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Sirt2 suppresses inflammatory responses in collagen-induced arthritis

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ABSTRACT

Arthritis is a common autoimmune disease that is associated with progressive disability, systemic complications and early death. However, the underling mechanisms of arthritis are still unclear. Sirtuins are a NAD^+-dependent class III deacetylase family, and regulate cellular stress, inflammation, genomic stability, carcinogenesis, and energy metabolism. Among the sirtuin family members, Sirt1 and Sirt6 are critically involved in the development of arthritis. It remains unknown whether other sirtuin family members participate in arthritis. Here in this study, we demonstrate that Sirt2 inhibits collagen-induced arthritis (CIA) using *in vivo* and *in vitro* evidence. The protein and mRNA levels of Sirt2 significantly decreased in joint tissues of mice with CIA. When immunized with collagen, Sirt2-KO mice showed aggravated severity of arthritis based on clinical scores, hind paw thickness, and radiological and molecular findings. Mechanically, Sirt2 deacetylated p65 subunit of nuclear factor-kappa B (NF-κB) at lysine 310, resulting in reduced expression of NF-κB-dependent genes, including interleukin 1β (IL-1β), IL-6, monocyte chemoattractant protein 1(MCP-1), RANTES, matrix metalloproteinase 9 (MMP-9) and MMP-13. Importantly, our rescue experiment showed that Sirt2 re-expression abated the severity of arthritis in Sirt2-KO mice. Those findings strongly indicate Sirt2 as a considerably inhibitor of the development of arthritis.

1. Introduction

Arthritis is a chronic inflammatory disease that leads to progressive joint destruction. Arthritis occurs world-wide, and its prevalence among adults is approximately 1% but varies across racial and ethnic groups [1]. Arthritis is characterized by synovial inflammation and hyperplasia ("swelling"), autoantibody production and systemic features, including cardiovascular, pulmonary, psychological, and skeletal disorders [2]. During the last decades, advances in understanding the pathogenesis of this disease have fostered the development of new therapeutics, with improved outcome. However, critical issues remain unresolved. The cause of arthritis is still largely unknown, and the prognosis is still poor.

The sirtuins are a highly conserved family of NAD^+-dependent enzymes that regulate lifespan in lower organisms. During the past decade, the mammalian sirtuins have been connected to an widening circle of activities that encompass cellular stress resistance, inflammation, genomic stability, carcinogenesis, and energy metabolism [3]. The mammalian sirtuin family has seven members, from Sirt1 to Sirt7. Previous literatures have shown different subcellular localizations for each family member, with Sirt6 and Sirt7 being nuclear proteins, Sirt3, Sirt4 and Sirt5 mitochondrial proteins, whereas Sirt1 and Sirt2 being both in the nucleus and the cytoplasm, in a cell-and tissue-dependent manner [4].

Recently, Niederer et al. [5] reported that Sirt1 was constitutively over-expressed in synovial tissues in patients with arthritis. TNFα-induced overexpression of Sirt1 in arthritis synovial cells contributed to chronic inflammation by promoting pro-inflammatory cytokine production and inhibiting apoptosis [5]. What's more, Sirt1 is mediator of human and mice chondrocyte survival [6–8]. Sirt1 expression decreases with the development of osteoarthritis and the reduction of Sirt1 in chondrocytes causes chondrocyte hypertrophy and cartilage matrix loss [9]. In addition, a current work contributed by Lee et al. [10] evidenced that Sirt6 was critically essential for the development of arthritis. Sirt6 overexpression significantly suppressed inflammatory responses and bone destruction in collagen-induced arthritic mice. Those findings support the notion that sirtuins are critical important in arthritis and other sirtuin family members may play certain roles in arthritis.

Accumulating data indicates that both Sirt1 and Sirt6 can physically interact with and regulate NF-κB [11,12], a pivotal transcriptional factor that regulates arthritis by modulating inflammatory gene expression and cell survival. Sirt1 interacts with and deacetylates p65, leading to decrease in NF-κB activation, whereas Sirt6 interacts with p65 on the chromatin and deacetylates local H3K9,

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repressing inflammatory gene expression [11,12]. As described above, Sirt3, Sirt4 and Sirt5 are mitochondrial proteins. They have no chance to interact with NF-κB physically. Therefore, Sirt2 and Sirt7 are more likely to participate in arthritis *via* interacting with NF-κB. However, only Sirt2 was reported to physically interact with and regulate NF-κB activation by deacetylating p65 [13]. Therefore, we hypothesized that Sirt2 might participate in arthritis by regulating NF-κB.

