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Cyr61 Induces IL-6 Production by Fibroblast-like Synoviocytes Promoting Th17 Differentiation in Rheumatoid Arthritis

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Cysteine-rich protein 61 (Cyr61)/CCN1 is a product of an immediate early gene and functions in mediating cell adhesion and inducing cell migration. We previously showed that increased production of Cyr61 by fibroblast-like synoviocytes (FLS) in rheumatoid arthritis (RA) promotes FLS proliferation and participates in RA pathogenesis with the IL-17–dependent pathway. However, whether Cyr61 in turn regulates Th17 cell differentiation and further enhances inflammation of RA remained unknown. In the current study, we explored the potential role of Cyr61 as a proinflammatory factor in RA pathogenesis. We found that Cyr61 treatment dramatically induced IL-6 production in FLS isolated from RA patients. Moreover, IL-6 production was attenuated by Cyr61 knockdown in FLS. Mechanistically, we showed that Cyr61 activated IL-6 production via the αvβ5/Akt/NF-κB signaling pathway. Further, using a coculture system consisting of purified CD4+ T cells and RA FLS, we found that RA FLS stimulated Th17 differentiation, and the pro-Th17 differentiation effect of RA FLS can be attenuated or stimulated by Cyr61 RNA interference or addition of exogenous Cyr61, respectively. Finally, using the collagen-induced arthritis animal model, we showed that treatment with the anti-Cyr61 mAb led to reduction of IL-6 levels, decrease of Th17 response, and attenuation of inflammation and disease progression in vivo. Taken together, our results reveal a novel role of Cyr61 in promoting Th17 development in RA via upregulation of IL-6 production by FLS, thus adding a new layer into the functional interplay between FLS and Th17 in RA pathogenesis. Our study also suggests that targeting of Cyr61 may represent a novel strategy in RA treatment. The Journal of Immunology, 2012, 188: 5776–5784.

Human rheumatoid arthritis (RA) is a systemic inflammatory disease that involves hyperplasia of synovial tissues and structural damage to cartilage, bone, and ligaments (1). The cause and pathogenesis of RA are still unclear. Th17 cells are now believed to play a crucial role in the lesions (2, 3). Synovial macrophages have been found to be particularly effective at promoting Th17 differentiation suggesting that optimal generation of Th17 cells is fueled by the inflammatory milieu present in the local microenvironment of RA (4). Elevated production of IL-17 has been observed in RA patients (5). IL-17 is reported to induce the production of proinflammatory cytokines, such as TNF-α and IL-1, in many local tissue cell types, such as macrophages and fibroblasts, and the upregulation of RANK ligand in osteoclasts, leading to stimulation of the activity of matrix metalloproteinases, matrix catabolism, and bone resorption (6, 7). Elevated production of IL-17 has also been observed in the collagen-induced arthritis (CIA) murine model (8). Mice deficient in IL-17 are protected from the development of CIA (9), and blockade of IL-17 with specific Abs can ameliorate symptoms in CIA (10), supporting a critical role of IL-17 in the development of arthritis symptoms.

IL-6 is abundantly present in the synovial fluid (SF) of RA patients and is thought to mediate many of the local and systemic effects of this disease (11, 12). Moreover, it is now clear that IL-6 is an essential cytokine involved in inducing the differentiation of Th17 cells in both humans and mice (13, 14). In the presence of IL-1β (in human) or TGF-β (in mouse), IL-6 activates the expression of the critical Th17 transcription factor RORc (in human) or RORγt (in mouse) through the Jak/STAT3 pathway in Th17 cell development (13, 15). IL-6 is induced by many inflammatory cytokines, such as IL-1β, TNF-α, and IFN-γ (16, 17). However, whether cysteine-rich protein 61 (Cyr61), a member of the CCN protein family that we have previously shown to be abundantly produced by RA fibroblast-like synoviocytes (FLS) (18), plays any role in IL-6 production and the inflammation processes of RA has not yet been explored.

