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Estradiol Promotes M1-like Macrophage Activation through Cadherin-11 To Aggravate Temporomandibular Joint Inflammation in Rats

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Macrophages play a major role in joint inflammation. Estrogen is involved in rheumatoid arthritis and temporomandibular disorders. However, the underlying mechanism is still unclear. This study was done to verify and test how estrogen affects M1/M2-like macrophage polarization and then contributes to joint inflammation. Female rats were ovariectomized and treated with increasing doses of 17β-estradiol for 10 d and then intra-articularly injected with CFA to induce temporomandibular joint (TMJ) inflammation. The polarization of macrophages and expression of cadherin-11 was evaluated at 24 h after the induction of TMJ inflammation and after blocking cadherin-11 or estrogen receptors. NR8383 macrophages were treated with estradiol and TNF-α with or without blocking cadherin-11 or estrogen receptors, to evaluate the expression of the M1/M2-like macrophage-associated genes. We found that estradiol increased the infiltration of macrophages with a proinflammatory M1-like predominant profile in the synovium of inflamed TMJ. In addition, estradiol dose-dependently upregulated the expressions of the M1-associated proinflammatory factor inducible NO synthase (iNOS) but repressed the expressions of the M2-associated genes IL-10 and arginase in NR8383 macrophages. Furthermore, estradiol mainly promoted cadherin-11 expression in M1-like macrophages of inflamed TMJ. By contrast, blockage of cadherin-11 concurrently reversed estradiol-potentiated M1-like macrophage activation and TMJ inflammation, as well as reversed TNF-α–induced induction of inducible NO synthase and NO in NR8383 macrophages. The blocking of estrogen receptors reversed estradiol-potentiated M1-like macrophage activation and cadherin-11 expression. These results suggested that estradiol could promote M1-like macrophage activation through cadherin-11 to aggravate the acute inflammation of TMJs. The Journal of Immunology, 2015, 194: 2810–2818.

The temporomandibular joint (TMJ) is a synovial joint that is frequently affected by osteoarthritis and rheumatoid arthritis (RA) (1). Temporomandibular disorders (TMDs) are an assorted set of clinical conditions characterized by pain in the TMJ and masticatory muscles (2). TMDs often show inflammatory changes, including capillary hyperemia, increased vascularity, fibrosis, synovial hyperplasia, and infiltrated inflammatory cells (3–5). Similar to RA, TMDs are approximately twice as prevalent (and more severe) in women than in men (6, 7). Sex hormones, particularly estrogens, are reported to be involved in TMD (6, 8–10). We also previously reported that estrogen aggravates TMJ inflammation through the induction of proinflammatory cytokines in the synovial membrane (11). However, the mechanism underlying estrogen-aggravated TMJ inflammation has not been fully understood.

Macrophages play a major role in joint inflammation by contributing to the synovial hyperplasia (12) and they act as the main producers of key inflammatory mediators, such as TNF-α (13). Depending on their present environmental cues, macrophages can assume a spectrum of activation states ranging from classically activated M1 inflammatory macrophages to various alternatively activated M2 macrophages that are involved in immune regulation and tissue repair (14, 15). M1 macrophages are generally proinflammatory based on their production of TNF-α, IL-1, IL-6, and inducible NO synthase (iNOS) (15, 16). In contrast, M2 macrophages are more likely to adapt to an anti-inflammatory role characterized by the production of IL-10 (17, 18), and have a key role in wound healing and resolution of inflammation (15). Moreover, previous studies showed that estrogens are involved in the modulation of macrophages in autoimmune diseases, such as RA or systemic lupus erythematosus (19). Therefore, we hypothesized that estrogen might induce macrophage activation and M1/M2 polarization in joint inflammation, which may provide new insights into female-predominant diseases.

Cadherin-11 is a well-known cell adhesion molecule that is responsible for tissue morphogenesis and architecture (20, 21). In joints, cadherin-11 is mainly expressed in the fibroblast-like synoviocytes and plays an essential role in synovial inflammation and arthritis pathology (22, 23). Furthermore, cadherin-11 is also expressed in the alveolar macrophages of patients with pulmonary fibrosis (24). Meanwhile, estradiol can regulate...
cadherin-11 expression in human endometrial stromal cells (25, 26) and in neurons of macaques (27). Therefore, we further hypothesized that estrogen might promote M1/M2 macrophage dichotomy and upregulate cadherin-11 in TMJ inflammation to aggravate joint inflammation.

