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α-Enolase Expressed on the Surfaces of Monocytes and Macrophages Induces Robust Synovial Inflammation in Rheumatoid Arthritis

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α-enolase (ENO1) is a multifunctional glycolytic enzyme expressed abundantly in the cytosol. It has been implicated in autoimmune and inflammatory diseases. Serum Abs against ENO1 were reported in rheumatoid arthritis (RA). Cell-surface expression of ENO1 has been found to be increased rapidly in response to inflammatory stimuli, but its expression and function have not been reported in RA. In this study, we show that cell-surface expression of ENO1 is increased on monocytes and macrophages isolated from RA patients but not on those from osteoarthritis patients, and Ab against ENO1 can stimulate these cells to produce higher amounts of proinflammatory mediators, such as TNF-α, IL-1α/β, IFN-γ, and PGE2 via p38 MAPK and NF-κB pathway. The frequency of ENO1-positive cells in synovial fluid mononuclear cells was higher than PBMCs. ENO1-positive cells were also found in the inflamed synovium from RA patients and arthritic ankle tissues of mice with collagen-induced arthritis. Taken together, these findings suggest that Abs against ENO1 present in RA sera may stimulate monocytes and macrophages expressing cell-surface ENO1 and contribute to production of proinflammatory mediators during the effector phase of synovial inflammation. The Journal of Immunology, 2012, 189: 365–372.

Rheumatoid arthritis (RA) is a well-known chronic inflammatory disease characterized by severe synovial inflammation in multiple joints accompanied by destruction of bone and cartilage (1). Many types of cells participate in the tissue destruction associated with RA (2). Moreover, based on the histologic features of tissues from RA patients, macrophages and lymphocytes are key infiltrates in inflamed subsynovium (3–5), and proinflammatory cytokines, such as TNF-α, IL-1α, IL-6, and IL-18, produced by these infiltrating cells are known to be related to the pathogenesis of RA (5, 6).

Abbreviations used in this article: ChIP, chromatin immunoprecipitation; CIA, collagen-induced arthritis; CII, type II collagen; ENO1, α-enolase; FLS, fibroblast-like synovocyte; OA, osteoarthritis; RA, rheumatoid arthritis; SFMC, synovial fluid mononuclear cell; ST, synovial tissue; TXA, tranexamic acid; VPLCK, D-Val-Phe-Lys chloromethyl ketone.

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α-enolase (enolase-1 [ENO1]) is a multifunctional protein that was first identified as a key component of the glycolytic pathway. ENO1 is ubiquitously expressed in the cytosol and it is also found on the cell surface as a plasminogen-binding receptor (7–9). PMA stimulated hematopoietic cells, such as neutrophils, lymphocytes, and monocytes, to express higher amounts of ENO1 on their surface (10, 11). Inflammatory stimuli such as LPS rapidly up-regulated cell-surface expression of ENO1 on human blood monocytes and U937 monocytes by rapid translocation of ENO1 to the cell surface from cytosol (12). These results imply that upregulated surface expression of ENO1 in hematopoietic cells may have an important role in the inflammatory process.

Abs against ENO1 have been found in a variety of autoimmune and inflammatory diseases such as systemic lupus erythematosus, RA, systemic sclerosis, Behçet’s disease, ulcerative colitis, Crohn’s disease, primary sclerosing cholangitis, and retinopathy (13–19). Serum levels of Abs against ENO1 are increased in 25–66% of RA patients (14, 16). However, the pathological role of these autoantibodies is not completely understood. We hypothesized that mononuclear cells derived from RA patients express higher amounts of ENO1 on their surface and Abs against ENO1 in their sera could stimulate these cells to produce inflammatory cytokines. In the current study, we found that cell-surface expression of ENO1 is increased in monocytes and macrophages isolated from RA patients but not on those from osteoarthritis (OA) patients, and Ab against ENO1 can stimulate these cells to produce higher amounts of proinflammatory mediators such as TNF-α, IL-1α/β, IFN-γ, and PGE2 via the p38 MAPK and NF-κB pathway.

