as the main cause for type I endometrioid cancer (1). However, in a recent 14-year-long cohort study that included 1,017 women with an intact uterus and ages 50 to 69 years, Cherry and colleagues found that the risk of endometrial cancer in women exposed to long-term unopposed estrogen was no higher than that in women with no such exposure (2). In another study, Simon and colleagues showed no increased risk of endometrial hyperplasia and carcinoma in 541 postmenopausal women undergoing treatment with unopposed 10 μg estradiol vaginal tablets for 1 year (3). These findings suggest that mechanisms other than unopposed estrogen exposure may exist to promote the development of estrogen-driven endometrial cancer.

Epidemiologic studies have revealed a strong association between endometrial cancer and insulin resistance (IR) as well as inflammation (4–7), and inflammation is one of the most significant clinical manifestations of IR (8). In agreement with this clinical finding, macrophage infiltration, a specific manifestation of chronic inflammation, was found to be positively correlated with development of many kinds of cancers, including endometrial cancer (9, 10). The mechanism(s) involved in the association of infiltrating macrophages and cancer development is still unclear. Studies indicate that macrophages might be involved in regulation of sex hormone receptors in hormone-related cancers: in prostate cancer, infiltrating macrophages can upregulate androgen receptor (AR) expression and enhance the oncogenic activity of AR (11, 12), and in breast cancer, obesity-induced chronic inflammation can activate estrogen receptor (ER) in cancer cells (13). On the basis of the above findings, we hypothesized that infiltrating macrophages might be involved in the development of endometrial cancer by regulating ER expression or activity in endometrial cells.
Macrophages are widely distributed innate immune cells that play indispensable roles in the innate and adaptive immune response to pathogens and in-tissue homeostasis. There are different types of macrophages that play differing roles in the development of cancer (14, 15). These include the classically activated (M1) macrophage and the alternatively activated (M2) macrophage subsets. M1 macrophages are thought to have the ability to kill pathogens and tumor cells by secreting high levels of proinflammatory cytokines (16). In contrast, M2 macrophages, of which CD163, CD204, and CD206 are the major markers, are reported to inhibit immune response and promote cancer development (17). There are also reports that subdivide M2 macrophages into different subtypes (15–19). Because the exact subtypes and function of M2 macrophages remain unclear (20), we will only discuss in this paper the role of CD68+CD163+ macrophages (CD68 is the common marker for macrophages) in the progression of endometrial cancer.

There are several mechanisms involved in activation of ER, including genetic mutations and epigenetic alterations (21); and inflammation is reported to be involved in epigenetic modulation. In gastrointestinal smooth muscle, cytokines take part in epigenetic control of the smooth muscle phenotypic switch (22). Inflammation (both pro- and anti-inflammatory processes) is thought to mediate exercise-induced DNA methylation (23). Yet it is not clear whether macrophages can activate ER through epigenetic modulation of secreted cytokines.

In the present study, we evaluated the effects of CD68+CD163+ macrophages on estrogen-driven endometrial cancer proliferation and further investigated the molecular mechanism(s) underlying ER expression as regulated by CD68+CD163+ macrophages.

Materials and Methods

Ethics statement
This study complied with the tenets of the Helsinki Declaration, and was approved by the Medical Ethics Committee of the Obstetrics and Gynecology Hospital of Fudan University (Shanghai, P.R. China, hereafter referred to as ‘Ob&Gyn Hospital’). All patients signed an informed consent form before participating in the study.

Patient collection and serum hormone analysis
We collected two groups of patients for serum hormone analysis. The first group was a total of 411 cases including 64 cases of disordered proliferative endometrium (DPE), 159 simple hyperplasia (SH), 65 complex hyperplasia (CH), 31 endometrial atypical hyperplasia (EAH), 53 endometrial cancer, and 39 healthy controls. Patient information is seen in Supplementary Table S1. Serum estradiol, progesterone, and sex hormone-binding globulin (SHBG) levels were analyzed among these patients. The second group was composed of 34 endometrial cancer patients and 34 age-paired healthy controls, and serum estradiol levels were compared between them. ‘Age-paired’ herein implies that every endometrial cancer patient had one healthy control of the same age or at least within an error range of no more than 1 year. Patient inclusion and exclusion criteria are seen in Supplementary Materials and Methods.