In this study, we explored whether estrogen could affect M1/M2 macrophage dichotomy and potentiate cadherin-11 to aggravate joint inflammation. The scheme for the animal experiments is illustrated in Fig. 1A. The 17β-estradiol (E2) administration and histological evaluation of TMJ inflammation were performed as previously described in detail (11). In brief, rats were randomly divided into four groups with five rats in each group, namely, control, sham, 0 μg-E2, and 80 μg-E2 groups. The rats were bilaterally ovarioctomized or sham ovarioctomized. One week later, the ovarioctomized rats were s.c. injected for 10 d with E2 (Sigma) at daily 0.5 h before the induction of TMJ inflammation, respectively. On the 10th day of estradiol treatment, TMJ inflammation was laterally ovariectomized or sham ovariectomized. One week later, the ovariectomized or sham ovariectomized animals had free access to food and water.

### Estradiol administration and induction of TMJ inflammation

The scheme for the animal experiments is illustrated in Fig. 1A. The 17β-estradiol (E2) administration and histological evaluation of TMJ inflammation were performed as previously described in detail (11). In brief, rats were randomly divided into four groups with five rats in each group, namely, control, sham, 0 μg-E2, and 80 μg-E2 groups. The rats were bilaterally ovarioctomized or sham ovarioctomized. One week later, the ovarioctomized rats were s.c. injected for 10 d with E2 (Sigma) at daily 0.5 h before the induction of TMJ inflammation, respectively. An estrogen receptor–specific antagonist, ICI 182,780 (Sigma), was administered as previously described (11). In brief, another two sham-ovarioctomized groups and two 80 μg-E2 groups (n = 5 for each group) were i.p. injected twice with the vehicle or ICI 182,780 (500 g per rat in the 0 μg-E2 groups). The rats were bi-daily ovariectomized or sham ovarioctomized. One week later, the ovarioctomized rats were injected with E2 (Sigma) at daily doses of 0 or 80 μg per rat in the 0 μg-E2 and 80 μg-E2 groups, respectively. On the 10th day of estradiol treatment, TMJ inflammation was induced by injecting 50 μl CFA (Sigma) into the upper compartment of bilateral TMs. The effectiveness of ovarioctomy and estradiol replacement was confirmed, as shown in Supplemental Fig. 1.

### Intra-articular injection of anti–cadherin-11 Ab and application of estrogen receptor antagonist

Following the same E2 administration schedule, an additional two sham-ovarioctomized groups and two 80 μg-E2 groups (n = 5 for each group) received intra-articular injections twice with the isotype IgG or anti–cadherin-11 Ab (10 μg; sc-30314; Santa Cruz) at 24 h before and 0.5 h before the induction of TMJ inflammation, respectively. An estrogen receptor–specific antagonist, ICI 182,780 (Sigma), was administered as previously described (11). In brief, another two sham-ovarioctomized groups and two 80 μg-E2 groups (n = 5 for each group) were i.p. injected twice with the vehicle or ICI 182,780 (500 μg per rat) at 24 h before and 0.5 h before the induction of TMJ inflammation, respectively.

### Histology and immunohistochemistry

TMs were removed en bloc, fixed in 4% paraformaldehyde, deminer- alized in 15% EDTA, and embedded by paraffin. The embedded TMs sections were sectioned (5 μm) and stained with H&E, and we histologically evaluated the TMJ inflammation using previously described methods (11). Sections were subjected to Ag retrieval with 0.125% trypsin and 2% sodium hypochlorite at 37°C for 30 min. The sections were blocked with 5% BSA for 30 min at room temperature and incubated overnight at 4°C with the antibodies or isotype IgG in DMEM/F12 supplemented with 20% charcoal-stripped FBS. The sections were incubated with one or two Abs (anti–IL-4 [1:100, sc-33560; Santa Cruz], or anti–IL-10 [1:100, sc-365858; Santa Cruz]), iNOS (1:100; ab15323; Abcam). After extensive washing with PBS, the sections were incubated with HRP-conjugated secondary Ab and ECL detection. The Journal of Immunology 2811

### Immunohistochemistry

Sections were incubated for 1 h at room temperature with the respective FITC-conjugated or tetramethylrhodamine isothiocyanate–conjugated secondary Ab (1:200; Jackson Immunoresearch Laboratories). Nuclei were counterstained with DAPI. Confocal microscopic images were acquired using a Zeiss laser scanning microscope (LSM 510), and the images were processed with the LSM 5 Release 4.2 software.

### Isolation of PBMCs and polarization of primary macrophages and cell treatments

Rat PBMCs were separated from the buffy coat by density gradient centrifugation with ficoll-hypaque (tbd science, Tianjin, China). the cells were washed three times with PBS and resuspended in RPMI 1640 with 2% fetal bovine serum (FBS), 10% FBS, and 1% Antibiotic-Antimycotic. Freshly isolated PBMCs were incubated and allowed to adhere for 1 h. After the nonadherent cells were discarded, the adherent cells were continuously cultured at 37°C and 5% CO2 for 5 d and then treated for another 4 d with a combination of 500 U/ml IFN-γ (PeproTech) and 10 ng/ml LPS (Sigma-Aldrich) or with 10 ng/ml IL-4 (PeproTech). After culturing, the M1 and M2 macrophages were identified as the macrophage marker CD68 and M1 marker iNOS or the M2 marker scavenger receptor CD163, respectively (15, 28).

