이학박사 학위논문

에놀레이즈-1의 새로운 리간드인 아포지질단백질 B가 류마티스 관절염에 미치는 영향

Apolipoprotein B binds to enolase-1

and aggravates inflammation

in rheumatoid arthritis

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Abstract

Apolipoprotein B binds to enolase-1 and aggravates inflammation in rheumatoid arthritis

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Background: Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by systemic inflammatory process that eventually leads to joint destruction. Monocytes and synovial macrophages are key players in the inflammatory process of RA. Enolase-1 (ENO1) is a multifunctional glycolytic enzyme located in

the cytoplasm of cells. It is also present on the cell surface as plasminogen receptor. The majority of cells expressing ENO1 in peripheral blood mononuclear cells (PBMCs) and synovial fluid mononuclear cells (SFMCs) derived from RA patients are known to be CD14-positive monocytes and macrophages.

Objective: The objective of this study was to identify novel ENO1 binding protein present in RA synovial fluid and determine the functional role of interaction between apolipoprotein B (apoB), a novel ligand of cell surface expressed ENO1, and ENO1 in RA.

Methods: ENO1 binding protein present in RA synovial fluid (SF) was identified by affinity-based mass spectrometry analysis. Interaction between ENO1 and apoB was evaluated by ligand blotting assay, ligand binding assay, surface plasmon resonance (SPR), and confocal microscopy. Production levels of pro-inflammatory cytokines in PBMCs from RA or healthy control (HC) after stimulation with apoB were evaluated by enzyme-linked immunosorbent assay (ELISA). Signaling pathways involved in the inductions of cytokines after stimulation with apoB were identified by using specific inhibitors. Pro-inflammatory effect of apoB was evaluated using K/BxN serum transfer arthritis mouse model.

Results: Characterization of physical interaction between ENO1 and

apoB showed that apoB was a novel ligand of ENO1. Interaction between surface ENO1 and apoB induced higher levels of pro-inflammatory cytokines in RA PBMCs compared to that in HC PBMCs. Intracellular p38 MAPK and NF-κB pathways were found to be key signaling pathways upon ENO1 activation. In K/BxN serum transfer arthritis mouse model, apoB aggravated arthritis severity. Moreover, apoB derived peptides showed agonistic or antagonistic actions.

Conclusion: ApoB is a novel ligand of ENO1. It might enhance chronic inflammation in RA patients.

Keywords: Enolase-1, apolipoprotein B, rheumatoid arthritis, inflammation, cytokine, monocyte

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Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by persistent synovitis with joint destruction, systemic inflammation, and production of autoantibodies [1]. Both monocytes and synovial macrophages are involved in the initiation and perpetuation of inflammatory response by promoting leukocyte adhesion and migration, matrix degradation, and angiogenesis. Infiltrating macrophages and lymphocytes in inflamed synovium produce cytokines such as tumor necrosis factor (TNF)－α, interleukin (IL)－1, IL－6, and IL－18 that contribute to articular and extra－articular inflammation [2, 3].

Activity of immune cells is largely regulated by a fine balance between stimulatory and inhibitory receptors on the cell surface. A novel stimulatory receptor on monocyte is enolase－1 (ENO1). ENO1 is a multifunctional glycolytic enzyme that acts as 2－phospho－D－glyceate hydrolase in the cytoplasm of cells [4]. However, ENO1 serves as a binding site for plasminogen when it is translocated to the cell surface [5－7]. Surface ENO1 expression of various cell types, including immune cells and cancer cells, is dependent on cell

activation status [8-12]. For example, human peripheral blood monocytes can markedly upregulate ENO1 on their surface after stimulation with paramethoxyamphetamine or lipopolysaccharide possibly due to rapid translocation of cytosolic ENO1 to the cell surface [12, 13].

Previous study has reported that peripheral blood mononuclear cells (PBMCs) from RA patients express more ENO1 on their surface compared to those from healthy control, and elicite higher inflammatory response including the production of TNF-α, IL-1α/β, IL-18, interferon (IFN)-γ, and prostaglandin E2, after stimulation with anti-ENO1 antibody [11].

Plasminogen is a well-known ligand of surface ENO1 [5, 6]. Other physiologic and pathologic ligands of surface ENO1 have not been fully elucidated yet. One potential candidate is apolipoprotein B (apoB). It contains regions homologous to Kringle 2 domains of plasminogen, which can bind to the C-terminal lysine residues of ENO1 [5, 6]. ApoB is a major component of low density lipoproteins (LDL), very low density lipoproteins (VLDL) and chylomicrons.

Interestingly, apoB and cholesterol are enriched in synovial fluid (SF) of RA patients [14]. It has been reported that SF/serum ratio

of apoB in RA patients is significantly higher than that in osteoarthritis patients [15]. ApoB alone or as part of LDL might contribute to higher prevalence of cardiovascular disease (CVD) in RA patients [16−18]. Moreover, a recent report has shown that LDL from RA patients is altered in its chemicophysical property with higher fragility. It can promote inflammatory response [19]. Accordingly, this study hypothesized that apoB might be able to bind to ENO1. Such interaction between apoB and ENO1 might contribute to both articular and systemic inflammation, thus accelerating atherosclerosis in RA patients.

Therefore, the objective of this study was to determine whether apoB was a novel specific ligand of ENO1 and whether the interaction between apoB and ENO1 could potentiate inflammatory response and aggravates arthritis in a murine arthritis model.

Materials and methods

Patient samples

SF was obtained from RA patients (n = 5), and osteoarthritis (OA) patients (n = 5). RA was diagnosed according to 2010 American College of Rheumatology / European League Against Rheumatism classification criteria [20]. Peripheral blood was collected from healthy volunteers (n=9), and RA patients (n=38). This study was approved by Institutional Review Board (IRB) of Seoul National University Hospital (IRB No 1702-057-831). PBMCs were isolated from heparinized blood by density gradient centrifugation using Ficoll-paque plus gradient (GE Healthcare Biosciences, Uppsala, Sweden). CD14-positive monocytes were isolated from PBMCs of RA patients using monocyte isolation kit II (MACS, Miltenyi Biotech, CA, USA).

Ligand blotting

Equal amounts of ENO1 (Prospec, Ness-Ziona, Israel), apoB100 (Calbiochem, CA, USA), and plasminogen (Molecular Innovations Inc, MI, USA) were loaded on Bolt 4 - 12 % Bis-Tris Plus gels (Life technologies, NY, USA). Proteins were transferred onto polyvinylidene difluoride (PVDF) membrane using iBlot dry blotting

system (Life technologies). After blocking with 5 % skim milk in PBS with 0.05 % Tween-20 (PBST), the membranes were incubated with ENO1 or without ENO1, followed by staining with mouse anti-ENO1 antibody (1 : 1000, Abnova, Taipei, Taiwan). These membranes were then incubated with horseradish peroxidase (HRP)-labelled anti-mouse IgG antibodies (Jackson Immuno Research Laboratories, PA, USA). Bands were visualized by enhanced chemiluminescence using Luminata Forte Western HRP Substrate (Millipore, MA, USA).

Ligand binding assay

Each well of a 96-well maxiSorp microtiter plate (NUNC, Roskilde, Denmark) was coated with apoB or plasminogen protein (0.2 nM to 10 nM) in 50 mM sodium carbonate coating buffer (pH 9.6) at 4 ˚C overnight. After washing with PBST, and blocking with PBS containing 1 % bovine serum albumin (BSA), 10nM of ENO1 solution was added. Binding of ENO1 was detected with mouse anti-ENO1 antibody (1:1000 dilution) and HRP-conjugated anti-mouse IgG antibody (1:5000 dilution). Detection was performed using tetramethylbenzidine (TMB, KPL, MD, USA). Absorbance value was measured at wavelength of 450 nm on a microplate reader (VersaMax, Molecular Devices, CA, USA).

Confocal microscopy

U937cells were fixed 4 % paraformaldehyde for 15 minutes at room temperature (RT), blocked with 1 % BSA in PBS for 30 minutes at RT, and incubated with apoB for 1 hour at RT. After cells were incubated with mouse anti-ENO1 antibody and goat anti-apoB antibody (Life technologies) for 1 hour at RT, they were incubated with Alexa Fluor 488 conjugated donkey anti-mouse IgG (Life technologies) and phycoerythrin (PE) conjugated donkey anti-goat IgG (BD biosciences, CA, USA) for 1 hour at RT. Images were captured using a Leica TCS SP8 STED and analyzed with Leica LAS AF Lite.

Surface plasmon resonance (SPR)

Kinetic and equilibrium constants of the interaction between ENO1 and apoB or plasminogen were analyzed with SPR measurements. SPR, an optical detection technique that could enable the the observation of binding between a potential ligand molecule and a surface-immobilized target molecule. The main advantage of this technology is that binding studies can be performed directly since labeling is not required. In addition, kinetics of target and analyte interaction can be determined [21, 22]. SPR measurements were performed with a BIAcore 3000 system (GE Healthcare Biosciences,

Uppsala, Sweden). Immobilization of ENO1 on a sensor chip CM5 (carboxymethylated dextran surface, BIAcore) was carried out using amine coupling kit (BIAcore) in a continuous flow of HBS−N buffer (10 mM HEPES, pH = 7.4) containing NaCl (150 mM). After immobilization, various concentrations of plasminogen or apoB (2.5 to 20 ug/ml) were injected for 120 seconds at a flow rate of 30 μl/ minutes. Dissociation from the sensor surface was monitored for 480 seconds at the same flow rate. HEPES buffer (pH 8.0) was used as running buffer. Binding events were measured at 25 ℃. Data were analyzed using the Biacore 3000 Evaluation software (GE Healthcare) with 1:1 binding model to calculate association rate constant K_a ($M^{-1}S^{-1}$), dissociation rate constant K_d (S^{-1}), and equilibrium dissociation constant K_D for the interaction between ENO1 and apoB or plasminogen.

