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

**Xiao-Xing Kou,*,† Chen-Shuang Li,* Dan-Qing He,* Xue-Dong Wang,* Ting Hao,† Zhen Meng,† Yan-Heng Zhou,* and Ye-Hua Gan†**

**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

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Abbreviations used in this article: E2, 17β-estradiol; iNOS, inducible NO synthase; RA, rheumatoid arthritis; TMD, temporomandibular disorder; TMJ, temporomandibular joint.

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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 polarization through potentiating cadherin-11 to aggravate joint inflammation.

In this study, we explored whether estrogen could affect M1/M2 macrophage dichotomy and upregulate cadherin-11 in TMJ inflammation, and tested whether estradiol-potentiated macrophage activation could be reversed by blocking cadherin-11.

## Materials and Methods

### *Animals*

Adult female Sprague Dawley rats (180–200 g) were used in this study. The experimental protocols were approved by the Animal Use and Care Committee of Peking University and were consistent with the Ethical Guidelines of the International Association for the Study of Pain. Rats were housed under a controlled temperature (22° ± 1°C) with a 12-h light/dark cycle; the 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 ovariectomized or sham ovariectomized. One week later, the ovariectomized rats were s.c. injected for 10 d 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 TMJs. The effectiveness of ovariectomy 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-ovariectomized 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 administrated as previously described (11). In brief, another two sham-ovariectomized 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*

TMJs were removed en bloc, fixed in 4% paraformaldehyde, demineralized in 15% EDTA, and embedded by paraffin. The embedded TMJ blocks 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 20 μg/ml Proteinase K solution at room temperature for 20 min. The sections were blocked with 5% BSA for 30 min at room temperature and incubated overnight at 4°C with Abs against rat CD68 (1:300; MCA341GA; Serotec), cadherin-11 (1:100, sc-6463; Santa Cruz), iNOS (1:100; ab15323; Abcam). After extensive washing with PBS, the sections were incubated with HRP-conjugated secondary Abs and visualized using diaminobenzidine (Zhongshan Golden Bridge Biotechnology, Beijing, China).

### *Immunohistofluorescence staining*

Sections were incubated overnight at 4°C with one or two Abs of anti-CD68 (1:300), anti-iNOS (1:100), anti–cadherin-11 (1:100), anti-CD163 (1:100, sc-33560; Santa Cruz), or anti–IL-10 (1:100, sc-365858; Santa Cruz). After extensive washing with PBS, the sections were incubated for 30 min 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 mM L-glutamine, 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% $CO_2$ 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) alone, to polarize the M1 and M2 macrophages, respectively (28, 29). The M1 and M2 macrophages were characterized by the macrophage marker CD68 and the 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 (T 5944; Sigma). The cadherin-11 Ab (1 μg) or ICI 182,780 (1 μM) was added to the culture medium at 0.5 h before treatment with TNF-α for 24 h. After the incubation, the culture medium was collected to measure the NO and IL-10 levels.

Fibroblast-like synoviocytes were isolated from the synovial membrane of TMJs from 6-wk-old rats as previously described (30). Synoviocytes ($2 \times 10^3$ cells/well, passages 4–6) 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 ($1 \times 10^4$ cells/well) were added to the synoviocytes with fresh medium and the cells were cocultured for 2 d in the absence or presence of 10 ng/ml TNF-α (T5944; Sigma) or 10 ng/ml IL-4 (PeproTech) and E2 ($10^{-8}$ M). Immunofluorescence staining of the cultured cells was performed using the same method as described in the previous section *Immunohistofluorescence Staining*.

### *Western blot analysis*

Western blot analysis was performed as previously described in detail (31). In brief, cell lysates were obtained 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 cadherin-11 (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′-TGACAGGATGCAGAAGGA-GA-3′/5′-TAGAGCCACCAATCCACACA-3′; rat iNOS sense/antisense, 5′-GAGTGAGGAGCAGGTTGAGG-3′/5′-CCAAGGTGTTGCCCTTT-TT-3′; rat IL-1β, sense/antisense 5′-CACCTCTCAAGCAGAGCACAG-3′/5′-GGGTTCCATGGTGAAGTCAAC-3′; rat TNF-α sense/antisense, 5′-CCAGGTTCTCTTCAAGGGACAA-3′/5′-CTCCTGGTATGAAATGG-CAAATC-3′ (11). The following primers were designed with the Primer Premier 5.0 software and commercially synthesized: rat cadherin-11 sense/antisense, 5′-TCCAACCAGCCAATAGTTACAGT-3′/5′-ATCACAATGG-GCAGGAGGTAGAG-3′; rat IL-10 sense/antisense, 5′-CACTGCTATGT-TGCCTGCTCTTAC-3′/5′-GGGTCTGGCTGACTGGGAAG-3′; rat arginase sense/antisense, 5′-TTGATGTTGATGGACTGGAC-3′/5′-TCTCTGGCT-TATGATTACCTTC-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.

