Effects and mechanisms of potent caspase-1 inhibitor VX765 treatment on collagen-induced arthritis in mice

Y. Zhang, Y. Zheng

Department of Rheumatology and Immunology, Beijing Chao-Yang Hospital, Capital Medical University, Beijing, China.

Abstract

Objective

VX765, a potent and selective caspase-1 inhibitor, inhibits the release of IL-1, IL-18 and IL-33. In this study we investigated the effect of VX765 treatment on collagen-induced arthritis (CIA).

Methods

Twenty-four mice were randomly divided into three groups of 8: Normal (wild-type), CIA and VX765 (CIA with VX765 treatment) groups. Mice in the VX765 group received intraperitoneal injection of VX765 (100 mg/kg, twice daily) starting at the day of the booster immunisation (week 3) for a duration of 4 weeks. At the end of experiments (week 7), joints clinical scores, radiographic scores and histologic scores were evaluated. Serum IL-1β, IL-18 and IL-33 levels were assessed by ELISA.

Results

VX765 prophylactic treatment significantly reduced joints clinical scores, suppressed bone marrow oedema and synovitis at the early stage of CIA, prevented bone erosion in progressive CIA, and decreased histologic scores and serum cytokine levels.

Conclusion

VX765 prophylactic treatment ameliorated the severity and progression of CIA. These findings suggest that caspase-1 is a potential therapeutic target for RA treatment.

Key words

rheumatoid arthritis, caspase-1 inhibitor, pro-inflammatory cytokines, VX765

Yongfeng Zhang, MM
Yi Zheng, MM

Please address correspondence to:
Yi Zheng,
Department of Rheumatology and Immunology,
Beijing Chao-Yang Hospital,
Capital Medical University,
8 Gongren Tiyuchang Nanlu,
Chaoyang District,
Beijing 100020, China.
E-mail: scitougao@sina.com

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Introduction

Rheumatoid arthritis (RA), a systemic autoimmune disorder, is a chronic, progressive inflammatory polyarthritis. Pro-inflammatory cytokines, such as IL-1β, IL-18, IL-33 and IL-6, play important roles in the pathogenesis of RA. IL-1β is associated with joint damage. Clinical studies on RA patients have shown that intracellular IL-1β expressions in $CD34^+$ cells increased compared to controls. IL-1β levels were correlated with the expression of C-reactive protein (CRP), reactive oxygen species (ROS), and Pulse Wave Velocity (PWV). These new findings suggest that IL-1β is also related with system involvement, such as atherosclerosis and vascular damage (1). Preclinical data has provided evidence that P2X7 is an upstream signalling pathway of IL-1β, IL-18 andIL-33. P2X7 antagonist in inhibits cytokines release, suppresses synovial inflammation and radiographical damage, and reduces mechanical hyperalgesia (2). IL-1β has also been linked to autoinflammatory disorders, such as cryopyrin-associated periodic syndromes (CAPS) and familial Mediterranean fever (FMF). In the past decade, many clinical studies have confirmed that the anti-IL-1β therapy in CAPS achieved clinical remission, modified the natural history and the outcomes (3). Therefore, IL-1β is a therapeutic target in the treatment of inflammatory diseases.

Caspase-1, formerly known as interleukin (IL)-1-converting enzyme, is a cysteine protease. Activated caspase-1 is a major upstream regulator of pro-inflammatory cytokines by cleaving pro-IL-1β, pro-IL-18 and pro-IL-33 into biologically active pro-inflammatory cytokines, and involves in the development and progression of RA (4-7). IL-1 inhibitor (soluble IL-1 receptor and IL-1 receptor antagonist) and IL-6 monoclonal antibodies can reduce synovitis severity and prevent or delay cartilage damage, and have been used in the clinical treatment of RA (8-10). However, some patients are not responsive or have strong immune response to the treatment of biological agents. In addition, the short half-life and expensive cost of biological agents prevent the wide application of these protein products. Non-peptide small-molecule inhibitors induce less immune response and exhibit good pharmacokinetic properties. Thus, blockage of the upstream signalling pathway of the pro-inflammatory cytokine through the application of small-molecule compounds will be a new means for the treatment of RA (11, 12).

VX-765, a small-molecule specific inhibitor of caspase-1, is a prodrug that requires esterase cleavage to form VRT-043198 after oral administration. VRT-043198 binds to pro-IL1-β, pro-IL-18 and pro-IL-33 to inhibit their activation (13). To date, at least three caspase 1 inhibitors, including Pralnacasan (VX-740), IDN-6556 and VX-765, have entered clinical evaluation. Studies have shown that long-term use of VX-740 causes liver toxicity and IDN-6556 is a broad-spectrum caspase inhibitor. VX-765 is a caspase-1 specific inhibitor and has stable pharmacokinetics, thus exhibits the most potent inhibitory effects (14, 15). Current studies on the inhibition of caspase-1 mainly focus on the treatment of epilepsy; however, the impact of inhibition of caspase-1 on the RA is still unclear.