Cyr61 is a product of an immediate early gene and is known to function in mediating cell adhesion and inducing cell migration (19). Cyr61 enhances growth factor-induced DNA synthesis in fibroblasts and endothelial cells (20, 21). As a secreted extracellular matrix protein, Cyr61 could elicit diverse functions by binding to different types of integrin molecules such as αvβ3, αvβ5, αHbβ3, and α6β1 on different cells (22–24). We reported previously that the expression of Cyr61 is greatly enhanced in FLS
from RA patients and that this increased expression of Cyr61 in turn acts further to stimulate FLS proliferation (18). Notably, we also found that IL-17 produced by Th17 cells is a major signal that is responsible for stimulating the production of Cyr61 by FLS. These results not only reveal for the first time to our knowledge that Cyr61 contributes to hyperplasia of synovial lining cells but also establish a new mechanism of the functional link between Th17 cells and FLS in RA, whereby the Th17 cells act to promote FLS proliferation through stimulating the expression of Cyr61 in these cells (18). Nevertheless, whether Cyr61 has any effect on Th17 differentiation and plays roles in the inflammation process of RA remains unknown.

In this study, we found that Cyr61 stimulated IL-6 production by FLS via the Cyr61/uvß5/Akt/NF-κB signaling pathway. Increased IL-6 in turn promoted Th17 differentiation. Blocking of Cyr61 action with an mAb (093G9) reduced IL-6 production by FLS, attenuated Th17 response, and ameliorated disease progression in CIA mice. In conclusion, Cyr61 plays a critical role in stimulating IL-6 expression by FLS in RA and contributes to Th17 cell differentiation and thus is likely a key molecule involved in the inflammation process of RA. Targeting of Cyr61 may be a novel therapeutic strategy for RA.

Materials and Methods

Animals

Six- to eight-week-old male DBA/1J mice were purchased from the Shanghai Laboratory Animal Center, Chinese Academy of Science. Mice were maintained under pathogen-free conditions. All experiments were performed according to the animal care and use committee guidelines.

Patients and specimens

A total of 48 RA patients (8 men and 40 women, aged 36–70 y, mean ± SD 53 ± 9 y) were included in the study. The disease duration of RA patients was 14 ± 9 y. The diagnosis of RA fulfilled the revised criteria by the American College of Rheumatology (25). The control subjects were 35 osteoarthritis (OA) patients fulfilling the diagnosis criteria of OA proposed by Altman (26). Synovial tissues were obtained from patients and FLS were cultured and identified as reported previously (18). The FLS were freshly isolated, and the cells were used for experiment at three to six passages. SF, serum specimens, and supernatants were collected as reported previously (18). All study protocols and consent forms were approved by the Institutional Medical Ethics Review Board of the Shanghai Jiao Tong University School of Medicine.

CD4+ T cell cocultured with FLS

CD4+ T cells (2 × 10^6), purified from healthy individual PBMC with Dynabeads Untouched Human CD4 T Cells kit (Invitrogen, Life Technologies) according to the manufacturer’s instructions, were cocultured with 2 × 10^5 FLS plated in 24-well plates stimulated with 5 μg/ml Cyr61 protein (PeproTech, Rocky Hill, NJ) or the Cyr61 gene was knocked down in a Transwell system. Abs to CD3, CD28, and rhIL-1β, anti–IFN-γ, and anti–IL-4 Ab (BD Biosciences) were added into the coculture system according to previous reports for Th17 differentiation (27).

Establishment and treatment of CIA

CIA was induced as described previously (28). Briefly, male DBA/1J mice were injected intradermally with 150 μg chicken type II collagen (Chondrex, Redmond, WA) in 0.05 M acetic acid emulsified in FCA. Booster injections were administered on day 21 with a total of 75 μg collagen II in Freund’s incomplete adjuvant. Joint inflammation was evaluated on a scale of 1–4 (29), with a maximum clinical score of 16 per mouse. Mice were treated with control IgG or anti-Cyr61 mAb 093G9 generated in our laboratory (200 μg/mouse) i.p. twice a week when the clinical score reached 2.

H&E staining and magnetic resonance imaging assay

Mice joints were removed from sacrificed CIA mice and fixed in 10% phosphate-buffered formalin, decalcified in 10% EDTA, embedded in paraffin, and stained with H&E for light microscopy. The magnetic resonance imaging (MRI) experiments were carried out at 7 T on a Varian NMR system (Varian) equipped with a 160-mm bore-diameter magnet, with maximum 400 mT/m gradients. The mouse was positioned on its side with its left hind paw held at a right angle on a horizontal plate, which was similar to the spontaneous posture of the treated mice. A rapid phased-array surface coil was used for data acquisition, and the radio frequency surface coil was positioned horizontally just above the mouse joint. The following parameters were used for optimized MRI imaging analysis: gradient echo sequence with fat suppression; FOV, 30 × 30 mm²; matrix size, 256 × 256; voxel size, 117 × 117 mm²; thickness, 0.8 mm; TR/TE, 195/5 ms; flip angle, 45°; NEX, 4 (30).