Materials and Methods

Patients and specimens

Heparinized peripheral blood was collected from healthy volunteers (n = 10), RA patients (n = 35), and OA patients (n = 14). Synovial fluids were obtained from RA patients (n = 18). Synovial tissue (ST) samples were obtained from patients undergoing total knee replacement surgery or ar-

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**EN01 stimulation**

PBMCs and SFMCs (3 × 10^6/ml) from RA patients or FLSs were stimulated with anti-ENO1 mAb (1 μg/ml) or plasminogen (Molecular Innovations, Novi, MI) at room temperature with gentle rotation for 1 h. MOPC-21 (1 μg/ml, Sigma-Aldrich) was used as an isotype control Ab. Cells were transferred to 24-well plates and incubated for 12 h at 37°C in a 5% CO2 humidified incubator.

**ELISA**

After EN01 stimulation with anti-ENO1 mAb or plasminogen, supernatants were collected, and TNF-α, IL-1α/β, IFN-γ, IL-18, and PGE2 levels were measured using ELISA kits following the manufacturer’s instructions. Human TNF-α, IL-1α/β, and IFN-γ ELISA kits were purchased from R&D Systems, human IL-18 from MBL (Nagoya, Japan), and human PGE2 from Cayman Chemical (Minneapolis, MN). To assess the role of plasminogen on the production of proinflammatory mediators, the lysine analog tranexamic acid (TXA; Sigma-Aldrich) and specific inhibitor of plasmin α-Va-Val-Phe-Lys chloromethyl ketone (VPLCK; Calbiochem) were used. RA PBMCs were stimulated with plasminogen for 12 h in the presence or absence of TXA or VPLCK. TNF-α and IL-1β levels of supernatant were determined by ELISA.

**Probing of the signaling pathways involved in cytokine production**

Specific inhibitors of NF-κB (BAY11-7082, PI3K (LY294002), ERK (PD98059), p38 MAPK (SB203580), and JNK (SP600125) were purchased from Sigma-Aldrich. These inhibitors were used to identify the signaling pathways involved in the inductions of cytokines by EN01 stimulation with anti-ENO1 mAb. PBMCs or SFMCs were pretreated with inhibitors or DMSO (vehicle control) for 1 h and washed twice with media. Then cells were stimulated with anti-ENO1 mAb (1 μg/ml) and incubated for a further 12 h. TNF-α and IL-1β levels of supernatant were determined by ELISA. To exclude unspecified effect of inhibitors, an additional experiment was performed using p38 MAPK inhibitor (SB203580) and its control compound (SB202474) purchased from Calbiochem.

**Western blot analysis**

After EN01 stimulation with anti-ENO1 mAb, cells were homogenized with lysis buffer, and total protein concentrations were measured using Bio-Rad Protein Assay Kits (Bio-Rad, Hercules, CA). Equal amounts of protein were resolved using 10–12% polyacrylamide-SDS gels and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk for 1 h, washed with 0.05% Tween-20 in PBS, and then incubated with primary Ab at 4°C overnight. Primary Abs for phospho-p38 MAPK (Cell Signaling Technology, Danvers, MA), p38 MAPK (Santa Cruz Biotechnology) and B-actin (Sigma-Aldrich) were diluted 1:1000, 1:200, and 1:4000 with 0.05% Tween-20 in PBS, respectively. Membranes were then washed and incubated with HRP-conjugated secondary Ab (1:5000; Cell Signaling Technology), and treated using an ECL detection kit (GE Healthcare, Buckinghamshire, U.K.).