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## 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) groups, 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

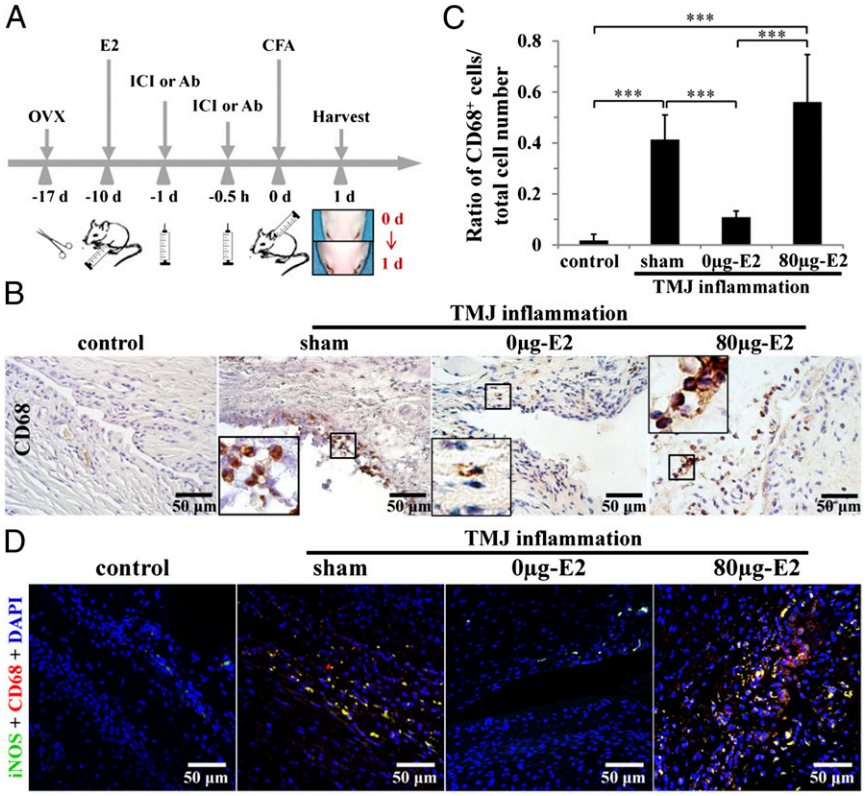


**FIGURE 1.** Estradiol promoted accumulation and activation of M1 macrophages in inflamed TMJs. (**A**) Scheme for animal experiments. (**B**) Immunohistochemistry of CD68 in infiltrated mononuclear cells of inflamed TMJs 24 h after induction of TMJ inflammation. The inflamed TMJs of the 80 μg-E2 groups presented the highest number of $CD68^+$ cells around the synovial membrane, followed by the sham, 0 μg-E2, and control groups. Female rats were sham-ovariectomized; otherwise, rats were ovariectomized and respectively dosed with 0 or 80 μg E2. Large boxed areas show higher-magnification views of the small boxes. (**C**) Quantification of the infiltrated $CD68^+$ macrophages of inflamed TMJs in (B). Cells were counted in five high-power fields per sample. Data are presented as the mean ± SD ratio of $CD68^+$ cells to the total number of cells counted around the synovial layer ($n = 5$). ***$p < 0.001$. (**D**) Evaluation of M1-like macrophages activated by immunohistofluorescence. The inflamed TMJs of the 80 μg-E2 group had the highest cells coexpression of the CD68 (red) and the M1 marker iNOS (green); the sham group had less expression, whereas hardly any expression was detected in the 0 μg-E2 group. Nuclei were counterstained with DAPI (blue). The merged yellow signal indicates M1 macrophages. Ab, cadherin-11 Ab; ICI, ICI 182,780; OVX, ovariectomized.

