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siRNA-mediated *c-Rel* knockdown ameliorates collagen-induced arthritis in mice

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ABSTRACT

Previous studies have shown that inflammatory mediators involved in the development of rheumatoid arthritis (RA) are regulated by the Rel/nuclear factor-κB (Rel/NF-κB) transcription factor family. c-Rel, a member of the Rel/NF-κB family that is preferentially expressed by immune cells, is a risk factor for several inflammatory diseases including RA. In the current study, we investigated whether targeting c-Rel can be used to treat collagen-induced arthritis, an animal model for RA. c-Rel specific siRNA (siRel) delivered by nanoparticles was used to knockdown the expression of c-Rel. Our results showed that siRel treatment significantly ameliorated collagen-induced arthritis. Further study revealed that c-Rel expression in the dendritic cells and macrophages from mice treated with siRel was significantly down-regulated. Consistent with the phenotypical result, the expression of inflammatory cytokines TNF-α, IL-1β, IL-6, IL-12 and IL-23 by peritoneal macrophages and splenocytes were significantly decreased. In addition, attenuated systemic and collagen-specific Th1 and Th17 immune responses were observed. Furthermore, we found that the expression of inflammatory cytokines was significantly down-regulated and the infiltration of $CD3^+$ T cells and $F4/80^+$ macrophages was markedly reduced in hind paws of mice treated with siRel. Collectively, our study provides strong evidence that siRNA-mediated c-Rel knockdown can suppress the development of collagen-induced arthritis in mice. Therefore, blocking c-Rel may represent an attracting strategy for the treatment of human rheumatoid arthritis.

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovial inflammation, cartilage and bone destruction [1–3]. The incidence of RA increases with age, and women are more susceptible to the disease than men. Approximately 0.5–0.8% of people suffer from RA worldwide [1,2]. Although the etiology of RA is complicated, it is believed that susceptibility genes together with environmental insults, epigenetic modifications, or post-translational modifications give rise to the activation of autoimmune response during the development of RA [4–8]. Activated dendritic cells and macrophages migrate into the normally sparsely populated synovial compartment and secret inflammatory cytokines and chemokines, which further activate endothelial cells and attract autoreactive T and B cells to accumulate into the synovial compartment. Accumulated T cells, B cells, monocytes, macrophages and local activated fibroblasts ultimately trigger osteoclast generation through the receptor activator of nuclear factor κB ligand (RANKL) and RANK pathway [1]. Cumulative evidence suggests that TNF-α, IL-6, IL-1β, IL-12, IL23, IL-17 and IFN-γ play critical roles in the inflammatory milieu of the synovial compartment [9–13]. More importantly, most of these inflammatory mediators are regulated by the Rel/nuclear factor-kB (Rel/NF-κB) transcription factor family [14–19]. Thus, Rel/NF-κB is a potential target for therapeutic intervention in RA.

Anti-inflammatory drugs that have been widely used clinically such as methotrexate and glucocorticoids are at least partially suppressing the Rel/NF-κB activation through the inhibition of IκBα degradation or the induction of IκB synthesis [20,21]. However, because most Rel/NF-κB proteins are ubiquitously expressed and are involved in a variety of biological processes not related to autoimmunity, nonspecific inhibition of the whole Rel/NF-κB proteins have significant side effects when used for a long period to control chronic inflammation. Unlike other

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members of the NF-κB family that are ubiquitously expressed, c-Rel is preferentially expressed by inflammatory cells [22,23]. Moreover, c-Rel regulates the expression of inflammatory cytokines including TNF-α, IL-6, IL-1β, IL-12, IL23, IFN-γ and IL-17A [24–27]. c-Rel deficient mice do not suffer from developmental problems or infectious diseases and are resistant to the development of collagen-induced arthritis (CIA), suggesting that c-Rel is required for RA development [28]. In addition, genome-wide association studies (GWAS) have also uncovered that the *Rel* locus, encoding c-Rel, is a risk factor for RA [29].

In the current study, we evaluated the anti-inflammatory effects of c-Rel specific siRNA delivered by nanoparticles during the development of CIA. Our results showed that siRNA-mediated c-Rel knockdown can significantly down-regulate the expression of multiple inflammatory cytokines and suppress the development of CIA. Therefore, c-Rel may be a potential therapeutic target for the treatment of RA.

2. Materials and methods

2.1. Animals

8 to 10-week-old female DBA/1 mice were used in the experiments and kept under pathogen-free conditions at the animal core facility of the Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences. All procedures were preapproved by the Institutional Animal Care and Use Committee.