Collagen-induced arthritis (CIA) is a widely used experimental model of polyarthritis that exhibits many histopathological manifestations in common with RA. IL-1β, IL-18 and IL-33 play important roles in the pathogenesis of CIA. Caspase-1 is a key enzyme in the activation of these pro-inflammatory factors (16, 17). In this study, we intraperitoneally injected VX765 into mice with CIA and investigated the impact of caspase-1 inhibition on the onset and progression of CIA. This study is the first to report that prophylactic treatment by VX765 prevents/delays the progression of CIA in mice.

Materials and methods

Materials

VX765 was purchased from Selleck Chemicals LLC (U.S.A.). DBA/1 mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. Twenty-four male DBA/1 mice, 4 weeks of age, weighing ~ 20g, were used in this study. This research was approved by Animal Ethical Committee. Bovine type II collagen, Freund's

complete adjuvant and Freund's incomplete adjuvant were purchased from Chondrex (U.S.A.). ELSIA kit was purchased from Shanghai BlueGene LLC.

CIA mouse model

CIA mouse model was created in accordance with previously published protocols (18). In brief, bovine type II collagen (10 mg) was dissolved in 0.1 mmol/l acetic acid at a concentration of 3 mg/ml. Equal volume of bovine type II collagen and complete Freund's adjuvant were mixed and emulsified on ice. The final concentration of emulsion was 1.5 mg/ml. Immunisation was given at day 0. Each mouse received a 100 μl collagen intradermal injection at the base of the tail. Booster immunisation was given at the week 3. Each mouse received a 50 μl collagen intradermal injection at the base of the tail. Arthritis developed at 4-5 weeks after immunisation. Joint damage occurred at week 7 after immunisation.

Experimental design

Twenty-four mice were divided into three groups of 8: VX765 prophylaxis group (VX765), CIA model group (CIA) and wild-type control group (Normal). The VX765 was dissolved in 0.9% saline (a cloudy suspension). Mice received intraperitoneal injection of VX765 (100 mg/kg), twice a day. Because the solution was a suspension, we used intraperitoneal injection instead of intravenous injection. VX765 treatment started at the day of the booster immunisation and lasted for 4 weeks. The onset time of CIA was within 4 weeks after booster immunisation, therefore, the VX765 treatment lasted for 4 weeks. CIA and Normal groups did not receive VX765 treatment. Mice from all the groups were euthanised at the week 7 after the initial immunisation. All experimental procedures were carried out in accordance with the Animal Ethical Committee.

Clinical scores evaluation

After the booster immunisation (week 3) the onset of arthritis was monitored daily and arthritis scores were recorded. CIA scoring criteria were as follows: 0 point, no joint swelling; 1 point, 1-2 swollen toes; 2 points, ≥3 swollen toes; 1 point each for palm or wrist or ankle swelling. The highest score for each paw was 4 points, and the highest total score for each mouse was 16 points. In addition, mice were weighed weekly and the body weights were recorded after booster immunisation.

Image evaluation

At the end of experiment (week 7), Micro-CT (Siemenseinveon CT) coronal imaging was performed in mice two hind paws, with resolution of 30 μm, field of view 54.6 mm × 27.4 mm, slice thickness of 29.86 μm, inter-slice gap of 29.86 μm, and scan time of 28 min and 18 sec.

Inveon™ Acquisition Workplace (IAW) software were used for image processing. Radiological scores were as the follows: 0 point, no bone damage; 1 point, swelling and tissue oedema; 2 points, joint erosion; 3 points, bone erosion and osteophyte formation. The total radiological score from both hind paws represents the total radiological score of each mouse.

At the end of the experiment (week 7), magnetic resonance imaging (MRI) coronal images were acquired from mouse two hind paws. The images were processed using Pharmascan 7T Imaging System (BRUKER, Germany) and Paravision Version software with matching 20 mm coil. The scan sequences were T1fat-suppressed + contrast-enhanced sequences. The acquisition parameters were as follows: repetition time of 269.9 milliseconds, echo time of 8 milliseconds, field of view 25 mm, slice thickness of 0.25 mm, inter-slice gap of 0.25 mm, and scan time of 5 min and 45 sec. The contrast agent, Gadopentetate Dimeglumine Injection, was injected via the tail vein at a volume of 100 μl. MRI bone marrow oedema was defined as abnormal high signals in the bone marrow tissues. Synovitis was defined as high signals at joint space without clear signal boundary (19, 20). Mice were injected intraperitoneally with 10% chloral hydrate before MRI.