**Chromatin immunoprecipitation assay**

Chromatin immunoprecipitation (ChIP) assays were based on a previously described protocol (20). Briefly, EN01-stimulated cells by anti-ENO1 mAb were fixed with formaldehyde and homogenized using lysis buffer. After shearing chromatin by sonication, a fraction (1%) of the sheared chromatin was set aside as input DNA. DNA samples were immunoprecipitated with rabbit anti-p65 Ab (Santa Cruz Biotechnology) at 4°C overnight. Rabbit IgG (Santa Cruz Biotechnology) was used for isotype control of rabbit anti-p65 Ab. Immunocomplexes were collected using salmon sperm DNA-agarose A slurry and eluted with elution buffer. Protein–DNA crosslinks were severed by heating at 65°C for 4 h. After RNase and proteinase K digestion, DNA was isolated by phenol/chloroform extraction. The same set of input DNA and ChIP DNA were subjected to semi-quantitative PCR. The TNF-α promoter–specific primers used for regions containing NF-κB binding motif were 5’-CCCTCCAGTCCTAGGCTCACCTAC-3’ (forward) and 5’-GGGAAAAAGATCCATTACAGGCAG-3’ (reverse), and IL-1β promoter–specific primers used for regions containing NF-κB binding motif were 5’-AAGTTCAGGGTTCAGGGTCAG-3’ (forward) and 5’-AGCTCAGAATTTCTGCTC-3’ (reverse).

**Statistical analysis**

Data are presented as means ± SDs. One-way ANOVA followed by Newman–Keuls multiple comparison test was used to compare three or
more groups. The $p$ values <0.05 were considered statistically significant. Statistical tests were carried out using GraphPad InStat version 5.01 (GraphPad, La Jolla, CA).

Results

Cell-surface expression of ENO1 on mononuclear cells derived from RA patients

The cell-surface expression of ENO1 on mononuclear cells was examined by flow cytometry analysis. Cell-surface expression of ENO1 was very low in PBMCs from healthy volunteers, but increased by Con A stimulation (Fig. 1A). In contrast, RA PBMCs and SFMCs were found to express ENO1 on their cell surfaces (Fig. 1A). The frequency of ENO1-positive cells was higher in RA PBMCs ($n = 42$; mean ± SD, 24.42 ± 13.19%) and RA SFMCs ($n = 20$; 42.15 ± 18.69%) than OA PBMCs ($n = 14$; 11.71 ± 5.35%) (RA PBMCs and RA SFMCs versus OA PBMCs: $p < 0.01$, $p < 0.001$ respectively; Fig. 1A, 1B), and the frequency of ENO1-positive cells was higher among RA SFMCs than among RA PBMCs ($p < 0.001$; Fig. 1B). These results suggest that ENO1 is readily expressed on the surfaces of mononuclear cells of both blood and inflamed joint tissues in RA patients. Because PBMCs and SFMCs consist of many types of immune cells, we examined which cellular subsets are mainly responsible for ENO1 expression by staining with immune cell-specific markers for CD3, CD14, CD19, and CD56. The majority of cells expressing ENO1 in PBMCs and SFMCs derived from RA patients were found to be CD14-positive monocytes and macrophages (RA PBMCs, 91.17 ± 6.23%; RA SFMCs, 67.46 ± 29.05%; Fig. 1C).

ENO1 expression in the arthritic ankle tissues of mice with collagen-induced arthritis and in the STs of RA patients

Recent study has reported that inflammatory cell invasion into acutely inflamed lung is mediated by increased cell-surface expression of ENO1 (12). Therefore, we examined ENO1 expression in the arthritic ankles tissues of mice with collagen-induced arthritis (CIA). CIA is the most widely used model for RA and a chronic erosive inflammatory disease affecting peripheral joints, and the tissue distribution and histopathology of the destruction process mimic that of RA (21). In concordance with our flow cytometry results (Fig. 1), ENO1-positive cells were found in arthritic ankle tissues ($n = 5$), but not in normal control mice ($n = 4$; Fig. 2A). We also examined ENO1 expression in STs from OA and RA patients, and as was found in CIA mice, ENO1 was found to be highly expressed in RA STs ($n = 34$), but not in OA tissues ($n = 32$; Fig. 2B).

