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IKKε aggravates inflammatory response via activation of NF-kB in rheumatoid arthritis

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Abstract. – OBJECTIVE: Rheumatoid Arthritis (RA) is a chronic systemic autoimmune disease, whereas its cause still remains elusive. Typical pathological manifestations of RA include persistent synovitis and bone degeneration in the surrounding joints. Although the incidence of RA is high in population, currently there have been no effective cures for it. The purpose of this study is to investigate the therapeutic effects and main mechanism of IKKε (inhibitor of nuclear factor kappa-B kinase ε) in collagen II induced- Rheumatoid Arthritis (CIA) mice model.

MATERIALS AND METHODS: IKKε$^{-/-}$ and wild-type (WT) littermate control mice were intraperitoneally injected with 5 mg/kg collagen II monoclonal antibody cocktail (Cab) for 5 days. After that, the nociception threshold and clinical rheumatoid arthritis articular damage score of mice were evaluated. After 5 days-CAb treatment, serum levels of a series of inflammatory cytokines including interleukin-6 (IL-6), IL-1β, tumor necrosis factor-α (TNF-α) and interferon (IFN) were detected with enzyme-linked immunosorbent assay (ELISA) in both groups. Besides, Real-time reverse transcription polymerase chain reaction (Real-time RT-PCR) was used to evaluate the expression of these inflammatory cytokines in plantar tissues. In addition, Western blot was performed to investigate the protein levels of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B) signaling pathway. Moreover, WT mice receiving CAb were further applied with or without IKK inhibitor amlexanox (25 mg/kg) to investigate the expression of the above-mentioned inflammatory cytokines.

RESULTS: Our work showed that IKKε$^{-/-}$ mice with CIA displayed less nociception and suppressed inflammatory response than WT mice. Meanwhile, the clinical rheumatoid arthritis articular damage scores were significantly decreased in IKKε$^{-/-}$ mice. The levels of TNF-α, IL-1β, IL-6 in serum and plantar tissues in IKKε$^{-/-}$ mice were significantly lower than those in WT mice. Besides, NF-κB expression in IKKε$^{-/-}$ mice was significantly decreased. Similarly, the same phenotype was observed in WT mice administrated with IKKε inhibitor amlexanox as that of IKKε$^{-/-}$ mice, indicating that inflammatory and nociception responses were remarkably decreased than those of the negative controls.

CONCLUSIONS: IKKε plays an important role in promoting nociception and inflammatory response in CIA. Our research demonstrated that knockout of IKKε may serve as a new direction for clinical prevention and treatment of rheumatoid arthritis. IKKε inhibitor amlexanox may become a new drug for the treatment of rheumatoid arthritis.

Key Words:
RA, IKKε, CIA, Amlexanox.

Introduction

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease characterized by non-suppurative inflammation in joints and surrounding tissues. It mainly affects synovium and usually leads to joint deformity and muscle atrophy in the late stage[1,2]. With an incidence of 0.2-0.4% in China, RA has become one of the major causes of loss of working ability, disability and huge financial burden among the elderly[3]. Unfortunately, there have been no efficient therapies for RA; then, further explorations are required. Current clinical treatment remains in control of inflammation and treatment of sequelae, so immunosuppressive agents are still widely used in spite of their severe side effects[4]. Therefore, it is of great importance to investigate the possible pathogenesis of RA and find more effective therapies for RA patients. In the past decades, much progress has been made