Histological evaluation

At the end of the experiment (week 7), mice four paws were harvested and fixed in 10% formalin for 48 hours, followed by EDTA decalcification for six weeks, and then embedded in paraffin, sectioned at the thickness of 4 μm and stained with HE. Pathological assessments were conducted in accordance with RA synovitis scores. Pathological scores of synovitis included: synovial lining layer hyperplasia, the degree of inflammation and angiogenesis in the lower layer of synovial lining layer. Lining layer hyperplasia score system was as follows: 0 point, less than 3 layers; 1 point, 3–4 layers; 2 points, 5–7 layers; 3 points, more than seven layers. Inflammation scores: 0 point, no inflammatory cells; 1 point, sparse inflammatory cells; 2 points, diffused distribution of inflammatory cells; 3 points, the formation of lymphoid follicles or germinal centres. Angiogenesis scores: o point, no angiogenesis; 1 point, mild angiogenesis; 2 points, moderate angiogenesis; 3 points, severe angiogenesis. The total pathological score of each paw was the cumulative sum of the three categories. The total pathological score of each mouse was the sum of the pathological scores of the four paws (21, 22).

Cytokine level assessment

At the end of the experiment, mice were euthanised and orbital blood samples were collected. Peripheral blood IL-1β, IL-18 and IL-33 levels were measured and analysed using ELSIA kit (Kit sensitivity of 1pg/ml).

Statistical analysis

Data analysis was performed using SPSS11.5 statistical software. The serum cytokines levels and body weight between the CIA group and the VX765 groups were compared using Student's-test; data were expressed as mean ± SD. The clinical scores, radiographic scores and pathological scores between the CIA group and the VX765 group were compared using the Mann-Whitney test; data were expressed as median. $p<0.05$ was considered statistically significant.