Serum estradiol and progesterone level were measured with chemical luminescence immunoassay (Beckman Coulter) and SHBG with electrochemiluminescence assay (Roche).

Endometrial sample collection and IHC
Thirty cases of normal endometrium in the proliferative phase, 34 hyperplasia, 16 EAH, and 40 endometrial cancer cases were collected. Patient collection and information are seen in Supplementary Material and Methods and Supplementary Table S2. Inclusion and exclusion criteria were the same as mentioned above. Sections from the tissues were used for IHC assessment of CD68 and CD163. Tissue microarray was also generated from these tissue samples for IHC staining for IL17A, ten-eleven translocation 1 (TET1), 5-hydroxymethylcytosine (5-hmC), and ERTa. Tissue microarray blocks were constructed using a Manual Tissue Arrayer 1 (Becher Instruments.) according to the manufacturer’s instructions.

IHC and semiquantitative optical analysis was performed as described previously (24) and seen in Supplementary Materials and Methods. Primary antibodies to CD68, CD163, and IL17A were purchased from Abcam, ERTa and TET1 from Sigma-Aldrich, and 5-hmC from Active Motif. Rabbit immunoglobulin G alone was used as a negative control.

Cell culture and proliferation evaluation
The human endometrial adenocarcinoma cell line Ishikawa and HEC-1-A was kindly provided by Dr. Yu Yinhu (MD Anderson Cancer Center, Houston, TX) and Dr. Wei Lihui (Peking People Hospital, Beijing, China) respectively. Cells were cultured in DMEM/F12 (Gibco BRL) supplemented with 10% FBS (Gibco BRL).

The human acute monocytic leukemia cell line (THP-1) was purchased from the ATCC and cultured in RPMI 1640 (Gibco BRL) with 10% FBS.

Cells were required to be starved in opti-MEM (Invitrogen) without FBS for 18 to 24 hours before initiating the experiment. The CCK8 kit (Dojingo) was used to evaluate cell proliferation following the manufacturer’s instructions.

Drug treatment
Starved cells reaching 80% confluence were treated with 17β-estradiol (E2), IL17A, or the selective ER antagonist Fulvestrant (ICI 182,780), which were all purchased from Sigma-Aldrich. Cells were treated with 10−9 M 17β-estradiol or 50 ng/mL IL17A if not specifically indicated. IL6 and CXCL11 were purchased from Pepro Tech, USA; IL10 and TGFβ1 from Sigma-Aldrich. IL17 receptor (IL17R) antibody was from R&D Systems.

siRNA, plasmid construct, and transient transfection
The TET1 small interfering RNA (si-TET1; Dharmaco) was transfected into HEC-1-A cells in the presence of Hiperfect transfection reagent (Dharmacon). The si-Con served as a transfection control.

The plasmid pcDNA-ESR1 (ESR1 is the gene encoding ERα protein, the plasmid was kept in our lab), pcDNA-TET1-flag (kindly provided by Prof. Zhao Shimin, Fudan University), or pPB-TET1 plasmid (kindly provided by Prof. Shi Yujiang, Harvard University, Cambridge, MA) was transfected into endometrial cancer cells using Lipofectamine™ (Invitrogen) according to the manufacturer’s protocol.
Western blot analysis and real-time PCR

Western blot analysis was conducted as previously described (25). The primary antibodies to GAPDH, β-tubulin, and β-actin were purchased from Epitomics company. ERα, ERβ, TET1, p-AKT, AKT, and cyclin D1 antibodies from Abcam company. RNA extraction and real-time PCR were performed as previously described (26). The primers were synthesized by Invitrogen Biotechnology Corporation (China; Supplementary Table S3).

Immunofluorescence analysis

Immunofluorescence analysis was carried out as previously described (27). CD68 and CD163 primary antibodies and fluorescence was detected using confocal microscopy. 4′, 6-Diamidino-2-phenylindole (DAPI) was used to identify cell nuclei. Fluorescence was detected using confocal microscopy (Leica TCS SP8 Configurable Confocal).