Competitive binding between apoB and plasminogen for ENO1

To investigate competitive binding between apoB and plasminogen for ENO1, 96−well maxiSorp microtiter plate was coated with 2 µg/ml of ENO1. Fixed concentration of plasminogen (10 µg/ml) and increasing concentration of apoB (0.156 to 10 µg/ml, 2−fold serial dilution) were co−incubated in each well of ENO1 coated plate. In addition, fixed concentration of apoB (10 µg/ml) and increasing concentration of plasminogen (0.156 to 10 µg/ml, 2−fold serial

dilution) were co-incubated in each well of ENO1 coated plate. Binding of plasminogen or apoB to ENO1 was detected with anti-plasminogen antibody or anti-apoB antibody, followed by detection using TMB substrate.

ENO1 stimulation

Purified PBMCs ($5x10^6$ cells/ml) from RA patients or healthy volunteers were stimulated with apoB protein or apoB derived peptides (Anygen, Korea) [23] in RPMI 1640 supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. They were then incubated at 37 ℃ in a 5 % CO_2 incubator for 24 hours.

siRNA

All siRNAs were purchased from SantaCruz Biotechnology (TX, USA). Target sequence of siRNA was a pool of three different siRNA duplexes (5'-GAGAUGGAUGGAACAGAAAtt-3', 5'-GUACCGCUUCCUUAGAACUtt-3', and 5'-CGCUUCCUUAGAACUUCUAtt-3') for human ENO1. The sequence of control siRNA was 5'-CCAGGGUUCCUAAUCGGAUUUGCUA-3'. Neon (Life technology) was used to transfect siRNA into monocytes of RA in accordance with the manufacturer's instruction.

Cytokines ELISA

After stimulation with apoB protein or apoB derived peptides, culture

supernatants were collected, levels of IL-1β, IL-6, and TNF$-\alpha$ in supernatants were measured using ELISA kits for these cytokines (BD Bioscience).

Examination of signaling pathways

Specific inhibitors of NK$-\kappa$B (BAY 11$-$7082), PI3K (LY294002), ERK (PD98059), p38 MAPK (SB203580), and JNK (SP600125) were purchased from Sigma$-$Aldrich (MO, USA). PBMCs from RA were pretreated with inhibitors or DMSO (negative control) for 1 hour. After wash with media, PBMCs were stimulated with apoB and incubated for 24 hours. Levels of IL-1β and IL-6 were measured by ELISA.

Western analysis for phospho$-$p38 MAPK

After stimulation with apoB, proteins from cell extracts were separated by Bolt 4$-$12 % Bis$-$Tris Plus gels and transferred onto PVDF membranes using an iBlot dry blotting system (Life technologies). After blocking with 5 % BSA in PBS, membranes were probed with phosphorylated p38 MAPK antibody or p38 MAPK antibody at 1:1000 dilution. Afterwards, the membranes were incubated with HRP$-$labelled secondary antibody. Target proteins were visualized with Enhanced Chemiluminescence.

K/BxN serum transfer arthritis mouse experiments

BALB/c mice were purchased from Orient Bio Inc. (Kyunggi, Korea). C57BL/6 wild-type mice were purchased from Jackson Laboratory (ME, USA). LDL receptor (LDLR) knock-out mice were provided by Dr. Goo Taeg Oh (Ewha Womans University, Seoul, Korea). All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University Hospital Biomedical Research Institute (IACUC No. 15-0058-C2A0).

To investigate the role of ENO1 in murine arthritis model, six-week-old male BALB/c mice were immunized with 50 µl of K/BxN mice sera by intraperitoneal injection on days 0 to induce arthritis as described previously [24]. Ankle thickness, clinical sore, and surface ENO1 expression on PBMCs were measured on days 0, 2, 5, 8, 12, and 16 after K/BxN serum transfer. To determine whether the interaction between surface ENO1 and apoB contributed to disease pathogenesis, six-week-old male BALB/c mice were immunized with 50 µl of K/BxN mice sera by intraperitoneal injection on day 0. ApoB was then intravenously injected on day 2 and day 7. LDLR knock-out mice and C57BL/6 wild-type mice and were immunized with K/BxN mice sera by intraperitoneal injection on days 0 and 4 to induce arthritis. Thickness values of both ankles were measured every other day using a caliper. Clinical score was determined for each paw using the following 0-3 point scale: 0 point, no swelling: 1 point, one digit swelling or mild swelling of the foot and ankle with the foot maintaining its original V shape: 2 point, long edges of foot

were parallel with each other with the disappearance of the normal V shape: 3 point, inversion of the V shape by expansion of the ankle and hindfoot to greater than the width of forefoot. Scores of four limbs were added together to give a clinical score with a maximum of 12 points. Mice were sacrificed on day 16. Serum levels of IL-1β, IL-6, and TNF$-\alpha$ were measured using multiplex ELISA kits (Bio-Rad, CA, USA).

Inhibition of cytokine secretion by blocking peptides

Purified PBMCs ($5\text{x}10^{6}$ cells/ml) from RA patients were treated with inhibitory peptides (IP1, IP2, or IP3), stimulatory peptide (SP2), or apoB protein for 24 hr at 37 ℃ in a 5 % CO_2 incubator.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 5.01 (GraphPad, CA, USA). Results are presented as means ± SEM. Differences between two groups were assessed by Mann-Whitney U tests and Wilcoxon signed rank test as appropriate. All reported p-values were two-sided. Statistical significance was considered at $p < 0.05$.

Result

ApoB is a specific ligand of ENO1

To identify potential ligands in RA synovial fluid for ENO1, mass spectrometry (MS) analysis was performed for proteins isolated from pooled synovial fluid of patients with RA or OA by affinity chromatography using ENO1 as receptor. Among ENO1 interacting proteins, apoB was highly enriched in RA SF compared to that in OA SF (signal to noise, STN = 34.71, p < 0.0001). Plasminogen, a known ENO1 ligand, was abundant in RA synovial fluid (STN = 7.29, p = 0.0004). To confirm the interaction between ENO1 and apoB, immobilized apoB and plasminogen were incubated with recombinant ENO1, followed by detection with anti-ENO1 antibody. It was found that ENO1 could bind to both apoB and plasminogen **(Figure 1a)**. In ligand binding assay, ENO1 exhibited a higher affinity for apoB than that for plasminogen **(Figure 1b)**. In SPR, the association rate constant Ka (rate of complex formation, recognition) of ENO1 with apoB was Ka, 1.499×10^{6} $M^{-1}S^{-1}$ which was 4-fold greater than that of ENO1 with plasminogen (Ka = 3.559×10^{5} $M^{-1}S^{-1}$). The equilibrium dissociation constant K_{D} (binding affinity) of ENO1 with plasminogen was 1.505×10^{-7}M which was 3-fold greater than that of apoB (K_{D} = 4.694×10^{-7} M) **(Figure 1c)**. Under fluorescence confocal microscopy, apoB and ENO1 co-localized on the cell surface

of U937 cells, a human monocytic cell line with constitutive expression of ENO1 on cell surface **(Figure 1d)**. Taken together, these results demonstrate that apoB is a specific ligand of surface ENO1.

Competitive binding between apoB and plasminogen for ENO1

ENO1 has binding sites for plasminogen. It acts as a receptor of plasminogen. To investigate whether plasminogen and apoB might share the same binding sites for ENO1, a competition assay was performed. When ENO1 was incubated in a fixed concentration (10 µg/ml) of plasminogen and then increasing concentrations (0.156 to 10 µg/ml) of apoB **(Figure 2a),** more apoB were found to bind to ENO1. Similarly, increasing concentrations of plasminogen inhibited the binding between apoB and ENO1 binding. However, such inhibition was to a lesser degree compared to that of apoB for the binding between plasminogen and ENO1**(Figure 2b)**. These findings suggest that both plasminogen and apoB can bind to ENO1 in a competitive fashion, although apoB has a higher affinity for ENO1 compare to plasminogen.

Surface ENO1 mediates production of inflammatory cytokines upon binding to apoB

Whether apoB binding to cell surface ENO1 could elicit inflammatory response was then evaluated next. PBMCs isolated from RA patients or healthy controls (HC) were treated with apoB for 24 hours. In response to apoB treatment, PBMCs from RA produced more IL-1β (1913.0 ± 164.1 in RA vs. 285.4 ± 157.8 pg/ml in HC, p = 0.024), IL-6 (10016.0 ± 2016.0 in RA vs. 1270.0 ± 806.9 pg/ml in HC, p = 0.048), and TNF$-\alpha$ (56.9 ± 20.8 in RA vs. 0.3 ± 0.3 pg/ml in HC, p = 0.036) than PMBCs from HCs (Figure 3).