Results

The effects of VX765 treatment on the incidence of CIA

There was no mortality in mice in any group during the experiment. The inci-

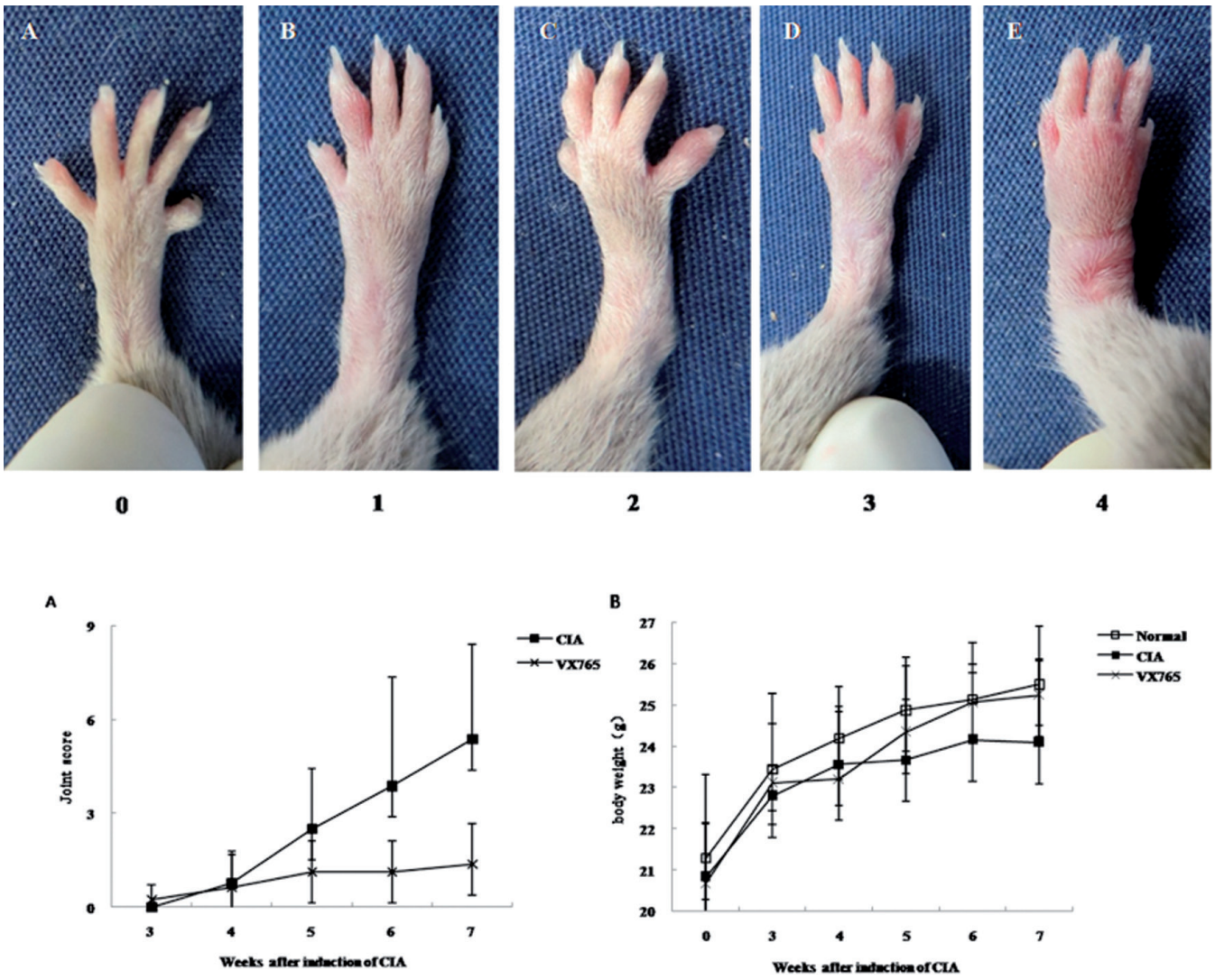

Fig. 1. The clinical scores of arthritis and body weight.
A, The clinical scores of arthritis in mice after immunisation. The clinical scores of arthritis in CIA group increased sharply three weeks after immunisation and peaked at seven weeks after immunisation. There was no significant increase in the clinical scores of arthritis in VX765 group four weeks after immunisation. The clinical scores of arthritis in VX765 group were significantly lower than the CIA group 7 weeks after immunisation (p=0.010). **B**, The body weight in three groups of mice. The body weight in the Normal mice increased stably. The body weight in CIA group decreased at four weeks after immunisation and decreased again at six weeks after immunisation. The body weight in the VX765 group decreased at three weeks after immunisation; however, the body weight increased stably at four weeks after immunisation and reached Normal group level at seven weeks after immunisation. The body weight of CIA and VX765 groups were overall lower than Normal group, the more severe the arthritis, the slower the weight gain in mice

dence of CIA in CIA group and VX765 group accelerated at the weeks 4–5, and then stabilised at the week 5. There were 7 mice (7/8) in CIA group and 6 mice (6/8) in VX765 group had arthritis at the week 7. The clinical scores of arthritis in CIA group and VX765 group gradually increased after week 3, and the increase in CIA group was statistically significant (Fig. 1A). The clinical scores of arthritis were significantly lower in the VX765 group (median: 1 point) than the CIA group (median: 6.5 points) (p=0.010) at week 7 (Fig. 5A). The body weight in CIA and VX765 groups decreased at week 3-5 (the acute phase of arthritis), however, there was a steady increase of the body weight in the VX765 group at week 5. There was no significant difference in the body weight between the VX765 group (25.23±1.67g) and the CIA group (24.09±1.98g) (p>0.05) at week 7 (Fig. 1B). The mice in VX765 group were active, had normal diet, fur was smooth and shiny, and moved easily; whereas, the mice in the CIA group had weight loss, had fur loss and dull fur, and were fatigued with less movement. Three weeks after immunisation, acute small ulcers formed at the injection site in the CIA group and these ulcers became hard crust at week 4.

The effects of VX765 treatment on the radiographic changes in joints of CIA mice

Micro-CT scan was performed at week 7 on the hind paws of mice in CIA and VX765 groups. The scans showed that CIA group had severe bone resorption and erosion; whereas, there was no significant bone destruction in VX765 group. The radiographic scores in the VX765 group (median: 1 point) were