Dot blot for detection of 5-hmC

Total DNA was extracted using a QIAamp DNA Mini Kit (QIAGEN). One μl DNA (500 ng/μl) was dropped on nitrocellulose membranes and irradiated by a UV lamp (2,000 w) for 15 minutes to cross-link DNA, and then incubated with 5-hmC primary antibody (Abcam). Secondary antibody (Jackson) was applied and detected by ECL reagent, with methylene blue stain as a loading control.

HMeDIP assays

HMeDIP assays were conducted as previously described (28). 5-hmC antibody was purchased from Active Motif, rabbit IgG was used as negative control. DNA fragments pulled down were evaluated by real-time PCR with ESR1 promoter fragment primer (Supplementary Table S3).

Statistical analysis

SPSS 18.0 (IBM SPSS Software) was used for statistics. Non-parametric tests, Student t test, paired t test, one-way or two-way ANOVA were used for appropriate analyses. The P value was set to 0.05 (two-tailed) as the significance test level. Each experiment was repeated at least three times. Error bars indicated SD in the graphs.

Results

Serum estradiol levels are not elevated in patients with endometrial hyperplastic diseases or type I endometrial carcinoma

Because long-term estrogen stimulation without progesterone protection is believed to be the main mechanism in the carcinogenesis of endometrial cancer, we first evaluated serum estradiol levels in both healthy controls and patients with various endometrial disorders, including DPE, SH, CH, EAH, and endometrioid cancer (Fig. 1A1). We found that the serum estradiol levels in patients with DPE, SH, CH, and EAH were not different from those of normal controls. Serum estradiol levels in endometrial cancer patients were lower than those in the other groups. We further subdivided endometrial cancer patients according to pathologic grade as endometrioid cancer grade 1 (G1), grade 2 (G2), or grade 3 (G3; Supplementary Fig. S1A1), and found that serum estradiol levels were lower in all three endometrial cancer subgroups compared with that of controls.

We found a difference in the average age among groups of patients studied, with that of endometrial cancer patients older than the control group, and with the average age of CH patients younger than that of the controls (Fig. 1B1 and Supplementary Fig. S1B1). Because ovarian function is significantly affected by age, we stratified patients according to age to eliminate this possible cause of bias. The results showed serum estradiol levels as well as age distribution in endometrial cancer patients between 25 years and 40 years were not different from other groups (Fig. 1A2 and 1B2 and Supplementary Figs. S1A2 and S1B2). Serum estradiol level in endometrial cancer patients between 41 years and 55 years was lower than other groups (Fig. 1A3 and Supplementary Fig. S1A3). However, the average age of this group was still higher than in other groups (Fig. 1B3 and Supplementary Fig. S1B3).

To further eliminate possible bias caused by age and to confirm our findings, we analyzed another group of 34 endometrial cancer patients and compared them with 34 age-paired healthy volunteers (Fig. 1C and Supplementary Fig. S1C). No significant difference in serum estradiol levels was found between the two groups.

The above findings indicated that serum estradiol levels were not elevated in endometrial cancer and endometrial hyperplasia patients. There are many factors affecting serum estradiol activity; among them, serum SHBG is one of the most important factors. SHBG can bind and deactivate serum estradiol and lower the active circulating estradiol. We found that serum SHBG was lower in EAH and endometrial cancer groups compared with other groups (Fig. 1D and Supplementary Fig. S1D). But there were no significant differences in E2/SHBG ratios among any of the groups except for an increased E2/SHBG ratio in the SH group (Fig. 1E and Supplementary Fig. S1E).

A lack of progesterone protection is another key mechanism in endometrial carcinogenesis. But we did not find differences in serum progesterone levels among patients in any of the groups (Fig. 1F and Supplementary Fig. S1F).

Results of serum sex hormone analysis indicated that circulating estradiol was not elevated in patients with endometrial cancer or its precancerous diseases. However, increased local estradiol levels in endometrium or increased estradiol sensitivity of endometrial cells might be a key mechanism(s) in the carcinogenesis of endometrial cancer. We thus focused on the possible mechanisms of increased local estradiol sensitivity.