Here in the present study, we reported that Sirt2 is critical for the development of collagen-induced arthritis (CIA). Sirt2-KO mice developed a more severe arthritic phenotype when immunized with collagen. Mechanically, Sirt2 deacetylated p65K310 and inhibited NF-κB-dependent inflammatory genes expression.

2. Materials and methods

2.1. Animals

Sirt2-knockout (Sirt2-KO) B6.129 mice were purchased from Jackson Lab. The male Sirt2-KO B6.129 mice were crossbred with female DBA/1 mice for five generations to obtain Sirt2-KO DBA/1 mice. Mice were housed in individually ventilated cages in the specific pathogen-free animal facility at the Animal Center (Qilu Hospital, Shandong University). All experimental animals used in this study were maintained under the protocol approved by the Institutional Animal Care and Use Committee at Shandong University.

2.2. Collagen-induced arthritis

8–12 weeks old DBA/1 mice were divided into four groups: (1) WT + mock, (2) WT + CIA, (3) Sirt2-KO + mock, (4) Sirt2-KO + CIA. CIA was induced as described previously [14]. For rescue experiment, Sirt2-KO mice received a single intraarticular (i.a.) delivery of 10 μl of adenovirus (3×10^9 pfu) expressing Sirt2 or GFP to each ankle joint on day 20 (the day before secondary immunization). Clinical arthritis scores were evaluated using a scale of 0–4 for each limb as described previously [14]. Hind paw thickness was measured with an electric caliper placed across the ankle joint at the widest point. An increase in diameter of the arthritic ankle at specific time points over that of day 0 was defined as the paw thickness index, and this value is presented as a percentage [10]. On day 45, all the mice were sacrificed and joint tissues and serum samples were harvested from each animal for end-point histology.

2.3. Micro-CT

Radiography was performed by using a small animal *in vivo* micro-CT scanner (eXplore CT 120 MicroCT; GE Healthcare) at 50-μm resolution. The 3D bone microarchitecture was generated by Microview software (GE Healthcare).

2.4. Adenovirus, infection and treatment of cells

The adenovirus constructs of Sirt2, Sirt2HY and GFP were gifts from Prof. Xi Wu of Second Military Medical University (Shanghai, China). Human arthritic fibroblast-like synoviocyte (FLS) or mouse FLS (2×10^6) were infected with either 100 multiplicity of infection of Ad-GFP, Ad-Sirt2 or Ad-Sirt2HY for 48 h before treated with TNF-α (10 ng/ml, Sigma) for 3 h. All treatments were performed in serum-free medium.

2.5. Quantitative real time RT-PCR (q-PCR)

Mice paws were snap-frozen in liquid nitrogen and mechanically homogenized in buffer for total RNA extraction. Total RNA of joint tissues or FLS was prepared with TRIzol (Invitrogen). cDNA was synthesized from 1 μg of RNA with One Step RT-PCR Kit (TaKaRa). q–PCR was performed with the SYBR Green (TaKaRa) detection method on an ABI-7500 RT-PCR system (Applied Biosystems). The primers were listed in Supplementary Table 1.

2.6. Cytokine expression analysis by ELISA

Serum levels of IL-1β, IL-6, TNF-α, MCP-1, IL-10, IL-17 and IL-33 were examined by using Quantikine ELISA kits (R&D Systems) according to the manufacturer's protocol.

2.7. Statistical analysis

Statistical differences among groups were determined using either Student's *t* test (for two groups) or one-way ANOVA (for more than two groups) using Graph-Pad Prism Software. The values were expressed as mean ± SEM of at least three independent experiments. *p* values of less than 0.05 were considered statistically significant. Additional information on materials and methods is available in Supplementary information.

3. Results

3.1. Sirt2-deficiency increases the severity of collagen-induced arthritis (CIA)

To study the role of Sirt2 in the development of arthritis, we first analyzed Sirt2 expression level in the joint tissues of arthritic mice. Sirt2 protein and mRNA levels were markedly reduced compared to control mice when immunized with collagen (Fig. 1A and B; Supplementary Fig. 1), suggesting that Sirt2 may play certain roles in arthritis.