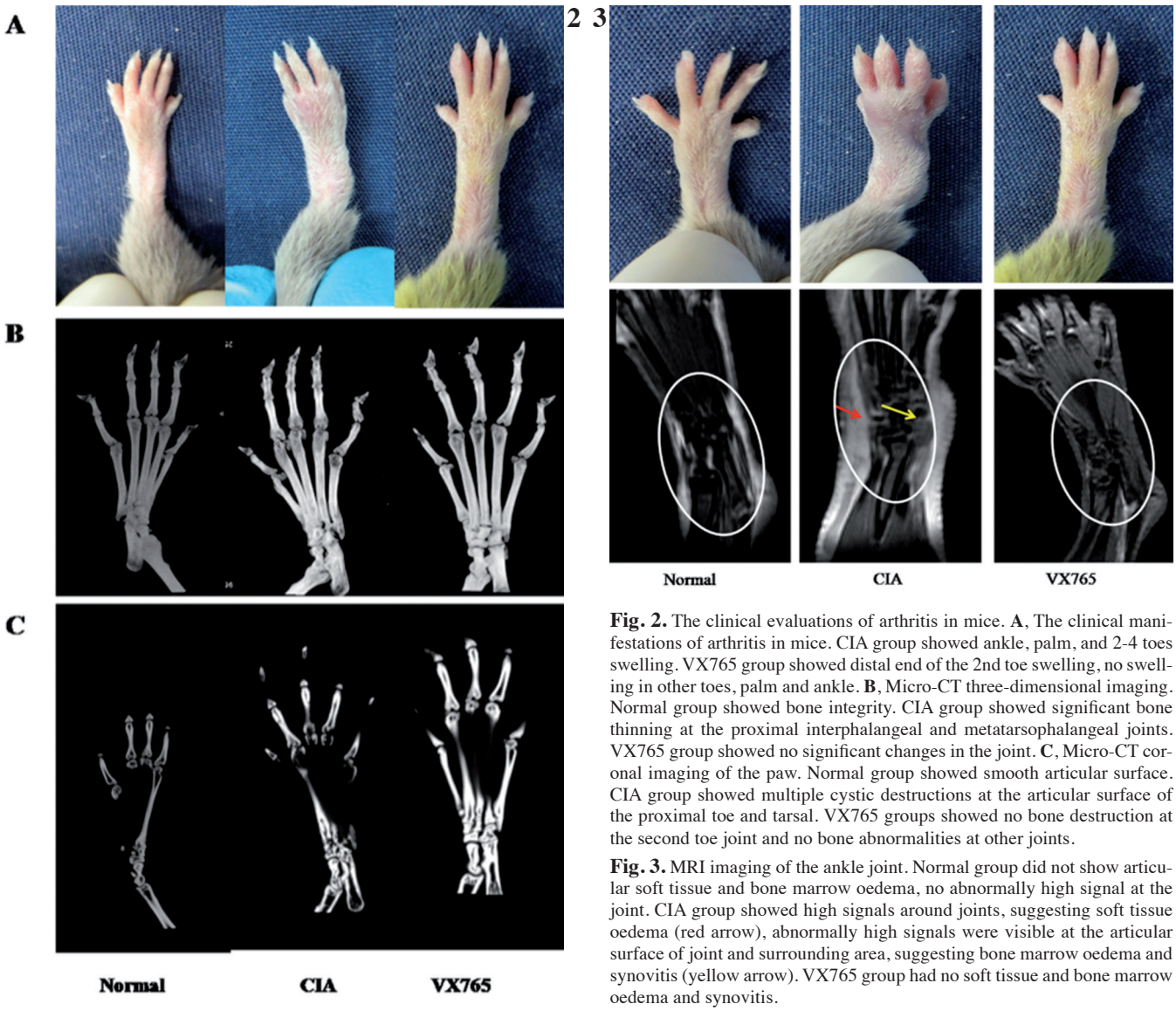

Fig. 2. The clinical evaluations of arthritis in mice. **A**, The clinical manifestations of arthritis in mice. CIA group showed ankle, palm, and 2-4 toes swelling. VX765 group showed distal end of the 2nd toe swelling, no swelling in other toes, palm and ankle. **B**, Micro-CT three-dimensional imaging. Normal group showed bone integrity. CIA group showed significant bone thinning at the proximal interphalangeal and metatarsophalangeal joints. VX765 group showed no significant changes in the joint. **C**, Micro-CT coronal imaging of the paw. Normal group showed smooth articular surface. CIA group showed multiple cystic destructions at the articular surface of the proximal toe and tarsal. VX765 groups showed no bone destruction at the second toe joint and no bone abnormalities at other joints.

Fig. 3. MRI imaging of the ankle joint. Normal group did not show articular soft tissue and bone marrow oedema, no abnormally high signal at the joint. CIA group showed high signals around joints, suggesting soft tissue oedema (red arrow), abnormally high signals were visible at the articular surface of joint and surrounding area, suggesting bone marrow oedema and synovitis (yellow arrow). VX765 group had no soft tissue and bone marrow oedema and synovitis.

significantly lower than the CIA group (median: 3.5 Points) (p=0.038) (Fig. 2, Fig. 5B). Normal group had intact articular bone structures. MRI scans showed that there was a large area of abnormally high signals in soft tissues in CIA group, suggesting the soft tissue oedema. In addition, there were abnormally high signals near the articular surface of the ankle joint and periarticular area in CIA group, indicating the bone marrow oedema and synovitis. The soft tissue/bone marrow oedema and synovitis in VX765 group was significantly less severe than the CIA group (Fig. 3). In the Normal group, the tarsal, metatarsal distal tibia and surrounding soft tissue were intact, no soft tissue and/or bone marrow oedema and no synovial thickening.

The effects of VX765 treatment on the joint pathology in CIA mice

Mice were euthanised at week 7 and four paws were harvested for the pathological analysis. The furs were removed from the mouse paws for gross observation. We found that there was significant swelling of subcutaneous tissues and synovial membrane in the CIA mice; whereas, there was no significant soft tissue swelling in VX765 mice (Fig. 6). Histological analysis showed that there were significant lining cell proliferation, neutrophil diffusion, lymphocyte infiltration, and visible angiogenesis in proliferating synovial membranes in the CIA group. VX765 group did not show inflammatory cell infiltration, synovial cell proliferation and angiogenesis. The pathological scores of VX765 group (median: 1 point) were significantly lower than the CIA group (median: 5.5 points) (p=0.000) (Fig. 4, Fig. 5C).

