Interleukin-19 blockade attenuates collagen-induced arthritis in rats

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Abstract

Objectives. RA is the most common form of inflammatory arthritis. IL-19 acts as a pro-inflammatory cytokine involved in the pathogenesis of RA. We investigated whether anti-IL-19 antibody treatment would modulate the severity of the disease in a CIA rat model.

Methods. We generated a CIA model by immunizing rats with bovine type II collagen. CIA rats were s.c. treated with anti-IL-19 antibody 1BB1. The effects of 1BB1 on CIA rats were determined by hind-paw thickness, severity score, bone destruction, BMD and cytokine production, which were evaluated using radiological scans, micro-CT, real-time quantitative PCR and ELISA. To analyse gene regulation by IL-19, rat synovial fibroblasts (SFs) were isolated and analysed for the expression of TNF-α, IL-1β and RANK ligand (RANKL).

Results. In vivo, IL-19 was highly expressed in the synovial tissue and SFs isolated from CIA rats. 1BB1 significantly ameliorated the severity of arthritis by decreasing hind-paw thickness and swelling; prevented bone destruction and bone loss; inhibited the expression of TNF-α, IL-1β, IL-6 and RANKL in synovial tissue; and decreased the production of IL-6 in serum. In vitro, IL-19-induced TNF-α, IL-1β, IL-6 and RANKL expression in CIA SFs.

Conclusions. Specifically blocking IL-19 inhibited pro-inflammatory cytokine production and prevented bone destruction in CIA rats. These findings provide evidence that IL-19 is a novel target, and that anti-IL-19 antibody may be a potential target to ameliorate the severity of RA.

Key words: IL-19, synovial fibroblast, collagen-induced arthritis.

Introduction

IL-19 is a cytokine in the IL-10 family, which includes IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IL-28 and IL-29 [1]. One major cellular source of IL-19 is monocytes, in which lipopolysaccharide, GM-CSF, TNF-α and IL-6 up-regulate IL-19 expression [2, 3]. IL-19 binds to IL-20R1/IL-20R2, a hetero-receptor complex that activates signal transducer and activator of transcription (STAT)3 and induces cell proliferation, decreases synovial cell apoptosis [4-6]. Acutely induced IL-19 in systemic inflammation may promote lung and other tissue injury in mice undergoing endotoxic shock [7]. IL-19 also alters the balance of Th1 and Th2 cells in favour of Th2 cells [8]. The chronic expression of IL-19 is correlated with Th2 cytokine production in patients with uraemia, and IL-19 is expressed alongside pathogenesis of asthma, psoriasis and RA [6, 9-11].

RA is a chronic, systemic inflammatory autoimmune disease. Intense inflammation occurs in synovial joints, infiltrating the normally delicate synovial membranes with mononuclear phagocytes, neutrophils and lymphocytes. Well-established studies have revealed pivotal roles of inflammatory cytokines, such as TNF-α, IL-1β and IL-6 in the pathogenesis of RA [12]. Synovial fibroblasts (SFs) play a crucial role in the pathogenesis of RA through the synthesis of various proteases, chemokines and cytokines, such as TNF-α, IL-1β, IL-6 and IL-20 [13-15]. Various molecules, such as M-CSF and RANK ligand (RANKL), are expressed in the inflamed synovium to stimulate osteoclastogenesis and bone resorption [16-18]. Patients with RA manifest irreversible cartilage damage, bone destruction and poor functional outcomes [19], which indicates that preventing bone damage is
important for anti-arthritis therapy. Therefore, it is necessary to find novel targets to develop new therapeutic agents against bone destruction and bone loss in patients with RA.

CIA is an experimental autoimmune model of human RA that is widely used for studying disease processes as well as for evaluating possible therapeutic agents. Endogenous IL-10 has been shown to partly suppress the production of TNF-α and IL-1β by synovial macrophages and synoviocytes in an animal model of CIA and was confirmed to have beneficial effects in this model [20–22].