To test whether Sirt2 deficiency contributes to arthritis disease, we used Sirt2-KO mice (Fig. 3A shows the efficiency of knockout) in this study. No idiopathic arthritis was observed in our study in both WT and Sirt2-KO mice. However, when Sirt2 was knocked out, mice suffered arthritis as early on day 23 (two days after secondary immunization), while the WT mice occurred arthritis from day 25 (Fig. 1C). Even though all WT and Sirt2-KO mice developed arthritis, Sirt2-KO group reached a 100% cumulative incidence on day 34 whereas WT group reached a 100% cumulative incidence on day 37 (Fig. 1D). Those findings indicated that Sirt2-deficiency facilitated the development of collagen-induced arthritis. However, for the reason that all WT and Sirt2-KO mice developed arthritis before the termination of experiment, we could not conclude whether Sirt2-deficiency affected the cumulative incidence.

Furthermore, arthritis scores and hind paw thickness were evaluated every five days from day 20 to day 45. We found that Sirt2-KO mice developed more severe swelling, erythema and joint rigidity in the hind paws (Fig. 1D–F and data not shown). Significant differences in the arthritis scores and hind paw thickness were observed from day 25 (Fig. 1D and E). We next evaluated structural changes in the hind paws on day 45 before the mice were sacrificed. The micro-CT images of hind paws in Sirt2-KO arthritic mice showed typical changes, including articular destruction, joint displacement, and irregular bony proliferation cloaking the entire ankle region. However, WT arthritic mice showed markedly less bone destruction in the hind paws (Fig. 1F). Those data indicated that Sirt2 considerably participated in arthritis and Sirt2-KO significantly promoted the development of pathologic arthritic disease and increased disease severity.

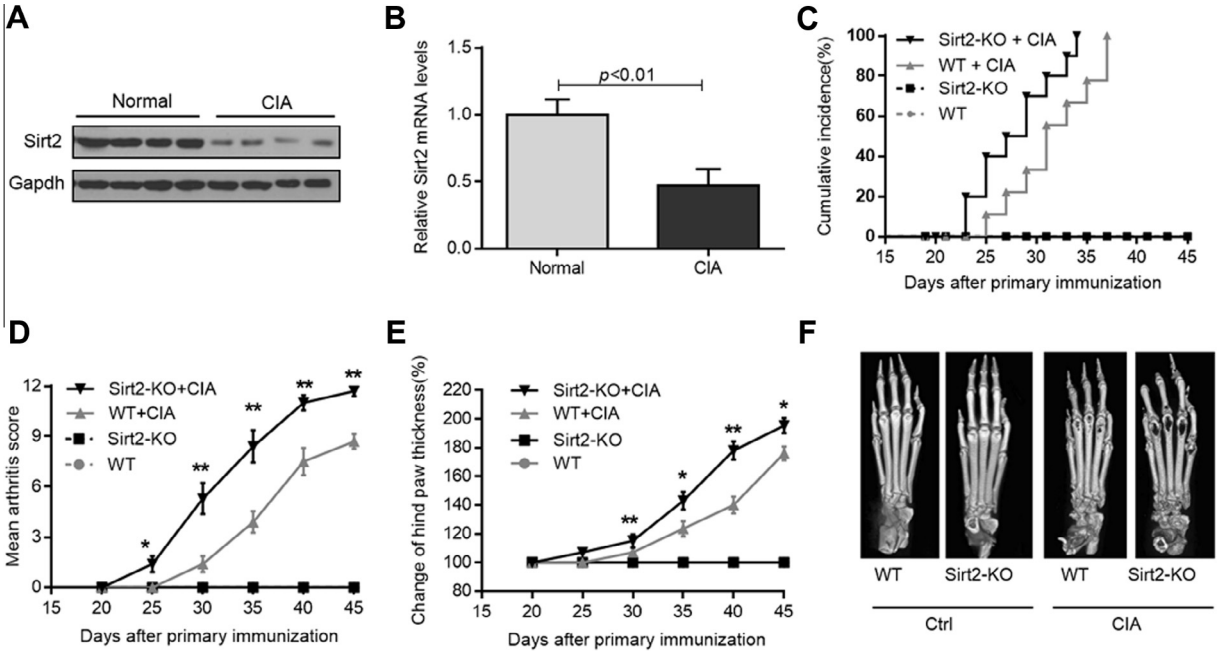

Fig. 1. Sirt2-deficiency aggravates severity of collagen-induced arthritis (CIA). DBA/1 mice were immunized intradermally with type II collagen (CII) on day 0 and were given a booster by intradermal injection with CII on day 21. Joint tissues were prepared from normal or CIA mice on day 45 and subjected to western blot (A) and q-PCR (B) analysis. WT and Sirt2-KO DBA/1 mice were immunized as before. (C) The cumulative incidence of arthritis. (D) The mean arthritis score. (E) The change of hind paw thickness. (F) The representative micro-CT images of the hind paws on day 45. $n = 10$ in each group. $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs.* WT + CIA.

