Master of Science

Negative regulation of NLRP3 inflammasome activation by DPP-4 in macrophages

대식세포의 NLRP3 염증복합체 활성 조절에 관여하는 DPP-4 역할

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Negative regulation of NLRP3 inflammasome activation by DPP-4 in macrophages

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Abstracts

Dipeptidyl peptidase-4 (DPP-4, also known as CD26) cleaves and inactivates several chemokines and cytokines, mediating inflammation and immune function. In immune system, DPP-4 mainly plays a role in T cell activation and co-stimulation and is involved in memory immune-senescence. Here, we hypothesized that DPP-4 may negatively regulate NLR family pyrin domain containing 3 (NLRP3) inflammasome activation in macrophages. To investigate this possibility, the potential function of DPP-4 in regulating lipopolysaccharide (LPS)-induced NLRP3-dependent inflammasome activation in macrophages was examined. To investigate the role of DPP-4 in activation of the NLRP3 inflammasome, LPS-primed bone marrow-derived macrophages (BMDMs) of wild type (WT, C57BL6/J) and DPP-4 knockout (KO) mouse were challenged with adenosine triphosphate (ATP). IL-1β and IL-18 expressions were evaluated by immunoblot and enzyme-linked immunosorbent assay (ELISA) assay in cell lysates and supernatants, respectively. We observed that deficiency of DPP-4 in macrophages promotes NLRP3 inflammasome activation. Furthermore, the release of IL-1β and IL-18 proteins in peritoneal lavage fluid were compared between WT and DPP-4 KO mice by ELISA in LPS-induced sepsis model. M1 macrophage-related gene expression of peritoneal macrophage from these mice was analyzed by qRT-PCR. DPP-4 deficiency enhanced the secretion of IL-1β and IL-18 into peritoneal lavage and augmented the transcript levels of macrophage-related genes. Thus, our findings suggest that DPP-4 negatively regulates activation of NLRP3 inflammasomes *in vitro*, and LPS-induced sepsis model *in vivo*, by promoting M1 macrophage profile.

Keywords: DPP-4, NLRP3, inflammasome, inflammatory cytokines

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Introduction

Dipeptidyl peptidase-4 (DPP-4; also known as CD26) exists either as a type II transmembrane protein or soluble form. It functions as a serine protease that inactivates incretin hormones to inhibit insulin secretion and to promote diabetes mellitus[1]. DPP-4 has enzymatic activity and can modulate intracellular signaling not only via proteolytic cleavage of N-terminal dipeptides from variety of substrates, but also via direct interaction with key regulatory molecules, thereby exerting immunoregulatory function. Increasing evidence suggest that an important role of DPP-4 in T cell activation and co-stimulation by binding with adenosine deaminase (ADA) through co-stimulatory mechanism. It is also involved in memory T cell generation and immune senescence[1-4]. In immune system, DPP-4 activates T cell activation and elevated DPP-4 is associated with various inflammatory diseases, suggesting the involvement of DPP-4 in immune regulation. However, the precise role of DPP-4 deficiency in macrophage-associated inflammation is still unclear[5-7].

Inflammasome activation is induced by several pathogen-derived or environmental factors and involved in the pathogenesis of sepsis and type 2 diabetes mellitus (T2DM). The best characterized inflammasome is NLRP3 inflammasome (NACHT, LRR, and PYD domains containing protein 3) and there is evidence for the NLRP3 inflammasome activation by islet amyloid polypeptide to enhance IL-1β generation in T2DM[8,9,30]. The NLRP3 inflammasome is an innate immune sensor that can be activated by pathogenic signals such as pathogen-associated molecular patterns (PAMPs) to induce inflammation[8-10]. Recent studies have demonstrated the key role of NLRP3 inflammasome in inflammatory responses. NLRP3 inflammasome activation requires double activation signal: priming and activation. PAMPs are the molecules associated with groups of pathogens, and most of them are recognized by Toll-like receptors (TLRs) [8,12]. Lipopolysaccharide (LPS) is the major pathogen of PAMPs and results in the activation of NF-κB and upregulation of pro-IL-1β and NLRP3 protein expression[13,14]. Then the inflammasome complex is activated by various inducers such as nigericin and adenosine triphosphate (ATP). Activation of the NLRP3 inflammasome leads to interact with apoptosis-associated speck-like protein (ASC) and release of pro-inflammatory cytokines, IL-1β, which is generally dependent on caspase-1. As a result, it contributes to pathological inflammatory diseases such as Parkinson's disease, Alzheimer's disease and sepsis[9,12,15,16].

In this study, we aimed to characterize the function of DPP-4 in macrophage and NLRP3 inflammasome activation. Our findings demonstrate that DPP-4 negatively regulates NLRP3 inflammasome activation and pro-inflammatory cytokine secretion.

Materials and Methods

Animals

6-8 weeks old male Wild Type (C57BL6/J, WT) and DPP4$^{-/-}$ (DPP4$^{-/-}$; DPP-4 KO) mouse were purchased from The Jackson Laboratory (Maine, USA) and maintained in specific pathogen-free conditions. All animal protocols were performed according to the guidelines for care and approved by the institutional ethics committees at Asan Medical Center (Seoul, Republic of Korea).

Dipeptidyl peptidase (DPP-4) activity assay

DPP-4 was detected by using the Dipeptidyl peptidase IV (DPP-4) Activity Assay Kit (ab204722) purchased from Abcam. The assay is based on the detection of released quenched fluorescent group AMC (7-Amino-4-Methyl Coumarin) from substrate via the action of DPP-4. It was detected at Ex/Em=360/460nm.