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in discovering the pathogenesis of RA and it is reported that the pathological changes may be related to the abnormal expression of inflammatory cytokines IL-6 and TNF-α in blood circulation[5]. In addition, previous clinical studies have shown that the NF-κB/IKKε signaling pathway is associated with the activity of RA[6]. Synoviocytes expressing IKK2 in RA could promote the activation of NF-κB[7]. Moreover, it has been reported that NF-κB activation is triggered by the phosphorylation of regulatory protein IκB, which consists of four different IκB kinases (IKKs), namely IKKα, IKKβ, IKKε and TANK-binding kinase 1 (TBK1). The specific role of IKKα and IKKβ in NF-κB activation has been confirmed, but the IKKε regulatory pathway remains uncertain[8]. We hypothesized that IKKε stimulated the NF-κB signaling pathway by inducing the activation of inflammatory cytokines, thereby exacerbating the inflammatory response of CIA. In this work, the collagen II and LPS (lipopolysaccharide) induced-rheumatoid arthritis model was used to screen and evaluate anti-inflammatory molecules, thus imitating RA in mice[9]. We found that IKKε could stimulate CIA development, increase RA-induced nociception response, and exacerbate the destruction of joint and foot in mice through promoting secretion of inflammatory cytokines via NF-κB signaling pathway. Administration of IKKε inhibitor amlexanox could reverse the exacerbated RA phenotype, which further confirms the role of IKKε in RA induction. Overall, our finding suggests that IKKε plays an important role in CIA and may be a potential therapeutic target for RA. The inhibitor amlexanox may become a new drug for the effective treatment of RA.

Materials and Methods

Animals

IKKε$^{-/-}$ mice were obtained from Jackson Lab (West Grove, PA, USA) and housed in SPF (specific pathogen free) animal facility. All the mice were caged individually. WT and IKKε$^{-/-}$ mice between 8-9 weeks were used for experiments (n=6-8). This study was approved by the Animal Ethics Committee of Capital Medical University Animal Center.

CIA Mice Model

Male WT and IKKε$^{-/-}$ mice (Model Animal Research Center of Nanjing University, Nanjing, China) between 8-10 weeks were intraperitoneally injected daily with 5 mg/kg collagen II monoclonal antibody cocktail (Abcam, Cambridge, MA, USA) for 5 days. Besides, lipopolysaccharide (LPS, Sigma-Aldrich, St. Louis, MO, USA) was injected intraperitoneally on the first day. Blood samples were collected by orbital venous plexus for the first five days. On the fifth day, mice were sacrificed by carbon dioxide asphyxiation. Plantar tissues were frozen in liquid nitrogen and stored at -80°C. For amlexanox (Sigma-Aldrich, St. Louis, MO, USA) administration, WT mice injected with CAb for 5 days were divided into 2 groups, then treated with 25 mg/kg amlexanox for 2 weeks. After that, blood and plantar tissues were obtained again for further experiments.

Clinical Rheumatoid Arthritis Articular Damage Score Evaluation

Mice joint involvement was assessed by a macroscopic scoring system before and during the injection of CAb. A score of 11-15 points represented serious joint injury, involving all the feet and toes; 6-10 points represented that more than two joints were involved; 1-5 points indicated that two or fewer joints were involved; 0 points meant non-arthritis performance.

Nociception Threshold Examination

Before and after injection of CAb into mice, the sensitivity of the mice to the nociception response was measured using an electronic pressure detector (BD Biosciences, San Jose, CA, USA).

Western Blotting

After collecting the liver, the total protein was extracted, separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, Merck Millipore, Billerica, MA, USA) and transferred to polyvinylidene difluoride (PVDF, Merck Millipore, Billerica, MA, USA) membrane. After 2 h blocking at room temperature, the immunoblots were incubated overnight with the following primary antibodies: anti-NF-κB (diluted 1:1000, Cell Signaling Technology, Danvers, MA, USA) and anti-β-actin (diluted 1:1000, Cell Signaling Technology, Danvers, MA, USA). On the next day, the immunoblots were washed in Tris-buffered saline and Tween 20 (TBST) for three times and incubated with the secondary antibody (horseradish peroxidase (HRP)-labeled IgG antibody diluted 1:10000, Cell Signaling Technology, Danvers, MA, USA). Chemiluminescence was used for detecting the protein bands.

Table I. RT-qPCR primer pairs.