3.2. Sirt2-deficiency increases pro-inflammatory cytokine levels

Pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6 play pivotal roles in the pathogenesis of arthritis, and they affect each other. We therefore measured pro-inflammatory cytokine levels in the serum by ELISA. Compared to WT arthritic mice, significantly increase in IL-1β, IL-6, TNF-α, IL-17, IL-33 and MCP-1 was observed in the serum of Sirt2-KO arthritic mice (Fig. 2A–F). Since arthritis is a systemic inflammatory disease, we wanted to know the inflammatory status of local tissues. Therefore, we analyzed the mRNA levels of pro-inflammatory cytokines (especially those controlled by NF-κB) in joint tissues by q-PCR. Consistent with the serum ELISA results, the relative mRNA levels of IL-1β, TNF-α and MCP-1 were markedly increased in Sirt2-KO arthritic mice (Supplementary Fig. 2). In the joint tissues, synovial fibroblasts are key players in joint damage through secreting matrix metalloproteinase (MMPs) and cathepsins [15]. We found that the levels of MMP-9 and MMP-13 were significantly up-regulated in Sirt2-KO arthritic mice (Supplementary Fig. 2).Taken together, those findings demonstrated that Sirt2-deficiency up-regulated pro-inflammatory cytokines and MMPs.

3.3. Sirt2 deacetylates p65 and regulates TNF-α-induced expression of inflammatory genes

Our results have strongly demonstrated that Sirt2 was a critical regulator in arthritis. However, the underling mechanism remains unclear. In the synovial cells of patients with arthritis, activation of the NF-κB pathway leads to the transactivation of a multitude of responsive genes that contribute to the inflammatory phenotype, including TNF-α from macrophages, MMPs from synovial fibroblasts and chemokines that recruit immune cells to the inflamed pannus [2]. This is largely a consequence of activation of the 'canonical' NF-κB pathway that involves heterodimers of p50/p65 [16]. Three works have shown that Sirt2 interacts with and deacetylates p65 (K310) subunit of NF-κB, and that Sirt2 regulates inflammation [13,17,18]. Therefore, we wanted to know whether Sirt2 participated in CIA by regulating NF-κB. To test this hypothesis, we analyzed protein levels of acetylated p65 (K310) and total p65 of the joint tissues of normal and CIA mice. We found that CIA significantly increased the acetylation level of p65, whereas the total protein level was not affected. When Sirt2 was knocked out, the acetylation level of p65 was significantly increased in both normal and CIA mice (Fig. 3A). Those findings implicated that Sirt2 regulated NF-κB at basal and CIA conditions by regulating acetylation of p65.

To confirm this conclusion, we used an *in vitro* arthritic model, in which we treated Sirt2-KO mouse FLS with TNF-α, a classical activator of NF-κB. We found that TNF-α decreased the level of Sirt2 and hyperacetylated p65 at lysine 310 (Fig. 3B). However, IL-17, another reported activator of NF-κB did not appear to decrease Sirt2 level or increase p65 acetylation rate in our system (data not shown). We next infected Sirt2-KO mouse FLS with Ad-GFP, Ad-Sirt2 or Ad-Sirt2HY (catalytic mutant) before TNF-α treatment. Sirt2 overexpression inhibited TNF-α-induced p65 hyperacetylation, but this effect was not observed in mouse FLS-overexpressed with Sirt2HY (Fig. 3C). Taken together, our data demonstrated that Sirt2 regulated NF-κB by deacetylating p65 at lysine 310 in joint tissues and mouse FLS.

As Sirt2 could deacetylate p65 and p65 acetylation is essential for the activation of NF-κB, we further investigated whether Sirt2 overexpression could inhibit NF-κB-dependent gene expression in Sirt2-KO mouse FLS. q-PCR results showed that Sirt2 overexpression significantly reduced the expression of TNF-α-induced NF-κB-dependent genes, including IL-1β, IL-6, RANTES, MCP-1, MMP-9 and MMP-13 (Fig. 3D–I), all of which are important for the development of arthritis. Similar results were observed in human arthritic FLS (Supplementary Fig. 3). Those results demonstrated that Sirt2 inhibited TNF-α-induced NF-κB-dependent gene expression.