Cell cultures

The bone marrow-derived macrophage (BMDMs) preparation methods were as follows: bone marrow cells were extracted from femurs and tibias of 7-week-old male C57BL/6J (WT) and DPP4 knockout (KO) mice. Then cells were cultured at 2×10^6 cells/ml in 10-cm cell culture plates in the a-MEM containing 10% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin (P/S) in the presence of human M-CSF (30ng/ml). BMDMs (3×10^5 cells) were cultured overnight in 6 well plates and then primed with 100ng/ml LPS from E. coli strain O26:B6 (Sigma, St. Louis, MO) for 4h before stimulation with 5Mm ATP (Sigma, St. Louis, MO) for 30min. All cells were cultured under a humidified 5% s(*v*/*v*) CO_2 atmosphere at 37 °C.

Quantitative real-time PCR

Total RNA was extracted from cells using QIAZOL reagent (Qiazen) following the manufacturer's instructions. RevertAid First strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to synthesize cDNA from RNA. qPCR analysis was performed in optical 96-well white plates using SYBR Green PCR master mix (Roche, Penzberg, Germany) and the Light Cycler 480 Real-time PCR Detection System (Roche), in

accordance with the manufacturer's instructions. Gene expression was normalized to that of glyveraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as an internal control. The relative expression of the target genes was calculated by the standard curve method using the target Ct values and the Ct value for GAPDH. The sequences of the oligonucleotide primes used were as follows: 5'-ATT ACC CGC CCG AGA AAG G-3' (mouse NLRP3 Forward), 5'-TCG CAG CAA AGA TCC ACA CAG-3' (mouse NLRP3 Reverse); 5'-ACA AGG CAC GGG ACC TAT G-3' (mouse casapase-1 Forward), 5'-TCC CAG TCA GTC CTG GAA ATG-3' (mouse caspase-1 Reverse); 5'-CTT GTC AGG GGA TGA ACT CAA AA-3' (mouse ASC Forward), 5'-GCC ATA CGA CTC CAG ATA GTA GC-3' (mouse ASC Reverse); 5'-AAA TAC CTG TGG CCT TG-3' (mouse IL-1β Forward), 5'-TTA GGA AGA CAC GGA TTC-3' (mouse IL-1β Reverse); 5'-GAC TCT TGC GTC AAC TTC AAG G-3' (mouse IL-18 Forward), 5'-CAG GCT GTC TTT TGT CAA CGA-3' (mouse IL-18 Reverse); 5'-GTT CTC AGC CCA ACA ATA CAA GA-3' (mouse iNOS Forward), 5'-GTG GAC GGG TCG ATG TCA G-3' (mouse iNOS Reverse); 5'-GGA GTA CCA TAG CTA CCT GG-3' (mouse IL-6 Forward), 5'-CTA GGT TTG CCG AGT AGA TC-3' (mouse IL-6 Reverse); 5'-TGG TTT GCC ATC GTT TTG CTG-3' (mouse IL-12β Forward), 5'-ACA GGT GAG GTT CAC TGT TTC T-3' (mouse IL-12β Reverse); 5'-AGT GAC AAG CCT GTA GCC-3' (mouse TNF-a Forward), 5'-AGG TTG ACT TTC TCC T GG-3' (mouse TNF-a Reverse); 5'-CTC CAA GCC AAA GTC CTT AGA G-3' (mouse ARG-1 Forward), 5'-AGG AGC TGT CAT TAG GGA CAT C-3' (mouse ARG-1 Reverse); 5'-CAG GTC TGG CAA TTC TTC TGA A-3' (mouse Ym1 Forward), 5'-GTC TTG CTC ATG TGT GTA AGT GA-3' (mouse Ym1 Reverse); 5'-GGT CTC AAC CCC CAG CTA GT-3' (mouse IL-4 Forward), 5'-GCC GAT GAT CTC TCT CAA GTG AT-3' (mouse IL-4 Reverse); 5'-TGG CCT TCC GTG TTC CTA C-3' (mouse GAPDH Forward), 5'-GAG TTG CTG TTG AAG TCG CA-3' (mouse GAPDH Reverse); 5'-GCT CTT ACT GAC TGG CAT GAG-3' (mouse IL-10 Forward), 5'-CGC AGC TG AGG AGC ATG TG-3' (mouse IL-10 Reverse);

Western blot analysis

Proteins were loaded onto sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel, and then electrophoretically transferred onto polyvinylidene fluoride (PVDF) membrane (Bio-Rad). Non-specific interactions were blocked using 5% skim milk in Tris-buffered saline (20mM Tris/HCl, pH 7.6, 150mM NaCl, and 0.1% Triton X-100) for 1h. The blots were probed with antibodies at 4°C overnight and incubated with the appropriate HRP-conjugated secondary antibody. Signals were visualized using Enhanced Chemiluminescence

(ECL) substrates. The protein expression was normalized to endogenous β-actin. The following primary antibodies were used: anti-β-actin Ab (Sigma-Aldrich), anti-NLRP3 Ab (Adipogen), anti-caspase-1 (Adipogen), anti-ASC (Santa cruz Biotechnology), anti-IL-1β (R&D), anti-p-ERK (Cell signaling technology), anti-ERK (Cell signaling technology), anti-p-JNK (Cell signaling technology), anti-JNK (Cell signaling technology), anti-p-p38 (Cell signaling technology), anti-p38 (Cell signaling technology), anti-p-AKT (Cell signaling technology), anti-p-IκBα (Cell signaling technology), anti-IκBα (Cell signaling technology), anti-NF-κB (Cell signaling technology), anti-DPP4 (Invitrogen)

Caspase-1 activity assay

The activity of casapase-1 was detected by using Caspase-1 Assay kit (ab39412, Abcam), following the manufacturer's instructions. All assays were performed in triplicated in three independent experiments.