Real-time PCR analysis

Total RNA extracted from cells and real-time PCR were performed as previously reported (18). The primers used in this study are shown in Supplemental Table I.

Proliferation assay

Proliferation assay was performed as described previously (31). Briefly, mouse mononuclear cells (1 × 10⁶ per well) from spleens or lymph nodes treated with 093G9 or control IgG were incubated in the presence or absence of CH (20 μg/ml). Cultures were maintained at 37°C in 5% CO₂ and pulsed with 1 μCi[^3H]thymidine 16 h before harvest.[^3H]thymidine incorporation was measured as count pulse per minute.

Western blot analysis

Protein immune blotting was performed as described previously (18). In brief, tissue or cell lysates was separated by SDS-PAGE electrophoresis followed by transfer to polyvinylidene fluoride membranes (Millipore) at 30 V overnight. The expression of Cyr61 and the activation of STAT3, Jak1, Akt, and NF-κB was analyzed using specific Abs (Cell Signaling Technology, Beverly, MA). After washing with PBS, the membranes were incubated with HRP-conjugated goat anti-mouse IgG at room temperature for 1 h followed by washing with PBS. The target proteins were examined with an ECL system (Millipore) and visualized with autoradiography film.

Confocal laser scanning fluorescence microscopy assay

NF-κB nuclear translocation in FLS was studied with confocal laser scanning fluorescence microscopy (LSM510; Zeiss, Jena, Germany) technique as described previously (18).

ELISA detection

Sera of mice, SF, and sera of RA and OA patients and supernatants of cell culture were collected and diluted for the measurement of IL-6, IL-1β, and TGF-β by ELISA (R&D Systems) according to the manufacturer’s recommendations. A standard curve was performed for each plate and used to calculate the absolute concentrations of the indicated cytokines.

Flow cytometric analysis

Single-cell suspensions from CIA mice spleen and lymph nodes were isolated. CD4+ T cells cocultured with FLS were collected after 4 d. For surface markers staining, fluorescence conjugated CD4, CD130 Abs were used. We performed intracellular staining for IL-4, IL-10, IL-17, and IFN-γ after stimulation with PMA (50 ng/ml) and ionomycin (1 μg/ml; Sigma) for 5 h in the presence of GolgiPlug (BD Biosciences) according to the manufacturer’s protocol. Flow cytometry was performed using a FACSCalibur cytometer and analyzed using CellQuest software (BD Biosciences).

Statistical analysis

All experiments were performed in triplicate. The difference among groups was determined by ANOVA analysis, and comparison between two groups was analyzed by the t test using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA). A value of p < 0.05 was considered statistically significant.