To further identify the functional role of the interaction between ENO1 and apoB, RA PBMCs were transfected with ENO1-specific siRNA (ENO1-siRNA) or control siRNA (Con-siRNA). When surface ENO1 expression was down-regulated after transfection with ENO1-specific siRNA (Figure 4a), production of inflammatory cytokines by RA PBMCs in response to apoB stimulation was decreased (Figure 4b-d).

Similarly, apoB-induced IL-1β and IL-6 production was suppressed in the presence of ENOblock, non-substrate analogue that could directly binds to ENO1 and inhibits its activity [25] (Figure 5). Taken together, these results suggest that interaction between apoB and ENO1 on cell surface can induce intracellular inflammatory cascades and that the pro-inflammatory response of PBMC to apoB depends on the expression of functional ENO1.

Downstream of apoB signaling involves p38 MAPK and NF-kB pathways

To elucidate the downstream signaling pathways ENO1, PBMCs from RA patients were pretreated with LJ294002 (PI3K-inhibitor), PD98059 (ERK1/2-blocker), SB203580 (p38 MAPK-inhibitor), SP600125 (JNK-inhibitor), and BAY11-7082 (NF-κB inhibitors) followed by stimulation with apoB for 24 hours. The production of IL-1β and IL-6 was suppressed by pretreatment with SB203580 (p38 MAPK inhibitor) and BAY11-7082 (NF-κB inhibitor), but not by PI3K, ERK or JNK inhibitors (Figure 6a to 6b). After stimulation with apoB, p38 MAPK phosphorylation level was increased in a time-dependent manner (Figure 6c). These results suggest that surface ENO1 activation involves intracellular p38 MAPK and NF-κB pathways.

Surface ENO1 expression is increased during development of arthritis in murine arthritis model.

To investigate the role of ENO1 in murine arthritis model, arthritis was induced by intraperitoneal injection of 50 μl of K/BxN sera on days 0. Surface ENO1 expression on PBMCs was evaluated on days 0, 2, 5, 8, 12, and 16 after K/BxN serum transfer. Ankle thickness

and arthritis score were increased progressively over this period (**Figure 7a, 7b**). Surface ENO1 expression on PBMCs was also increased (**Figure 7a**). ENO1 expression was associated with arthritis severity such as ankle thickness (**Figure 7d to 7e**).

ApoB deteriorates arthritis in K/BxN sera transfer arthritis mouse model.

In this study, it was hypothesized that apoB might play a role in inflammation induction by binding to ENO1. To determine whether the interaction between surface ENO1 and apoB might contributes to disease pathogenesis, K/BxN serum and apoB protein were administered to BALB/c mice and the severity of arthritis was then evaluated. Administration of apoB alone without K/BxN serum did not induce arthritis. Overall arthritis score and ankle thickness were substantially higher in mice treated with both K/BxN serum and apoB than those treated with K/BxN serum alone (**Figure 8a**). Serum levels of IL-1β, IL-6, and TNF$-\alpha$ were increased in mice treated with K/BxN serum and apoB (**Figure 8b**).

When LDL receptor (LDLR) is absent or defective, as in familial hypercholesterolemia, plasma levels of apoB and LDL cholesterol are known to be markedly elevated [26]. LDL is associated with apoB and bind to LDL receptor. To determine whether apoB activates immune cells not by LDLR, K/BxN sera were administered to LDLR knockout mice. Arthritis score and ankle thickness were increased in

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C57BL/6 wild type mice after immunization with K/BxN sera. As expected, arthritis score and ankle thickness were markedly higher in LDLR knockout mice compared to those in wild type mice (Figure 9a). Serum levels of IL-1b, IL6 and TNF were all higher in the LDL-R knockout mice than in the wild type (Figure 9b). Taken together, these results demonstrated that apoB could deteriorate inflammatory arthritis in murine arthritis model. Such effect of apoB was not through LDL receptor.

Effect of cell surface ENO1 on induction of inflammatory cytokines by stimulation with apoB derived peptides

To identify potential interacting regions between ENO1 and apoB, MS-based binding epitope mapping assay and peptide microarrays were performed as described previously[23]. Five apoB derived peptides were identified to be able to bind to ENO1 through peptide arrays. To evaluate the agonistic or antagonistic actions of apoB derived peptides through, interaction with ENO1, PBMCs from RA patients and HC were incubated for 24 hours with apoB derived ENO1 binding peptides (BP) or non-binding peptide (NBP). Peptides that stimulated the production of pro-inflammatory cytokines was named stimulatory peptides (SPs). Those that inhibited the production of pro-inflammatory cytokines were named inhibitory peptides (IPs). PBMCs from RA patients stimulated with BPs produced significantly

more pro-inflammatory cytokines than those stimulated with NBP, including IL-1β (1128.0 $\pm$ 350.5 pg/ml by SP2 vs. 256.0 $\pm$ 73.77 pg/ml by NBP, p < 0.001), IL-6 (41747 $\pm$ 4729 pg/ml by SP2 vs. 16788 $\pm$ 3154 pg/ml by NBP, p = 0.004), and TNF-α (893.1 $\pm$ 537.5 pg/ml by SP2 vs. 34.9 $\pm$ 27.4 pg/ml by NBP, p = 0.016) (**Figure 10a**). SP1 tended to induce pro-inflammatory cytokines production. However, IP 1, IP2, or IP3 fail to induce any measurable cytokine production. In addition, PBMCs from RA patients responded to SP2 more rigorously than PBMCs from HC with higher production of IL-1β (1128.0 $\pm$ 350.5 pg/ml in RA vs. 538.3 $\pm$ 199.8 pg/ml in HC, p = 0.047) and IL-6 (41747 $\pm$ 4729 pg/ml in RA vs. 11352 $\pm$ 3657 pg/ml in HC, p = 0.001). Production of TNF-α (893.1 $\pm$ 537.5 pg/ml in RA vs. 74.2 $\pm$ 40.9 pg/ml in HC, p = 0.072) tended to be higher in PBMCs of RA patients compared to that in PBMCs of HC in response to treatment with SP2.

Monocytes and macrophages are known to be majority of ENO1 expressed cells in RA PBMCs [11]. Therefor whether monocytes and macrophages were main cells for the production of IL-1β, Il-6, and TNF-α was determined in this study. CD14 positive cells were isolated from PBMCs of RA patients. These CD14 positive cells or CD14 negative cells were then incubated with apoB derived peptides (**Figure 10b**). After incubation, culture supernatants were collected, and the levels of IL-1β, IL-6, and TNF-α were measured by

ELISA. Results showed that levels of IL-1β, IL-6, and TNF$-\alpha$ were significantly higher in supernatants of CD14 positive cells, but not in CD14 negative cells, upon ENO1 stimulation with apoB derived peptides, suggesting that these cytokines were produced by CD14 positive cells after the ligation of apoB to surface expressed ENO1.

  SP2 is different from IP2 only in two amino acids at the C$-$terminal. To define the functional motif within SP2 (KNKYGMVAQVTQ), three alanine substitution forms (A1, KNKYGMVAQVAQ, A2, KNKYGMVAQVTA, and A3, KNKYGMVAQVAA) and two truncated forms (T1, KNKYGMVAQVT and T2, KNKYGMVAQV) at residues T (Threonine) and Q (Glutamine) of SP2 were used **(Figure 11)**. Produced cytokines in response to treatment with modified SP2 (i.e., alanine substitution or truncation) were dramatically decreased. This suggested that T and Q are crucial to the agonistic property of SP2.

Effect of inhibitory peptides on por$-$inflammatory cytokines in PBMC from RA

Since IP1, IP2, and IP3 failed to induce any inflammatory response after binding to ENO1, whether IP1, IP2, and IP3 could serve as antagonist or inhibitor to ENO1 were investigated. PBMCs from RA patients were co$-$treated with peptides IP1, IP2, or IP3 and SP2 (stimulatory peptide, **Figure 12a**) or apoB protein **(Figure 12b)**. These IPs, but not NBP, decreased SP2$-$induced production of IL-1β and IL-6. SP2$-$induced TNF$-\alpha$ production tended to be reduced

by treatment with IPs. Furthermore, these IPs inhibited the production of cytokines (IL−1β, IL−6, and TNF−α) in response to apoB. These results suggest that IPs might be able to inhibit apoB induced pro−inflammatory cytokine production.
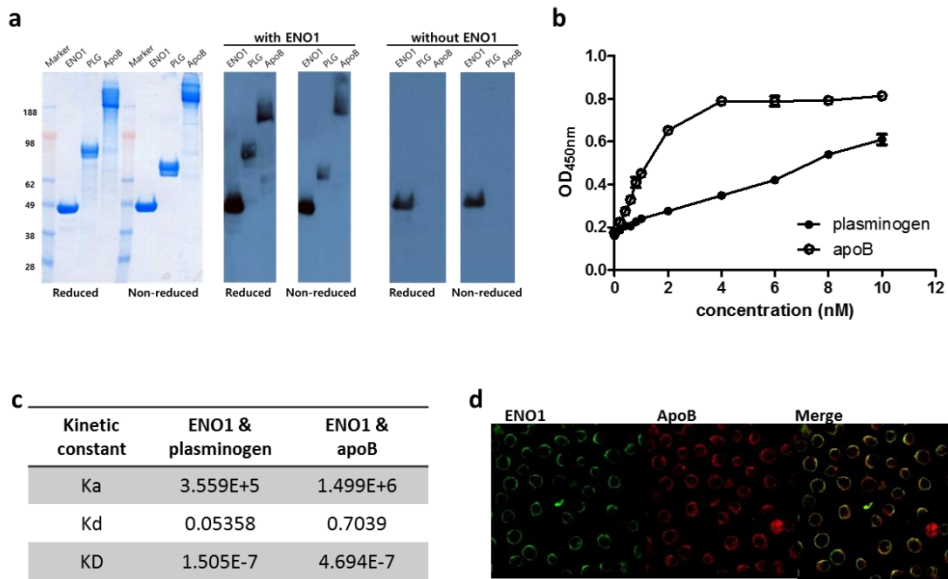
Figure 1. ApoB is a specific ligand of ENO1.