The effects of VX765 treatment on the peripheral blood cytokines

The serum IL-1β (2.05±0.98 pg/ml), IL-18 (74.84±41.93 pg/ml) and IL-33 (140.51±40.10 pg/ml) levels in the VX765 were significantly lower than that of the CIA group (6.89±2.38 pg/ml, 129.46±48.03 pg/ml, and 269.69 ± 99.02 pg/ml, respectively) (p<0.001, p=0.014, and p=0.001, respectively) (Fig. 6).

Discussion

Caspase-1 is the key enzyme in NLRP3-inflammasome signalling pathway and is mainly involved in the activation

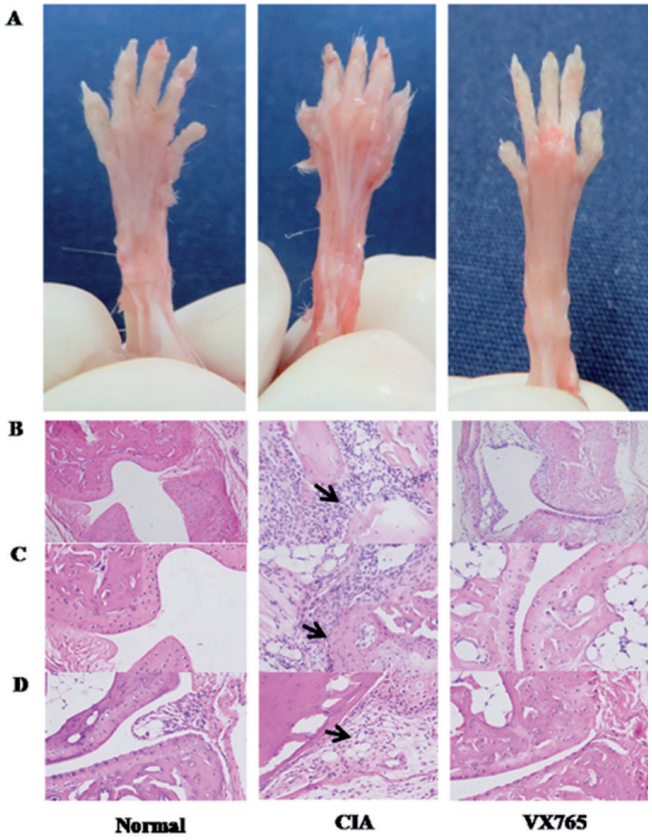

Fig. 4. Joint pathology. **A**, Gross pathology of mouse paw joint. Normal group had normal subcutaneous tissue and synovial membranes. CIA group showed a significant swelling of soft tissue and synovial joint. VX765 group showed no swelling of articular soft tissue and synovial membranes. **B**, HE staining of mouse metatarsophalangeal joint. Normal group had intact articular surface, no inflammatory cell infiltration. CIA group showed diffused infiltration of inflammatory cells into the joint space and surrounding tissues. VX765 did not show inflammatory cell infiltration. **C**, Normal group showed normal cartilage structure below 3rd layer of the synovial lining cells. CIA group showed significant proliferation of synovial lining cells, invasion of synovial membrane into articular cartilage, the damage of normal cartilage structure, and the formation of pannus. VX765 showed normal articular cartilage structure without synovial lining cell proliferation. **D**, Normal group showed no angiogenesis in synovial tissues. CIA showed visible angiogenic at the proliferating synovial membranes, and diffused perivascular inflammatory cell infiltration. VX765 group showed no synovial proliferation and angiogenesis (HE staining 20 × 10).