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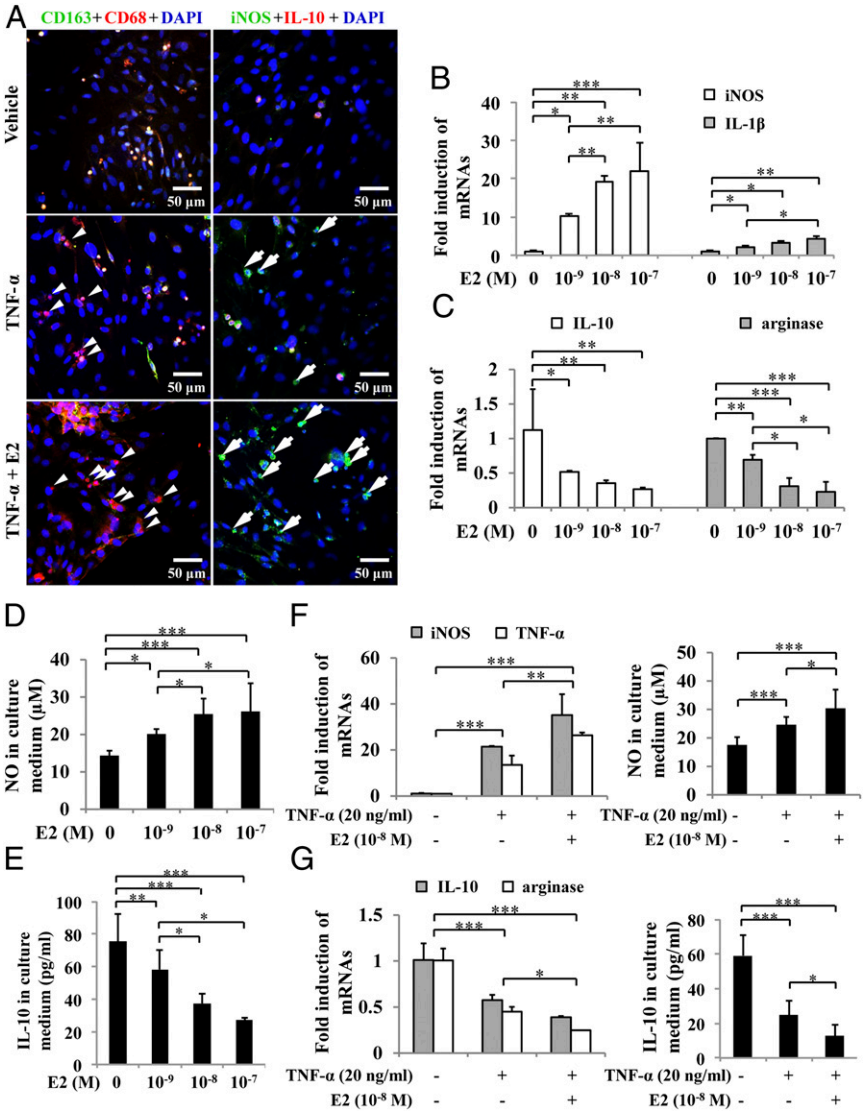


**FIGURE 2.** Estradiol promoted M1 markers but repressed M2 markers in NR8383 macrophages. (**A**) Immunofluorescence of double immunostaining for CD68 and M2 marker CD163 or for M1 activation marker iNOS and M2 activation marker IL-10 in NR8383 macrophages cocultured with synoviocytes. Treatment with E2 decreased the proportion of $CD163^+$ M2 cells and decreased the proportion of $IL\text{-}10^+$ cells, whereas TNF-α induction of $iNOS^+$ monocytes was further increased. Nuclei were counterstained with DAPI (blue). Cells were treated with 20 ng/ml TNF-α alone or combined with $10^{-8}$ M E2 for 24 h. Arrowheads indicate the $CD68^+CD163^-$ macrophages; arrows indicate the $iNOS^+IL\text{-}10^-$ macrophages. (**B**–**E**) Estradiol potentiated M1-like macrophages and repressed M2-like macrophage-associated genes. The mRNA expressions were assessed by real-time PCR; secretions of NO and IL-10 in the culture media were assessed by ELISA and Griess reagent, respectively. Cells were treated with increasing doses of E2 for 24 h. (**F** and **G**) Estradiol potentiated the TNF-α–induced upregulation of M1 markers and repression of M2 markers. Cells were treated with TNF-α alone or together with E2 for 24 h ($n = 5$). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

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. In 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 $iNOS^+IL\text{-}10^-$ immunostaining) and M2 macrophages (induced by IL-4, identified by $CD68^+CD163^+$ and $iNOS^-IL\text{-}10^+$ 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.