Macrophage infiltration is positively correlated with development of endometrial hyperplasia and endometrioid cancer

On the basis of the findings that macrophage infiltration is correlated with cancer development (14, 15) and can upregulate androgen receptor in prostate cancer (12), we hypothesized that macrophage infiltration might play roles in enhancing estradiol sensitivity in endometrium. Although M2 macrophages are reported to facilitate tumor progression (29), the subtype and function of M2 macrophage are far from clear (15–19). We focused on CD68+ (macrophage-specific marker) and CD163+ (possible M2 macrophage marker) macrophages only in this study. Using serial sections of endometrial tissue samples, IHC staining (Fig. 2A) showed that the infiltration pattern in different endometrial lesion was similar between CD68+ cells and CD163+ cells. CD68+ cells and CD163+ cells were found mostly in tissue...
mesenchyme. And macrophages also presented in necrotic spot in endometrial cancer lesion. Because necrotic spot presents only in cancer lesion, we focused on the infiltrated macrophages in mesenchyme only. Infiltration of CD68$^+$ cells and CD163$^+$ cells increased gradually from normally proliferative endometrium, hyperplasia to endometrial cancer, which was the highest in EAH and endometrioid adenocarcinoma G1, and decreased when the cancer lesions progressed to grade 2 and grade 3. Immunofluorescence staining confirmed that CD63-expressing cells in endometrial lesion also express CD68 (Fig. 2B). These results indicated that macrophage infiltration is positively correlated with carcinogenesis of endometrioid adenocarcinoma, and most of these macrophages are possible CD68$^+$CD163$^+$ M2 macrophages. The highest infiltration of CD68$^+$CD163$^+$ macrophages in EAH and endometrioid adenocarcinoma G1 suggested that CD68$^+$CD163$^+$ macrophages might play important roles in helping endometrial cells attaining their malignant properties in the early stage of carcinogenesis.

**CD68$^+$CD163$^+$ macrophages enhance estradiol-driven endometrial cancer cell proliferation**

To test our hypothesis that infiltrating macrophages might play roles in upregulating estradiol sensitivity in endometrium, we asked whether CD68$^+$CD163$^+$ macrophages could enhance estradiol-driven endometrial cancer cell proliferation.

We first induced CD68$^+$CD163$^+$ macrophages from THP-1 cells (Supplementary Materials and Methods and Supplementary Fig. S2). Macrophages are terminally differentiated and cannot proliferate, so we cocultured CD68$^+$CD163$^+$ macrophages with endometrial cancer cells to evaluate whether they could stimulate proliferation of endometrial cancer cells. Two different endometrial cancer cell lines, Ishikawa and HEC-1-A cells, were used and cocultured with CD68$^+$CD163$^+$ macrophages in different cell-number ratios. As shown in Fig. 3A, CD68$^+$CD163$^+$ macrophages significantly stimulated proliferation in both endometrial cancer cell lines.

We then asked whether CD68$^+$CD163$^+$ macrophages promoted endometrial cancer cell proliferation by secreting cytokines. We
Figure 2.
CD68⁺ macrophage and CD163⁺ macrophage infiltration is correlated with endometrial hyperplasia and endometrioid adenocarcinoma. A, IHC staining of macrophage-specific marker CD68 and M2 macrophage-specific marker CD163 in endometrial tissue samples. Number of cases (n) in each group is marked. B, immunofluorescence staining shows infiltrating macrophages expressed CD68 and CD163 simultaneously. CD68 (green) and CD163 (red) were expressed in cytoplasm of infiltrating macrophages. 4', 6-Diamidino-2-phenylindole (DAPI; blue) was used as a DNA dye to indicate cell nuclei. Magnified pictures of the representative area were shown below. PP, normal endometrium in proliferative phase; H, hyperplasia; G1, endometrioid adenocarcinoma grade 1; G2, endometrioid adenocarcinoma grade 2; G3, endometrioid adenocarcinoma grade 3.
cultured endometrial cancer cells with only conditioned medium (CM) from CD68^+CD163^+ macrophages, and the results showed that CM also elicited a positive and significant effect on the proliferation of both Ishikawa and HEC-1-A cells (Fig. 3B). Consistent with the increased proliferation, the expression of cyclin D1, an indicator of cell proliferation, was also upregulated by CM in endometrial cancer cells in a time-dependent manner (Fig. 3C).