The rat macrophage NR8383 cell line was cultured in phenol red–free DMEM/F12 with 20% charcoal-stripped FBS. Cells were treated with indicated concentrations of E2 or TNF-α (10 units/ml) or IFN-γ (500 units/ml) for 24 h. After the incubation, the culture medium was collected to measure the NO and IL-10 levels.

Fibroblast-like synovocytes were isolated from the synovial membrane of TMs from 8- to 10-week-old rats as previously described (30). Synovocytes were cultured in DMEM/F12 (Life Technologies) containing 15% charcoal-stripped FBS and 1% Antibiotic-Antimycotic. Synovocytes (5 × 10^5 cells/well) were plated in 12-well plates (corning costar) and cultured for 3 d with phenol red–free DMEM/F12 Nutrient Mix (Life Technologies) containing 15% charcoal-stripped FBS (hyclone). NR8383 macrophages (1 × 10^5 cells/well) were added to the synovocytes with fresh medium and the cells were cultured for 3 d in the absence or presence of 10 ng/ml TNF-α (TS944; Sigma) or 10 ng/ml IL-4 (PeproTech) and E2 (10^-9 M). Immunofluorescence staining of the cultured cells was performed using the same method as described in the previous section Immunohistochemistry.

### Western blot analysis

Western blot analysis was performed as previously described in detail (31). In brief, cells were harvested by incubating with a denaturing lysis buffer (Applygen technologies, Beijing, China). Equal protein quantities were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. After blocking with 5% nonfat milk in TBS containing 0.1% Tween 20, the membranes were incubated overnight at 4°C with the antibodies (1:1000) and β-actin (1:1000; Santa Cruz) Abs. The blots were developed with an HRP-conjugated secondary Ab and ECL detection.

### Quantitative real-time PCR

Total RNA was isolated from cells with Trizol reagent (invitrogen) according to manufacturer’s instructions. Reverse transcription and real-time PCR were performed as previously described in detail (32). The published sequences of the commercially synthesized primers are as follows: rat β-actin sense/antisense, 5'-TGACAGGATGAGAAGGAGA-3'/5'-TTAGGCGCACAATCCACACACACACAC-3'; rat iNOS sense/antisense, 5'-GAAGTGGAGGAGCCGTTAGG-3'/5'-CCAAAGGTTGTCCTTCTTTT-3'; rat IL-1β sense/antisense 5'-CACCCTCTCAACGACAGCAGAC-3'/5'-GGGGTTCATGTGAATCAAC-3'; rat IL-1α sense/antisense, 5'-CCAGGTTTCCTCTCAAGGGGACAA-3'/5'-CTCTCTGATGAAATGCGAATCC-3' (11). The following primers were designed with the Primer Premier 5.0 software and commercially synthesized: rat cadherin-11 sense/antisense, 5'-TCCAACCACGACATGTCATT-3'/5'-ATCCAATGCGGCGAGGAG-3'; rat arginase sense/antisense, 5'-TTGGACTGAGTGACTGGGAAAG-3'; rat iNos sense/antisense, 5'-ATCAGTTGAGCCGAGGCTG-3'; rat iNos sense/antisense, 5'-CATTGCTGATGTGTCCTGCTTTACAC-3'/5'-GGGTTCTGGTGACTGGGAAAG-3'; rat iNos sense/antisense, 5'-TTTGGACTGAGTGACTGGGAAAG-3'/5'-CTCTCTGATGAAATGCGAATCC-3'. The efficiency of the newly designed primers was confirmed by sequencing.

### IL-10 and NO secretion assay

The IL-10 and NO levels in the culture medium were quantified with an ELISA kit (R&D Systems) and Griess reagent (R&D Systems), respectively, according to manufacturer’s instructions.

### Statistical analysis

Statistical analysis was performed with SPSS 13.0. All data were presented as mean ± SD and assessed via the independent two-tailed Student t test or one-way ANOVA. The p values <0.05 were considered to be statistically significant.
Results

**Estradiol promoted accumulation and activation of M1-like macrophages in inflamed TMJs**

To identify macrophages among the infiltrated mononuclear cells in the synovium of inflamed TMJ, we immunostained TMJ sections with CD68, which is a macrophage marker related to lysosomal glycoproteins (33). The inflamed TMJs from the sham group presented with a higher number of CD68+ macrophages, representing up to 43% of the cells around the synovial membrane compared with the control group ($p < 0.001$). In contrast, such features were barely detected in the 0 μg-E2 group. Moreover, the number of CD68+ macrophages was more significantly increased in the 80 μg-E2–treated groups, which comprised up to 56% of the cells around the synovial membrane, compared with the sham ($p < 0.01$) and 0 μg-E2 ($p < 0.001$) (Fig. 1B, 1C), indicating that estrogen contributed to macrophage accumulation in inflamed TMJs.