FLSs play an important role in the pathogenesis of RA (22, 23). Therefore, we also investigated the surface expression of ENO1 in FLSs from RA patients. ENO1 was not found to be expressed on

![FIGURE 1. Cell-surface expression of ENO1 on monocytes and macrophages from RA patients. (A) Cell-surface expression of ENO1 on PBMCs and SFMCs from OA or RA patients. PBMCs or Con A-activated PBMCs from healthy volunteers ($n = 10$) were used as negative or positive controls. Cell-surface ENO1 was stained with FITC-conjugated mAb to ENO1 and analyzed by flow cytometry. (B) Percentages of ENO1-positive cells on total numbers of PBMCs from OA patients ($n = 14$) and RA patients ($n = 42$) and of SFMCs ($n = 20$) from RA patients. Symbols represent individual patients. Bars represent mean values. (C) CD3, CD14, CD19, and CD56 expressions in ENO1-positive cells in RA PBMCs and SFMCs. Left panel, Representative data of RA PBMCs. Values shown in the right panel are the means ± SDs of independent experiments from 12 different RA patients. **$p < 0.01$, ***$p < 0.001$.](http://www.jimmunol.org/10.4049/jimmunol.1700689)
supernatants were then determined by ELISA as described in Materials and Methods. ENO1 mAb. RA PBMCs (n = 34) (B) were investigated by immunohistochemical staining (original magnification ×400). Scale bars, 50 μm.

Induction of TNF-α, IL-1α/β, IL-18, IFN-γ, and PGE2 production by ENO1 stimulation with anti-ENO1 mAb

In RA PBMCs and SFMCs, most of the ENO1-positive cells were monocytes and macrophages (Fig. 1C). Monocytes and macrophages represent a predominant cell lineage in the pannus and play important roles in chronic inflammation (4). Furthermore, key inflammatory cytokines in RA, such as, TNF-α and IL-1β, are known to be produced by activated monocytes and macrophages.

Thus, we investigated whether cell-surface expression of ENO1 could mediate the production of proinflammatory mediators, such as TNF-α, IL-1α/β, IL-18, IFN-γ, and PGE2 in PBMCs and SFMCs. When PBMCs and SFMCs from RA patients were stimulated with anti-ENO1 mAb, the levels of TNF-α (anti-ENO1 versus control, RA PBMCs, 9780.0 ± 520.5 versus 467.2 ± 20.2 pg/ml; p < 0.001; RA SFMCs, 12760.0 ± 193.5 versus 1715.0 ± 257.3 pg/ml; p < 0.001), IL-1α (anti-ENO1 versus control, RA PBMCs, 1535.2 ± 52.6 versus 20.1 ± 1.0 pg/ml; p < 0.001; RA SFMCs, 2756.2 ± 92.0 versus 2.0 ± 1.0 pg/ml; p < 0.001), IL-1β (anti-ENO1 versus control, RA PBMCs, 7604.2 ± 157.4 versus 166.7 ± 10.4 pg/ml; p < 0.001; RA SFMCs, 9240.0 ± 485.4 versus 805.0 ± 225.2 pg/ml; p < 0.001), IL-18 (anti-ENO1 versus control, RA PBMCs, 104.8 ± 20.4 versus 1.4 ± 0.1 pg/ml; p < 0.001; RA SFMCs, 54.7 ± 5.0 versus 12.9 ± 1.8 pg/ml; p < 0.001), and IFN-γ (anti-ENO1 versus control, RA PBMCs, 190.0 ± 10.0 versus 80.0 ± 26.5 pg/ml; p < 0.01; RA SFMCs, 530.0 ± 105.4 versus 313.3 ± 32.1 pg/ml; p < 0.05) were dramatically increased (Fig. 3A, 3B). In addition, PGE2 production (anti-ENO1 versus control, RA PBMCs, 10935.1 ± 314.3 versus 743.1 ± 66.9 pg/ml; p < 0.001; RA SFMCs, 12003.3 ± 607.0 versus 343.9 ± 60.9 pg/ml; p < 0.001) was also found to be significantly increased by ENO1 stimulation with anti-ENO1 mAb (Fig. 3A, 3B).