(a) Ligand blotting assay. ENO1, plasminogen (PLG), and apoB were subjected to SDS−PAGE (left) and transferred to PVDF membranes. These membranes were then incubated with ENO1 protein (middle) or without ENO1 protein (right) followed by incubation with anti−ENO1 antibody. ENO−1 could bind to plasminogen (lane 2 in each blot) and apoB (lane 3 in each blot). (b) 96−well maxisorp plate coated with increasing concentrations (0.2 nM – 10 nM) of plasminogen (●) or apoB (○) and incubated with ENO1. Binding of ENO1 to apoB or plasminogen was detected with anti−ENO1 antibody. Compared to plasminogen, apoB had higher affinity for ENO1. (c) Sensograms. Various concentrations (2.5 to 20 μg/ml) of plasminogen or apoB were injected over sensorchip surfaces coupled

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to ENO1. Binding affinity of apoB or plasminogen for ENO1 was calculated as described in Materials and Methods (d) Co-localization of apoB and ENO on the cell surface. U937 cells were incubated with apoB and then stained with antibodies against ENO1 and apoB. Confocal images showed that apoB (red) was co-localized with cell surface expressed ENO1 (green). (Yellow in merged images)
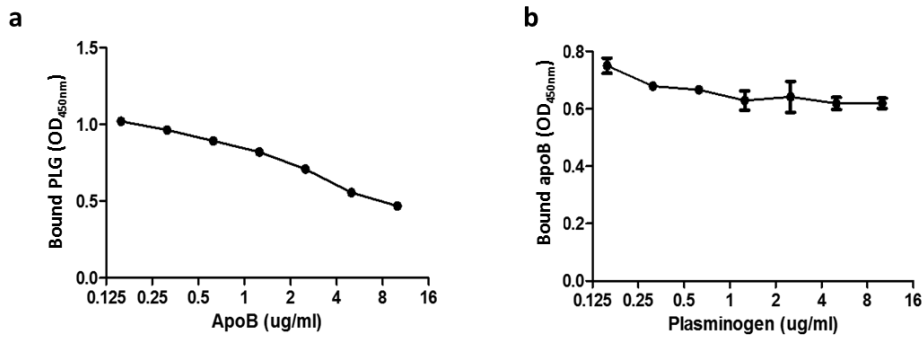
Figure 2. Competitive binding of apoB and plasminogen to ENO1.

To investigate competitive binding between apoB and plasminogen for ENO1, tapoB and plasminogen were incubated in ENO1 coated plate. Fixed concentration (10 μg/ml) of plasminogen and various concentrations (0.156 − 10 μg/ml) of apoB were co-incubated in each well of ENO1 coated plate. Bound plasminogen (a) was detected with anti-plasminogen antibody. By contrast, fixed concentration (10 μg/ml) of apoB and various concentrations (0.156 − 10 μg/ml) of plasminogen were incubated in ENO1 coated plate, and then bounded apoB (b) was detected with anti-apoB antibody. ApoB had higher affinity for ENO1 than plasminogen.
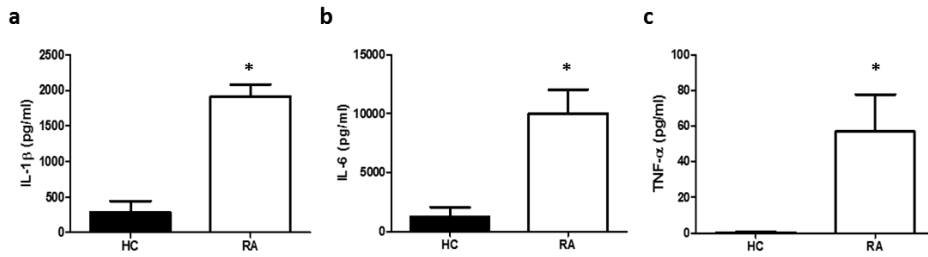
Figure 3. ApoB induces pro-inflammatory cytokines in PBMCs from RA patients.

PBMCs from heathy controls (HC, n = 3) or RA patients (n = 6) were stimulated with apoB. PMBCs from RA produced higher levels of IL-1β (a), IL-6 (b), and TNF-α (c) than PBMCs from HC. Data are presented as mean $\pm$ SEM. *, $p < 0.05$.
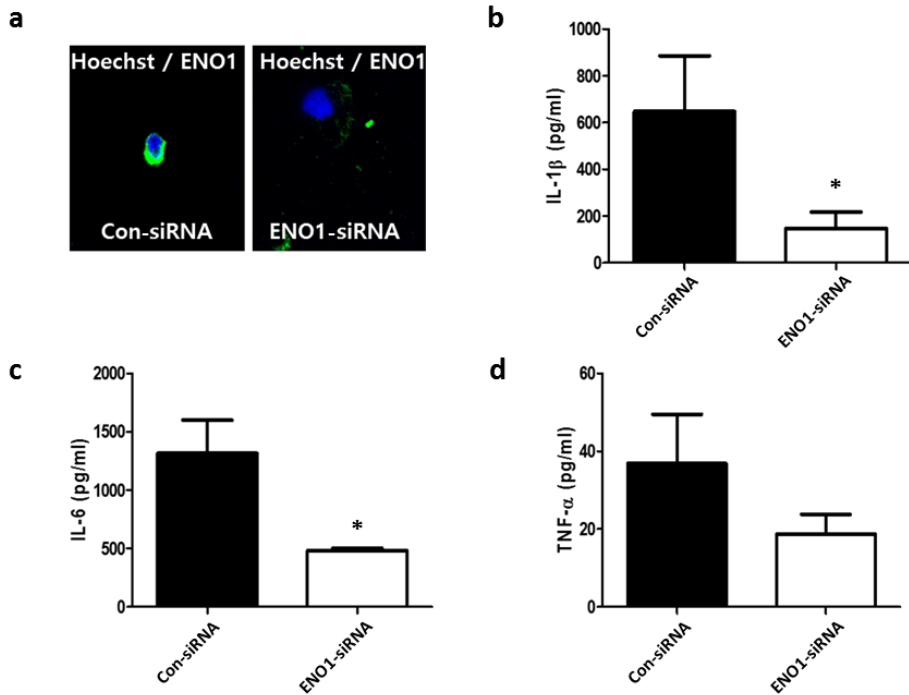
Figure 4. Reduction of pro-inflammatory cytokines by transfection with ENO1-siRNA

(a) CD14+ cells from RA patient were transfected with either ENO1-siRNA or control-siRNA (con-siRNA). The efficiency of ENO1-siRNA mediated down-regulation of surface ENO1 was determined by confocal microscopy at 24 hours after transfection. CD14 positive cells from RA patient (n = 3) were transfected with ENO1-siRNA or control-siRNA followed by treatment with apoB for 24 hours. Lower ENO1 expression was associated with decreased production of IL-1β (b), IL-6 (c), and TNF-α (d). Data are presented as mean $\pm$ SEM. $*$ p, < 0.05.
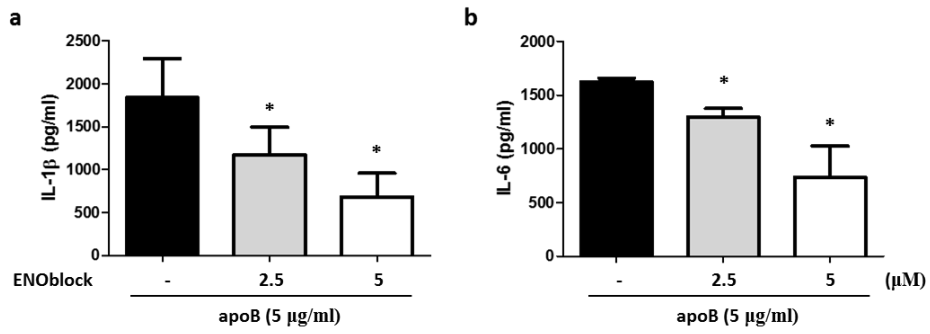
Figure 5. Reduction of pro-inflammatory cytokines by treatment with ENOblock