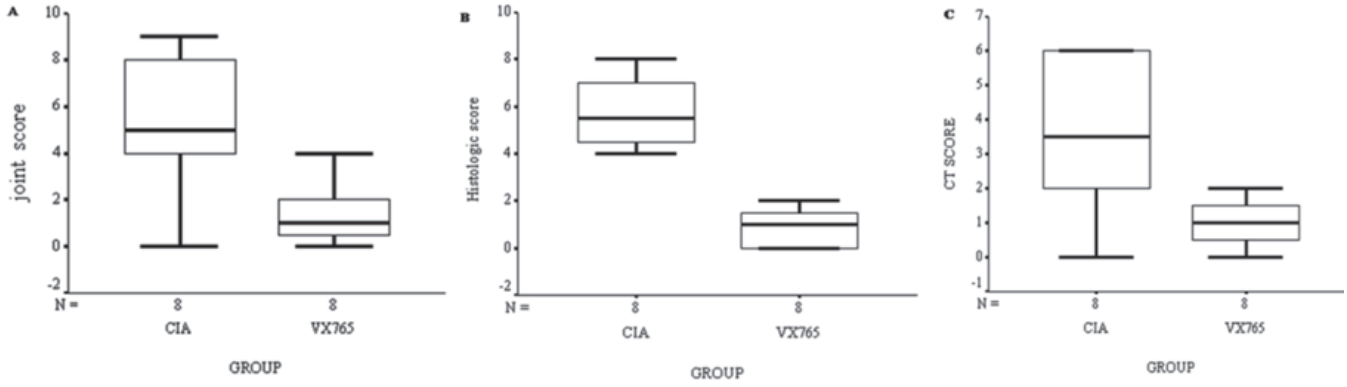

Fig. 5. The clinical scores and imaging scores. **A**, The clinical scores of the joint arthritis in CIA group and VX765 group seven weeks after immunisation. The clinical scores in the VX765 group were significantly lower than the CIA (p=0.010). **B**, The Micro-CT joint scores in the CIA group and VX765 group 7 weeks after immunisation. The Micro-CT joint scores in the VX765 group were significantly lower than the CIA group (p=0.0038). **C**, The pathological scores in CIA group and VX765 group seven weeks after immunisation. The pathological scores in the VX765 group were significantly lower than CIA group (p=0.000).

of pro-inflammatory cytokines, such as IL1-β, IL-18 and IL-33. Under physiological conditions, caspase-1 stimulates cell survival response, controls intracellular bacterial growth, and regulates inflammatory cytokine production. Under pathological conditions, the formation of NLRP3-inflammasomes converts precursor caspase-1 into cleaved caspase-1 via proximity-induced autoactivation. Active caspase-1 cleaves cytokine precursor, mediates inflammatory response and induces apoptosis (23). Clinical studies and animal models of epilepsy have demonstrated that VX765 treatment suppresses the release of IL1-β and IL-18 by inhibiting caspase-1 activation, thus prevents the onset of disease. Meanwhile, VX765 has completed a phase II trial in patients with psoriasis and phase IIa clinical trials in patients with localisation-related epilepsy and generalised epilepsy, and shows promising results (15, 24). The study of familial cold autoinflammatory syndrome (FCAS), an autoimmune inflammatory disease, indicates that VX765 can significantly reduce IL1-β and IL-18 secreted from human peripheral blood monocytes *in vitro* (25). To date, the effects of VX765 treatment on the CIA mouse model are still unclear. This study reports that VX765, a cas-

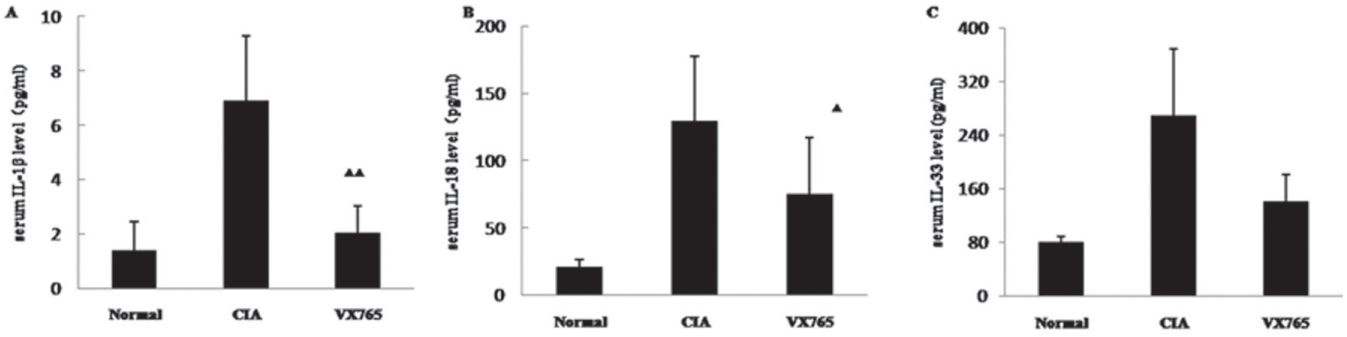

Fig. 6. The serum cytokine levels. **A**, Serum IL-1β level in VX765 group was significantly lower than CIA group (p<0.001). **B**, Serum IL-18 level in VX765 group was significantly lower than CIA group (p=0.014). **C**, Serum IL-33 level in VX765 group was significantly lower than CIA group (p=0.001). ▲: p<0.05, compared with CIA group; ▲▲: p<0.01, compared with CIA group.

pase-1 specific inhibitor, ameliorated the severity and progression of arthritis in CIA mice, significantly reduced joint radiographic and histological scores, and reduced peripheral cytokine levels. Pro-inflammatory cytokines, such as IL-1β, IL-18 and IL-33 show a clear relationship with the pathogenesis of RA. Cytokine inhibitors have been widely used in clinical practice. However, few studies focus on caspase-1 and RA. This study shows that VX765 prophylactic treatment (twice daily) in CIA mice significantly decreased the incidence and severity of arthritis. Clinical evaluation shows that arthritis scores in VX765 group were significantly lower than the CIA group, indicating VX765 treatment can decrease arthritis severity. The body weight in VX765 mice gradually increased and was similar to the Normal mice at the end of treatment. Moreover, VX765-treated mice were active during the experiment, indicating VX765 did not pose adverse effects on the general state of life in mice.