Name	Forward	Reverse
mGAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
mIL-1β	TGGACCTTCCAGGATGAGGACA	GTTCATCTCGGAGCCTGTAGTG
mIL-6	TACCACTTCACAAGTCGGAGGC	CTGCAAGTGCATCATCGTTGTTC
mTNF-α	GGTGCCTATGTCTCAGCCTCTT	GCCATAGAACTGATGAGAGGGAG
mIFN-γ	CAGCAACAGCAAGGCGAAAAAGG	TTTCCGCTTCCTGAGGCTGGAT

Real-Time Quantitative PCR

Total RNA was extracted by TRIzol (Invitrogen Carlsbad, CA, USA). cDNA synthesis was performed by the reverse transcription according to the manufacturer's instructions. qRT-PCR was performed to detect the relative gene expression levels. All experiments were repeated three times. Primer sequences are shown in Table I.

Serum Cytokines Detection

The serum of mice was collected 8 h and 24 h after APAP treatment. ELISA was performed according to the protocol of ELISA kit (Bio Legend, San Diego, CA, USA). The OD (optical density) values at A562 nm and A450 nm wavelength were measured by microplate reader (Bio-Rad, Hercules, CA, USA). The cytokines concentrations were calculated according to the standard curve of concentration and absorbance values.

Statistical Analysis

All experiments were repeated at least three times. Data are expressed as mean ± SE. The Student's *t*-test was used to compare the differences between the two groups. One-way ANOVA followed by Least Significant Difference (LSD) was used to determine differences among multiple groups. All statistical analyzes were performed using Graphpad Prism (v6.0, La Jolla, CA, USA). $p < 0.05$ was considered statistically significant.

Results

IKKε Depletion Increases the Nociception Threshold in CIA Mice

First we evaluated the effect of IKKε depletion on the nociception threshold in CIA mice model by an electronic pressure nociception detector. 100 mg LPS were injected on the first day. Next, the control groups of WT and IKKε$^{-/-}$ mice were injected with 0.5% methylcellulose, while the treatment groups were intraperitoneally injected with 0.5% methylcellulose and 5 mg/kg CAb for 5 days, and 100 mg LPS were injected on the first day. As shown in Figure 1, the nociception threshold was significantly lower in the CIA groups than that in the control groups 5 days after CIA, suggesting that the establishment CIA model was successful. Surprisingly the nociception threshold of IKKε$^{-/-}$ CIA mice was similar to that of WT control ($p > 0.05$). In addition, the nociception threshold in the IKKε$^{-/-}$ CIA group was significantly higher than that in the WT CIA group (Figure 1). These data demonstrated that IKKε depletion could increase the nociception threshold in CIA mice.

IKKε Depletion Decreases the Joint Involvement of RA Mice

Next, we evaluated the severity of RA in mice by the macroscopic score system via scoring the joint activity and plantar stiffness degree.

The results showed that the score of CIA group in WT and IKKε$^{-/-}$ mice was significantly higher than that in the control group. Besides, in the CIA group, the score of IKKε$^{-/-}$ mice was lower than

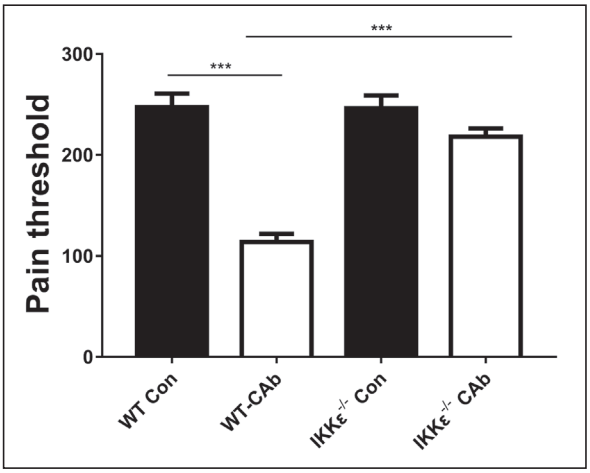

Figure 1. IKKε depletion increases the nociception threshold in CIA mice. Five days after CAb treatment, the sensitivity of WT and IKKε$^{-/-}$ mice to nociception was examined by electronic pressure nociception detector. ***$p < 0.001$. n = 6-8. Data are expressed as means ± SEM.

that in WT mice (Figure 2). With the extension of modeling time, joint activities were gradually relieved while the degree of joint involvement was reduced, suggesting that IKKε was related to the RA severity.