To further confirm the enhancing effect of estrogen on cadherin-11 expression in macrophages, we pretreated NR8383 macrophages with E2 before stimulation with TNF-α. As shown in

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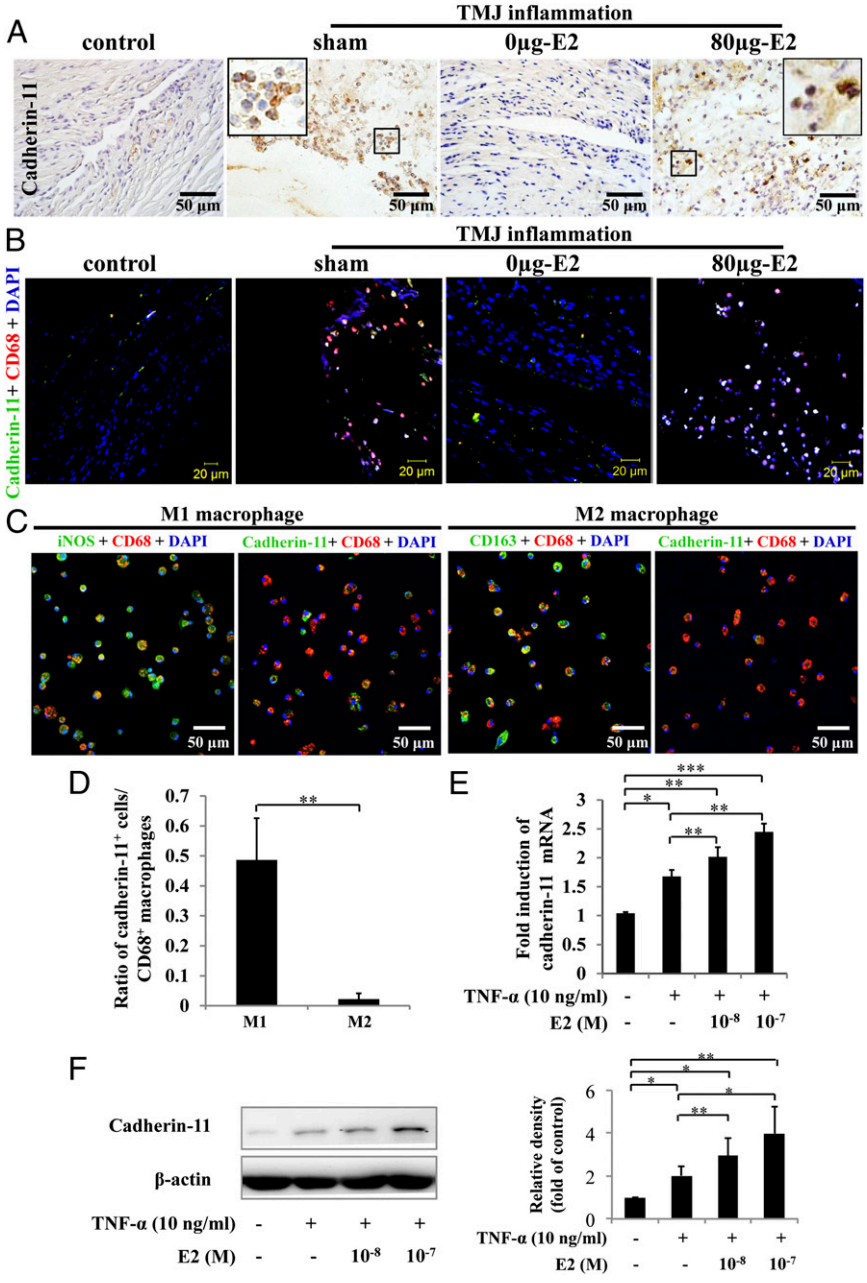


**FIGURE 3.** Estradiol upregulated cadherin-11 in M1 macrophages. (**A**) Immunohistochemistry of cadherin-11 in infiltrated mononuclear cells of inflamed TMJs at 24 h after induction of TMJ inflammation. Immunostaining of cadherin-11 was detected in most of the mononuclear cells in the eroded and broken 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).