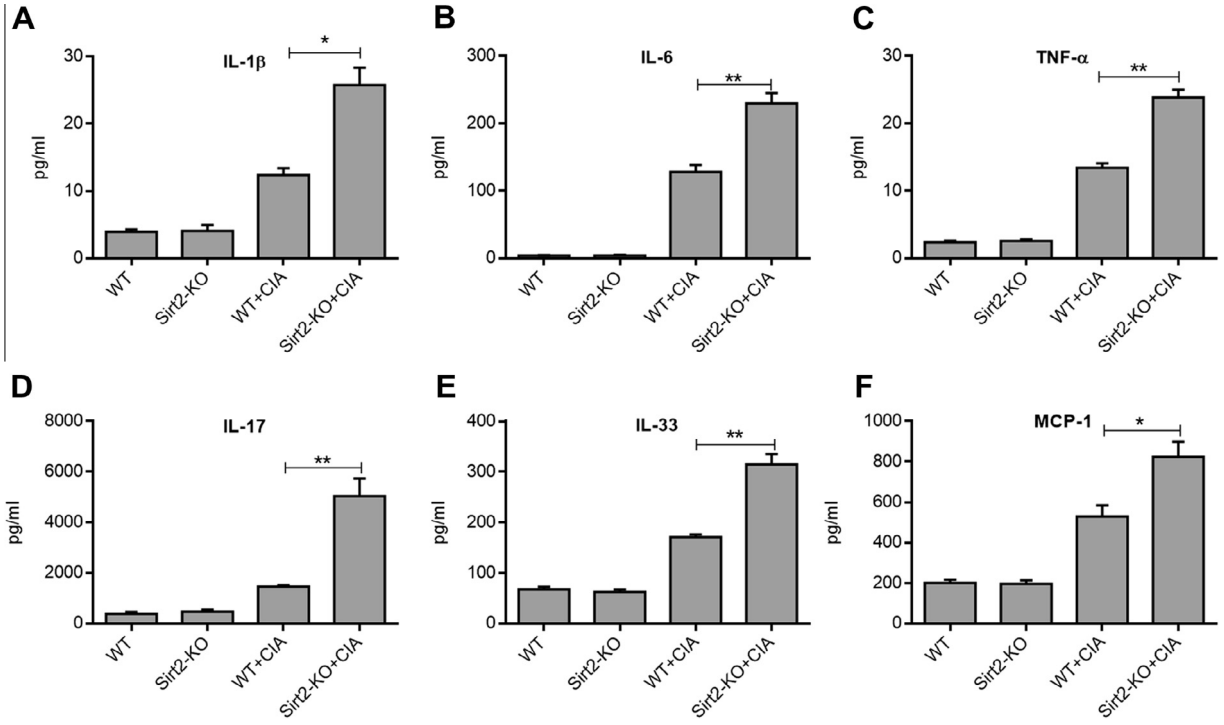

Fig. 2. Sirt2-deficiency up-regulates serum levels of pro-inflammatory cytokines. WT and Sirt2-KO DBA/1 mice were immunized as before. On day 45, serum samples were collected from normal and CIA mice. Various arthritis-associated cytokines in the serum were measured by specific ELISA assay. (A) Interleukin 1β (IL-1β); (B) IL-6; (C) TNF-α; (D) IL-17; (E) IL-33; (F) Monocyte chemoattractant protein 1(MCP-1). $n = 5$ in each group. $^{*}p < 0.05$ and $^{**}p < 0.01$.

3.4. Sirt2 rescue reduces severity of CIA in Sirt2-KO mice

Our findings have shown that Sirt2 is important for the development of arthritis and Sirt2 deficiency increases the severity of collagen-induced arthritis. We next investigated whether Sirt2 rescue could block the development of arthritis and decrease the severity. Therefore, we performed rescue experiments by using a single intraarticular delivery of 10 μl of adenovirus (3×10^9 pfu) expressing Sirt2 to each ankle joint of Sirt2-KO mice on day 20 post primary immunization (the day before secondary immunization). Sirt2 overexpression was observed after Ad-Sirt2 injection into the joint tissue mice, and it maintained for more than two weeks (Supplementary Fig. 4).

Our rescue results showed that Sirt2 re-expression significantly reduced the mean arthritis scores and hind paw thickness in Sirt2-KO mice (Fig. 4A and B). In addition, our histopathologic analysis using hematoxylin and eosin staining of ankle joints also revealed a marked reduction of cell infiltration, synovial hyperplasia and bone erosion in mice injected with Ad-Sirt2 (Fig. 4C). Further assessment by micro-CT showed that Sirt2 rescue abated bone destruction (Fig. 4D). Finally, we performed ELISA assay to detect the cytokine levels in serum, and we found that Sirt2 rescue significantly reduced the levels of pro-inflammatory cytokines (Supplementary Fig. 5). Those results demonstrated that Sirt2 rescue markedly repressed the development and severity of arthritis in Sirt2-KO mice.