Cytokines play pivotal roles in the pathogenesis of RA. IL-10 is known for its anti-inflammatory and regulatory function on different cell types involved in the pathogenesis of RA. Studies suggest that most of the IL-10 family cytokines are involved in RA [6, 15, 21, 23]. IL-19 acts as a pro-inflammatory cytokine and promotes joint inflammation in RA by inducing IL-6 production and decreasing synovial cell apoptosis [6], which indicates that IL-19 could be the target of a therapeutic drug. We therefore evaluated whether IL-19 is involved in the pathogenesis of CIA and targeting IL-19 will ameliorate the severity of the disease in a CIA rat model.

Materials and methods

Assessing the severity of CIA

All animal experiments were conducted according to the protocols based on the National Institutes of Health standards and the guidelines for the care and use of experimental animals. The research procedures were approved by the animal ethics committee of National Cheng Kung University in Taiwan. We established the animal model of CIA using the same protocol as previously described [15]. Eight-week-old male Sprague-Dawley rats were immunized using an emulsion composed of equal parts of Freund’s complete adjuvant containing 4 mg/ml heat-killed Mycobacterium tuberculosis (Arthrogen-CIA; Chondrex, Redmond, WA, USA) and bovine type II collagen (CII) solubilized at 2 mg/ml in 0.05 M acetic acid. The rats received an intradermal injection of 200 μl of emulsion (200 μg of bovine CII) into the dorsum. Booster doses were administered on Day 8, with s.c. 100-μl injections of the same emulsion into the base of the tail. The onset of CIA was between Days 11 and 13 after the first immunization. The severity of arthritis in each hind paw was monitored and scored on a scale of 0–5, where 0: no redness or swelling; 1: slight swelling in the ankle or redness in the foot; 2: progressive swelling, inflammation and redness from the ankle to the midfoot; 3: swelling and inflammation of the entire foot, not including the toes; 4: swelling and inflammation of the entire foot, including the toes; and 5: swelling and inflammation of the entire foot, with loss of mobility. The severity of arthritis in each rat was determined independently and blindly by four individual observers, and the average of their scores was calculated. The thickness of the rats’ hind paws was measured with a digital vernier caliper. Using a radiograph equipped with a direct digital imaging system, we X-rayed the ankle joints of rats after they had been given a general anaesthetic (pentobarbital; Sigma-Aldrich, St Louis, MO, USA) on Day 25. Bone destruction in the ankle joint was scored on a scale of 0–3, where 0: no swelling or bone damage; 1: mild bone damage; 2: moderate bone erosion; and 3: severe bone erosion. All experiments were repeated three times.

Generating mouse mAb against IL-19

Anti-IL-19 antibody 1BB1 [9] is an anti-human-IL-19 mouse mAb that cross-reacts with rats. We previously reported [7, 24] that 1BB1 neutralized the activity of IL-19. 1BB1 hybridoma cells were cultured in DMEM (Invitrogen Life Technologies) containing 10% FBS and 1% penicillin/streptomycin. 1BB1 was purified from mouse ascites using protein A chromatography.

Immunohistochemistry

Paraffin-embedded tissue samples were used for immunohistochemistry (IHC) staining with anti-IL-19 (1BB1), at 4°C overnight as previously described [24]. Incubating paraffin tissue sections with mouse immunoglobulin (Ig)G1 isotype (clone 11711; R&D Systems) instead of primary antibody was the negative control. We used 3 μg/ml as the working concentration for each primary antibody and for the control mouse IgG1.

Treatment

The rats were divided into two CIA groups and one healthy control group (n = 5 each). The CIA groups were given one of the following treatments three times a week: mouse IgG (mIgG) (Chemicon International, Temecula, CA, USA) (5 mg/kg injected s.c.), or 1BB1 (5 mg/kg, s.c.). The treatment was started on Day 9 after the initial immunization with bovine CII.