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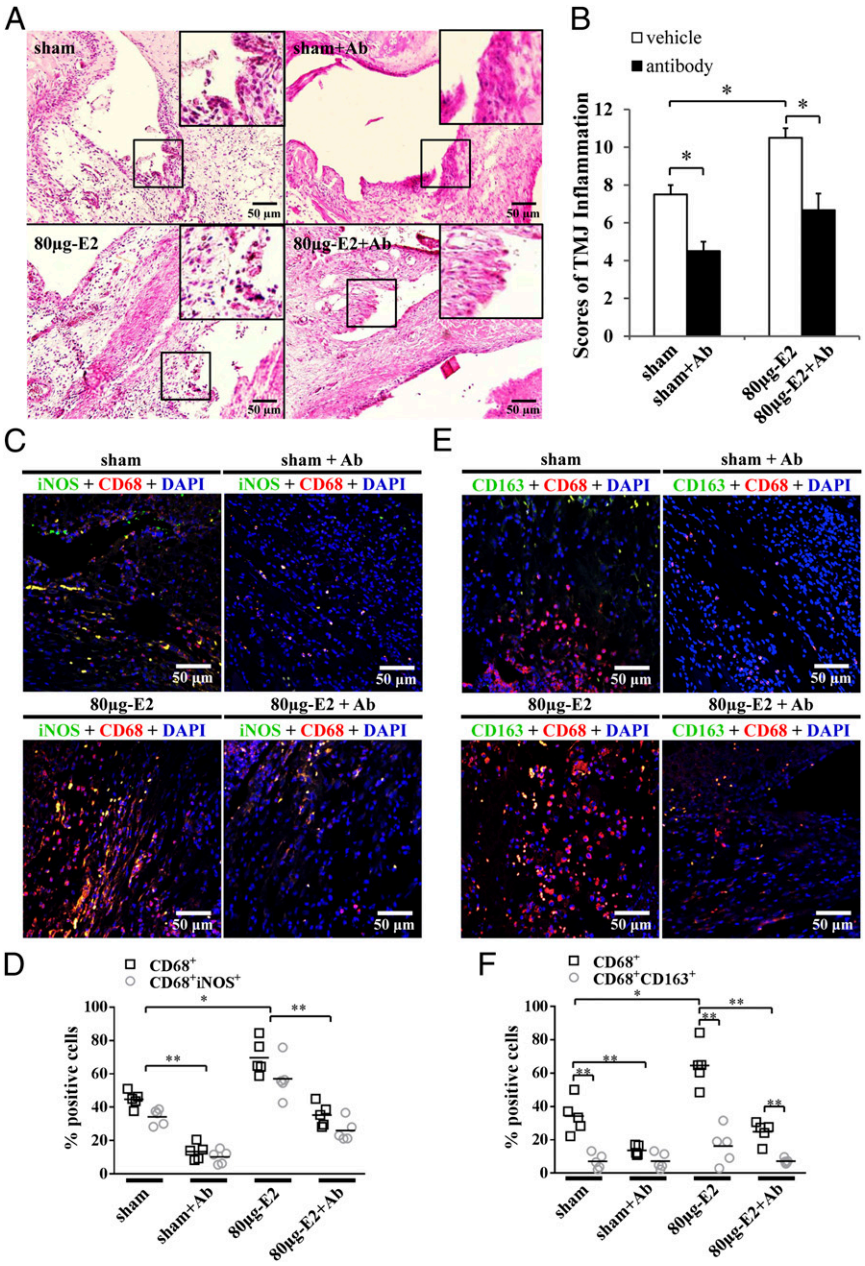


**FIGURE 4.** Blocking cadherin-11 partially reversed E2-potentiated joint inflammation and M1 macrophage activation. (**A**) Representative photomicrographs of TMJ histopathology. Pretreatment with the cadherin-11 Ab inhibited the features of joint inflammation, including the proliferation of synoviocytes and infiltration of mononuclear cells in the synovial membrane of the inflamed TMJs of the sham group. Pretreatment also mitigated erosion of the synovial membrane and severe infiltration of mononuclear cells in the intimal lining and sublining layer of the inflamed TMJs of the 80 μg-E2 group. The large boxed areas show higher-magnification views of the small boxes. (**B**) Blocking cadherin-11 attenuated estradiol-potentiated TMJ inflammation. The TMJ inflammation scores were evaluated in the same groups as in (A), as previously described in detail (11) ($n = 3$). (**C** and **D**) Immunohistofluorescence of M1 macrophages in inflamed TMJs. Pretreatment with the cadherin-11 Ab significantly reversed the infiltration of $CD68^+$ and $CD68^+$ $iNOS^+$ (M1) macrophages in the sham group, as well as the E2-potentiated infiltration of $CD68^+$ and $CD68^+iNOS^+$ macrophages in the 80 μg-E2 group. (**E** and **F**) Immunohistofluorescence of M2 macrophages in inflamed TMJs. The proportion of $CD68^+CD163^+$ macrophages (M2) was not affected by blocking cadherin-11. 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 positive cells to the total number of cells counted around the synovial layer ($n = 5$). $*p < 0.05$, $**p < 0.01$. Ab, cadherin-11 Ab.