Results

Cyr61 induced IL-6 production by FLS of RA patients

IL-6 is abundantly present in SF of RA patients, and a number of cell types are known to produce IL-6, including macrophages and fibroblasts (1, 11). We have previously shown that Cyr61, an extracellular matrix protein secreted by FLS, plays an important role in promoting FLS proliferation (18). To explore further whether...
Cyr61 may also stimulate IL-6 production by FLS, we set up an in vitro cell culture system using FLS isolated from RA patients. We first analyzed the cytokine profile of these patients (SF and serum samples from RA and OA patients) and found that the levels of IL-1β and IL-6 were higher in RA SF than in OA SF, but the serum levels of both cytokines were very low in both RA and OA patients (Fig. 1A), consistent with other reports (11, 32). However, TGF-β levels were higher in sera of RA and OA patients than those in SF (Fig. 1A). After confirming that RA SF contained higher levels of IL-6 and IL-1β, we next tested the potential effect of Cyr61 on the expression of these two cytokines by FLS of RA patients. The results showed that Cyr61 significantly stimulated IL-6 and IL-1β mRNA expression in FLS in a dose-dependent manner and reached a peak at 2 h (Fig. 1B, 1C), whereas the expression of TGF-β was not enhanced in response to Cyr61 stimulation (Fig. 1B). Consistent with these observations, ELISA assay showed that concentration of IL-6 in FLS culture supernatant was significantly increased upon addition of exogenous Cyr61, whereas the level of TGF-β did not show much alteration. Notably, although IL-1β mRNA expression in FLS was moderately increased by Cyr61 treatment, the concentration of IL-1β in FLS culture supernatant was too low to be detected (Fig. 1D), suggesting that FLS might not be a significant source of IL-1β in RA SF (11). To examine further the autocrine role of Cyr61 in the regulation of IL-6 expression by FLS, we used a specific small interfering RNA against Cyr61 to knock down Cyr61 expression in FLS. To probe further the downstream signaling pathways, we used known inhibitors of several pathways, including PDTC (inhibitor of NF-κB activation), SB203580 (inhibitor of p38 MAPK), and PD98059 (inhibitor of ERK1/2). The results showed that Cyr61-stimulated IL-6 mRNA and protein expression in FLS was markedly decreased in the presence of the NF-κB inhibitor. In contrast, inhibition of p38 MAPK and ERK1/2 activities showed no effect on Cyr61-induced IL-6 production (Fig. 2C). Further analysis showed that Cyr61 treatment led to a dramatic increase in the phosphorylation level of the NF-κB p65 subunit in FLS (Fig. 2D) and enhanced NF-κB nuclear translo-

**FIGURE 1.** Cyr61 promoted IL-6 production in RA FLS. (A) IL-6, IL-1β, and TGF-β levels in SF and serum from RA patients (n = 20) and OA patients (n = 15) detected by ELISA. OA-SF (●), RA-SF (▲), OA-sera (▼), and RA-sera (●). (B) IL-6, IL-1β, and TGF-β mRNA expression in RA FLS stimulated by Cyr61 (0.62, 1.25, 2.5, 5, 10 μg/ml) determined by real-time PCR using a housekeeping gene as an endogenous control. (C) IL-6, IL-1β, and TGF-β mRNA expression in RA FLS stimulated by Cyr61 (0, 2, 4, 6, 8 h) determined by real-time PCR. (D) IL-6 (▼), IL-1β (▲), and TGF-β (●) protein levels in supernatant were evaluated by ELISA. FLS were seeded into 12-well plates (1 × 10^5 per well). The FLS were stimulated with Cyr61 protein (10, 5, 2.5, 1.25 μg/ml) for 48 h. (E) IL-6, IL-1β, and TGF-β mRNA expression in Cyr61-knockdown FLS was detected by real-time PCR. (F) The protein levels of IL-6, IL-1α, and TGF-β in FLS culture supernatant treated with SiCyr61 (small interfering RNA against Cyr61, black bar) or SiNC (small RNA of negative control, open bar) were measured by ELISA. Data represent the mean ± SEM of at least three independent experiments. **p < 0.01.

Cyr61 induced IL-6 production in FLS via αvβ5/Akt/NF-κB signaling pathway

Previous studies have identified several integrins as the cell surface receptors for Cyr61 in different cell types (20, 22–24). We thus examined the mRNA expression profile of the integrin family in RA FLS and found high levels of expression for integrin αv, β1, and β5 (Fig. 2A). However, only the anti-αvβ5 Ab could block Cyr61-induced IL-6 production (Fig. 2B and data not shown), suggesting that αvβ5 is likely the major receptor on FLS that mediates the effect of Cyr61, which is consistently with our previous reports (18). To probe further the downstream signaling pathways, we used known inhibitors of several pathways, including PDTC (inhibitor of NF-κB activation), SB203580 (inhibitor of p38 MAPK), and PD98059 (inhibitor of ERK1/2). The results showed that Cyr61-stimulated IL-6 mRNA and protein expression in FLS was markedly decreased in the presence of the NF-κB inhibitor. In contrast, inhibition of p38 MAPK and ERK1/2 activities showed no effect on Cyr61-induced IL-6 production (Fig. 2C). Further analysis showed that Cyr61 treatment led to a dramatic increase in the phosphorylation level of the NF-κB p65 subunit in FLS (Fig. 2D) and enhanced NF-κB nuclear translo-

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Western blot. The data represent one of the three independent experiments. *significance of p < 0.05, **significance of p < 0.01, ***significance of p < 0.001.