In vivo septic shock model

To induce septic shock, LPS (10mg/kg) was injected into the peritoneal cavity of 6-8 weeks old male WT and DPP-4 KO mice (n=6/group), and then the mouse health status was monitored at specific intervals. At 6h later the injection, 5ml of PBS was injected into the peritoneal cavity and then, the fluid was separated. An ELISA was used to measure the IL-1β and IL-18 secretion level in peritoneal lavage fluid of mice.

Enzyme-Linked Immunosorbent Assay (ELISA)

The secreted levels of IL-1β and IL-18 protein in cultured sup of BMDMs and peritoneal lavage were measured by specific sandwich ELISA system, following the manufacturer's protocol.

Statistics

GraphPad Prism 5.0 software was used for Student's t-test analysis. The data are presented as the mean ±SD of triplicate measurements in a representative experiment. Statistical analysis was performed using an unpaired two-tailed t test. Values of $p<0.05$ were considered statistically significant. Values of $p<0.05$, $p<0.005$, $p<0.0005$ are designated by *, **, and ***, respectively. s

Results

Expression of DPP-4 is decreased by LPS-induced inflammation in macrophages.

To determine whether DPP-4 is affected by inflammation response, we examined the gene expression of DPP-4 in macrophage followed by LPS treatment. Bone marrow-derived macrophages (BMDMs) isolated from ICR mice were left unstimulated (control) or treated with LPS (10ng/ml) for 4h and DPP-4 mRNA expression was analyzed by qRT-PCR. LPS treatment markedly reduced the DPP-4 mRNA expression (Figure 1A). We then compared plasma DPP-4 activity between WT and DPP-4 KO mice and confirmed that DPP-4 activity was considerably reduced in DPP-4 KO mice when compared to WT mice (Figure 1B).

DPP-4 deficiency upregulates NLRP3 inflammasome expression in BMDMs.

The NLRP3 inflammasome is activated by endogenous danger signals including ATP, nigericin, and monosodium urate (MSU). Activation of the NLRP3 inflammasome leads to the production of inflammatory cytokines such as IL-1β and IL-18, which are generally dependent on caspase-1[8,9,12]. To explore the role of DPP-4 in the regulation of NLRP3 inflammasome activation in macrophages, lipopolysaccharide (LPS)-primed BMDMs were exposed to ATP. We found that DPP-4 KO BMDMs with LPS priming and ATP stimulation increased the mRNA expression of IL-1β compared with those in WT BMDMs (Figure 2A). However, the mRNA expression levels of NLRP3, caspase-1, and ASC were not significantly different between WT and DPP-4 KO BMDMs (Figure 2A). Western blotting results also revealed that the protein levels of pro-caspase-1 and pro-IL-1β were upregulated in cell lysate and supernatant from DPP-4 KO BMDMs compared to that from WT BMDMs. (Figure 2B). These results suggest that DPP-4 might play a role in regulation of NLRP3 inflammasome activation.

Loss of DPP-4 promotes NLRP3 inflammasome-mediated IL-1β and IL-18 secretion in macrophages.

The activity of the NLRP3 inflammasome in macrophages was previously reported in pathologies such as sepsis and Parkin's disease[9,10,17]. In addition to upregulation of NLRP3-related gene expression, the secretion of IL-1β and IL-18 is a known hallmark of inflammasome activation[9,18], which is considered to mediated caspase-1 activation. Intrigued by these observations, we assessed IL-1β and IL-18 release from BMDMs followed by LPS priming and ATP stimulation by ELISA. IL-1β (Figure 3A) and IL-18 (Figure 3B) secretion was increased in DPP-

4 KO BMDMs when compared with WT BMDMs, indicating that DPP-4 may be a potent regulator for NLRP3 inflammasome activation

Depletion of DPP-4 improves NLRP3 inflammasome activation by increasing M1 macrophage gene expression *in vitro*.

NLRP3 inflammasome activation leads to caspase-1 activation, which inactivates precursors into the mature active form[8,9,22]. To determine whether DPP-4 affects caspase-1 activity in BMDMs primed with LPS and stimulated with ATP, we compared caspase-1 activity in WT and DPP-4 KO BMDMs and found that DPP-4 deficiency has no significant effect on caspase-1 activation (Figure 4A).

The Mitogen-Activated Protein Kinase (MAPK) signaling pathways are considered as the classical pathways that regulate the inflammatory response[19-21]. The activation of MAPKs leads to the mass release of inflammatory factors such as TNF-a and IL-1β, consequently accelerating the inflammatory processes[20]. To assess the mechanism of NLRP3 inflammasome activation in DPP-4 KO BMDMs, we investigated the MAPK pathway-related protein level by exposing the BMDMs from WT and DPP-4 KO mice to LPS to activate TLR4 signaling. We observed that LPS treatment increased phosphorylation of p-ERK in DPP-4 KO BMDMs after 60 min (Figure 4B).