4. Discussion

The data presented here reveals a novel role for Sirt2 as an arthritis suppressor. Firstly, we found that Sirt2 decreased in the joint tissues of mice with CIA. By using *in vivo* and *in vitro* evidence, we have demonstrated that Sirt2 deficiency led to severe arthritic phenotype in CIA. Mechanically, Sirt2 repressed acetylation of p65 at lysine 310 and inhibited expression of inflammatory genes. Importantly, Sirt2 rescue inhibited arthritis development and reduced the severity in Sirt2-KO mice.

Arthritis is a frequent chronic inflammatory disease. It undoubtedly belongs to autoimmune diseases with a complex network of interactions between adaptive and innate immunity. Improved knowledge of the numerous factors involved in arthritis led to considerable advances over the last 15 years. The introduction of targeted treatments deeply changed the face of the disease, and secondarily produced crucial information on the mechanisms underlying arthritis [19].

Sirtuins are a family of NAD^+-dependent class III deacetylase that are reported to deacetylate both histone and non-histone proteins. Several members of sirtuin family were demonstrated to participate in inflammation. Especially, current studies have shown that Sirt1 and Sirt6, two predominantly nuclear members, play considerable roles in human and mouse arthritis models *via* regulating inflammation, chondrocyte survival and osteoclasts differentiation [5–8,10]. Resveratrol, a potential activator of Sirt1, was reported to inhibit TNF-α-induced inflammation in fibroblast and modulate mouse collagen-induced arthritis [20,21]. Interestingly, both Sirt1 and Sirt6 can interact with NF-κB and deacetylate or destabilize it respectively, resulting in neutralized NF-κB activation and reduced inflammation [11,22].

Sirt1 can physically interact with the p65 subunit of NF-κB and inhibits transcription by deacetylating p65 at lysine 310 [12,23]. p65 acetylation has an important role in the regulation of NF-κB-dependent transcription for a subset of target genes. Loss of Sirt1 leads to hyperactive NF-κB signaling and expression of its downstream genes [22]. Both Sirt1 and Sirt2 are located in cytoplasm and nucleus, and Sirt2 was also reported to physically interact with and deacetylate p65 at lysine 310 [13,17], implicating that Sirt2 may play a similar role as Sirt1 in arthritis and other inflammatory diseases.

Indeed, we firstly found that Sirt2 mRNA and protein levels decreased in the joint tissues of mice with CIA (Fig. 1). This prompted