**FIGURE 2.** Signaling pathways involved in Cyr61 regulation of IL-6 production in RA FLS. (A) Integrins involved in IL-6 production regulated by Cyr61. Integrin expression profiles in RA FLS treated with Cyr61 or BSA were evaluated by real-time PCR. (B) Inhibition of Cyr61-stimulated IL-6 expression by anti-αvβ5 mAb assessed by real-time PCR. The FLS were pretreated with anti-αvβ5 Ab (20 μg/ml) (Millipore, Billerica, MA) or control IgG for 1 h. After that, Cyr61 protein (5 μg/ml) was added to the culture system for another 2 h. The FLS were harvested to detect the expression of IL-6 by real-time PCR. (C) Effect of inhibitors of signaling pathways on Cyr61-induced IL-6 expression. RA FLS were treated with 4 μM PDTC, 1 μM PD98059, or 10 μM SB203580 in combination with Cyr61 (5 μg/ml) for 4 h (shaded bars). Cyr61 expression was evaluated by real-time PCR. Control (open bars); Cyr61 (no inhibitors, black bars). (D) Phosphorylation of NF-κB was detected by Western blot. Lane 1: unstimulated FLS. Lane 2 and lane 3, stimulated with Cyr61 (5 μg/ml) for 10 min and 30 min, respectively. (E) Nuclear translocation of NF-κB was monitored by confocal fluorescence microscopy. Top panels, Unstimulated FLS. Bottom panels, Stimulated with Cyr61 (5 μg/ml) for 60 min. NF-κB was detected by FITC–anti-p65 (green). Nuclei were stained with DAPI (blue). Merged picture shows NF-κB translocation into the nucleus. Original magnification ×600. (F) Phosphorylation of Akt was detected by Western blot. The data represent one of the three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

**Cyprinid-stimulated FLS promoted Th17 differentiation in vitro**

Considering that IL-6 is critical for Th17 differentiation and its expression was elevated in RA FLS stimulated by Cyr61, we hypothesized that this Cyr61-stimulated production of IL-6 by FLS might in turn promote Th17 differentiation. To this end, we cocultured RA FLS with purified CD4+ T cells in a Transwell system. The results showed that the expression of RORc, a critical transcription factor of human Th17, was significantly increased by coculturing with Cyr61-stimulated FLS compared with that in control BSA-treated FLS, as was Th17-associated cytokine IL-17 (Fig. 3A). The expression of IL-22 (34) was also increased. To corroborate this result further, we performed a similar coculture experiment using Cyr61-knockdown FLS, and results showed that knockdown of Cyr61 reduced the expression of RORc, IL-17, and IL-22 in the cocultured CD4+ T cells (Fig. 3B). We further examined the proportion of IL-17+ cells generated in this coculture system by intracellular cytokine staining. The results showed that Cyr61-stimulated FLS significantly enhanced the differentiation of IL-17–producing Th17 cells, whereas knockdown of Cyr61 in FLS or blocking of IL-6 by IL-6 neutralizing Ab reduced the stimulatory effect of FLS on Th17 differentiation (Fig. 3C, 3D and Supplemental Fig. 1). These data indicate that Cyr61-stimulated FLS facilitated Th17 differentiation in vitro.

**Blocking of Cyr61 ameliorated inflammation of CIA mice and downregulated Th17 population in vivo**

As we found that Cyr61-stimulated FLS promoted Th17 differentiation in vitro, we asked whether Cyr61 indeed plays a role in Th17 cell differentiation in vivo. We treated CIA mice with an anti-Cyr61 mAb (named 093G9), which was generated by our group (35) and shown to block the effect of Cyr61 on the proliferation of and IL-6 production by FLS (Fig. 4A, 4B). As shown in Fig. 5A, the inflammatory score of CIA mice treated with 093G9 was significantly lower than that of CIA mice treated with control IgG. Consistently, histological examination showed that the joints of mice treated with control IgG were infiltrated with leukocytes and exhibited synovial hyperplasia, pannus formation, cartilage destruction, and bone erosion, which are characteristic features of CIA. In contrast, these features were ameliorated by mAb 093G9 treatment. MRI analysis showed that in control IgG-treated mice,
the surrounding tissues were squeezed by joint inflammation and edema, and these symptoms were improved in 093G9-treated mice (Fig. 5A). Furthermore, the proliferation of T cells isolated from 093G9-treated mice in response to in vitro CII Ag challenge was significantly inhibited compared with that of the control IgG-treated group (Fig. 5B). Thus, treatment with 093G9 significantly reduced the inflammation phenotype of the CIA mice.