Previous studies reported that activation of NLRP3 inflammasome is regulated by the anti-inflammatory factors including NF-κB[19,21,23-25]. To investigate whether NLRP3 inflammasome was regulated by M1/M2 macrophage polarization, we compared gene expression levels of M1 or M2-like macrophage between WT and DPP-4 KO BMDMs by qRT-PCR. The mRNA expressions of M1 macrophage marker genes such as iNOS, IL-6, IL-1β, and TNF-a were enhanced in DPP-4 KO BMDMs compared to WT BMDMs. However, the M2 macrophage marker genes such as ARG-1, Ym1, and IL-4 were modestly decreased in DPP-4 KO BMDMs (Figure 4C). Taken together, these results suggest that DPP-4 deficiency may attenuate NLRP3 inflammasome activation by increasing M1 macrophage gene expression *in vitro*.

DPP-4 deficiency enhances LPS-induced NLRP3 inflammasomes *in vivo* by promoting M1 macrophage profile.

NLRP3 inflammasome activation plays an important role in septic shock[26-27]. To confirm the effect of DPP-4 in activation of NLRP3 inflammasomes *in vivo*, we investigated the role of DPP-4 deficiency in LPS-induced septic shock mouse model. WT and DPP-4 KO mice (n=3/group) were intraperitoneally (i.p.) injected with LPS (10mg/kg) or Phosphate-Buffered Saline (PBS). It has been known that intraperitoneal injection of LPS into mice elicits NLRP3-dependent production and secretion of IL-1β and IL-18. After 6h of LPS injection, we harvested the peritoneum and measured IL-1β and IL-18 protein levels in peritoneal lavage fluid in both WT and DPP-4 KO mice. We found that IL-1β (Figure 5A) and IL-18 (Figure 5B) secretion were significantly increased in the peritoneal lavage fluid from DPP-4 KO mice. Furthermore, we isolated peritoneal macrophage and cultured with ATP (5mM) for 30min. DPP-4 protein level was decreased in peritoneal macrophage from DPP-4 KO mice compared with those of WT and the protein level of NLRP3 was significantly upregulated in DPP-4 KO and this increase was further enhanced by ATP (Figure 5C). To investigate whether DPP-4 deficiency enhances the expression of M1-related genes, we isolated peritoneal macrophage from WT and DPP-4 KO mice peritoneum and compared mRNA expression of these genes. M1 macrophage related-gene expression was significantly upregulated in peritoneal macrophage from DPP-4 KO mice when compared with that of WT mice (Figure 5D).

Collectively, these results suggest that DPP-4 may negatively regulate LPS-induced NLRP3-indlammasome activation by promoting M1 macrophage profile *in vivo*.

Discussion

Dipeptidyl peptidase-4 (DPP-4) has enzymatic activity that cleavages N-terminal of either L-proline or L-alanine dipeptides from numerous chemokines, resulting in both active and inactive fragments of substrates[1-3]. In immune system, DPP-4 can modulate intracellular signaling by directly interacting with regulatory molecules. It mainly plays a role stimulating effect on T cells and secretion of TGF-β[4]. Although the role of DPP-4 in immune response has been reported by various investigations based on its expression on T cells[4-6], the role played by DPP-4 in primary macrophage with respect to LPS-induced inflammation is still uncertain.

The NLRP3 is an immune sensor that detects host immune response from endogenous signals and environmental factors, resulting in the formation and activation of NLRP3 inflammasome. And the NLRP3 inflammasome consists of a sensor (NLRP3), an adaptor (ASC, also known as PYCARD) and an effector (Caspase-1). These assembly of the NLRP3 inflammasome leads to production and release of the pro-inflammatory cytokines IL-1β and IL-18 dependent on caspase-1[8-10].

In this study, We observed that DPP-4 deficiency increased IL-1β secretion upon to NLRP3 inflammasome activation in macrophages by promoting M1-like macrophage profile. Our data show that the mRNA expression level of DPP-4 was downregulated in Bone marrow-derived macrophages (BMDMs) from ICR mice with LPS, suggesting that DPP-4 was involved LPS-induced inflammation in mouse macrophages (Figure 1). The NLRP3 inflammasome has been generally activated by ATP and leads to the production of inflammatory cytokines like IL-1β and IL-18, which are dependent on caspase-1[8]. We found that cultured sup in DPP-4 KO BMDMs with LPS priming and ATP stimulation increased the protein level of casapase-1 and IL-1β compared with WT BMDMs (Figure 2). These findings suggest that DPP-4 might play a key role in the immune response.

Given that IL-1β and IL-18 release were another hallmark of inflammasome activation[9,18], we assessed the amounts of IL-1β and IL-18 protein by ELISA. The results showed that IL-1β and IL-18 secretion were increased in DPP-4 KO BMDMs cultured supernatants with LPS priming and ATP stimulation, suggesting that DPP-4 may be a potent regulator for NLRP3 inflammasome activation (Figure 3).

To assess the mechanism of NLRP3 inflammasome activation on DPP-4 KO BMDMs, first we explored the caspase-1 activity assay[8,9,12]. However, we observed DPP-4 deficiency has no significant effect compared in WT

BMDMs (Figure 4A). The production of IL-1β is regulated by various inflammasomes, including NLRP3, AIM2, and NLRC4 inflammasomes[11], further studies will be necessary to understand the detailed underlying mechanisms that activates from pro-caspase-1 to mature caspase-1 form. Caspases independently sense intracellular LPS of TLR4 by binding to LPS[22]. Non-canonical inflammasome involves caspase-11 in mice and caspase-4/5 in humans, rather than caspase-1[22,27,28]. Casapase-4/5/11 induce a pyroptosis through the processing of Gasdermin D(GSDMD), and pannexin-1, which is a protein channel that releases ATP from the cell. Further studies will be involved in processing of caspase-4/5/11 activation[27-29]. Indeed, we showed that the mRNA levels of M1 macrophage-related marker gene, such as iNOS, IL-1β, and TNF-a were enhanced in DPP-4 KO BMDMs compared to WT BMDMs, indicating that the deficiency of DPP-4 enhances NLRP3 inflammasome activation by increasing M1 macrophage level *in vitro* (Figure 4C).