IKKÐ Aggravates the Inflammatory Response by Promoting Inflammatory Cytokines Secretion

After confirming the involvement of IKKε in the pathogenesis of RA, we examined the expression of inflammatory cytokines in plantar tissue and serum of WT and IKKε$^{-/-}$ mice since the pathological changes in RA may be related to the abnormal expression of inflammatory cytokines.

Results showed that mRNA expression levels of IL-6, IL-1β and TNF-α in IKKε$^{-/-}$ mice CIA group were significantly lower than those in WT CIA group, while IFN-γ (interferon-γ) expression difference was not significant between the two groups of CIA mice (Figure 3A). Next, we validated the protein expression of IL-6, IL-1β and TNF-α in mouse serum by ELISA. Results showed that the serum levels of these three inflammatory cytokines in IKKε$^{-/-}$ mice CIA group were also significantly lower than those in WT CIA group (Figure 3B). These data indicated that the deletion of IKKε would help to reduce the inflammatory response of RA.

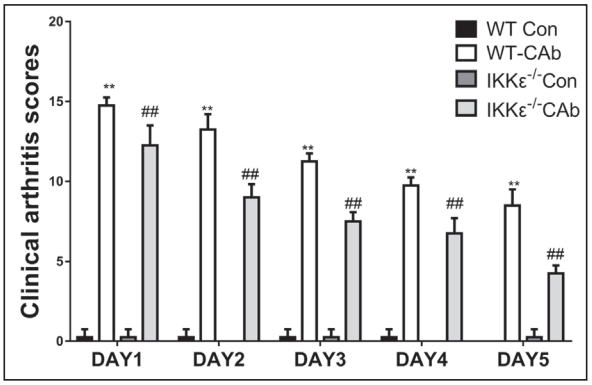

Figure 2. IKKε depletion decreases the joint involvement of RA mice. After administration of CAb, the joint involvement and plantar tissue stiffness of WT and IKKε$^{-/-}$ mice were observed, macroscopically scored, and recorded daily. ***p < 0.001. n = 6-8. Data are expressed as means ± SEM.

IKKε Initiates Inflammatory Response Via Activating NF-κB Signaling Pathway

To further investigate the underlying mechanism of IKKε's involvement in RA inflammatory response, we examined the activation of classical inflammation and transcriptional signaling pathways. As shown in Figure 4, the protein level of NF-κB in IKKε$^{-/-}$ mice CIA group was higher than that in WT CIA group, indicating that IKKε$^{-/-}$ mice exerted a suppressed inflammatory response. The above