micro-CT

The micro-CT analyses of the tibia metaphysis were done on a system (1076 micro-CT-40; Skyscan, Aartselaar, Belgium) equipped with a high-resolution, low-dose X-ray scanner. The X-ray tube was operated with photon energy of 50 kV, current of 200 μA and exposure time of 1200 ms through a 0.5-mm-thick filter. The scanning conditions were set at 35-mm width, 35-μm pixels. After standardized reconstruction of the scanned images, the data sets for each tibia sample were resampled with software (CTAn; Skyscan) to orient each sample in the same manner. Data from each slice were converted to binary data using a threshold obtained using discriminant analysis in which the pixel values in the histograms of background and bone were assumed to be normally distributed. We then chose the threshold as an intermediate pixel value lying on the tails of the two normal distributions. BMD, a three-dimensional bone characteristic parameter, was analysed in 30 consecutive slices. All experiments were repeated three times.
Detecting IL-19, TNF-α, IL-1β, IL-6 and RANKL in synovial tissue

Fresh synovial tissue from the knee joints of healthy and CIA rats was isolated by an orthopaedic surgeon. Histological analysis confirmed healthy and arthritic synovium. Total RNA was isolated. Reverse transcription was performed with reverse transcriptase according to the manufacturer’s protocol. An analysis of IL-19, TNF-α, IL-1β, IL-6 and RANKL was then done with gene-specific primers on a thermocycler. Quantification analysis of mRNA was normalized with rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the housekeeping gene. Relative multiples of change in mRNA expression was determined by calculating $2^{-\Delta\Delta Ct}$.

Measuring serum IL-6 in CIA rats

To measure IL-6 levels in serum, blood was collected from the healthy and CIA rats, and the supernatant was stored at −80°C for analysis. The serum was used to determine the expression of IL-6 with a commercially available rat IL-6 ELISA kit (R&D Systems) according to the manufacturer’s instructions.

Isolating and culturing rat SFs

Freshly isolated synovial tissue from CIA was collected, and histological analysis confirmed arthritic synovium. The synovial tissue was then finely minced into 2- to 3-mm pieces and digested by dispase (Roche, Mannheim, Germany) for 45 min at 37°C in DMEM. Isolated SFs were cultured in DMEM containing 10% FBS. All in vitro experiments were done with primary SF cultures between Passages 2 and 4.

Analysing TNF-α, IL-1β, IL-6 and RANKL expression in rat SFs

CIA rat SFs were plated for 12 h in DMEM with 10% FBS. SFs were kept in serum-free DMEM medium for 8 h, and then SFs were incubated with IL-19 (200 ng/ml) for 4 h to analyse TNF-α, IL-1β, IL-6 and RANKL expression. Total RNA was isolated (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed with reverse transcriptase according to the manufacturer’s protocol (Clontech, Palo Alto, CA, USA). TNF-α, IL-1β, IL-6 and RANKL expression was then amplified on a thermocycler (LC 480; Roche Diagnostics, Indianapolis, IN, USA), with SYBR Green (Roche Diagnostics) as the interaction agent. Quantification analysis of mRNA was normalized with rat GAPDH as the housekeeping gene. Relative multiples of change in mRNA expression was determined by calculating $2^{-\Delta\Delta Ct}$.

Statistical analysis

Commercial statistical software (Prism 5.0; GraphPad Software, San Diego, CA, USA) was used for the statistical analysis. A two-way analysis of variance non-parametric test (Kruskal-Wallis test) was used to compare the data between groups. Post hoc comparisons were done using Dunn’s multiple comparison test. Results are expressed means (s.d.). $P < 0.05$ was considered statistically significant.

Results

Up-regulation of IL-19 in a rat CIA model

Recent studies [6, 11] showed that IL-19 was highly expressed in rheumatoid joints, and was involved in the regulation of synovial inflammation in RA. In our rat CIA model, IL-19 was significantly up-regulated in synovial tissue isolated from the rats (Fig. 1A and B). We also used ELISA to analyse serum IL-19 and found no significant difference after the induction of CIA (data not shown). In addition, IHC staining showed that synovial tissue was stained with IL-19 and that the primary SFs expressed IL-19 (Fig. 1C and D). Therefore, IL-19 may be induced locally on the SFs of CIA rats.