Given that Cyr61 production by FLS constitutes a positive-feedback loop in which its production further stimulates FLS proliferation (18) and that anti-Cyr61 treatment significantly improved the inflammation phenotype of CIA mice, we next examined Cyr61 expression in local synovial tissues of these treated mice. The results showed that Cyr61 expression was significantly decreased at both mRNA and protein levels after 093G9 treatment (Fig. 5C). Analyses of inflammatory cytokine profiles of these mice showed that levels of TNF-α and IL-17 were reduced in spleens and lymph nodes of 093G9-treated mice (Fig. 5D). Consistent with this, the expression of RORγt was also reduced (Fig. 5D). Flow cytometry analysis confirmed a specific reduction in the Th17 population in spleens and lymph nodes of 093G9-treated CIA mice compared with that in the control IgG-treated mice (Fig. 5E).

Blocking of Cyr61 attenuated IL-6/IL-6R signaling pathway in vivo

As we had observed that Cyr61 was able to stimulate IL-6 production in FLS (Fig. 1) and facilitate Th17 cell differentiation in vitro (Fig. 3), and that blocking of Cyr61 with a specific mAb led to downregulation of the Th17 population in CIA mice in vivo (Fig. 5), we asked whether the effect of anti-Cyr61 on Th17 differentiation in vivo was due to the reduction of IL-6 levels. Indeed, IL-6 expression in synovial tissues was dramatically decreased in 093G9-treated CIA mice compared with that of control IgG-treated mice, whereas the expression of TGF-β was not altered (Fig. 6A). Moreover, the concentration of IL-6 in sera of 093G9-treated mice was also reduced compared with that of control mice (Fig. 6B). We next examined whether Cyr61 blockade impaired the IL-6 signaling pathway in T cells of 093G9-treated mice. The results showed that the expression of IL-6Rα and IL-6Rβ was reduced significantly in T cells of 093G9-treated CIA mice compared with that of control mice (Fig. 6C). Consistently, the proportion of CD4+gp130+ T cells in 093G9-treated CIA mice was also dramatically decreased as shown by the FACS assay (Fig. 6D). We also examined the Jak1/STAT3 signaling pathway downstream of IL-6 receptor activation (36, 37) and found that the phosphorylation of Jak1 and STAT3 was inhibited (Fig. 6E) in

**FIGURE 3.** CD4+ T skewed into Th17 when cocultured with FLS. (A) Profile of Th17 differentiation relevant factors, RORγt, IL-17, IL-21, IL-22, and IL-23 expression in CD4+ T cells cocultured with Cyr61-provoked FLS. (B) Profile of genes indicated as in (A) in CD4+ T cells cocultured with Cyr61-knockdown FLS. (C) The proportion of CD4+IL-17+ cells detected by intracellular staining among CD4+ T cells cocultured with Cyr61-stimulated FLS or Cyr61-knockdown FLS or no FLS. (D) Percentage of CD4+IL-17+ cells. Data represent the mean ± SEM of at least three independent experiments. *p < 0.05, **p < 0.01.

**FIGURE 4.** Anti-Cyr61 mAb 093G9 inhibits proliferation of FLS and IL-6 production by FLS of RA patients in vitro. (A) The proliferation of FLS was inhibited by anti-Cyr61 Ab 093G9 compare with control IgG. (B) Cyr61-stimulated production of IL-6 by FLS was inhibited by 093G9.

*p < 0.05, **p < 0.01, ***p < 0.001.
T cells of 093G9-treated mice. Consistent with the observed effect of Cyr61 in vitro (Fig. 3), blocking of Cyr61 in vivo using 093G9 also led to reduction in the expression of IL-21 (Fig. 6F). We thus conclude that Cyr61 blockage attenuated IL-6/IL-6R signaling in vivo. Together, Cyr61 produced by RA FLS can initiate a novel cross-talk between FLS and Th17 cells. Cyr61 acts to stimulate IL-6 production by FLS, and the increased IL-6 then acts to promote Th17 differentiation (Fig. 7).