ENOblock (2.5 μM and 5 μM) blocked IL-1β (a) and IL-6 (b) production in PBMCs from RA patients (n = 3) after stimulation with apoB. Data are presented as mean ± SEM. *, $p < 0.05$.
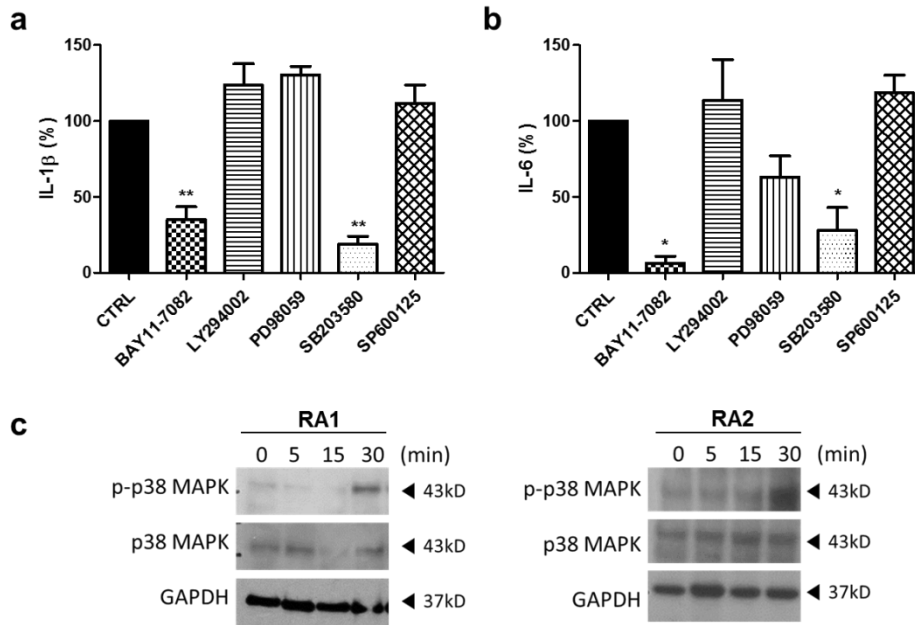
Figure 6. Induction of pro-inflammatory cytokines by apoB signaling via p38 MAPK and NF-kB pathways. PBMCs from RA were pretreated for 1 hour with BAY11-7082 (NF-κB inhibitor), LY294002 (PI3K inhibitor), PD98059 (ERK inhibitor), SB203580 (P38 MAPK inhibitor), and SP600125 (JNK inhibitor), followed by stimulation with apoB for 24 hours. The production of IL-1β (a) and IL-6 (b) was blocked in the presence of BAY11-7082 and SB203580. Data are presented as mean $\pm$ SEM. (c) Increased phosphorylation of p38 MAPK in PBMCs from RA patients after apoB stimulation. PBMCs from RA were stimulated with apoB for 5, 15, and 30 min. Changes of phosphorylated p38 MAPK levels were

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investigated using immunoblotting. At 30 min after activation, phospho−p38 MAPK levels were increased. Results are representatives of six independent experiments. ∗, $p < 0.05$; ∗∗, $p < 0.01$.
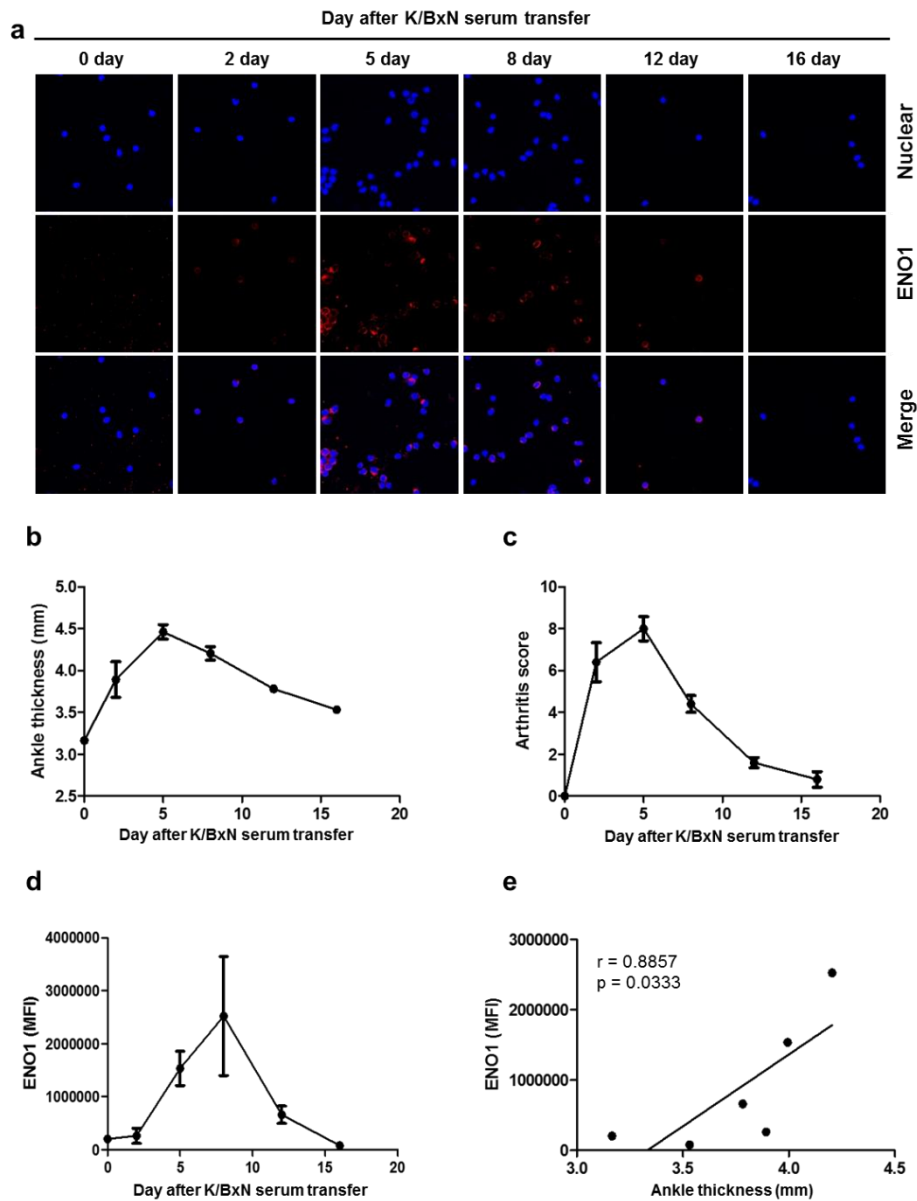
Figure 7. Increased surface ENO1 on PBMCs in K/BxN serum transfer arthritis model.

Balb/c mice were immunized with K/BxN mice sera on days 0 to induce arthritis. (a) Confocal microscopy showing increased surface ENO1 expression on PBMCs after induction of arthritis with transfer of K/BxN serum. (b) Ankle thickness and (c) clinical score measured on days 0, 2, 5, 8, 12, and 16 after K/BxN serum transfer. (d) Increase of ENO1 fluorescence intensity per cell after injection and peaked at day 8. (e) Expression levels of surface ENO1 were significantly correlated with ankle thickness.
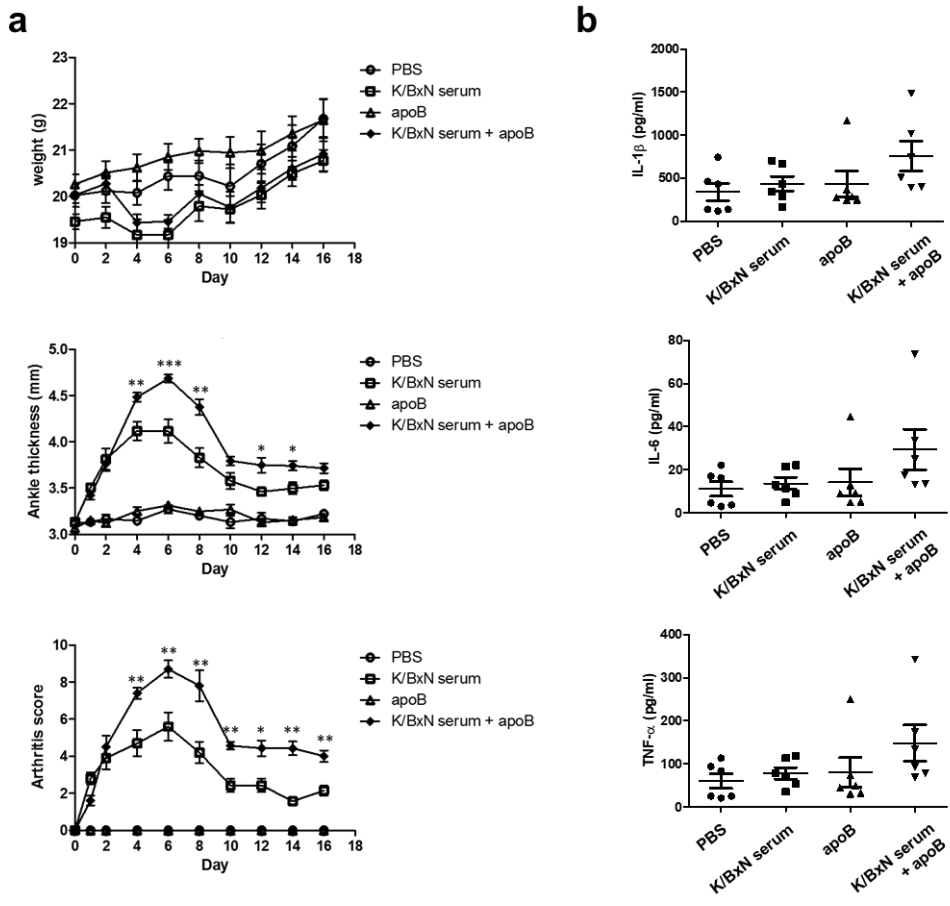
Figure 8. ApoB aggravates K/BxN serum transfer arthritis in mice.