Anti-IL-19 antibody 1BB1 ameliorated the severity of arthritis in a rat CIA model

Since IL-19 acts as a pro-inflammatory factor in a CIA rat model, we wanted to study whether an anti-IL-19 mAb, 1BB1, is therapeutic for reducing the severity of CIA. We have demonstrated the specific neutralization activity of the anti-IL-19 antibody, 1BB1, in both reactive oxygen species and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay [7, 24]. To determine whether interference with IL-19 activity by 1BB1 reduced inflammation and joint destruction in vivo, we examined the effects of 1BB1 in CIA rats. The treatment with 1BB1 started on Day 9 after the initial immunization with bovine CII and was performed three times a week. Hind-paw thickness was significantly ($P < 0.05$) lower in CIA rats treated with 1BB1 (5 mg/kg) than in control rats treated with mlgG (Fig. 2A). The severity scores were significantly different between the mlgG group and the 1BB1 group ($P < 0.05$) (Fig. 2B). These findings indicated that 1BB1 arrested the development and progression of inflammation and reduced hind-paw thickness in CIA rats.

Radiological analysis of the bones and joints of CIA rats

We radiologically evaluated the severity of cartilage damage and bone destruction in healthy rats and rats with CIA on Day 25 after the initial immunization. The hind-paw joints were severely damaged in mlgG control rats. The thickness of the hind paws and the bone destruction in mlgG groups consistently increased from Day 11 until the end of the study (Fig. 3A). The severity of bone destruction in CIA ankles was significantly ($P < 0.05$) lower in the 1BB1-treated group than in the mlgG-treated group (Fig. 3B). The results further confirmed that 1BB1 was potent in reducing the severity of arthritis in the hind paws of CIA rats.

1BB1 protected against bone destruction and increased bone density in CIA rats

Joint destruction and loss of bone density accompany the progression of CIA [25, 26]. To further test whether 1BB1 protects against bone destruction, we did micro-CT analyses of the tibias from CIA rats. The tibias from the mlgG
FIG. 1 Up-regulation of IL-19 in CIA rats. (A) Synovial tissue was taken from healthy controls and CIA rats 20 days after their initial injection with CII. Total RNA was isolated. RT-PCR was used to analyse the expression of IL-19 in synovial tissue. (B) The synovial tissue was taken from rats at the indicated time during the induction of CIA. Total RNA was isolated. Real-time qPCR was used to analyse the expression of IL-19 in synovial tissue. Data are represented as mean (s.d.). *P < 0.05 vs Day 1. Time of injecting CII (†). (C and D) IHC staining showed that IL-19 was strongly stained in synovial tissue and SFs derived from CIA rats but only slightly stained in healthy control rats (n = 5 per group). Positively stained cells of IL-19 (→). All experiments were performed three times with similar results.

FIG. 2 The effects of the anti-IL-19 antibody 1BB1 on reduction of hind-paw thickness and severity score in CIA rats. (A) Arthritis was induced in two groups of rats (n = 5 each) by intradermally injecting them with bovine CII on Day 0. Booster doses were given on Day 8. A third group contained healthy controls (n = 15). 1BB1 (5 mg/kg) was s.c. injected three times a week throughout the study. mlG-G-treated CIA rats were negative controls. Hind-paw thickness indicated the level of disease severity. (B) The severity score (n = 15) was determined according to the degree of joint swelling and erythema of the hind paws. Differences between the mlG- and 1BB1-treated groups are statistically significant. *P < 0.05 vs mlG controls. The initial date of treatment (†). All experiments were repeated three times. Each point is presented as means (s.d.) of 15 rats.

IL-19 blockade is a potential RA therapy
control group showed prominent bone destruction compared with the intact joints in the healthy controls (Fig. 4A). 1BB1 treatment clearly protected CIA rats against bone loss compared with the mIgG control group (Fig. 4A). BMD measurements showed that 1BB1 treatment in CIA rats significantly inhibited bone loss compared with the mIgG control group ($P < 0.05$; Fig. 4B). These results indicated that 1BB1 protected CIA rats not only by reducing the severity of arthritis but also by decreasing bone loss.