To identify the phenotype of these E2-promoted macrophages, double immunostaining for CD68 and iNOS was performed. As shown in Fig. 1D, most of the CD68+ macrophages (red) demonstrated high coexpression of the proinflammatory M1 marker iNOS (green); an orange fluorescent signal was present in the inflamed TMJs from the sham group. Furthermore, the iNOS and CD68 coexpressing macrophages were hardly detected in the 0 μg-E2 group, but their numbers were increased in the 80 μg-E2 group. These results indicated that E2 contributed to the activation of M1-like macrophages in inflamed TMJs.

**Estradiol promoted M1/M2 ratio of macrophages in NR8383 cells**

In the acute inflammation of TMJ model, M1-like macrophages appeared to be the major phenotype affected by estradiol treatment. We speculated that E2 might only promote the M1 polarization of macrophages, that is, increase the M1/M2 ratio. To mimic the environment of synovial inflammation, we cocultured NR8383 macrophages with synoviocytes and treated these cultures with TNF-α, which is one of the key signals of M1 macrophage activation (15). As shown in Fig. 2A, the anti-inflammatory M2 macrophage marker CD163 (green) was colocalized with CD68 (red; merged as orange fluorescence) in most of the macrophages in the vehicle group. After treatment with TNF-α, the proportion of CD68+CD163+ macrophages (red, as indicated by the arrowheads) was increased. Furthermore, treatment with E2 decreased the proportion of CD163+ cells. Double immunostaining of iNOS and IL-10 for M1 and M2 activation, respectively, in these cells showed that TNF-α–induced iNOS expression in the macrophages was further potentiated by E2 (Fig. 2A). However, IL-4–induced IL-10 expression was repressed by E2 (Supplemental Fig. 2A).

As shown in Fig. 2B and 2D, treatment with E2 also dose-dependently upregulated iNOS and IL-1β mRNA expression and NO secretion. Conversely, mRNA expression of the M2...
Macrophage-associated genes arginase and IL-10, as well as IL-10 secretion, was repressed by E2 in a dose-dependent manner (Fig. 2C, 2E). In addition, E2 could further potentiate TNF-α–induced upregulation of iNOS and TNF-α mRNA expression, as well as NO secretion (Fig. 2F). In contrast, arginase and IL-10 mRNA expressions, as well as IL-10 secretion, showed the opposite trend (Fig. 2G). All these results suggested that E2 increased the M1/M2 ratio of macrophages.

Estradiol promoted cadherin-11 expression in infiltrated M1-like macrophages

Cadherin-11 plays an essential role in synovial inflammation and arthritis pathology (22). We speculated that cadherin-11 might be involved in E2-mediated macrophage activation. As shown in Fig. 3A, the immunostaining of cadherin-11 was detected in some of the infiltrated mononuclear cells under the synovial membrane of the inflamed TMJs of the sham group, whereas no such feature was observed in the control TMJs. Ovariectomized rats of the 80 µg-E2 group, cadherin-11 was observed in most of the mononuclear cells in the erosion and broken synovial membrane, whereas no such feature was observed in the TMJs of ovariectomized rats that received 0 µg-E2. Therefore, E2 enhanced the expression of cadherin-11 in the infiltrated monocytes of the synovial membrane.