(a) K/BxN serum transfer mice (□) and K/BxN serum and apoB transfer mice (◆) were immunized with 50 μl of K/BxN mice sera by intraperitoneal injection on day 0 to induce arthritis. K/BxN serum and apoB transfer mice (◆) were then immunized with apoB (1 mg / kg) by intravenous injection on days 2 and 7. Weight, ankle thickness, and clinical score were measured every other day. Serum cytokine levels were measured on day 7 after K/BxN serum transfer (b).
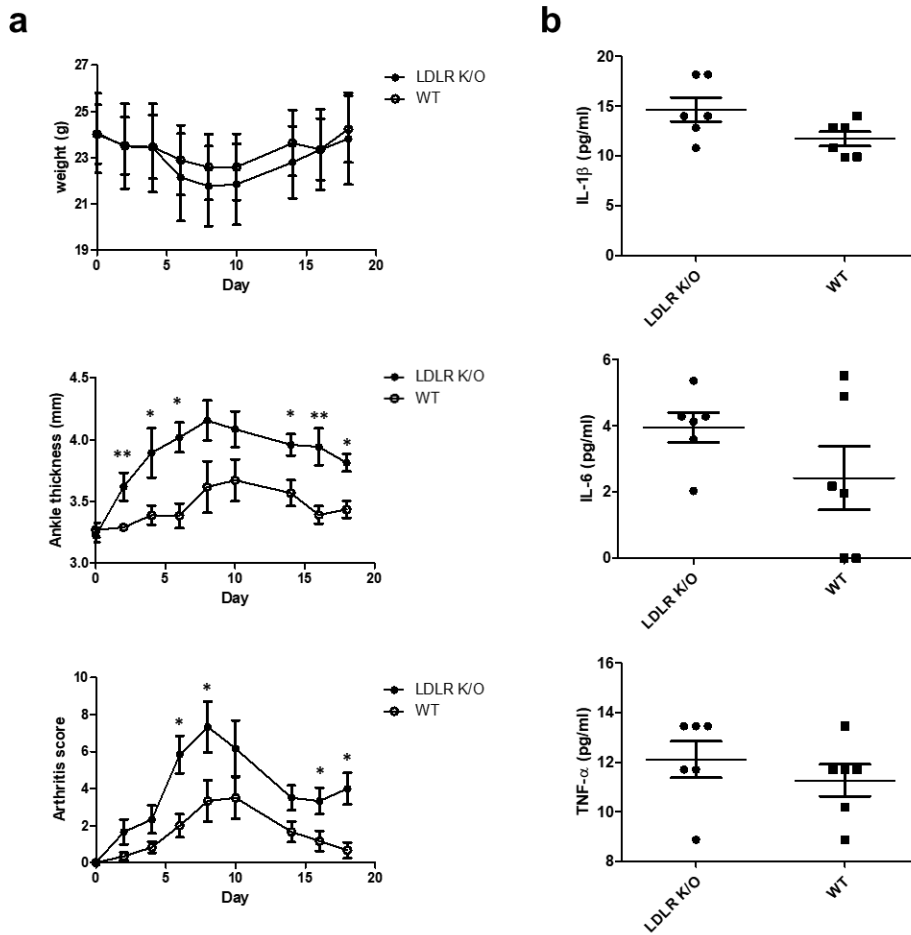
Figure 9. Development of more severe K/BxN serum transfer arthritis in LDLR knockout mice than that in wild type mice.

(a) Wild type mice (WT, ○) and LDLR knockout mice (LDLR K/O, ●) were injected intraperitoneally injected with 50 μl of K/BxN mice sera on day 0 and 4. Serum cytokine levels were measured on day 8 after K/BxN serum injection (b).
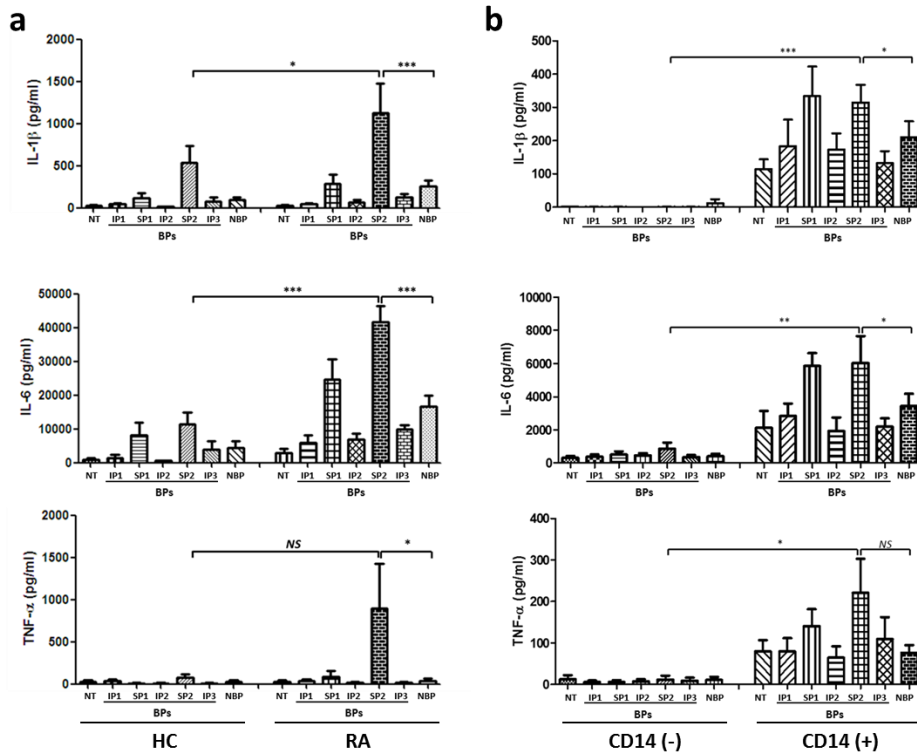
Figure 10. Induction of inflammatory cytokines in the PBMCs after stimulation of surface expressed ENO1 with apoB derived peptides.

(a) PBMCs from HC (n = 6) or PBMCs from RA (n = 6) were stimulated with apoB derived peptides. Levels of pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α in the culture supernatants were measured. (b) When CD14 negative cells (CD14(-)) and CD14 positive cells (CD14(+)) from RA patients were stimulated by apoB derived peptides and levels of inflammatory cytokines in culture supernatants were then determined. Levels of

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IL-1β, IL-6, and TNF$-\alpha$ in supernatants were measured by ELISA. NT, no treatment; BPs, binding peptides; NBP, non$-$binding peptide; SP, stimulatory peptide; IP, inhibitory peptide; Data are presented as mean $\pm$ SEM. $*$, $p < 0.05$; $**$, $p < 0.01$; $***$, $p < 0.001$.
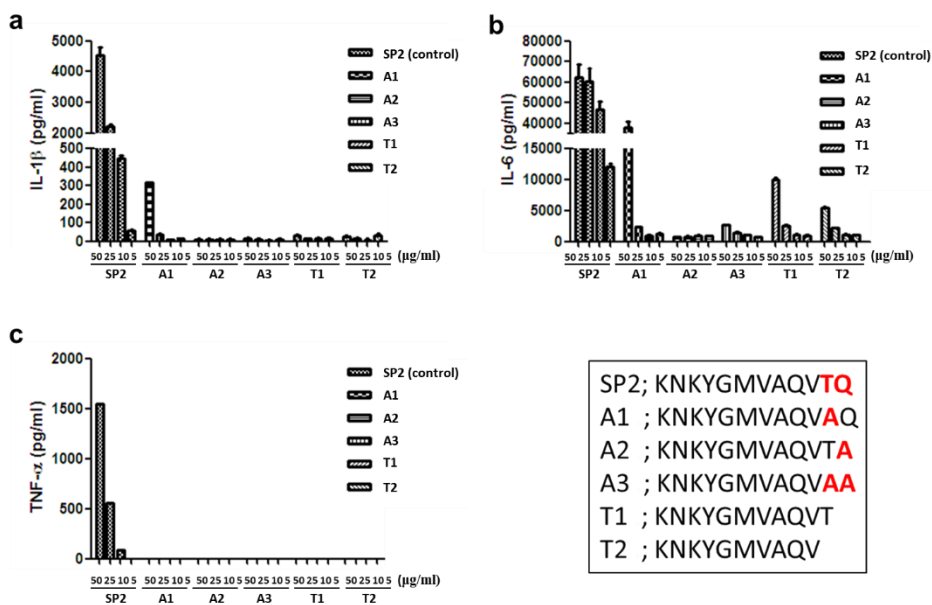
Figure 11. Reduction of inflammatory cytokines by alanine substitution or truncation of residues at T and Q of SP2.