To explore whether CD68^+CD163^+ macrophages were associated with estradiol sensitivity in endometrial cancer cells, we analyzed the relative proliferative rates of endometrial cancer cells in response to estradiol in the presence or absence of CD68^+CD163^+ macrophage CM (Fig. 3D). We found that although 17β-estradiol could stimulate Ishikawa cell proliferation, the proliferation was more significant after adding CM. It is notable because of the relatively weak expression of ERa in HEC-1-A cells (Supplementary Fig. S3), that the proliferative stimulation of estradiol alone on HEC-1-A cells was moderate (Fig. 3D). However, the addition of CM significantly enhanced the effect of estradiol-driven proliferation in HEC-1-A cells.
Cell proliferation was determined by CCK8 test. Transfection efficiency was evaluated by Western blot analysis. In summary, our findings above showed that CD68^+ CD163^+ macrophages or their conditioned medium could enhance estradiol-driven endometrial cancer cell proliferation. This indicated that CD68^+ CD163^+ macrophages may be involved in the regulation of the estradiol-driven, pro-proliferative pathway in endometrial cancer cells, and that this might be achieved through cytokines secreted by CD68^+ CD163^+ macrophages. There might be two possible mechanisms involved: (1) cytokines in CM secreted by CD68^+ CD163^+ macrophages upregulate ERα in endometrial cancer cells; and (2) macrophages secrete estrogen directly and promote endometrial cancer cell proliferation. Because we found in HEC-1-A cells (Fig. 3D) that cellular proliferation was not stimulated significantly even at high estradiol concentrations (indicating that higher estrogen concentrations might not be the key factor in promoting HEC-1-A cell proliferation), we now suggest that upregulated ERα expression might be the key mechanism in promoting endometrial cancer cell proliferation after addition of CM from CD68^+ CD163^+ macrophages.

ERα is an adaptor for CD68^+ CD163^+ macrophage-enhanced estradiol-driven proliferation

To further investigate the molecular pathway(s) underlying the proliferation stimulating effect of CD68^+ CD163^+ macrophages on endometrial cancer cells and to elucidate the role of ERs in this process, we treated endometrial cancer cells with the selective ERα antagonist fulvestrant (ICI 182,780). We found that ICI was able to inhibit the effect of CD68^+ CD163^+ macrophages on estradiol-induced proliferation in both Ishikawa and HEC-1-A cells in both contact coculture manner (Fig. 4A) and when the endometrial cancer cells were cultured with CM of CD68^+ CD163^+ macrophages (Fig. 4B). Indicating that CD68^+ CD163^+ macrophages might promote estradiol-driven endometrial cancer cell proliferation by activating ERs in endometrial cancer cells.

To validate the effect of CD68^+ CD163^+ macrophages on activating ERs in endometrial cancer cells, we analyzed the changes in ER expression in endometrial cancer cells upon the addition of CM. Western blot analysis demonstrated that CM of CD68^+ CD163^+ macrophages caused an upregulation in ERα expression but not in ERβ expression in both Ishikawa and HEC-1-A cells (Fig. 4C), and ERα expression was upregulated in a time-dependent manner in response to the addition of CM (Fig. 4D). In addition, overexpression of ERα in HEC-1-A cells enhanced estradiol-driven proliferation, similar to the effects of CM (Fig. 4E). Collectively, these results suggest that CD68^+ CD163^+ macrophages enhance estradiol-driven proliferation by increasing ERα expression in endometrial cancer cells.

CD68^+ CD163^+ macrophages upregulate ERα expression in endometrial cancer cells via IL17A

The fact that conditioned medium from CD68^+ CD163^+ macrophages was sufficient to enhance estradiol-driven
Figure 5.