MRI manifests in the early stage of RA as bone marrow oedema and synovitis; bone destruction occurs at the later stage of RA. Thus, early suppression of bone marrow oedema can prevent arthritis progression. In this study joint MRI radiography in CIA group showed significant soft tissue oedema, bone marrow oedema and synovitis; whereas, VX765 mice did not show visible bone marrow oedema and synovitis, suggesting VX765 treatment may prevent the onset of arthritis. Micro-CT scans further indicate that the CIA group had significant cystic destruction in the articular surface and joint space narrowing; whereas, VX765 group did not show significant bone destruction. The radiographic scores in VX765 group were significantly lower than the CIA group, indicating that VX765 preventive treatment suppresses bone destruction. This study evaluated the clinical and radiographic changes and found that VX765 preventive treatment can ameliorate the severity and progression of arthritis.

RA pathogenesis involves cellular and humoral immunity. The inflammatory cells and cytokines stimulate synovial lining layer cell proliferation and hypertrophy; the synovial lining layer can change from its normal state (1-3 layers) to more than l0 layers. Pannus formation in synovial cells stars from synovial edge and gradually expands to the whole articular cartilage surface, and disrupts the normal structure. Angiogenesis is an important factor in sustained synovitis in RA. Synovial hypertrophy caused local tissue hypoxia promotes angiogenesis, and angiogenesis in turn increases the delivery of growth factors and cytokines and enables sustainable inflammation and synovial hyperplasia. This study shows that joint tissues in CIA mice had visible diffused inflammatory cell infiltration, synovial hypertrophy, pannus formation, invasive destruction of articular cartilage and angiogenesis. The joint histological scores were significantly increased. VX765 mice had no significant joint pathological changes and the histology scores were significantly lower than the CIA mice. These results indicate that VX765 prophylactic treatment suppresses inflammatory cell infiltration, synovial lining cell proliferation, angiogenesis, and inhibits invasive pannus formation and cartilage damage.

On the other hand, inflammatory cells secrete many cytokines, such as interleukin family members (IL1-β, IL-18, and IL-33) and TNF-α (26, 27). These pro-inflammatory cytokines directly stimulate the proliferation of synovial cells and endothelial cells, or stimulate the release of collagenase, matrix metalloproteinases (MMPs) from synovial cells, chondrocytes and synovial fibroblasts, which results in aggravation of inflammation and the degradation of proteoglycan and collagen, eventually leads to joint structural damage (28, 29). Meanwhile, these pro-inflammatory cytokine are secreted to the periphery system, which lead to systemic inflammation. Thus, inhibition of pro-inflammatory cytokine activation is a key regulator of cytokine disorders. This study shows that serum IL1-β, IL-18 and IL-33 levels significantly increased in CIA mice, compared with Normal mice. VX765 prophylactic treatment significantly decreased serum IL1-β, IL-18 and IL-33 levels, compared with CIA group. These results indicate that VX765 suppressed IL1-β, IL-18 and IL-33 activation by specific inhibition of Caspase-1 activity, which in turn delayed the onset of arthritis and reduced arthritis severity.

The treatment of RA is advanced from the traditional non-selective immunosuppressive treatment to biological agents, such as TNF-α inhibitors (etanercept, adalimumab, etc.), IL-1 inhibition agent (anakinra), and IL-6

inhibitor (tocilizumab), that targeting pro-inflammatory cytokines. These treatments have achieved good results, but are limited in clinical application process. In recent years, the intracellular inflammation signalling pathways become the therapeutic targets in RA treatment. The inhibition of intracellular inflammation signalling pathways has achieved good therapeutic effect and safety, compared with the traditional MTX (30-33). VX765, small molecule inhibitors of caspase-1, induces less immune responses, has lower molecular weight, can easily cross the blood-brain barrier, and exhibits better pharmacokinetic properties. In this study, we used intraperitoneal injection of VX765 with a dose of 100 mg/kg, twice daily. This dose has been used in a variety of animal experiments and has been demonstrated to be safe and effective in experimental animals (13, 24).

Conclusion

This study demonstrates that VX765 treatment ameliorates the severity and progression of arthritis in CIA mice through the inhibition of IL1-β, IL-18 and IL-33 activation. This study suggests that caspase-1 may be a potential target for the treatment of RA.

Limitations of the study

In this study, we explored the impact of VX765 treatment on the disease severity and progression in CIA mice. However, this is an exploratory study and the sample size is small. In addition, the underlying mechanisms of VX765 treatment need to be explored in depth in the future.

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