*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 $IL\text{-}10^+$ 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 blockage 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

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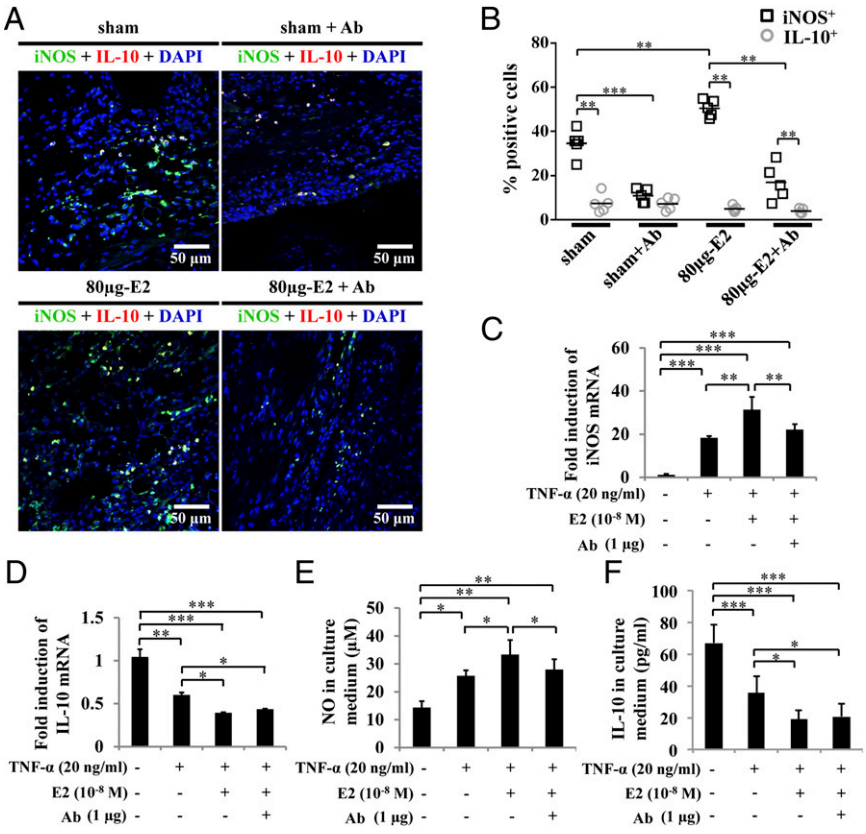


**FIGURE 5.** Blocking cadherin-11 partially reversed expressions and releases of M1 macrophage-associated proinflammatory cytokines. (**A** and **B**) Immunohistofluorescence for M1 marker iNOS and M2 marker IL-10 in inflamed TMJs. Pretreatment with the cadherin-11 Ab reversed iNOS expression and E2-potentiated iNOS expression in the infiltrated cells of the TMJs 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.

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 series sections showed that most of the infiltrated $CD68^+$ macrophages were colocalized with iNOS (merged as orange) in the synovial membrane of the sham and 80 μg-E2 groups (Fig. 6B). Moreover, treatment with ICI 182,780 reversed the infiltration of $CD68^+$ $iNOS^+$ cells and partially reversed cadherin-$11^+$ both in the sham and 80 μg-E2 groups (Fig. 6B, 6C), indicating that estrogen played an important role in the induction of cadherin-11 expression and M1-like macrophage activation in the synovium of inflamed TMJs.