The correlation between cadherin-11 and the infiltrated CD68+ macrophages during the acute inflammation of TMJs was assessed by immunohistofluorescence using confocal microscopy. Compared with the control group, the fluorescence signals of both cadherin-11 (green) and CD68 (red) were colocalized after the induction of TMJ inflammation in some of the infiltrated mononuclear cells in the sham group (merged as white signals); such features were barely observed in the 0 µg-E2 group. Moreover, the fluorescence signals of cadherin-11 and CD68 were further increased; 50% of the cadherin-11 stain was colocalized with the infiltrated CD68+ mononuclear cells in TMJs of the 80 µg-E2 group (Fig. 3B), indicating that E2 mainly induced cadherin-11 in the infiltrated CD68+ macrophages. In PBMC-derived M1 macrophages (induced by IFN-γ and LPS, identified by CD68+ iNOS+ and iNOS+IL-10+ immunostaining; Fig. 3C and Supplemental Fig. 2B), 48.62% of the macrophages expressed cadherin-11 after M1 polarization, whereas cadherin-11 expression was barely detected after M2 polarization (Fig. 3C, 3D), suggesting that cadherin-11 was mainly expressed in M1 macrophages.
Estradiol upregulated cadherin-11 in M1 macrophages. (A) Immunohistochemistry of cadherin-11 in infiltrated mononuclear cells of inflamed TMJs at 24 h after induction of TMJ inflammation. Immunostaining of cadherin-11 was detected in most of the mononuclear cells in the eroded and broken synovial membrane in TMJs of the 80 µg-E2 group, and fewer such features were detected in TMJs of the sham group, but no such feature was observed in TMJs of the 0 µg-E2 group. The large boxed areas show higher-magnification views of the small boxes. (B) Immunohistofluorescence of cadherin-11 and CD68 in the infiltrated macrophages of inflamed TMJs. Fluorescence signals of both cadherin-11 (green) and CD68 (red) were coexpressed (white) in most of the infiltrated mononuclear cells in TMJs of the 80 µg-E2 group, and fewer such features were detected in TMJs of the sham group, but no such feature was observed in TMJs of the 0 µg-E2 group. (C and D) Immunocytofluorescence of cadherin-11 in PBMC-derived primary M1 and M2 macrophages. Cadherin-11 was expressed mainly in M1 macrophages but was barely present in M2 macrophages. Nuclei were counterstained with DAPI (blue). Cells were counted in five high-power fields per sample. Data are presented as mean ± SD of the ratio of cadherin-11+ cells to PBMC-derived CD68+ cells (n = 3). (E and F) Estradiol upregulated cadherin-11 in NR8383 macrophages. Expression of cadherin-11 was evaluated by real-time PCR and Western blot analysis of NR8383 macrophages treated with TNF-α alone or combined with increasing doses of E2 for 24 h. β-Actin served as an internal control for equal loading of Western blot analysis. The right panel of the Western blots show the quantified cadherin-11 protein presented as the relative density compared with the control group; data were normalized against the β-actin expression in each sample. n = 3. *p < 0.05, **p < 0.01, ***p < 0.001.

Fig. 3E and 3F, increasing doses of E2 further potentiated TNF-α–induced expressions of cadherin-11 mRNA and protein in NR8383 macrophages.

 Estradiol-potentiated joint inflammation and M1-like macrophage activation partially depended on cadherin-11

At 24 h after induction of TMJ inflammation, histopathologic examination showed the features of joint inflammation, including the proliferation of synoviocytes and infiltrated mononuclear cells in the synovial membrane of the TMJs of the sham group. However, few of these features were observed in the TMJs of the sham group pretreated with the cadherin-11 Ab (Fig. 4A). Erosion of the synovial membrane and severely infiltrated mononuclear cells in the intimal lining and sublining layers were observed in the TMJs of the 80 µg-E2 group, whereas only moderate infiltration of mononuclear cells was observed in the TMJs of the 80 µg-E2 group pretreated with the cadherin-11 Ab (Fig. 4A). The scores of TMJ inflammation induced by CFA injection in the sham and 80 µg-E2 groups were also partially reversed by pretreatment with the cadherin-11 Ab (Fig. 4B).

To verify the effect of cadherin-11 on macrophage polarization during the acute inflammation of TMJs, we performed double immunostaining of CD68 and iNOS or CD163. Infiltration of CD68+ macrophages was observed in the synovial membrane of the sham and 80 µg-E2 groups, with most of the CD68+ macrophages expressing the M1 marker iNOS (Fig. 4C, 4D) and a few expressing M2 marker CD163 (Fig. 4E, 4F). Compared with the sham group, the number of CD68+ and CD68+iNOS+ M1-like macrophages was significantly increased in the synovial membrane of the 80 µg-E2 group. Moreover, blocking cadherin-11 in the sham group significantly reversed the infiltration of CD68+ and CD68+iNOS+ macrophages. Similarly, blocking cadherin-11 in the 80 µg-E2 group reversed E2-potentiated infiltration of CD68+ and CD68+iNOS+ macrophages (Fig. 4C, 4D). The proportion of anti-inflammatory CD68+CD163+ M2-like macrophages was not affected by blocking cadherin-11 (Fig. 4E, 4F).
Expression and release of M1-like macrophage-associated proinflammatory cytokines partially depended on cadherin-11

As shown in Fig. 5A and 5B, double immunostaining of iNOS and IL-10 showed that the number of iNOS+ cells was higher in the sham and 80 μg-E2 groups, but these groups had few IL-10+ cells. The 80 μg-E2 group also presented higher expression of iNOS compared with the sham group. Furthermore, blocking cadherin-11 reversed iNOS expression in infiltrated cells of the sham group and partially reversed estradiol-potentiated expression of iNOS in the 80 μg-E2 group. In addition, no significant changes in IL-10 expression were observed among the four groups.