The release of pro-inflammatory cytokine IL-1β by intraperitoneal injection of LPS *in vivo* is associated with the NLRP3 inflammasome activation[17,26]. We observed that the amounts of IL-1β (Figure 5A) and IL-18 (Figure 5B) were significantly increased in the peritoneal lavage fluid from DPP-4 KO mice by increasing M1 macrophage related-gene expression. Additionally, we should observe the mRNA level of M2 macrophage related-gene expression. Our results suggest that depletion of DPP-4 improved the activation of NLRP3 inflammasome activation in a mouse model (Figure 5). However, Further studies will be necessary to confirm that DPP-4 deficiency enhances M1 macrophages population by FACS analysis (Flow cytometry) and to assess the therapeutic potential of DPP-4 in the inflammasome activation, we should explore other inflammasome activation such as NLRC4 and AIM2 inflammasome or treat other materials related-NLRP3 inflammasome (nigericin and MSU).

In summary, our study suggest that DPP-4 may be a negative regulator of NLRP3 inflammasome activation in macrophage, suggesting DPP-4 as a potential therapeutic target for treatment of inflammatory diseases and sepsis model. These findings suggest that DPP-4 may play as a novel biomarker in acute immune response.

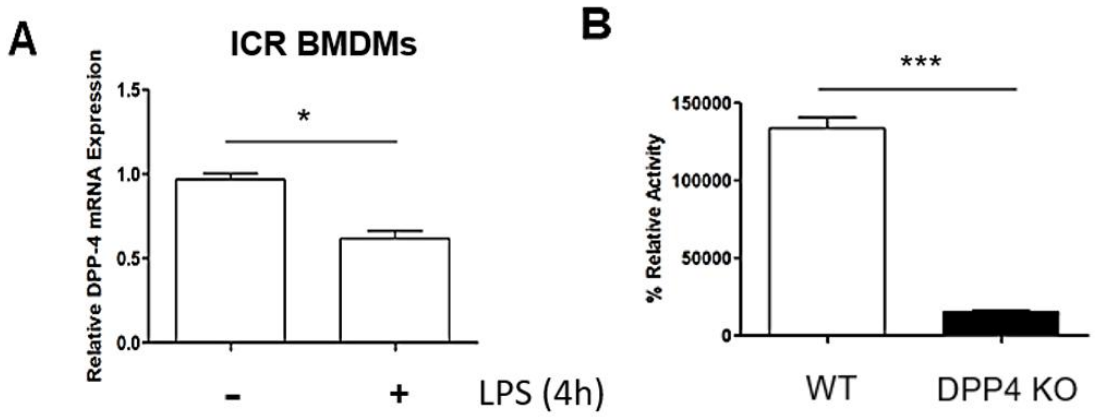
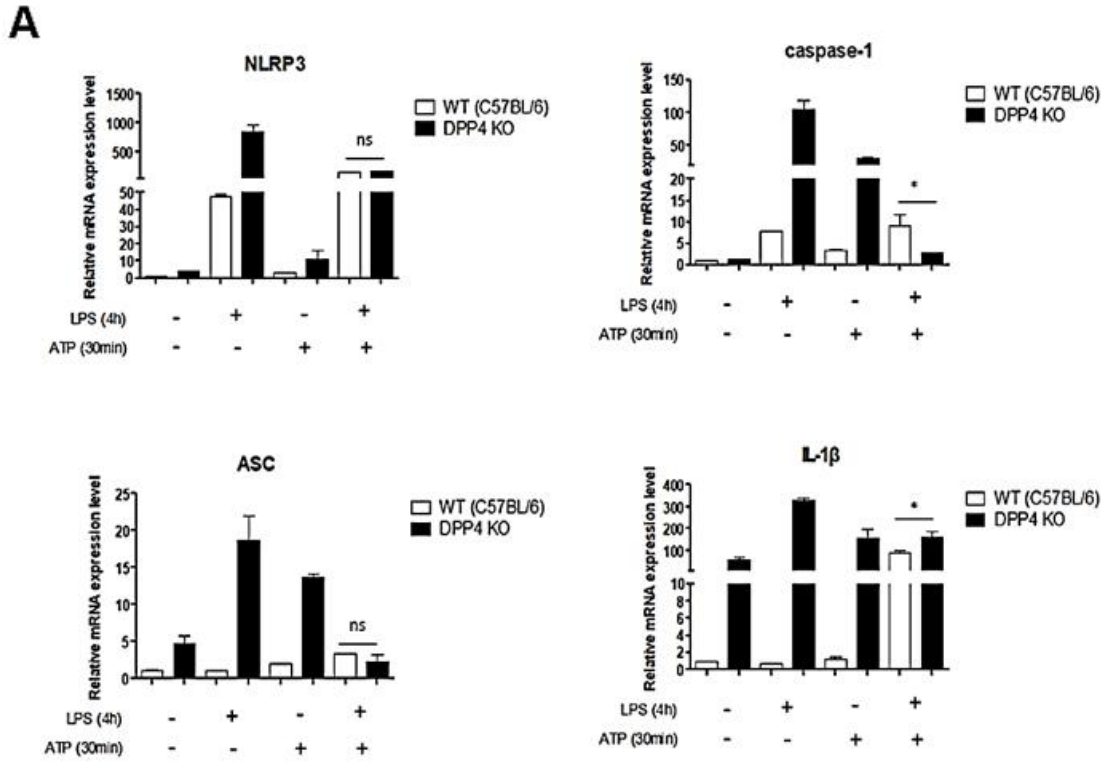


Figure 1. Expression of DPP-4 is decreased by LPS-induced inflammation in macrophages.