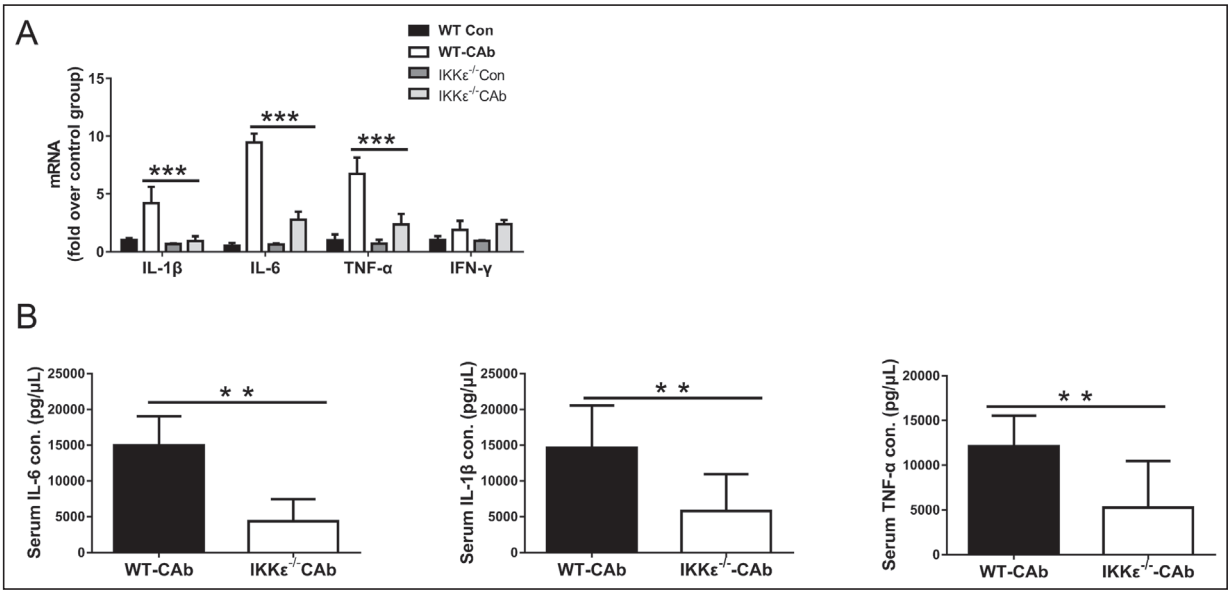

Figure 3. IKKε aggravates the inflammatory response by promoting inflammatory cytokines secretion. ***(A)*** mRNA levels of inflammatory cytokines were measured by qRT-PCR in the plantar tissues of WT and IKKε$^{-/-}$ mice five days after CAb treatment; ***(B)*** Serum levels of IL-1β, IL-6 and TNF-α were measured by ELISA. *p < 0.05; **p < 0.01; ***p < 0.001. n = 6-8. Data are expressed as means ± SEM.

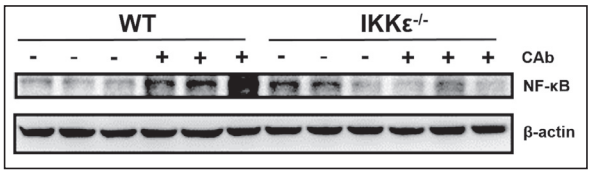

Figure 4. IKKε initiates inflammatory response via activating NF-κB signaling pathway. Five days after CAb or equivalent methylcellulose treatment of WT and IKKε$^{-/-}$ mice, the plantar tissues of mice were taken. The expressions of tissue NF-κB were detected by Western blot n=3.

results suggested that NF-κB could regulate inflammatory response of RA via modulating IKKε. Furthermore, IKKε could promote the secretion and expression of inflammatory cytokines by activating NF-κB.

Amlexanox Effectively Inhibits the Inflammatory Activity of IKKε in RA

In WT and IKKε$^{-/-}$ mice, we demonstrated that IKKε regulated the progression of RA by promoting the expression of inflammatory cytokines, decreasing the nociception threshold, and disrupting joint and plantar tissues.

Amlexanox has been reported to be a potent inhibitor of IKKε in recent years[10]. Therefore, amlexanox was applied in our model to investigate whether it can inhibit the activity of IKKε in WT mice, thus reducing the inflammatory response of RA. WT mice were randomly divided into three groups. The control group was given 0.5% methylcellulose, while the treatment and the therapy group were given the same dose of CAb. After that, the therapy group mice were given 25 mg/kg amlexanox for 2 weeks. All experiments mentioned above were then performed in these mice and corresponding samples. The results showed that the nociception thresholds of the therapy group and the control group had no significant difference. However, nociception thresholds of therapy group and control group were significantly higher than the CIA treatment group (Figure 5A). In addition, the joint involvement and activity gradually improved in mice administrated with amlexanox. After 2 weeks of therapy, mice restored the same joint activity as the control group (Figure 5B). Importantly, in serum and plantar tissues, the expressions of inflammatory cytokines including IL-6, IL-1β and TNF-α of the therapy group were significantly lower than those of the treatment group, indicating that amlexanox could effectively inhibit the inflammatory response of RA (Figure 5C-D). Finally, we verified the activation of the NF-κB signaling pathway. As shown in Figure 5E, the expression of NF-κB in the therapy group was significantly lower than that in the treatment group, which is consistent with that in IKKε$^{-/-}$ mice. These results further confirmed the pro-inflammatory effect of IKKε in RA and that amlexanox could reduce RA inflammatory response as a potent inhibitor of IKKε.