Pro−inflammatory cytokines such as IL−1β (a), IL−6 (b), and TNF−α (c) were reduced by alanine substitution (A1, A2, A3) or truncation (T1, T2) of residues at T and Q of SP2. Data are presented as mean ± SEM.
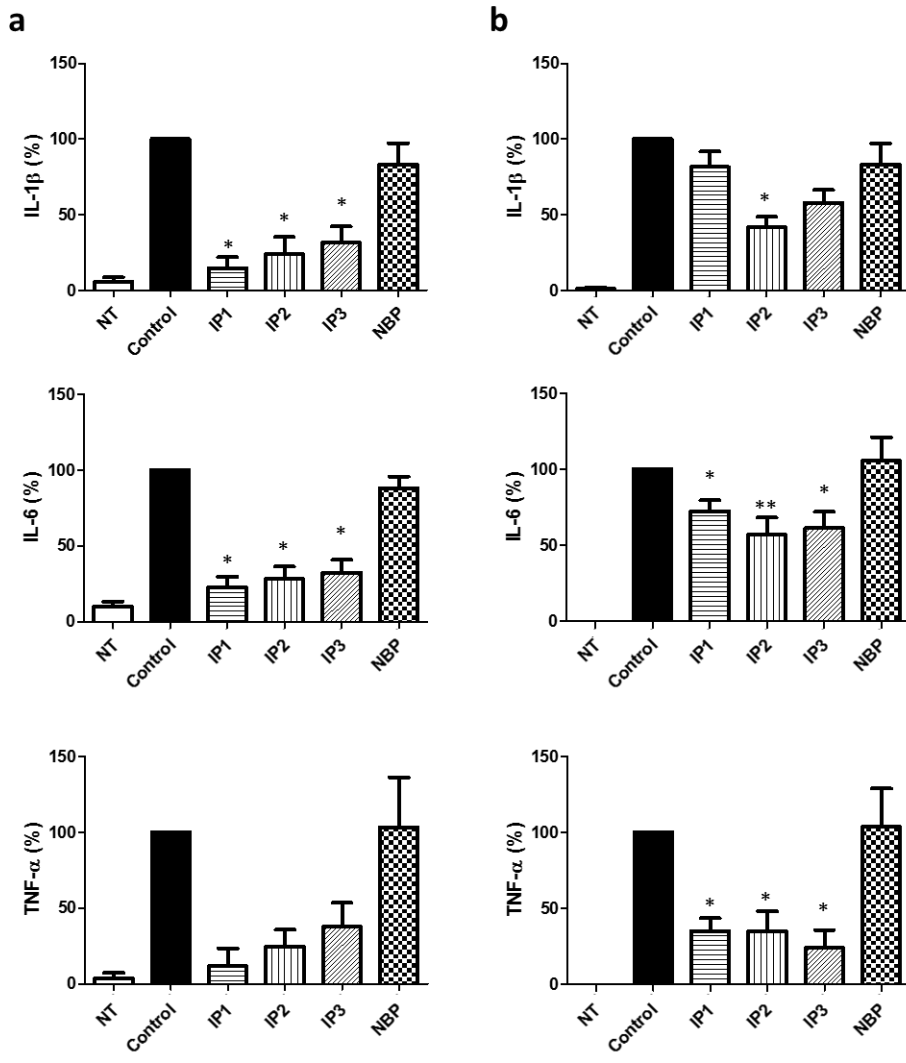
Figure 12. Inhibition of inflammatory cytokines in PBMCs after stimulation of surface expressed ENO1 with IPs.

PBMCs from RA were co-treated with IPs and either SP2 (a) or apoB protein(b). After incubation, levels of IL-1β, IL-6, and TNF-α in

supernatants were measured by ELISA. NT, no treatment; BPs, binding peptides; NBP, non-binding peptide; SP, stimulatory peptide; IP, inhibitory peptide; Data are presented as mean $\pm$ SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.
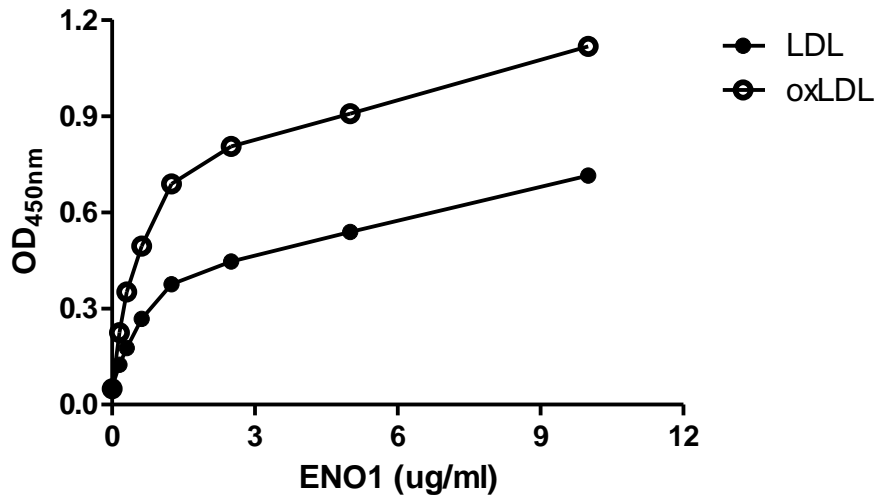
Supplementary Figure 1. Oxidized LDL (oxLDL) binds to ENO1 with a higher affinity than LDL.

The plate coated with LDL (●) or oxLDL (○) were incubated with increasing concentrations (0.156 to 10 μg/ml) of ENO1 by serial dilution. Binding of ENO1 to LDL or oxLDL was detected using anti−ENO1 antibody. Compared to LDL, oxLDL had higher affinity for ENO1.
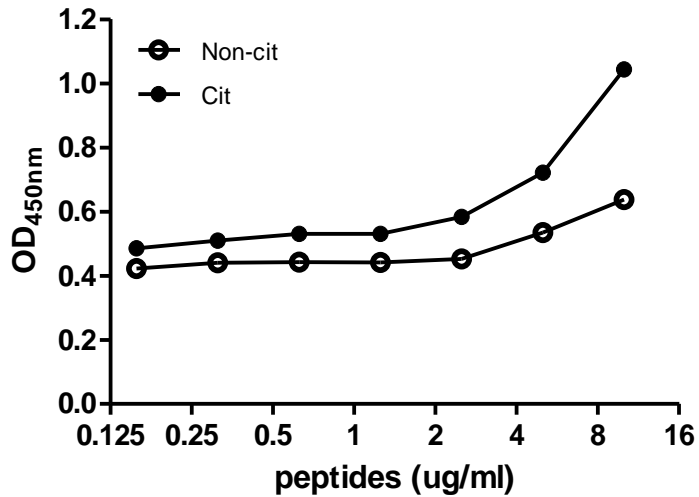
Supplementary Figure 2. ApoB binds to citrullinated ENO1 derived peptides with higher affinity than non-citrullinated ENO1 derived peptides.

Plate coated with citrullinated ENO1 derived peptides (Cit, ●) or non-citrullinated ENO1 derived peptides (Non-cit, ○) was incubated with apoB. Binding of apoB to citrullinated ENO1 derived peptides or non-citrullinated ENO1 derived peptides was detected using anti-apoB antibody. Compared to non-citrullinated ENO1 derived peptides, citrullinated ENO1 derived peptides had higher affinity for apoB.

Discussion

Results of this study demonstrated that apoB was a specific ligand of ENO1. Their interaction induced pro-inflammatory response in PBMCs from RA patients. In addition, it was found apoB derived peptides could interact with ENO1 as agonists or antagonists. ApoB signaling involved the activation of intracellular p38 MAPK and NF-κb pathways. In addition, surface ENO1 expression was correlated with arthritis severity. Moreover, apoB deteriorated arthritis in murine arthritis model. Taken together, these results suggest that apoB and ENO1 interaction might contribute to pro-inflammatory response in arthritis.

As a key enzyme of glycolysis, enolase is present in all cells. It exists in three major isoforms in mammals: muscle-specific ENO3, neuron-specific ENO2 and an ubiquitous ENO1 [5, 27]. ENO1 is a metalloenzyme that catalyzes the dehydration of 2-phospho-D-glycerate to phosphoenolpyruvate [27]. Apart from its cytosolic enzyme activity, ENO1 is present on the cell surface. Its expression can be markedly upregulated by translocation to cell surface during cell activation [12, 13, 28]. Since ENO1 lacks a signal sequence for endoplasmic reticulum-Golgi export pathway, it might be translocated to cell surface via non-classical secretory route [29,

30]. Surface ENO1 is known to be a physiologic receptor for plasminogen. ENO1 can enhance plasminogen activation by tissue-plasminogen activator. ENO1 also protects plasminogen from α2-plasmin inhibitor [6]. Therefore, ENO1 helps generate plasmin for intra- and extra-vascular fibrinolysis at site of active inflammation [12].

Recent study has demonstrated that ENO1 is upregulated on the surface of PBMCs and SFMCs isolated from RA patients compared to that from OA patients or healthy controls [11]. ENO1 ligation with anti-ENO1-antibody elicited significant inflammatory response in this study, suggesting that ENO1-activation might contributes to RA pathogenesis. To the best of our knowledge, the present study is the first one that clearly identifies apoB as a novel physiologic ligand of ENO1.