(A) Bone marrow-derived macrophages (BMDMs) from ICR mice were left unstimulated (control) or treated with LPS (10ng/ml) for 4h. Then mRNA expression level of dipeptidyl peptidase-4 (DPP-4) were analyzed by qRT-PCR. (B) Dipeptidyl peptidase-4 (DPP-4) activity was determined in WT (C57BL/6) and DPP-4 KO BMDMs utilizing DPP-4 activity assay kit. *p* values were calculated using Student's t-test. The data represent the mean ± standard deviation. * $p<0.05$, *** $p<0.005$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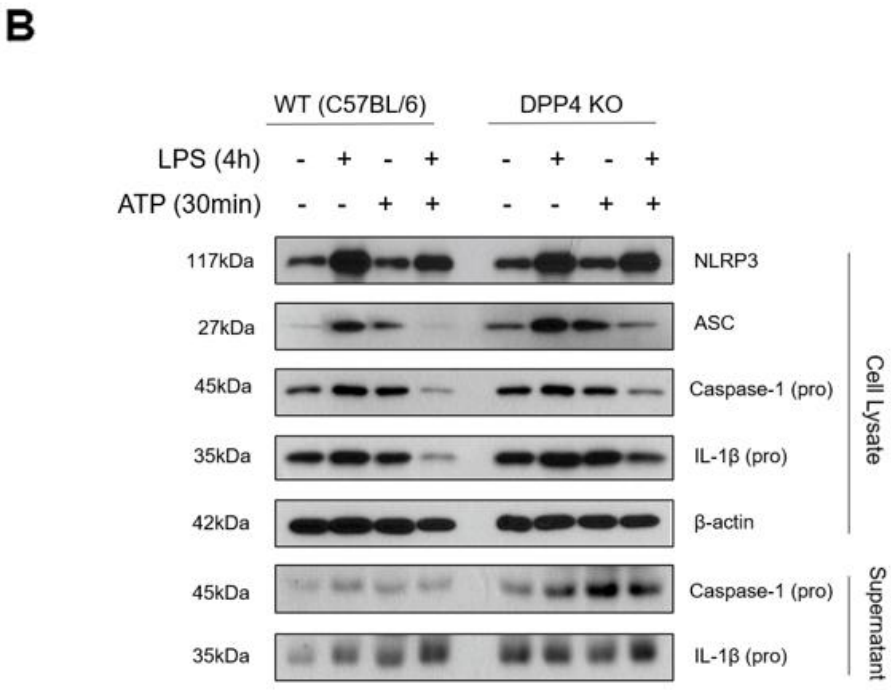
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45kDa
Caspase-1 (pro)
35kDa
IL-1β (pro)
42kDa
β-actin
Cell Lysate
45kDa
Caspase-1 (pro)
35kDa
IL-1β (pro)
Supernatant

Figure 2. DPP-4 deficiency upregulates NLRP3 inflammasome expression in BMDMs.

(A) WT and DPP-4 KO BMDMs were left unstimulated or treated with LPS (10ng/ml), ATP (5mM) alone or stimulated with LPS (10ng/ml) for 4h and then treated with ATP (5mM) for 30min. NLRP3, Caspase-1, ASC and IL-1β mRNA levels was analyzed by qRT-PCR. (B) Cell lysate and culture supernatants were immunoblotted for NLRP3 inflammasome-related components. * $p<0.05$ and ** $p<0.005$ compared with WT BMDMs. p values were calculated using Student's t-test. The data represent the mean ± standard deviation.

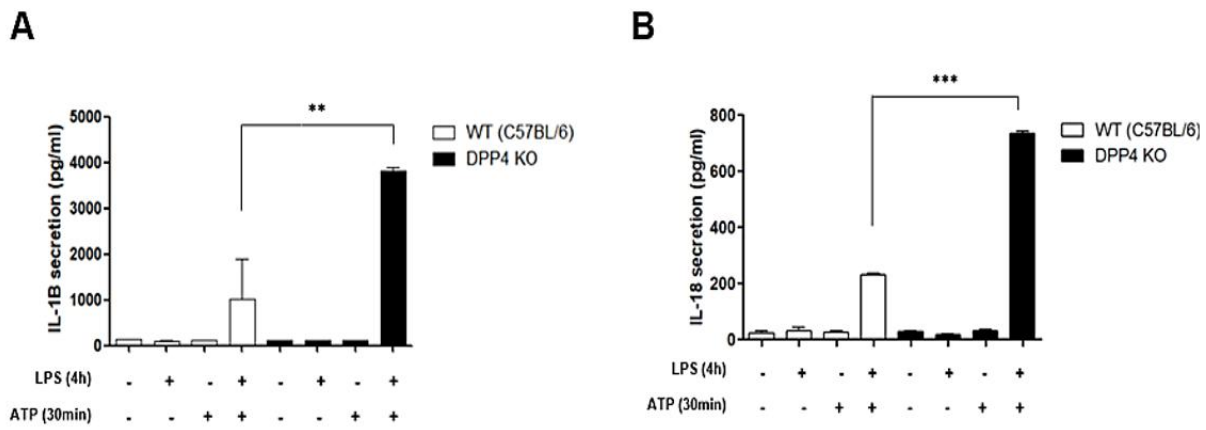

Figure 3. Loss of DPP-4 promotes NLRP3 inflammasome-mediated IL-1β and IL-18 secretion in macrophages.