2.2. Materials

TRIzol was purchased from Life Technologies (USA). M-MLV reverse transcriptase was obtained from Promega (USA). Collagenase was purchased from Sigma-Aldrich (USA). THUNDERBIRD SYBR qPCR mix was purchased from TOYOBO (Japan). Anti-CD3 and anti-CD28 were purchased from eBioscience (USA). ELISA kits for the detection of inflammatory cytokines TNF-α, IL-1β, IL-6, IL-12, IL-23, IL-17A and IFN-γ were also purchased from eBioscience (USA). Goat anti-Mouse IgG1 and IgG2a were purchased from Sigma-Aldrich (USA). Chicken type II collagen, incomplete and complete Freund's adjuvant were all purchased from Chondrex (USA). FITC-conjugated anti-CD11c, PE-conjugated anti-F4/80, APC-conjugated anti-CD3 and APC-conjugated goat anti-rabbit antibody were all obtained from Biolegend (USA). HRP-conjugated donkey anti-goat antibody and purified anti-c-Rel antibody were purchased from Santa Cruz Biotechnology (USA). siRNA that specifically targeting mouse c-Rel (siRel) (sense: 5′-CAACCGGAC AUACCCGUCUdTdT-3′, anti-sense: 5′-AGACGGGUAUGUCCGGUU GdTdT-3′) and negative control siRNA (siNC) (sense: 5′-UUCUCCGAACGUGUCACGUdTdT-3′, anti-sense: 5′-ACGUGACACG UUCGGAGAAdTdT-3′) were synthesized by Shanghai GenePharma Co. Ltd. (Shanghai, China). 2′-O-Methyl oligonucleotide modification was introduced to siRNA to make the modified antisense oligo more stable against general base hydrolysis and nucleases, as well as increase the melting temperature of duplexes.

2.3. Preparation of siRNA loaded PEG-PLL-PLLeu nanoparticles

Poly (ethylene glycol)-b-poly (L-lysine)-b-poly(L-leucine) (PEG-PLL-PLLeu) triblock polypeptide were synthesized as previously reported and dissolved in DEPC water (1 mg/ml) to form nanoparticles [30,31]. Desired amount of siRNA (siRel or siNC) in DEPC water were then mixed with equal volume of PEG-PLL-PLLeu nanoparticles by gentle pipetting. Nanoparticles loaded with siRNA were allowed to stand at room temperature for 30 min before use.

2.4. Induction and therapeutic treatment of CIA

CIA was induced in DBA/1 mice by intradermal immunization with chicken type II collagen (100 μg) emulsified in complete Freund's adjuvant at the base of the tail, followed by a booster immunization with chicken type II collagen (100 μg) emulsified in incomplete Freund's adjuvant. As soon as disease onset, mice were assigned to siNC or siRel group in a balanced manner, to achieve a similar distribution of severity and day of onset in each group. siNC or siRel (500 pmol) loaded nanoparticles were intraperitoneal injected every other day and continued for 22 days. The severity of CIA was assessed as previously described [32]. Briefly, the score was the cumulative score of all four paw scores on a scale of 0–16, where each paw was scored as follows: 0 = normal paw; 1 = one toe inflamed and swollen; 2 = more than one toe, but not entire paw inflamed and swollen, or mild swelling of entire paw; 3 = entire paw inflamed and swollen; 4 = very inflamed and swollen or ankylosed paw. To obtain histological profiles of the knees, hind paws were fixed in 10% formalin, embedded in paraffin, sectioned, stained with hematoxylin/eosin, and examined by microscopy.

2.5. Intracellular staining of c-Rel

To check the silencing effects of siRel treatment in vivo, peritoneal macrophages were isolated on day 22 after treatment and stained with PE-conjugated antibody to F4/80. Total cells from the spleen were also isolated on day 22 after treatment and stained with FITC-conjugated antibody to CD11c. Cells were then fixed with 4% formaldehyde for 30 min, permeabilized with perm buffer for 10 min and incubated for 30 min with a 1:100 dilution of anti-c-Rel antibody. Cells were then washed three times and incubated for 30 min in 1:200 dilution of APC-conjugated goat anti-rabbit antibody in perm buffer. Stained cells were washed three times and analyzed on CytoFLEX flow cytometry system (Beckman Coulter, Inc.). Data was analyzed with the FlowJo software.

2.6. Detection of inflammatory cytokines

For the detection of LPS induced cytokines, peritoneal macrophages and splenocytes were isolated on day 22 after treatment. Cells were then treated with LPS (1 μg/ml) for 24 h and culture supernatants were collected; For the detection of cytokines produced by T cells, total splenocytes and popliteal lymph node cells were cultured at 1×10^7/ml in complete RPMI 1640 culture medium either alone or in the presence of anti-CD3 (0.5 μg/ml) plus anti-CD28 (0.5 μg/ml) or heat denatured chicken type II collagen (50 μg/ml). Culture supernatants were collected 48 h later. Supernatants were analyzed for levels of IL-1β, TNF-α, IL-6, IL-17A, IFN-γ, IL-12 and IL-23 by quantitative enzyme-linked immunosorbent assay (ELISA) per manufacturer's recommendations.

2.7. Detection of serum autoantibody

In order to detect the anti–type II collagen antibodies in the serum, heat denatured chicken type II collagen (10 μg/ml) were coated on ELISA plates overnight at 4 °C. After blocking with 4% BSA, serum from treated mice were added in 1:2 dilution and incubated for 2 h at room temperature. Goat anti-mouse IgG1 or IgG2a were used at 1:750 dilution as the first detection reagent. HRP-conjugated donkey anti-goat antibody was used at 1:2000 dilution as the secondary detection reagent. The concentration of anti–type II collagen antibodies was displayed as optical density.

2.8. Analysis of inflammatory cell subsets in the paw

After sacrificing the mice on day 22 after treatment, paws were cut out and digested with 1 mg/ml collagenase in RPMI 1640 for 1 h at 37 °C. Cells were filtered through a cell strainer with a 70 μm nylon mesh, followed by washing in complete RPMI 1640 medium. Cells were then stained with PE-conjugated anti-F4/80 and APC-conjugated anti-CD3 and analyzed on CytoFLEX flow cytometry system.