To confirm that the cells responsible for cytokine production were mainly monocytes and macrophages, we sorted CD14-positive cells in PBMCs from RA patients. It was found that CD14-positive cells, but not negative cells significantly produced TNF-α upon ENO1 stimulation with anti-ENO1 mAb (mean ± SD, 1828.7 ± 148.2 versus 39.6 ± 9.9 pg/ml; p < 0.001, representative of three independent experiments). We also obtained the same result for SFMCs from RA patients (2715.6 ± 126.1 versus 39.6 pg/ml; p < 0.001) and STs from RA patients (2715.6 ± 126.1 versus 39.6 pg/ml; p < 0.001, representative of three independent experiments). To exclude the possibility that the increased cytokine production is caused by endotoxin contamination, the concentration of endotoxin in reagents used for the stimulation with anti-ENO1 Abs was measured by Limulus amebocyte lysate assay.

**FIGURE 3.** Increased productions of TNF-α, IL-1α/β, IFN-γ, and PGE2 in the PBMCs and SFMCs of RA patients after ENO1 stimulation with anti-ENO1 mAb. RA PBMCs (A) or SFMCs (B) were stimulated by anti-ENO1 mAb (1 μg/ml) or isotype Ab (MOPC-21, represented as CTRL on the figure) for 1 h as described in Materials and Methods and then incubated for a further 12 h. Levels of TNF-α, IL-1α/β, IL-18, IFN-γ, and PGE2 in culture supernatants were then determined by ELISA as described in Materials and Methods. Data are presented as the mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.
stimulation with Abs was <0.9728 EU/ml. It is generally known that *Limulus* amebocyte lysate reacts with endotoxin or LPS, which is a membrane component of Gram-negative bacteria. We confirmed that 1 EU/ml LPS in RA PBMCs could not induce TNF-α production (1 EU/ml LPS versus control, 446.4 ± 41.4 versus 525.5 ± 23.9 pg/ml). Therefore, all of the results by the stimulation of ENO1 were considered to be induced specifically by the ligation of anti-ENO1 Ab.

*Increases in the productions of TNF-α and IL-1β by ENO1 stimulation with plasminogen*

ENO1 is known as one of the receptor for plasminogen, a zymogen of the serine protease plasmin, and plasminogen was reported to be an important mediator of joint inflammation in RA (24–26). The interaction of plasminogen and ENO1 is mediated by binding of plasminogen kringle domains to the C-terminal lysine residues of ENO1. Then, the activation of plasminogen into plasmin by plasminogen activator and localization of the proteolytic activity of plasmin on cell surfaces are enhanced by the interaction between plasminogen and ENO1 (7–9). To assess whether binding of plasminogen to ENO1 could induce production of proinflammatory mediators like Ab against ENO1, ENO1-positive cells were stimulated with plasminogen. After plasminogen treatment, the levels of TNF-α were increased in a dose-dependent manner in RA PBMCs and SFMCs (plasminogen treatment versus control, \( p < 0.001 \), respectively; Fig. 4A, 4B), which concurs with our

**FIGURE 4.** Increased production of TNF-α in the PBMCs and SFMCs of RA patients after ENO1 stimulation with plasminogen. (A and B) RA PBMCs or SFMCs were stimulated for 12 h with various concentration of plasminogen as indicated. After incubation, changes in the levels of TNF-α in supernatants were determined by ELISA. Data are presented as the mean ± SD. (C and D) RA PBMCs were stimulated with plasminogen (50 μg/ml) in the presence or absence of 10 mM TXA or 12.5 or 25 μM VPLCK for 12 h. After incubation, the levels of TNF-α and IL-1 β in supernatants were determined by ELISA. Data are presented as the mean ± SD. (E and F) To clarify the binding sites of plasminogen and anti-ENO1 mAb, we did competition assay between plasminogen and anti-ENO1 mAb. RA PBMCs (1 × 10⁶ cells/ml) were pretreated with plasminogen (1 or 4 μg/ml) for 30 min and washed twice with PBS. Then cells were stained with FITC-conjugated anti-ENO1 mAb and analyzed by flow cytometry (E). RA PBMCs were stimulated with plasminogen (12.5 μg/ml) and/or anti-ENO1 mAb (1 μg/ml) simultaneously and then incubated for 12 h. Levels of TNF-α in culture supernatants were determined by ELISA. Data are presented as the mean ± SD (***\( p < 0.01 \), ****\( p < 0.001 \)) (F).
observation of stimulation of ENO1 with anti-ENO1 mAb (anti-ENO1 versus plasminogen 50 μg/ml treatment, RA PBMCs, 1444.8 ± 123.8 versus 1784.2 ± 52.5 pg/ml; RA SFMCs, 5111.3 ± 120.6 versus 4504.7 ± 358.0 pg/ml). Plasminogen binding to the C-terminal lysine residues of plasminogen receptors including ENO1 was reported to be inhibited by lysine analog, such as TXA (7, 8, 12). Plasminogen-induced TNF-α and IL-β production was suppressed by the treatment of TXA (Fig. 4C). In addition, we also used VPLCK, which blocks the catalytic center of plasmin (27). As a result, we also found the suppression of plasminogen-induced TNF-α and IL-β production by VPLCK (Fig. 4D). These results suggest that the binding of plasminogen to ENO1 and activation of plasminogen into plasmin is important in the plasminogen-mediated cytokine production.