1BB1 inhibited the production of cytokines and RANKL in synovial tissue

Excess production of pro-inflammatory cytokines such as TNF-$\alpha$, IL-1$\beta$ and IL-6 has been shown to play a crucial role in synovial cell activation and joint destruction in RA [27]. In addition, RANKL is considered an important regulator of bone erosion in RA [28]. 1BB1 significantly alleviated the severity of CIA in vivo in rats. To confirm whether the reduction of disease severity was associated with the inhibition of pro-inflammatory cytokines and RANKL production, we isolated the knee synovial tissue from CIA rats and used real-time qPCR and ELISA to analyse cytokines and RANKL expression. The synovial expression of TNF-$\alpha$, IL-1$\beta$, IL-6, IL-19 and RANKL (Fig. 5A-E) in CIA rats treated with 1BB1 was significantly lower than that in mIgG-treated CIA control rats ($P < 0.05$). 1BB1 also significantly ($P < 0.05$) inhibited IL-6 serum level compared with the mIgG control group (Fig. 5D). No significant difference was observed in the inhibition of serum levels of TNF-$\alpha$ or IL-1$\beta$ after 1BB1 treatment (data not shown). Taken together, the results indicated that IL-19 may be synergistic with TNF-$\alpha$, IL-1$\beta$ and IL-6 in triggering synovial inflammation in the synovial cavity of the CIA ankles. IL-19 was also involved in joint destruction by up-regulating RANKL.

IL-19 induced the expression of TNF-$\alpha$, IL-1$\beta$, IL-6 and RANKL in SFs

There is a complex cytokine network in the joints of RA. SFs play a pivotal role in the pathogenesis of RA through...
the synthesis of various cytokines, such as TNF-α, IL-1β and IL-6 [12, 27]. Our in vivo data demonstrated that 1BB1 decreased the expression of TNF-α, IL-1β, IL-6 and RANKL, which indicated that IL-19 may be involved in the inflammatory process of CIA. Recent evidence showed that IL-19 increased the production of IL-6 in synovial cells of RA patients. Therefore, we hypothesized that activated SFs may be a major source of those cytokines induced in response to IL-19 in the inflamed synovium. Therefore, we isolated primary cultures of SFs from CIA and healthy rats to analyse whether IL-19 up-regulated the gene expression of TNF-α, IL-1β, IL-6 and RANKL in SFs in vitro. IL-19 (200ng/ml) induced TNF-α, IL-1β and IL-6 expression in primary culture of SFs derived from CIA rats (Fig. 6A-C). Furthermore, our in vivo data demonstrated that 1BB1 protected against bone loss and inhibited RANKL, which indicated that IL-19 may be indirectly involved in osteoclastogenesis. Therefore, we investigated whether IL-19 alters RANKL expression in vitro. We found that RANKL expression was significantly higher in IL-19-treated SFs derived from CIA rats (Fig. 6D). These results suggested that IL-19 may be pivotal in inflammatory bone destruction because it induces SFs to produce TNF-α, IL-1β, IL-6 and RANKL.

Discussion

In this study, we showed that the specific IL-19 mAb 1BB1 neutralized IL-19 activity in vitro and in vivo and it has therapeutic potential for RA. 1BB1 reduced the expression of TNF-α, IL-1β, IL-6, IL-19 and RANKL; decreased the severity of CIA; and protected CIA rats against arthritic bone destruction. In addition, IL-19 induced TNF-α, IL-1β, IL-6 and RANKL expression in SFs, which suggested that IL-19 might be involved in inflammatory bone destruction through regulating pro-inflammatory cytokines. Therefore, we provided new evidence that IL-19 is involved in the pathogenesis of CIA and may be a new target for treating RA because anti-IL-19 antibody protected CIA rats against bone loss and reduced the severity of CIA.