Our hypothesis was apoB might be a natural ligand of surface ENO1 given its homologous sequences to plasminogen. Systematic screening of synovial fluid proteins using mass spectrometry in this study actually identified apoB as the protein with the highest affinity to ENO1 among ENO1 binding proteins. This finding is striking for several reasons: i) apoB is abundant in the serum as part of lipoproteins such as LDL and VLDL; ii) apoB is enriched in synovial fluid of RA patients [14, 15]; iii) LDL or modified LDL can induce monocytes to produce inflammatory response both *in vivo* and *in vitro*

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[16, 31-34]; and iv) LDL in RA patients exhibit altered physical property such as higher fragility [19]. Therefore, the pro-inflammatory property of RA-LDL might be mediated, by apoB, at least in part. Interestingly both LDL and oxLDL were able to bind to ENO1 in a dose dependent manner, although oxLDL exhibited higher affinity for ENO1 **(Supplementary Figure 1)**. Therefore, available apoB in free form or as a component of lipoproteins interact with surface ENO1. Local or systemic inflammation with accumulation of oxLDL might lead to the release of more apoB, which can augment inflammatory response by binding to surface ENO1 on activated monocytes at site of active inflammation such as joint and arteries.

 There are multiple regulatory mechanisms of ENO1 activation by apoB. The upregulation of ENO1 on cell surface might be a critical step in regulating the interaction between ENO1 and apoB since apoB is abundant in plasma. Furthermore, structural modification of apoB or apoB carrying lipoproteins during inflammatory response might be an additional regulatory mechanism. For example, LDL from RA patients are more fragile. They are susceptible to oxidation [19]. Therefore, apoB might be released from LDL or its stimulatory peptide-motif might be exposed on the surface of lipoproteins for a close interaction with ENO1. Indeed, oxLDL which is enriched in RA, can induce higher inflammatory response than native LDL [19, 35]. This could be supported by the fact that oxLDL showed stronger

interaction with ENO1 than native LDL **(Supplementary Figure 1)**.

Post-translational modification such as citrullination is critical in RA [36-38]. It might also influence the interaction between apoB and ENO1 [39-41]. Strikingly, citrullinated ENO1-derived peptides reported to be present in plasminogen binding site and membrane exposed loop within ENO1 (amino acid sequence, 250-272) [42, 43] were found to be able to bind to apoB more than non-citrullinated ENO1-derived peptides **(Supplementary Figure 2)**. Since citrullination of ENO1 decreases plasminogen binding capacity, leading to decreased fibrinolysis in RA [40], such citrullination might shift that ENO1 preferential interaction of ENO1 with apoB rather than plasminogen, thus contributing to enhanced inflammatory response in RA patients.

In murine arthritis model, ENO1 surface expression was correlated with disease activity. Exogenous administration of apoB exacerbated inflammatory arthritis. These results are consistent with previous finding showing that PBMCs from patients with active RA exhibit higher ENO1 expression and the binding of ENO1 with antibody can induce inflammatory response [11]. Accordingly, activation of ENO1 with antibody or apoB might lead to augmented immune response with higher disease activity. As such, apoB might be an important immune-modulator which links lipid-metabolism with local or systemic inflammation.

RA is associated with increased risk of cardiovascular mortality due to accelerated atherosclerosis [44, 45]. The inflammatory environment associated with RA, rather than traditional cardiovascular risk factors, has been postulated to be implicated in accelerated atherosclerosis in RA patients [46]. In atherosclerosis, monocytes will infiltrate atherosclerotic plaques, accumulate lipid-rich material, and differentiate into macrophage-type foam cells [47]. In atherosclerosis, LDL are accumulated within the arterial wall, attracting immune cells and leading to chronic inflammation [48]. This process involves autoimmune response to the protein moiety of LDL, apoB [49-51]. Taken together, monocytes with highly upregulated ENO1 on their surface might infiltrate and bind to apoB which accumulated within the arterial wall. Such association will contribute to the production of pro-inflammatory cytokines. Therefore, apoB might be a pathophysiologic link between RA and atherosclerosis via its interaction with ENO1 on immune cells.

Mice naturally have high levels of HDL but low levels of LDL, different from humans. However, apolipoprotein E deficient mice showed an increase in the plasma concentration of apoB and they spontaneously developed atherosclerosis. Interestingly, it has been reported that apolipoprotein E deficient mice develop more severe collagen induced arthritis than wild type mice [52-55]. Similarly, results of the present study showed that LDLR deficient mice with

markedly increased levels of apoB developed more severe arthritis than wild type mice upon transfer of K/BxN serum transfer. This finding suggests that apoB mediated induction of inflammatory response is not mediated by LDL receptor.

This study has several limitations. First, although interaction between ENO1 and apoB transmited inflammatory signals via activations of p38 MAPK and NF−κB pathways with subsequent production of inflammatory cytokines including IL−1β, IL−6, and TNF−α, key cytokines in arthritis [56], the upstream of p38 MAPK and NK−κB signaling was not identified in this study. Second, whether surface ENO1 activation might influence cellular energy metabolism [57, 58] was not determined in this study. Third, ENO1−apoB interaction might not be general feature of any inflammatory condition. It might not be specific for RA. Further studies are needed to clarify these points.

Conclusion

This study elucidated the receptor-ligand relationships between ENO1 and apoB and the biological significance of such interactions. Results of this study suggest that the interaction between ENO1 and apoB might enhance chronic inflammation in RA patients.

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List of abbreviation

ApoB	Apolipoprotein B
BP	Binding peptide
CVD	Cardiovascular disease
ELISA	Enzyme-linked immunosorbent assay
ENO1	Enolase-1
HC	Healthy control
HRP	Horseradish peroxidase
IFN	Interferon
IL	Interleukin
IP	Inhibitory peptide
LDL	Low density lipoproteins
NBP	Non-binding peptide
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PP	Positive signal peptide
RA	Rheumatoid arthritis
RT	Room temperature
SF	Synovial fluids
SFMC	Synovial fluid mononuclear cells
SP	Stimulatory peptide
SPR	Surface plasmon resonance

TNF	Tumor necrosis factor
VLDL	Very low density lipoproteins

에놀레이즈-1의 새로운 리간드인 아포지질단백질 B가 류마티스 관절염에 미치는 영향

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배경: 류마티스 관절염은 만성염증성 자가 면역 질환으로 관절 파괴와 관련된 복잡한 전신성 염증 과정을 특징으로 한다. 단핵 세포와 활막 대식세포는 류마티스 관절염의 염증에 중요한 세포이다. 에놀레이즈-1 은 세포질에서 포도당 분해 효소의 하나로서 세포 표면에서 플라스미노겐 수용체로도 알려져있다. 류마티스 관절염 환자에서 유래한 말초혈액과 활액 에서 에놀레이즈-1을 발현하는 세포의 대부분은 CD14 양성 단핵구로 알려져있다.

목적: 이 연구는 세포표면에 발현된 에놀레이즈-1의 새로운 리간드를

조사하고 새로운 리간드인 아포지질단백질 B와 에놀레이즈-1 사이의 상호 작용에 의한 생물학적 역할을 연구하는 것을 목표로 하였다.

방법: 에놀레이즈-1과 결합하는 단백질을 류마티스 관절염 환자의 활액을 이용하여 질량분석법으로 조사하였다. 리간드 블롯팅 분석, 리간드 결합 분석, 표면 플라스몬 공명 및 공초점 현미경에 의해 에놀레이즈-1과 아포지질단백질 B 사이의 상호 작용을 조사하였다. 아포지질단백질 B를 이용하여 에놀레이즈-1을 자극한 후 류마티스 관절염 환자군 또는 건강인 대조군의 말초혈액 단핵세포가 분비하는 염증성 사이토카인의 생산을 효소면역법으로 측정하여 평가하였다. 그리고 아포지질단백질 B를 이용한 에놀레이즈-1 자극에 의한 사이토카인의 유도에 관여하는 신호 전달 경로를 확인하기 위해 특정 억제제를 사용하였다. 그리고 생체 내 연구를 위해 K / BxN 혈청 전달 관절염 마우스 모델을 사용하였다.

결과: 리간드 블롯팅 분석, 리간드 결합 분석, 표면 플라스몬 공명 및 공초점 현미경 검사는 아포지질단백질 B가 에놀레이즈-1의 새로운 리간드임을 보여주었다. 표면 에놀레이즈-1과 아포지질단백질 B 사이의 상호 작용은 건강인 대조군 보다 류마티스 관절염 환자군의 말초혈액 단핵세포에서 더 높은 수준의 염증 유발성 사이토카인을 유도하였다. 아포지질단백질 B에 의한 표면 에놀레이즈-1 활성화는 세포 내 p38 MAPK 및 NF-κB 경로를 통하여 이루어졌다. 동물실험을 통하여 K / BxN 혈청 전달 관절염 마우스 모델에서 아포지질단백질 B에 의한 관절염의 중증도가 악화됨을 관찰할 수 있었다. 또한, 에놀레이즈-1과

상호작용하는 아포지질단백질 B의 활성 펩타이드 모티프를 확인하였다.

결론: 본연구는 에놀레이즈-1과 아포지질단백질 B 사이의 수용체 - 리간드 관계를 밝히고, 에놀레이즈-1과 아포지질단백질 B 의 상호작용이 류마티스 관절염 환자의 자가면역 염증반응을 항진시키는 중요한 요소임을 제시하였다.

주요어: 류마티스 관절염, 에놀레이즈-1, 아포지질단백질 B, 염증, 사이토카인, 단핵구

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