As we have already shown that both of anti-ENO1 mAbs and plasminogen induce proinflammatory cytokine production via the stimulation of membrane-bound ENO1 on RA PBMCs and SFMCs, we next investigated whether plasminogen and anti-ENO1 mAb share the binding site on membrane-bound ENO1 through the competition assay. When cells were stained with anti-ENO1 mAb after the pretreatment of plasminogen, there was no change in binding activity of anti-ENO1 mAb to membrane-bound ENO1 (Fig. 4E). In addition, when cells were simultaneously treated with plasminogen and anti-ENO1 mAbs, TNF-α production was additively increased (Fig. 4F). It suggests that plasminogen and anti-ENO1 mAb do not share the binding site on membrane-bound ENO1.

Induction of TNF-α and IL-1β by ENO1 stimulation with anti-ENO1 mAb via the p38 MAPK and NF-κB pathways

To identify the signaling pathways responsible for productions of proinflammatory mediators by stimulation of ENO1 with anti-ENO1 mAb, we used known inhibitors of several signaling pathways. Cell surface-expressed ENO1 on PBMCs and SFMCs derived from RA patients were stimulated with anti-ENO1 mAb after being pretreated with the indicated inhibitors. The productions of TNF-α and IL-1β were suppressed by SB203580 pretreatment (an inhibitor of p38MAPK) in RA PBMCs and SFMCs (anti-ENO1 versus SB203580 treatment, p < 0.001, respectively) and by BAY11-7082 pretreatment (an inhibitor of NF-κB activation) in RA PBMCs and SFMCs (anti-ENO1 versus BAY11-7082 treatment, p < 0.001, respectively). However, pretreatments with PD98059 (an inhibitor of ERK1/2), SP600125 (an inhibitor of JNK), or LY294002 (an inhibitor of PI3K activation) did not affect the productions of TNF-α or IL-1β (Fig. 5A, 5B). The cytotoxicity of each inhibitors at the concentration used in the current study was assessed by trypan blue dye exclusion assay. The viability and number of cells were not affected by the treat-