(A-B) WT and DPP-4 KO BMDMs were left unstimulated or treated with LPS (10ng/ml), ATP (5mM) alone or stimulated with LPS (10ng/ml) for 4h and then treated with ATP (5mM) for 30min. Supernatants were harvested and the amounts of IL-1β and IL-18 proteins were determined by ELISA. The data represent the mean ± SD of one among three biological replicates, with three technical replicates each *(** p<0.05, *** p<0.0005*; Student's t test).

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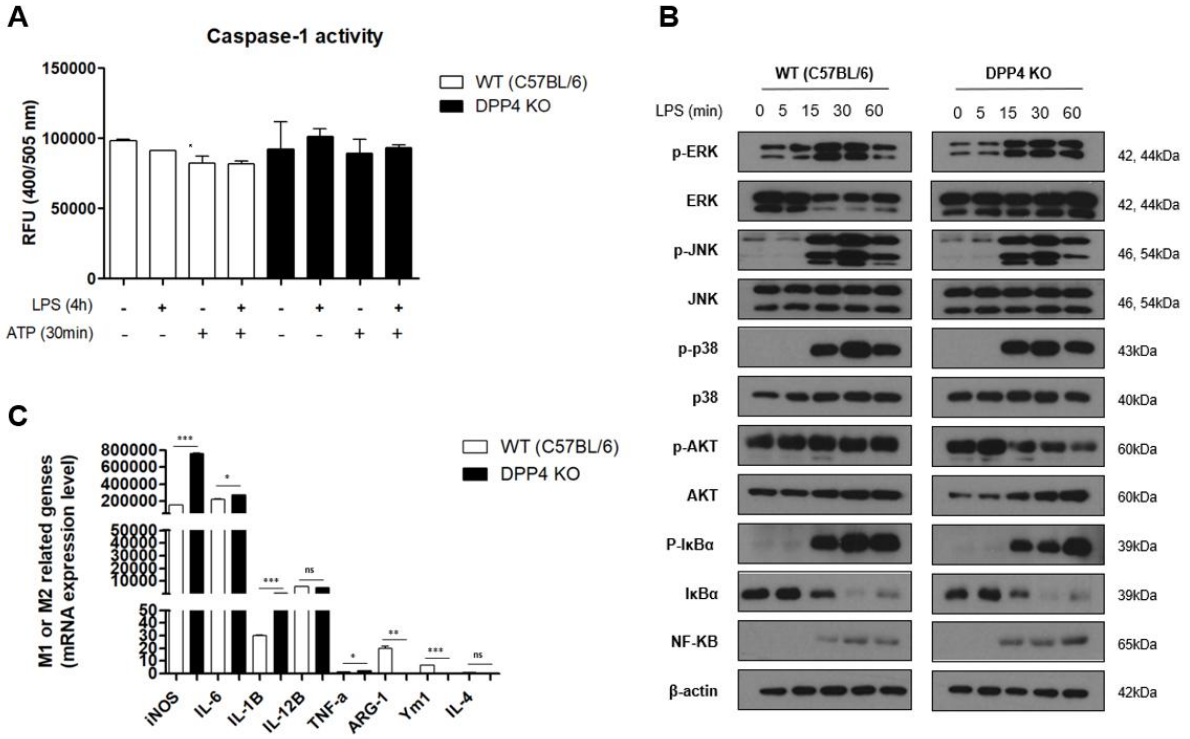

Figure 4. Depletion of DPP-4 induces NLRP3 inflammasome activation by increasing M1 macrophage gene expression *in vitro*.

(A) WT and DPP-4 KO BMDMs were left unstimulated or treated with LPS (10ng/ml), ATP (5mM) alone or stimulated with LPS (10ng/ml) for 4h and then treated with ATP (5mM) for 30min. Whole lysates were analyzed with Casapase-1 activity assay kit. (B) BMDMs from WT or DPP-4 KO mice were treated with LPS (10ng/ml) for indicated times. Cell extracts were immunoblotted for MAPK pathway-related proteins. Representative immunoblots of p-JNK, JNK, p-ERK, ERK, p-p38, p38, p-AKT, AKT, p-IκBα, IκBα, NF-κB, and β-actin are presented. β-actin was used as a loading control. (C) The mRNA levels of the M1-macrophage markers (iNOS, IL-6, IL-1β, IL-12β, and TNF-a) and the M2-macrophage markers (ARG-1, Ym1, and IL-4) were analyzed by qRT-PCR. The data represent the mean ± SD of one among three biological replicates, with three technical replicates each (* p<0.05, ** p<0.005, *** p<0.0005, ns: not significant; Student's t test).