Recent advances in the field of immunopathology of RA have oriented treatment targeting pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 [29, 30]. Anti-cytokine therapies: the TNF-α blocker etanercept (a soluble decoy receptor for TNF-α), the IL-1 blocker anakinra (an IL-1 receptor antagonist) and the IL-6 blocker tocilizumab (an anti-IL-6 receptor antibody), are considered important therapeutic progress in the treatment of RA acting at the level of cellular processes responsible for inflammatory bone destruction.

Fig. 4 Micro-CT analysis of the knee joints in CIA rats. (A) The tibia metaphysis was taken from healthy controls and CIA rats treated with 1BB1 (5mg/kg) or mIgG (5mg/kg) 25 days after their initial immunization with CII. Representative micro-CT photos are shown for each group. (B) Tibial BMD was measured. Data are represented as mean (S.D.). Differences between the treated and control groups are statistically significant. *P < 0.05 vs mIgG controls. All experiments were performed three times with similar results. Data are from a representative experiment.
These new therapies are active not only in modulating the disease inflammatory processes but also in stopping the radiological course of RA. A previous study [6] has shown that IL-19 induces IL-6 production on synovial cells isolated from patient’s rheumatoid synovium. In the present study, we demonstrated that IL-19 induced the expression of TNF-α, IL-1β and IL-6 in CIA SFs, which suggested that IL-19 was an upstream regulator of TNF-α, IL-1β and IL-6. IL-19 may also contribute to joint destruction indirectly by increasing TNF-α, IL-1β and IL-6 production. Furthermore, 1BB1 potently reduced TNF-α, IL-1β and IL-6 expression in synovial tissue in vivo, which indicated that treatment with 1BB1 might also inhibit other RA-associated pro-inflammatory cytokines altogether.

The RANKL–RANK signalling mechanism is the pathway of osteoclast formation and activity [16, 35]. TNF-α and IL-1β, which are secreted by SFs and activated T cells [36], regulate RANKL expression. We found that IL-19 acts on SFs and induced RANKL expression, which indicated that IL-19 might be an upstream regulator for RANKL expression in vivo. This result implies that IL-19 causes bone destruction during the progression of CIA through directly inducing RANKL or by indirectly inducing RANKL by up-regulating TNF-α and IL-1β. The potent inhibition of RANKL expression by 1BB1 in synovial tissue of CIA rats provided additional in vivo evidence. RANKL expression is also up-regulated in many malignant tumour cells, and is involved in cancer-associated bone destruction and tumour-induced osteolysis [37, 38]. Therefore, we speculated that anti-IL-19 antibody may be a new target for treating cancer-associated osteolysis and bone erosion.

RANKL stimulates osteoclast differentiation and increases bone resorption. Denosumab, a human mAb against RANKL, inhibits osteoclast-mediated bone resorption and has been developed for use in osteoporosis [39, 40]. Our results showed that 1BB1 protected CIA rats against bone loss and increased their BMD. Therefore, anti-IL-19 antibody shows promise as a therapeutic candidate for protection against osteoporosis-associated bone loss.

The pleiotropic cellular effects of IL-19 and other pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-1β, may act cooperatively to mediate the initial inflammatory response and lead to the progression of cartilage damage and bone destruction in the pathogenesis of RA. Therefore, targeting a single cytokine may not be sufficient for treating complex inflammatory diseases such as RA. We speculated that combined anti-cytokine treatment may provide a novel therapeutic cocktail for RA. Furthermore, in addition to IL-19, IL-20 and IL-22 are...
Fig. 6 IL-19 up-regulated TNF-α, IL-1β, IL-6 and RANKL in SFs. SFs were kept in serum-free DMEM for 8 h and treated with IL-19 (200 ng/ml) for 4 h. Total RNAs were isolated and subjected to real-time qPCR using primers specific for (A) TNF-α, (B) IL-1β, (C) IL-6 and (D) RANKL. *P < 0.05 vs untreated controls. All experiments were performed three times with similar results.

Rheumatology key messages
- Anti-IL-19 antibody significantly decreased the severity of CIA and prevented bone destruction in CIA rats.
- Anti-IL-19 antibody may be a potential new target for treating RA and inflammation-related bone diseases.

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