Similarly, E2-enhanced induction by TNF-α of the cadherin-11 mRNA and protein expression in NR8383 macrophages was partially reversed by treatment with ICI 182,780 (Fig. 6D, 6E). In addition, blocking estrogen receptors by ICI 182,780 partially reversed E2-enhanced induction by TNF-α of iNOS and TNF-α (M1 markers) mRNA expression, as well as NO secretion. In addition, blocking estrogen receptors partially reversed the inhibitory effect of E2 on arginase and IL-10 (M2 markers) mRNA expression and protein secretion (Supplemental Fig. 4). These data suggested that estrogen has an important role in cadherin-11 expression and cadherin-11–mediated M1-like macrophage activation.

## 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 hepa-

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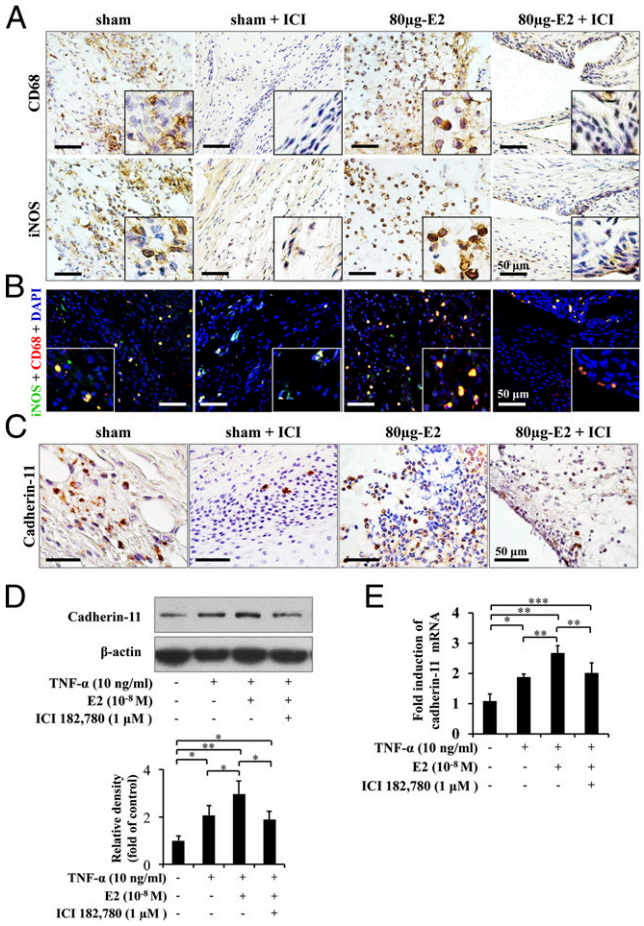


**FIGURE 6.** Blocking estrogen receptors partially reversed E2-potentiated M1 macrophage activation and cadherin-11 expression. (**A** and **B**) Immunohistochemistry and immunohistofluorescence of iNOS and CD68 of inflamed TMJs pretreated with ICI 182,780. Pretreatment with the estrogen receptor antagonist ICI 182,780 partially reversed the inflammation-induced and E2-potentiated infiltration of $CD68^+$ macrophages and the expression of iNOS in the infiltrated cells in the TMJs of the sham and 80 μg-E2 groups, respectively. Nuclei were counterstained with DAPI (blue) in (B). (**C**) Immunohistochemistry of cadherin-11 in inflamed TMJs pretreated with ICI 182,780. Cadherin-$11^+$ cells in the sham and 80 μg-E2 groups were partially reversed by ICI 182,780. (**D** and **E**) Western blot and real-time PCR analysis of cadherin-11 protein and mRNA expressions in NR8383 macrophages pretreated with ICI 182,780 before treatment with TNF-α and E2 for 24 h. β-Actin was served as an internal control for equal loading of Western blot analysis. The *lower panels* of the Western blots show the quantified cadherin-11 protein, which is presented as the relative density compared with the control group; data were normalized against the β-actin expression in each sample. $n = 3$. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

tocellular carcinoma growth. To the best of our knowledge, this study is the first to define an E2-potentiated M1-like macrophage activation profile in CFA-induced TMJ acute inflammation of a rat model. This E2-potentiated M1 activation could contribute to the perpetuation of joint inflammation and tissue breakdown. The importance of M1 activation was previously revealed in a clinical study where the synovial fluid levels of M1-derived cytokines were well correlated with joint inflammation in RA (39). Therefore, the promotion and accumulation of M1 macrophages by estrogen in inflamed joints may be an important mechanism underlying the sex differences of TMD and RA.

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.

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 cadherien-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 and arginase. 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.

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## Disclosures

The authors have no financial conflicts of interest.

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