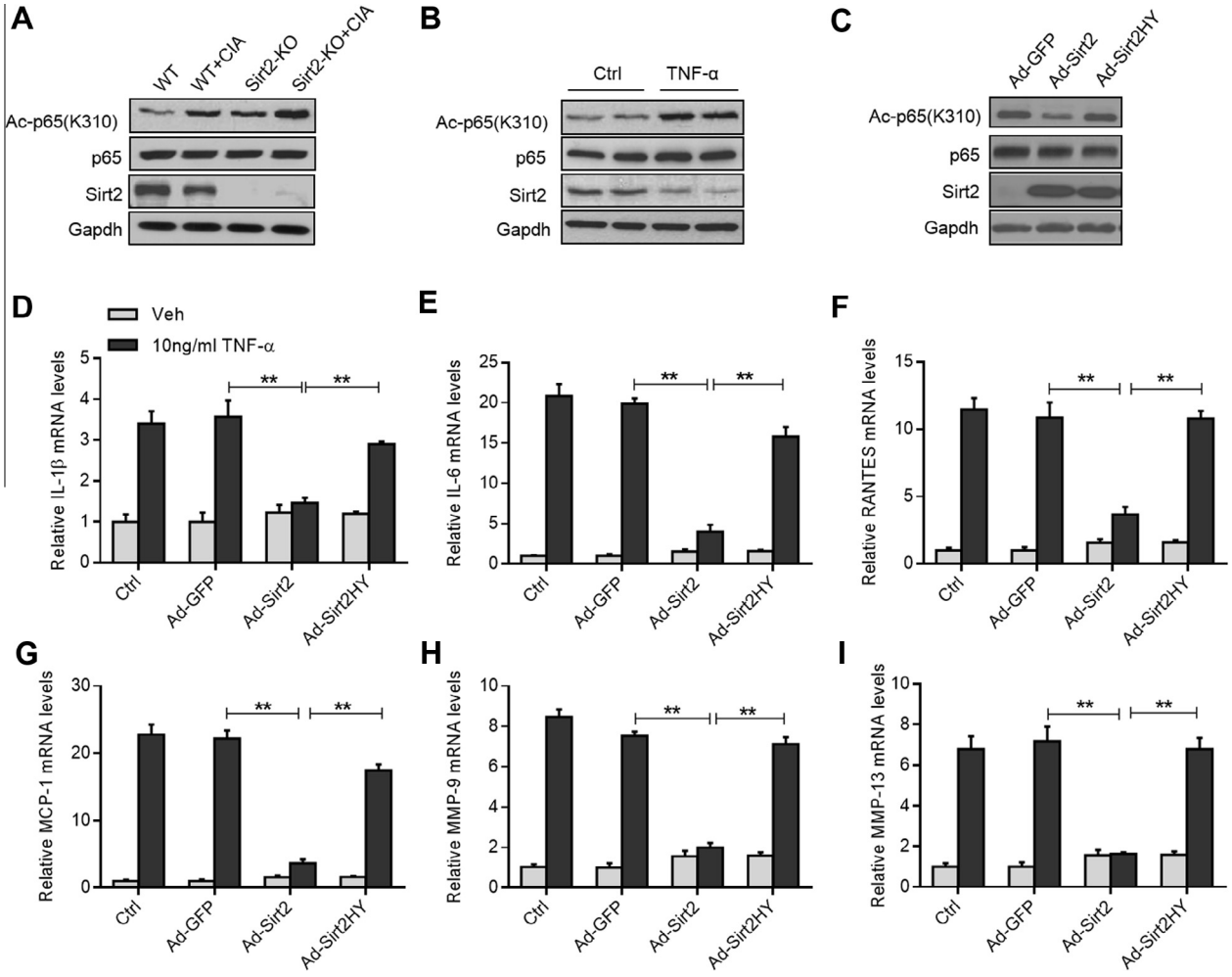

Fig. 3. Sirt2 deacetylates p65 and inhibits TNF-α-induced expression of pro-inflammatory genes. (A) Sirt2 deacetylates p65 at lysine 310 in CIA mice. On day 45, join tissues were collected from normal and CIA mice, and the total proteins were extracted and subjected to western blot analysis with indicated antibodies. (B) TNF-α inhibits Sirt2 expression and promotes acetylation of p65. Mouse FLS were treated with TNF-α (10 ng/ml) for 3 h. (C) Sirt2 inhibits p65 acetylation in FLS. Sirt2-KO mouse FLS were infected with 100 multiplicity of infection of Ad-GFP, Ad-Sirt2 or Ad-Sirt2HY for 48 h and then cells were treated with TNF-α (10 ng/ml) for 3 h. (D–I) Sirt2 inhibits pro-inflammatory gene expression in FLS. Sirt2-KO mouse FLS were treated as in (C). Total RNA was subjected to q-PCR analysis. (D) IL-1β; (E) IL-6; (F) RANTES; (G) MCP-1; (H) MMP-9; (I) MMP-13. $^{*}p < 0.05$ and $^{**}p < 0.01$.

us to investigate the role of Sirt2 in CIA, and our results showed that loss of Sirt2 aggravated the severity of arthritis with earlier incidence, more severe swelling, erythema, and joint rigidity in the hind paws (Fig. 1). Significantly, Sirt2-deficiency up-regulated pro-inflammatory cytokine levels both in serum and in joint tissues of mice with CIA. We showed that Sirt2-deficiency increased the concentrations of TNF-α, IL-1β, IL-6, IL-17, IL-33 and MCP-1 at the local tissue and/or systemic levels (Fig. 2 and Supplementary Fig. 1). These findings are in consistence with previous studies showing that inhibition of NF-κB by Sirt6 overexpression suppressed the production of pro-inflammatory cytokines in CIA mice [10]. Each cytokine up-regulates the production of the others [1,2,24,25]. Therefore, these suppressive effects of Sirt2 on pro-inflammatory cytokine production might ameliorate inflammatory responses and joint destruction in CIA mice. Moreover, Sirt2-deficiency developed more severity of progressive joint destruction (Fig. 1), this may be at least partly attributed to the disorder of MMPs, including MMP-9 and MMP-13 (Supplementary Fig. 1), as previous studies have demonstrated that MMPs are critical for FLS-induced joint damage [15].