To further confirm the effect of cadherin-11 on proinflammatory macrophages, we detected the M1/M2 macrophage-associated gene expression in NR8383 macrophages pretreated with the anti-cadherin-11 Ab before stimulation with TNF-α and E2. As shown in Fig. 5C–F, blocking cadherin-11 reversed TNF-α-induced and E2-potentiated iNOS mRNA expressions, as well as the NO secretion. However, TNF-α–induced and E2-potentiated decreases of IL-10 mRNA expression and secretion were not reversed by blocking cadherin-11.

To elucidate the mechanism through which cadherin-11 contributes to M1 macrophage activation, we found that after stimulating NR8383 macrophages with Cadherin-11–Fc for 30 min, the phosphorylation of Erk and NF-κB p65 could be detected by Western blot analysis (Supplemental Fig. 3A). Compared with the activation of Erk, p38, and NF-κB p65 by TNF-α, cadherin-11–Fc also stimulated the activation of Erk and NF-κB p65, but not of p38, in NR8383 cells. Moreover, TNF-α–induced and E2-potentiated activation of Erk and NF-κB p65 was partially repressed by blockade of cadherin-11 (Supplemental Fig. 3B).

Blocking estrogen receptors partially reversed E2-potentiated M1-like macrophage activation and cadherin-11 expression

After the induction of acute TMJ inflammation, immunohistochemistry staining showed that the estrogen receptor antagonist ICI 182,780 partially reversed the infiltration of CD68+ macrophage and iNOS expression in the infiltrated cells in the sham group, as
well as partially reversed E2-potentiated infiltration of CD68+ macrophage and iNOS expression in the 80 µg-E2 group (Fig. 6A). Double immunostaining of CD68 and iNOS in sections showed that most of the infiltrated CD68+ macrophages were colocalized with iNOS (merged as orange) in the synovial membrane of the sham and of 80 µg-E2 groups, respectively, whereas IL-10 expression was not affected. Nuclei were counterstained with DAPI (blue). Data are presented as mean ± SD of the ratio of positive cells to total cells counted around the synovial layer (n = 5). (C-F) mRNA expressions of iNOS and IL-10 and secretion of NO and IL-10 in NR8383 macrophages. Treatment with the cadherin-11 Ab reversed the TNF-α–induced and E2-potentiated iNOS mRNA expression and NO secretion. The mRNA expression was assessed by real-time PCR; IL-10 and NO levels in culture media were assessed by ELISA and Griess reagent, respectively. Cells were pretreated with the anti–cadherin-11 Ab before treatment with TNF-α and E2 for 24 h (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001. Ab, cadherin-11 Ab.

Discussion

In this study, we provided several lines of evidence to show that E2 potentiates cadherin-11 expression in macrophages, thereby contributing to M1 macrophage activation and aggravating acute joint inflammation. First, E2 increased the number of infiltrated macrophages with a predominant profile of proinflammatory M1-like macrophages in acute TMJ inflammation. E2 upregulated the expression of M1-associated proinflammatory cytokines but repressed the expression of M2-associated genes in NR8383 macrophages in a dose-dependent manner. Second, E2 promoted cadherin-11 expression, mainly in the M1 macrophages of inflamed TMJ and in NR8383 macrophages. Third, blocking cadherin-11 reversed E2-potentiated M1 macrophage activation and TMJ inflammation, as well as TNF-α–induced induction of iNOS and NO in NR8383 macrophages. Fourth, blocking estrogen receptors reversed E2-potentiated M1-like macrophage activation and cadherin-11 expression, as well as TMJ inflammation. These results demonstrated that estrogen could contribute to TMJ inflammation partially through cadherin-11 to modulate the M1/M2-like macrophage dichotomy.