Discussion

Rheumatoid arthritis (RA) is a systemic inflammatory autoimmune disease involving the entire body joints. RA has been affecting 1% of the world population, while the incidence of RA in women is as twice high as in men[10]. The most recent statistics show that the disability rate among RA patients in China is about 0.32-0.36%, which severely weakens the economic productivity in the society[11].

Classic characteristics of RA are the chronic synovitis of the joints. Other pathological features of RA includes autoantibody production, synovial cell proliferation, inflammatory cell infiltration (e.g., neutrophils and macrophages), angiogenesis, as well as bone and cartilage destruction, which eventually lead to the destruction and loss of function in the entire joints[12]. Advanced studies have been carried out in the exploration of clinical and fundamental mechanism of RA worldwide, therefore, RA patients nowadays have multiple therapeutic options[1,13,14]. However, these studies could not fully elucidate the pathogenesis of RA. Because the underlying mechanisms of RA occurrence have been found to vary, including genetic, immunomodulatory and environmental factors, currently no specific cure of RA was demonstrated enough potent[15-17].

IKKε has been proved to be correlated with the activity and severity of RA in a clinical study[6]. We confirmed that IKKε deficiency can effectively reduce the degree of RA nociception, joint involvement and tissue stiffness. In addition, several studies have suggested that IL-1β, IL-6, TNF-α and INF-γ are involved in the major inflammatory responses of RA[18-21]. Indeed, our work found that expressions of these inflammatory cytokines in IKKε$^{-/-}$ mice were significantly lower than those in WT mice, indicating that IKKε mediates the in-