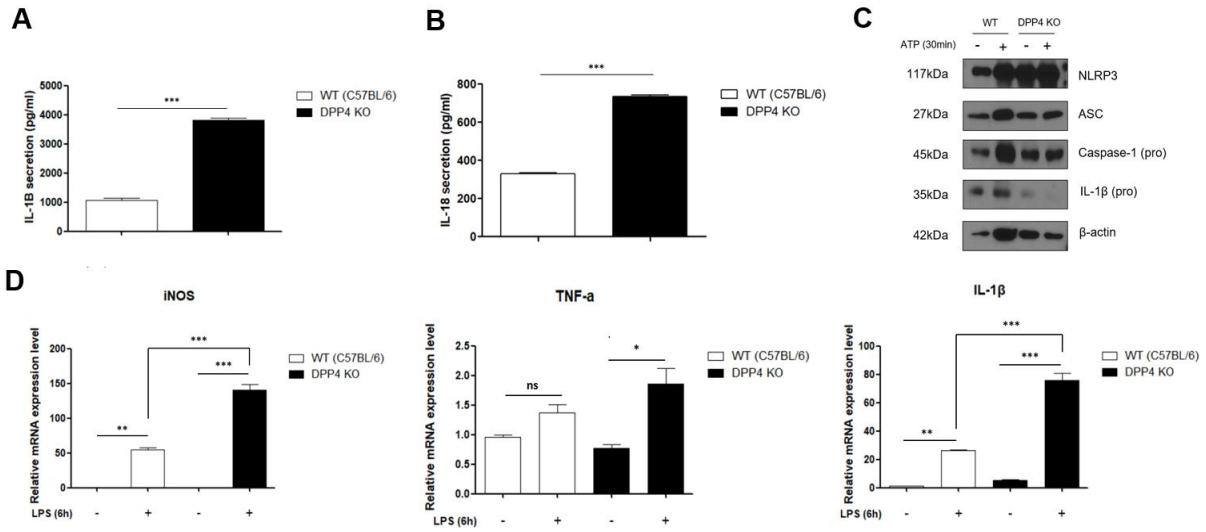

Figure 5. DPP-4 deficiency enhances LPS-induced NLRP3 inflammasomes *in vivo* by promoting M1 macrophage profile.

(A-B) WT or DPP-4 KO mice were intraperitoneally injected with PBS or LPS (10mg/kg) and left alone for 6h. Production of IL-1β and IL-18 in peritoneal lavage fluid 6h after intraperitoneal injection of LPS (10mg/kg of body weight) or PBS was determined utilizing ELISA in WT mice (n=3) and DPP-4 KO mice (n=3). (C) The protein expression of NLRP3, Casapase-1, ASC, IL-1β, and β-actin was assessed by western blot analysis. (D) The mRNA expression of M1 (iNOS, TNF-a, and IL-1β) macrophage markers were evaluated by qRT-PCR. The results were analyzed by one-way ANOVA followed by Turkey's test. The significance levels are indicated: * $p<0.05$, ** $p<0.005$, *** $p<0.0005$, ns: not significant.

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국문요약

디펩티딜 펩티다아제-4 (DPP-4)는 기질을 분해하고 염증 및 면역 기능에 상당한 영향을 미치는 여러 케모카인 (chemokine) 과 사이토카인 (cytokine)을 불활성화하는 프로테아제다. 이처럼 디펩티딜 펩티다아제-4 (DPP-4)는 면역반응 조절에 중요하다고 알려져 있는데, 대식세포에서의 역할은 잘 알려지지 않았다.

이 연구에서 디펩티딜 펩티다아제-4 (DPP-4)가 NLRP3 인플라마솜 활성화의 부정적인 조절하는 역할을 하며 나아가 패혈증 쇼크의 치료제로서의 목표를 할 수 있다는 것을 가정했다. 이러한 가능성을 조사하기 위해 골수유래 대식세포에서 NLRP3 의존성 인플라마솜 활성화를 조절하는 디펩티딜 펩티다아제-4 (DPP-4)의 기능을 연구하였다.

골수유래 대식세포에서 지질다당류 (LPS)에 의한 디펩티딜 펩티다아제-4 (DPP-4)의 발현이 억제되는 것을 확인했고, 디펩티딜 펩티다아제-4 (DPP-4) 이 결손된 골수유래 대식세포에서 NLRP3 인플라마솜 활성화와 관련된 유전자의 발현이 증가하는 것을 확인했다. 또한 효소면역 측정법을 통해 대식세포 밖으로 분비되는 인터류킨 1β (IL-1β)와 인터류킨 18 (IL-18)의 증가한 것을 확인해 디펩티딜 펩티다아제-4 (DPP-4)의 결핍이 NLRP3 인플라마솜 활성화를 촉진시키는 것을 확인했다. 이 결과와 동일하게 디펩티딜 펩티다아제-4 (DPP-4) 결핍 마우스의 LPS 유도 패혈증 모델에서 인터류킨 1β (IL-1β)과 인터류킨 18 (IL-18)의 발현이 증가했고, M1 대식세포 관련 유전자의 발현이 상당히 증가한 것을 확인했다.

따라서 본 연구 결과를 통해 디펩티딜 펩티다아제-4 (DPP-4)가 골수유래 대식세포에서 결핍이 일어나게 되면 NLRP3 인플라마솜 활성화가 유도되어 관련 유전자와 단백질의 발현이 증가한다. 그리고 실험동물 모델에서 동일한 결과를 확인했으며 이는 M1 대식세포 관련 유전자의 발현이 증가했기 때문임을 확인했다. 이를 통해, 골수유래 대식세포에서 DPP-4 가 M1 대식세포 집단을 촉진하여 NLRP3 인플라마솜의 활성화를 음성적으로 조절한다는 것을 증명했다.

핵심 키워드: DPP-4, NLRP3, 염증복합체, 염증성 사이토카인