Estrogen contributed to joint inflammation through the activation of M1 macrophages. Macrophages serve essential functions during the onset and maintenance of joint inflammation. The number of synovial macrophages is correlated with the clinical disease activity (34, 35); selective macrophage depletion has a strong anti-inflammatory effect in animal models of arthritis (36, 37). Generally, M1 macrophages are proinflammatory, whereas M2 macrophages are anti-inflammatory (15–18). We not only showed that E2 potentiated accumulation of macrophages (CD68+) among the infiltrated mononuclear cells in the synovium of the inflamed TMJs, but also identified that the accumulated macrophages were mainly M1-like macrophages. In contrast, M2-like macrophages barely appeared in the inflamed TMJs, which suggested that E2 mainly promoted M1 polarization in CFA-induced acute joint inflammation. Our results in NR8383 macrophages cocultured with synoviocytes further confirmed and expanded this speculation that E2 not only promoted M1 polarization and M1-associated genes TNF-α and iNOS expression, but also repressed M2 polarization and M2-associated genes IL-10 and arginase expression. Consequently, E2 increased the M1/M2 ratio. Our results of E2-induced inhibition of M2-like macrophage activation were consistent with those of a previous study (38), wherein E2 inhibited M2 macrophage activation to repress hepatic...
Inflammation plays an essential role in synovial inflammation and arthritis pathology (22). Given that macrophage infiltration or cadherin-11 overexpression can aggravate joint inflammation, the relationship of cadherin-11 overexpression to estrogen-potentiated macrophage infiltration can be inferred. We showed in this study that cadherin-11 could be further potentiated by E2 in the infiltrated macrophages of the inflamed TMJs. This observation was supported in NR8383 macrophages, wherein E2 also potentiated TNF-α–induced induction of cadherin-11. The upregulation of cadherin-11 by E2 in this study was similar to those of previous studies, wherein E2 can potentiate the stimulatory effects of progesterone on the cadherin-11 expression in endometrial stromal cells (25) or upregulate cadherin-11 in serotonin neurons (27). Subsequently, we tested whether E2-potentiated cadherin-11 expression could contribute to M1 activation. Results showed that E2-potentiated macrophage infiltration and M1 activation, as well as the severity of joint inflammation, partially depended on cadherin-11. Similar results were observed in NR8383 macrophages. These results were the first, to our knowledge, to demonstrate that estrogen potentiates cadherin-11 in infiltrated macrophages, which leads to M1 polarization and further aggravates joint inflammation.

However, the underlying mechanism of the contribution of cadherin-11 to M1-like macrophage activation in inflamed joints remains unclear. This mechanism may be related to the fact that cadherin-11 can stimulate the activation of Erk and NF-κB p65 in NR8383 macrophages. This hypothesis is consistent with a previous study that cadherin-11 directly induced cytokines, as well as activated MAPKs and the NF-κB pathway in synovial fibroblasts (40). NF-κB is a key regulator of proinflammatory cytokines, such as TNF-α and iNOS (41); both of them contribute to M1 macrophage polarization (15). This speculation could also be supported by the present results that blocking cadherin-11 partially reversed iNOS expression in the infiltrated macrophages of inflamed TMJs, as well as reversed E2-potentiated activation of Erk and NF-κB p65 in NR8383 macrophages. In addition, our previous study showed that E2 potentiates proinflammatory cytokines, including TNF-α and iNOS, via NF-κB activation in the synovial membrane (11). This phenomenon may also be related to the reasons why cadherin-11 is abundantly expressed in synoviocytes and is required for synoviocytes to produce the extracellular matrix, which modulates recruitment, activation, and retention of immune cells (40). Nevertheless, further study is required to elucidate the details of the contribution of cadherin-11 to M1-like macrophage activation in inflamed joints.

The estrogen receptor signaling pathway is important for the upregulation of cadherin-11 expression and M1-like macrophage activation in inflamed joints because the estrogen receptor antagonist ICI 182,780 partially blocked cadherin-11 expression and M1-like macrophage activation in the inflamed TMJs, as well as cadherin-11 expression in NR8383 macrophages. Furthermore, blocking the estrogen receptor also reversed E2-potentiated expression of the M1 markers TNF-α and iNOS, as well as reversed E2-repressed expression of the M2 markers IL-10 andarginase. Our proposed mechanism states that estrogen modulates the M1/M2 macrophage dichotomy through cadherin-11 during joint inflammation. However, much of this mechanism still needs to be elucidated.

In conclusion, E2 promotes M1-like macrophage polarization through cadherin-11 to aggravate joint inflammation. These results may improve our current understanding of the clinical predominance of females in TMD and RA patients.

Estrogen potentiated cadherin-11 in macrophages to contribute to M1-like macrophage activation and to aggravate joint inflammation. The overexpression of cadherin-11 in the synoviocytes plays an essential role in synovial inflammation and arthritis pathology (22). Given that macrophage infiltration or cadherin-11 overexpression can aggravate joint inflammation, the relationship of cadherin-11 overexpression to estrogen-potentiated macrophage infiltration can be inferred. We showed in this study that cadherin-11 could be further potentiated by E2 in the infiltrated macrophages of the inflamed TMJs. This observation was supported in NR8383 macrophages, wherein E2 also potentiated TNF-α–induced induction of cadherin-11. The upregulation of cadherin-11 by E2 in this study was similar to those of previous studies, wherein E2 can potentiate the stimulatory effects of progesterone on the cadherin-11 expression in endometrial stromal cells (25) or upregulate cadherin-11 in serotonin neurons (27).