CD68^+CD163^+ macrophages upregulate ERα expression in endometrial cancer cells via IL17A. A1, HEC-1-A cells stimulate cytokine expression in CD68^+CD163^+ macrophages. HEC-1-A cells were seeded in the upper chamber and CD68^+CD163^+ macrophages in the lower chamber of a Transwell system. After culture for 24 hours, CD68^+CD163^+ macrophages were harvested for inflammatory factor screening using real-time PCR. A2, CD68^+CD163^+ macrophages stimulate cytokine expression in HEC-1-A cells. HEC-1-A cells from the lower chamber were harvested for inflammatory factor screening. B, IL17A upregulates ERα expression in HEC-1-A cells. HEC-1-A cells were treated by IL6 (1 ng/mL), IL10 (20 ng/mL), IL17A (50 ng/mL), CXCL11 (1 ng/mL), and TGFβ1 (1 ng/mL) for 48 hours. Expression of ERα or ERβ was detected by Western blot analysis. C, IL17A promotes ERα expression in time- and dose-dependent manners. D, IL17R antibody compromises the effect of IL17A on ERα expression upregulation. HEC-1-A cells were treated with 1 μg/mL IL17R antibody and/or 50 ng/mL IL17A for 48 hours. E, IL17A promotes estradiol-driven proliferation. HEC-1-A cells were treated with 10^{-9} mol/L 17β-estradiol and/or 50 ng/mL IL17A. Cells treated with DMSO only were used as controls. Cell proliferation was evaluated by the CCK8 test at indicated time points. F, overexpression of ERα exerts the same proliferation-stimulating effect as does IL17A on estradiol-driven proliferation. HEC-1-A cells transfected with pcDNA-ESR1 plasmid or pcDNA3.0 were treated with 10^{-9} mol/L 17β-estradiol and/or 50 ng/mL IL17A for 48 hours before CCK8 test. G, IL17A and 17β-estradiol promotes ERα transcription. HEC-1-A cells were treated with 50 ng/mL IL17A and/or 10^{-9} mol/L 17β-estradiol for 24 hours. Relative ERα and ERβ mRNA fold-change was analyzed by real-time PCR. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
proliferation of endometrial cancer cells makes us hypothesize that such proliferative stimulation is mediated by cytokines secreted by CD68+CD163+ macrophages. To screen for possible cytokines that might mediate the proliferation-stimulating effect of CD68+CD163+ macrophages on endometrial cancer cells, we screened cytokines produced in CD68+CD163+ macrophages in the presence of HEC-1-A cells or vice versa. We found that IL6, IL10, IL17A, CXCL11, and TGFβ1 were the cytokines stably upregulated in both macrophages and HEC-1-A cells (Fig. 5A and 5A).

To decide on the key cytokine promoting estradiol-driven endometrial cancer cell proliferation, we further investigated the effect of all the five cytokines on ERα and ERβ expression in HEC-1-A cells. Intriguingly, all five factors were able to upregulate ERα but not ERβ expression, and IL17A demonstrated the most significant effect (Fig. 5B). Western blot analysis confirmed that IL17A upregulated ERβ expression in a time-dependent and dose-dependent manner (Fig. 5C). The effect of IL17A on ERα expression and transcription was compromised when we blocked IL17R with its specific antibody (Fig. 5D and Supplementary Fig. S4), indicating that IL17A upregulated ERα in endometrial cancer cells via IL17R.

To confirm the premise that IL17A upregulation of ERα is functional and can promote endometrial cancer cell proliferation, we used a cell proliferation test to see whether IL17A was sufficient to enhance estradiol-driven proliferation of endometrial cancer cells. As shown in Fig. 5E, both IL17A and a physiologic concentration (10^{-9} mol/L) of estradiol could stimulate the proliferation of HEC-1-A cells. This proliferation was significantly upregulated when cells were treated with IL17A and 17β-estradiol in combination. We then overexpressed ERα in HEC-1-A cells by plasmid transfection and found that the endometrial cancer cell proliferation was upregulated after ERα overexpression, and this cellular proliferation was comparable with those treated with 17β-estradiol and/or IL17A (Fig. 5F), suggesting that IL17A promotes HEC-1-A cell proliferation by activating the ERα signaling pathway.

To confirm this finding, we compared the activation of downstream cellular proliferation pathways after treatment with IL17A or by overexpressing ERα. As shown in Supplementary Fig. S5, IL17A treatment and overexpression of ERα activated the cellular proliferation pathway PI3K/AKT and cyclin D1 expression to a similar extent. Taken together, these findings confirmed that IL17A is involved in CD68+CD163+ macrophage-stimulated endometrial cancer cell proliferation by regulating the ERα pathway.