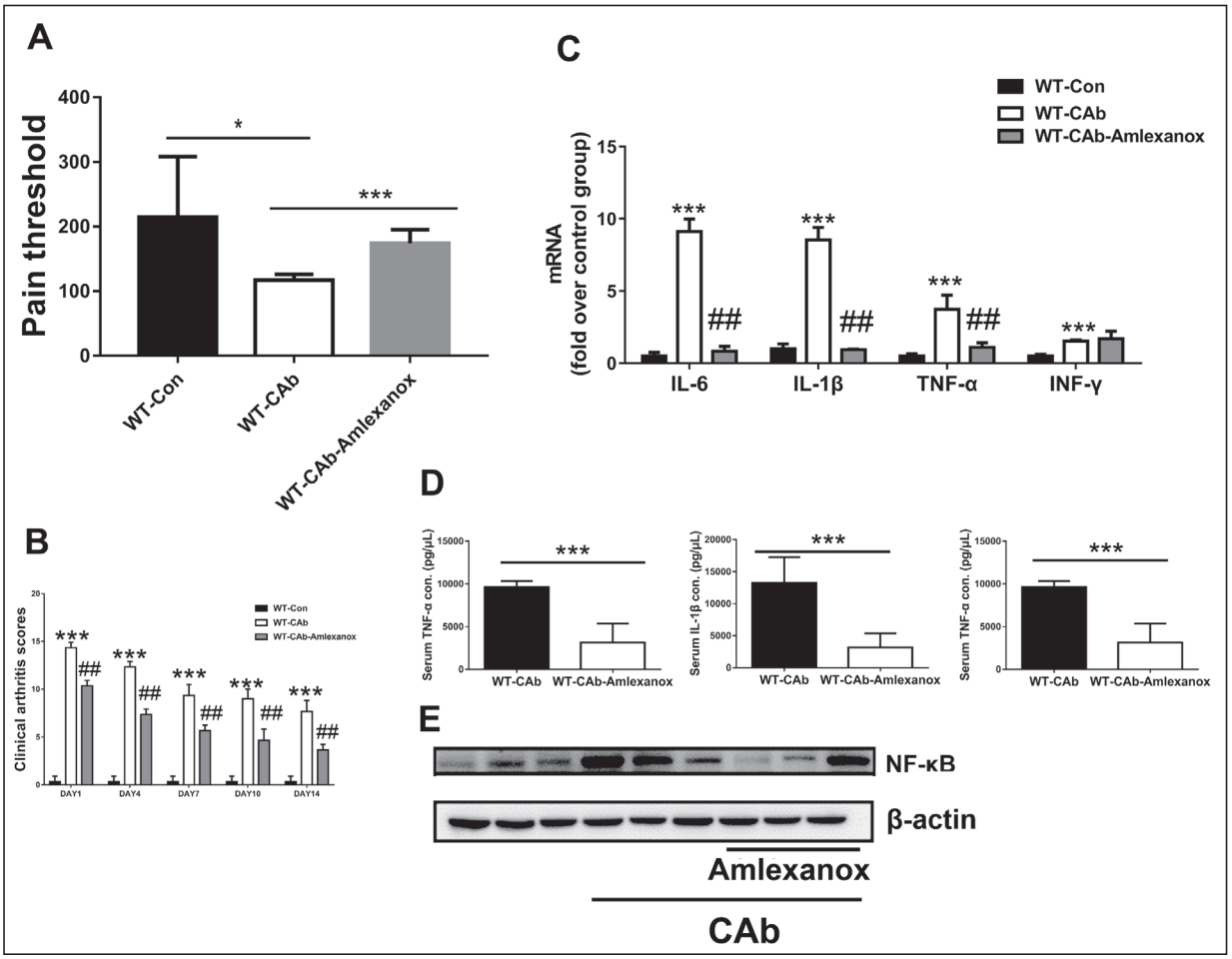

Figure 5. Amlexanox effectively inhibits the inflammatory activity of IKKε in RA. WT mice were randomly divided into three groups: the control group mice were injected with methylcellulose for 5 days; the treatment group and the therapy group mice were injected with 5 mg/kg CAb for 5 consecutive days, then the therapy group mice were given by gavage with 25 mg/kg amlexanox for 2 weeks. ***(A)*** The nociception threshold measured by electronic pressure nociception detector; ***(B)*** At regular intervals, the joint involvement and the stiffness were observed and macroscopic scored. $^{\#\#}p < 0.001$, compared with the WT CIA group; $^{**}p < 0.001$, compared with the WT control group ***(C, D)*** Expression levels of IL-1β, IL-6 and TNF-α in mice tissues and serum; $^{\#\#}p < 0.001$ *vs.* control group; $^{**}p < 0.001$ *vs.* WT CIA group; ***(E)*** NF-κB protein levels were analyzed by Western blot in the plantar tissues from three groups.

flammatory response of RA. Meanwhile, we also demonstrated that IKKε exerted an inflammatory response through activation of the NF-κB signaling pathway, which is consistent with previous findings, since NF-κB plays a role in RA[22-24]. Amlexanox is a listed drug for the treatment of diseases such as oral ulcers; however, in recent years, it is found to effectively inhibits IKKε activity[8,25]. Our results suggested that amlexanox administration could effectively alleviate joint nociception and inflammation in RA mice. In summary, we demonstrate that IKKε plays a key role in promoting inflammatory response in the CAb-induced RA model. Besides, the IKKε inhibitor amlexanox may be an anti-inflammatory potential drug for the treatment of RA possibly by targeting IKKε.

Conclusions

IKKε can promote the expression of pro-inflammatory cytokines including IL-1β, IL-6, TNF-α, by regulating the activation of NF-κB signaling pathway, thus mediating the inflammatory response of RA. IKKε inhibitor amlexanox could effectively inhibit inflammatory activation in RA.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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