For the mechanism, firstly we found that CIA down-regulated Sirt2, resulting in hyperacetylation of p65 at lysine 310 and activation of NF-κB in the joint tissues (Fig. 3). Using TNF-α-based inflammatory FLS model, we demonstrated that inflammatory stimulus reduced Sirt2 level and hyperacetylated p65, while Sirt2 but not Sirt2HY overexpression can inhibit p65 acetylation and activation of NF-κB. Those findings demonstrate that Sirt2 inhibits CIA by suppressing NF-κB activation. When this work was under preparation, two works reported the anti-inflammatory functions of Sirt2. Pais et al. [18] found that Sirt2 is an abundant deacetylase in the brain, and that Sirt2 is a major inhibitor of microglia-mediated inflammation and neurotoxicity. Using *in vitro* and *in vivo* models, they showed that Sirt2 functioned as a 'gatekeeper', preventing excessive microglial activation through p65K310 deacetylation. Upon infection, Sirt2 translocates from the cytosol to the chromatin of the host at the transcription start sites of a subset of genes [26]. Sirt2 mediated deacetylation of H3K18 and repressed gene expression when cells were infected with *Listeria monocytogenes*. This process was dependent on activation of the cell surface receptor Met and downstream phosphatidylinositol 3-kinase (PI3K)/AKT signaling [26]. Those findings in combination with the present data strongly indicated Sirt2 as inhibitor of inflammation. Both Pais's work and our data indicated that inflammatory factors (LPS, collagen and TNF-α) reduced the levels of Sirt2, which may account for the decrease in Sirt2 anti-inflammatory functions. However, how Sirt2 is down-regulated remains unknown. In our system, Sirt2 protein level was reduced as early as 3 h post TNF-α treatment. This is interestingly and

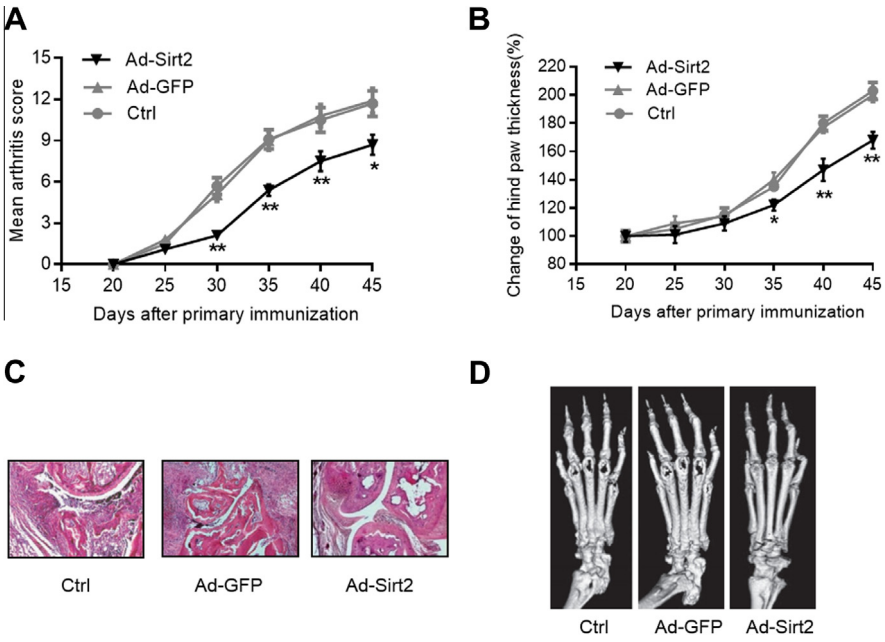

Fig. 4. Sirt2 rescue reduces severity of CIA in Sirt2-KO mice Sirt2-deficient DBA/1 mice were immunized intradermally with CII on day 0 and given a booster by intradermally injection with CII on day 21. The day before secondary immunization, Sirt2-KO mice received a single intraarticular delivery of 10 μl of adenovirus (3×10^9 pfu) or PBS to each ankle joint. (A) The mean arthritis score. (B) The change of hind paw thickness. (C) Hematoxylin and eosin staining of ankle joints. (D) The representative micro-CT images of the hind paws on day 45. $n = 10$ in each group. $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs.* Ad-GFP.

may suggest that Sirt2 is regulated at both transcriptional level and post-translational level depending upon the term conditions. Our further work should be carried out to explore the underling mechanisms by which Sirt2 is regulated at transcriptional and post-translational levels in inflammatory conditions.

In summary, this study demonstrates that the cytoplasmic sirtuin Sirt2 directly controls the activation of NF-κB at the level of post-translation and, hence, the development of arthritis and progressive joint destruction.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.153.

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