We then investigated the possible mechanism(s) involved in upregulation of ERα by IL17A. We first evaluated the effect of IL17A on ERα transcription with or without estrogen stimulation, and found that both IL17A and 17β-estradiol promoted ERα but not ERβ transcription in HEC-1-A cells, and that this effect was most significant when HEC-1-A cells were treated with IL17A and 17β-estradiol in combination (Fig. 5C). This indicated that IL17A might promote ERα expression in endometrial cancer cells by activating ERα gene (ESR1) transcription. We then investigated possible mechanism(s) involved in IL17A upregulation of ESR1 transcription.

TET1-mediated 5-hydroxymethylation of the ERα gene promoter is involved in IL17A upregulation of ERα expression

Abnormal DNA hydroxymethylation has been reported to be involved in the development of various cancers (30). We thereby used tissue microarray from clinical samples to evaluate a potential relationship among IL17A, ERα expression, and DNA hydroxymethylation. We evaluated TET1 and 5-hmC as the two markers for DNA hydroxymethylation. TET1 is one of the primary components of the ten-eleven translocation 5-methylcytosine dioxygenase family, and catalyzes the conversion of 5-methylcytosine (5-mC) to 5-hmC (31). Abnormal TET1 expression has been associated with the development of multiple types of cancer (30). 5-hmC, a modified cytosine base that facilitates gene expression, is a critical epigenetic marker of DNA hydroxymethylation (32). As is shown in Fig. 6A, there were parallel increases in the levels of IL17A, TET1, 5-hmC, and ERα when endometrial lesions developed from hyperplasia, and progressed from EAH to endometrial cancer. The immunostaining of IL17A, TET1, and 5-hmC showed the highest expression in EAH; and when the lesions progressed from endometrioid adenocarcinoma G1 to G3, the expression of TET1 and 5-hmC (as well as IL17A and ERα) decreased. IHC scores of the four markers were calculated by semiquantitative optical analysis (Supplementary Fig. S6). There were positive correlations between IL17A expression and TET1, 5-hmC, or ERα (Fig. 6B).

To further investigate the role of hydroxymethylation in IL17A-induced ERα expression, we measured the expression change of TET1 in endometrial cancer cells treated with IL17A. Western blot analysis revealed that IL17A upregulated TET1 expression in endometrial cancer cells in a time- and dose-dependent manner (Fig. 6C). When endogenous TET1 in the endometrial cancer cells was silenced by siRNA, the IL17A-induced ERα mRNA as well as protein expression was attenuated (Fig. 6D). On the other hand, overexpression of TET1 enhanced ERα expression even without IL17A treatment (Fig. 6E). These findings suggested that TET1 is involved in IL17A-induced ERα expression.
We then asked whether IL17A promoted ERα gene transcription through hydroxymethylation of the ERα gene promoter by TET1. We first detected the global level of 5-hmC in endometrial cancer cells treated with IL17A, and dot-blot analysis (Fig. 6F) showed that global DNA hydroxymethylation was upregulated after IL17A treatment. To further confirm the involvement of TET1 in the epigenetic regulation of ERα expression, we analyzed hydroxymethylation status of the ERα gene promoter region in HEC-1-A cells by silencing or overexpressing TET1. Primer for ERα gene promoter region was synthesized (Supplementary Table S3). HMeDIP assay (Fig. 6G) showed that silencing TET1 by siRNA compromised ERα gene promoter hydroxymethylation, whereas overexpression of TET1 in HEC-1-A cells resulted in significant increase of 5-hmC in ERα gene promoter region. Taken together, these results suggest that IL17A promotes ERα gene transcription through hydroxymethylation of the ERα gene promoter by TET1.

Discussion

It is well documented that type I endometrial cancer is hormone related, with unopposed estrogen stimulation as the primary oncogenic mechanism. Type I endometrial cancer has clear precancerous stages, from DPE, hyperplasia, atypical hyperplasia, and progresses onward to endometrial cancer; and estrogen stimulation is involved in every step of endometrial cancer development and progression (33, 34). Putting these unique features of endometrial cancer to our advantage, we examined...
both endometrial cancer and its precancerous diseases to seek a better understanding of endometrial cancer development. Serum hormone analysis showed that serum estradiol was not elevated in endometrial cancer and hyperplasia patients or even decreased in endometrial cancer patients after adjusting for age. In addition, the protective factors against estrogen stimulation, such as serum progesterone level, were not found to be decreased when endometrial lesions progressed from hyperplasia to cancer. These findings indicated that increased local estrogen sensitivity in endometrium, rather than increased circulating estrogen levels, might be the most important contributing factor to estrogen-driven endometrial tumorigenesis.