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**FIGURE 5.** Proinflammatory cytokine production by ENO1 stimulation with anti-ENO1 mAb via p38MAPK and NF-κB pathways. Suppressions of the ENO1-induced productions of TNF-α and IL-1β by specific inhibitors for p38MAPK and NF-κB. RA PBMCs (A) and SFMCs (B) were pretreated for 1 h with PD98059 (30 μM), SB203580 (40 μM), SP600125 (20 μM), LY294002 (5 μM), or BAY11-7082 (2.5 μM) before ENO1 stimulation with anti-ENO1 mAb or isotype Ab (1 μg/ml). DMSO was used as vehicle control. After 12 h, levels of TNF-α and IL-1β in culture supernatants were determined by ELISA. Data are presented as the mean ± SD. (C) Increased phosphorylation of p38 MAPK in PBMCs and SFMCs from RA patients after ENO1 stimulation. RA PBMCs or RA SFMCs were stimulated with anti-ENO1 mAb or isotype Ab (1 μg/ml) for 30, 60, and 120 min, and changes in phosphorylated p38 MAPK levels were examined by immunoblotting. Results are representative of four independent experiments. (D) Increased binding activity of NF-κB to the promoter regions of TNF-α and IL-1β upon ENO1 stimulation. Changes in the binding activity of NF-κB for the promoter region of TNF-α and IL-1β were investigated using ChIP assays. RA PBMCs were stimulated with anti-ENO1 mAb or isotype Ab (1 μg/ml) for 30 min and crosslinked using formaldehyde. Sonicated chromatin from cells was precipitated with anti–NF-κB p65 Ab and analyzed by PCR using primers containing consensus motif of NF-κB on TNF-α and IL-1β promoter, respectively. A fraction (1%) of the sonicated chromatin was set aside as input DNA before the Ab binding and used as chromatin reference for PCR. ***p < 0.001.
ment of inhibitors (Supplemental Fig. 2). Using commercially available control compound of SB203580, we confirmed SB203580 has no unspecific effects. The production of TNF-α and IL-1β was not suppressed by SB202474 pretreatment (control compound of SB203580) in RA PBMCs at the same concentrations of SB203580 used in our experiment (40 μM) (Supplemental Fig. 3). These results suggest that ENO1 regulates the production of proinflammatory cytokines via the p38 MAPK and NF-κB pathways. To confirm these results, we examined the activations of p38 MAPK and NF-κB. After ENO1 stimulation with anti-ENO1 mAb, p38MAPK phosphorylation was found to increase in a time-dependent manner in PBMCs and SFMCs derived from RA patients (Fig. 5C). Furthermore, binding of NK-κB to the promoter regions of TNF-α and IL-1β after stimulation of ENO1 with anti-ENO1 mAb was confirmed by ChIP assays on chromatin samples. Upon stimulation of ENO1 with anti-ENO1 mAb, the binding activity of NK-κB p65 was increased in consensus binding motif of NF-κB on TNF-α and IL-1β promoter regions (Fig. 5D).

Discussion

In the current study, cell-surface expression of ENO1 was found to be higher in PBMCs and SFMCs derived from RA patients than those derived from OA patients or healthy subjects (Fig. 1A), and the frequency of ENO1-positive cells among RA SFMCs was higher than among RA PBMCs (Fig. 1B). Furthermore, monocytes and macrophages were found to be the cells that predominantly expressed ENO1 in PBMCs and SFMCs (Fig. 1C). In addition, productions of TNF-α, IL-1α/β, IFN-γ, and PGE₂ were enhanced by ENO1 stimulation with anti-ENO1 mAb (Fig. 3) via the activations of p38 MAPK and NF-κB (Fig. 5). The present study is the first, to our knowledge, to show that the cell-surface expression of ENO1 is highly upregulated on monocytes and macrophages from RA patients, and upregulated expression of ENO1 plays an important role in the production of proinflammatory mediators by Ab against ENO1. Furthermore, our findings suggest that autoantibodies against ENO1 present in sera from RA patients may stimulate ENO1-positive monocytes and macrophages, and these cells contribute to the production of proinflammatory mediators during pathogenesis of RA.

Recent study has investigated the role of increased cell-surface expression of ENO1 in the migration of inflammatory cells into inflammatory sites (12). They demonstrate that inflammatory stimulation of monocytes cells induces rapid translocation of ENO1 to the cell surface, increasing local plasmin generation and thereby providing cells with enhanced migratory, transmigratory, and invasive properties, promoting their recruitment to the inflamed lung (12). Importantly, monocytes and macrophages are the majority of cells expressing ENO1 on their surface in RA patients. Taken together, cell-surface ENO1-expressed monocytes and macrophages may be accumulated in the inflamed RA synovium, possibly recruited from the circulation or resident macrophages newly expressing surface ENO1 induced by the proinflammatory milieu.