Subsequently, we tested whether E2-potentiated cadherin-11 expression could contribute to M1 activation. Results showed that E2-potentiated macrophage infiltration and M1 activation, as well as the severity of joint inflammation, partially depended on cadherin-11. Similar results were observed in NR8383 macrophages. These results were the first, to our knowledge, to demonstrate that estrogen potentiates cadherin-11 in infiltrated macrophages, which leads to M1 polarization and further aggravates joint inflammation.

However, the underlying mechanism of the contribution of cadherin-11 to M1-like macrophage activation in inflamed joints remains unclear. This mechanism may be related to the fact that cadherin-11 can stimulate the activation of Erk and NF-κB p65 in NR8383 macrophages. This hypothesis is consistent with a previous study that cadherin-11 directly induced cytokines, as well as activated MAPKs and the NF-κB pathway in synovial fibroblasts (40). NF-κB is a key regulator of proinflammatory cytokines, such as TNF-α and iNOS (41); both of them contribute to M1 macrophage polarization (15). This speculation could also be supported by the present results that blocking cadherin-11 partially reversed iNOS expression in the infiltrated macrophages of inflamed TMJs, as well as reversed E2-potentiated activation of Erk and NF-κB p65 in NR8383 macrophages. In addition, our previous study showed that E2 potentiates proinflammatory cytokines, including TNF-α and iNOS, via NF-κB activation in the synovial membrane (11). This phenomenon may also be related to the reasons why cadherin-11 is abundantly expressed in synoviocytes and is required for synoviocytes to produce the extracellular matrix, which modulates recruitment, activation, and retention of immune cells (40). Nevertheless, further study is required to elucidate the details of the contribution of cadherin-11 to M1-like macrophage activation in inflamed joints.
Disclosures
The authors have no financial conflicts of interest.

References
Supplementary Figure S1. Confirmation of the effectiveness of ovariectomy and estradiol replacement in female rats. The serum level of E2 in the ovariectomized group receiving 80μg-E2 replacement were higher (93.01 pg/ml) than that (22.75 pg/ml) in 0μg-E2 group (n = 5, p < 0.01), which mimicked the peak or nadir physiologic level of estrous cycle for normal female rats, respectively. Serum levels of 17beta-estradiol in the indicated groups were measured by radioimmunoassay using an Access Immunoassay System (Beckman Coulter).
Supplementary Figure S2. (A) Estradiol repressed M2 marker IL-10 in NR8383 macrophages. Immunofluorescence of double immunostaining for M1 activation marker iNOS and M2 activation marker IL-10 in NR8383 macrophages cocultured with synoviocytes. Treatment with E2 decreased proportion of IL-4-induced IL-10+ macrophages. Nuclei were counterstained with DAPI (blue). Cells were treated with 10 ng/ml IL-4 alone or together with 10^{-8} M E2 for 24 hours. Arrow heads indicate iNOS+IL-10- macrophages. (B) Immunocytocfluorescence of iNOS and IL-10 in PBMCs-derived primary M1 and M2 macrophages. M2 marker IL-10 was barely detected in iNOS+ M1 macrophages, and M1 marker iNOS was barely detected in IL-10+ M2 macrophages. Nuclei were counterstained with DAPI (blue).
**Supplementary Figure S3.** Cadherin-11 activates ERK and NF-κB p65 in NR8383 macrophages. (A) After NR8383 macrophages were serum-starved overnight and stimulated with or without Cadherin-11-Fc (2.5 or 5 μg/ml) or TNF-α (20 ng/ml) for 30 min, total cell lysates were analyzed for p-Erk1/2, p-p38 or p-p65 by western blotting. The phosphorylation of Erk and NF-κB p65 were detected after cadherin-11 treatment. (B) Serum-starved NR8383 macrophages were pretreated with or without cadherin-11 antibody in the presence or absence of the indicated concentrations of TNF-α and E2 for 60 min. TNF-α-induced and E2-potentiated activation of Erk and NF-κB p65 was partially repressed by blockage of cadherin-11. Total Erk and β-actin were used as loading controls.
Supplementary Figure S4. Blocking estrogen receptors reversed E2-potentiated induction by TNF-α of M1 macrophages markers and repression by TNF-α of M2 macrophages markers. (A) Expressions of M1 and M2 macrophages markers in NR8383 macrophages pretreated with or without ICI 182,780. mRNAs expressions were evaluated by real-time PCR. (B) Secretions of NO and IL-10 of NR8383 macrophages. IL-10 and NO in culture media were assessed by ELISA and Griess reagent, respectively. Cells were pretreated with ICI 182,780 before treatment with TNF-α and E2 for 24 hours (n = 3, *p < 0.05, **p < 0.01, ***p < 0.001).