Studies have shown the correlation of chronic inflammation and endometrial cancer (4, 35). But the correlation of inflammation and endometrial hyperplasia is still unclear. We demonstrated that the infiltrating macrophages were present at early stages of endometrial hyperplasia, and the degree of macrophage infiltration was positively correlated with the progression of endometrial hyperplasia to cancer. The lack of an increase in circulating estradiol levels and the presence of an increase in macrophage infiltration strongly suggested that infiltrating macrophages in endometrial lesions might contribute to the sensitization of endometrial cells to estrogen stimulation.

The mechanism(s) of macrophage infiltration and development of endometrial cancer is still unclear. It was reported that macrophages contribute to tumor development and progression by promoting cancer cell proliferation, invasion, metastasis, and angiogenesis; and by inhibiting cytotoxic T-cell activity (36–39). In the present study, we proposed a model of the relationship between the inflammatory microenvironment and estradiol-driven endometrial cancer cell proliferation (Fig. 7). In brief, monocyes are recruited into endometrial lesions and polarized into CD68⁺CD163⁺ macrophages. Infiltrating CD68⁺CD163⁺ macrophages upregulate inflammatory cytokines in endometrial lesions, including IL17A. The latter promotes TET1 expression in endometrial cancer cells. TET1-mediated hydroxymethylation of the ESRI gene promoter is involved in IL17A-induced ERα expression. Elevated ERα levels then increase estrogen sensitivity in endometrial cancer cells, which in turn stimulates endometrial cancer cell proliferation through the PI3K/AKT pathway.

It is interesting to note that we found in clinical samples that the expression of TET1, 5-hmC, and ERα was the highest in EAH and was then degraded gradually in endometrial cancer from G1 to G3. We suggest that the TET1-related 5-hmC modulation plays a role(s) in assisting cells in attaining cancerous properties under the stimulation of estrogen. When cells gain this property, estrogen is no longer the primary engine for cancer development, progression, and de-differentiation. The cancer cells then turn to other more-common stimulators for proliferation instead of estrogen. Our previous finding that endometrial cancer cell proliferation depends more upon the TGFβ-EGFR pathway when cells lose progesterone receptor and become progesterin resistant, supports this hypothesis (39). Such findings also suggest that higher expression of TET1 and 5-hmC might be indicators of well-differentiated tumors, producing an improved prognosis.

Our data showed that IL17A was upregulated in both macrophages and endometrial cancer cells in the coculture system. We suppose that infiltrating macrophages could upregulate IL17A in both macrophages and endometrial cells, which caused elevated IL17A in the microenvironment, leading to increased estrogen sensitivity in endometrium. It is possible that IL17A produced by both macrophages and endometrial cancer cells play roles in upregulating ERα in endometrial cancer cells. However, exogenous IL17A was sufficient to induce ERα and blocking IL17 receptor reduced ERα expression in endometrial cancer cells (Fig 5D), which indicates that IL17A originating from macrophages alone is sufficient to induce ERα in endometrial cancer cells.

In this paper, we showed significant upregulation of ERα protein in endometrial cancer cells after IL17A treatment. Our findings indicate that transcriptional mechanism plays role in IL17A-driven ERα upregulation. Nevertheless, other mechanisms such as post transcriptional regulation might also be involved. Studies have shown that ubiquitination regulation is deeply involved in ER turn over (40), which guarantees further study.

In conclusion, we found that increased estrogen sensitivity in the endometrium might be one of the key mechanisms underlying estrogen-driven carcinogenesis of the endometrium. Infiltrating macrophages play important roles in increased local estrogen sensitivity in endometrial lesion. Our findings provide a new perspective into the carcinogenesis of endometrial cancer, and contribute possible targets for the prevention and treatment of endometrial cancer. The concept that inflammation-induced estrogen sensitivity in the endometrium acts as a driver of type I endometrial cancer has implications for infiltrating macrophages as a prognostic biomarker of progression in this disease setting.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: X. Luo, X. Chen, Z. Zhang, Y. Feng
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