Macrophages play an important role in the pathogenesis of rheumatoid synovitis. They have been shown to produce various inflammatory mediators and interact with other cells or extracellular matrix macromolecules in the RA synovium (4). In favor of our hypothesis that increased expression of ENO1 on their surface drives the proinflammatory response stimulated by Abs against ENO1 in RA patients, we found that stimulation of cell-surface ENO1 on PBMCs and SFMCs of RA patients with anti-ENO1 mAb induced the productions of TNF-α, IL-1α/β, IFN-γ, and PGE₂. In addition, we confirmed that monocytes and macrophages in PBMCs or SFMCs are the major producers of TNF-α after stimulation of ENO1 with anti-ENO1 mAb. We observed that IL-18 production by PBMCs and SFMCs was elevated in RA patients (Fig. 3). IL-18 has been implicated to have a role in the pathogenesis of RA (28–30) to augment the activations of monocytes and macrophage and potently induce the production of IFN-γ, an effector cytokine for the progression of RA (4).

Plasminogen has been suggested to play an important role in joint inflammation of RA (24–26). Plasma levels of plasminogen are significantly increased in patients with RA. Moreover, the levels of plasminogen correlate with the degree of disease activity, being highest in those with severe disease (24). Because ENO1 has binding sites for plasminogen and acts as a receptor of plasminogen (7–9), we investigated whether plasminogen also induces the production of inflammatory mediators like anti-ENO1 mAb. As a result, we found plasminogen could induce cytokine production through the binding to ENO1 and plasmin-mediated signaling (Fig. 4C, 4D). However, plasminogen and anti-ENO1 mAb did not share the binding site on membrane-bound ENO1 (Fig. 4E, 4F). This result suggests that the production of proinflammatory mediators related to the pathogenesis of RA could be triggered by the interaction between anti-ENO1 Ab or plasminogen and ENO1 on the cell surfaces of monocytes and macrophages in RA patients.

In the current study, ENO1 was also found to be expressed on FLSs, one of the main cell lineages composing pannus. FLSs also play important roles in local inflammatory responses in the synovium of RA patients; for example, in the productions of IL-6 and IL-8 (31). Unlike PBMC and SFMC, ENO1 expression on cultured FLSs from RA patients was observed after TNF-α stimulation. Furthermore, TNF-α–induced ENO1 expression on RA FLSs was found to contribute to the production of IL-6 by stimulation with anti-ENO1 mAb (Supplemental Fig. 1). We are currently investigating other characteristics of ENO1-expressing FLSs in synovium. ENO1 was also highly expressed on the infiltrated neutrophils in ST of RA (Fig. 2). It was reported that although monocytes and neutrophils predominate in acute onset RA, the most typical feature of prolonged synovitis in chronic RA is characterized by the presence of large T cell and plasma cell infiltrates (32). Further investigation may be necessary regarding the exact role of ENO1-positive neutrophils.

We have shown the induction of ENO1 on PBMCs from healthy individuals by treatment with Con A (Fig. 1A). In addition, ENO1 expression was induced by proinflammatory mediators such as IL-1β, IL-6, PGE₂, or TNF-α (Supplemental Fig. 4). The frequency of ENO1-positive cells among SFMCs was higher than among PBMCs. It seems that surface expression of ENO1 is induced by the proinflammatory mediators under an inflammatory milieu in synovial joints.

The positivity of Abs against citrullinated ENO1 was reported to be significantly higher in RA patients than in healthy controls (33). Our assay could not discriminate whether ENO1 expressed in RA PBMC/SFMC are citrullinated or whether our ENO1 Ab preferentially binds to citrullinated ENO1 on the cell surface. However, we speculate that ENO1 is likely to be citrullinated after being translocated to the cell surface by enzymes such as extracellular peptidyl arginine deiminase.

Taken together, proinflammatory milieu in RA can induce ENO1 translocation to the cell surface from the cytosol of monocyte and macrophages. Abs against ENO1 can stimulate these cells to produce higher amounts of proinflammatory mediators such as TNF-α, IL-1α/β, IFN-γ, and PGE₂ via p38 MAPK and NF-κB pathway and contribute to the perpetuation of synovial inflammation in RA.
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Disclosures
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References