**Discussion**

CCN1/Cyr61 was the first identified member of the CCN family. It is a cysteine-rich protein of ~40 kDa encoded by a growth factor-inducible immediate-early gene (19, 24). Numerous studies have shown that Cyr61 regulates cell adhesion and cell migration. For instance, Cyr61 was shown to promote proliferation of endothelial cells and angiogenesis in vitro and in vivo (20, 38, 39). We recently reported that Cyr61 is overexpressed by RA FLS and stimulates FLS proliferation in an autocrine manner (18). Notably, the high expression of Cyr61 in RA FLS appears to be stimulated by IL-17. These results not only revealed for the first time to our knowledge that Cyr61 contributes to hyperplasia of synovial lining cells but also established a new link between Th17 cells and FLS in RA, whereby the Th17 cells act to promote FLS proliferation through stimulating the expression of Cyr61 in these cells.

Recently, it is known that FLS do not only provide the structural basis of organ composition and function as structure-building cells: under disease conditions such as inflammation, fibroblasts are critical switches that regulate the response to tissue injury, contribute to the resolution or chronicity of the organ-specific pathology, and determine the consequences of disease. In RA, FLS are a key part of the local immune system and, through the integration of signals from different sources, contribute to both disease initiation and perpetuation (1). Nevertheless, whether FLS-produced Cyr61 has in turn any effect on Th17 differentiation, as well as the mechanisms of Cyr61 in the inflammation process of RA, remain unclear.

In this study, we found that apart from its function to stimulate FLS proliferation, Cyr61 is able to induce IL-6 expression in these cells. Knockdown of endogenous Cyr61 led to reduced IL-6 expression in FLS, further substantiating the autocrine role of Cyr61 in inducing IL-6 expression by FLS. As IL-6 is a critical cytokine that functions in promoting Th17 differentiation (13, 40), we went on to test whether RA FLS could stimulate Th17 differentiation in a coculture system and found that it was indeed the case. Moreover, addition of exogenous Cyr61 or knockdown of endogenous Cyr61 enhanced or reduced the stimulatory effect of FLS on Th17 differentiation. Importantly, administration of a specific anti-Cyr61 Ab in CIA mice not only ameliorated the inflammation...
phenotype of these animals but also led to downregulation of the Th17 population in vivo. Mechanistically, we show that this was accompanied by a reduction of the IL-6/IL-6R signaling pathway in T cells of the anti-Cyr61–treated mice. Recently, it is observed that IL-21 is both a product and inducer of Th17 cells, providing a critical autocrine feedback loop in mice. However, human Th17 cells produce large amounts of IL-22, although mouse Th17 cells do not uniquely produce this cytokine. So, in our study, there was significant variation of IL-22 in human FLS treated with Cyr61, and the significant variation of IL-21 was observed in CIA mice treated with 093G9. IL-23 may be more important for the survival and population expansion of Th17 cells than for Th17 lineage commitment (15, 41). In our study, Cyr61-induced IL-6 may influence CD4+ T cell differentiation toward Th17 cells, but it may not affect the survival and population expansion of Th17 cells. In the current study, we failed to detect variation of IL-23 production both in vitro and in vivo. Collectively, the results suggest that Cyr61 produced by RA FLS can initiate a novel cross-talk between FLS and Th17 cells, whereby Cyr61 acts to stimulate IL-6 production by FLS, and the increased IL-6 then acts to promote Th17 differentiation (Fig. 7). This new cross-talk, together with the regulation of Cyr61 production in FLS by IL-17 that we had previously discovered, forms a novel feed-forward and malicious cycle that leads to mutual stimulation of FLS and Th17, two important cell populations in RA via Cyr61 promotion of IL-6 production. This malicious cycle may in turn play important roles in RA pathogenesis.

How does Cyr61 induce IL-6 production in FLS? Our results showed that among the candidate receptors for Cyr61, the levels of integrins αv, β1, and β5 were high on RA FLS. Nevertheless, only
the anti-cy5.5 Ab could block Cyr61-induced production of IL-6. Further analysis showed that Cyr61 treatment of FLS led to strong activation of Akt and NF-kB signaling. Inhibitor studies indicate that NF-kB activation plays an important role in Cyr61-induced IL-6 production. Notably, previous studies in other cell types also implicated that Cyr61 binding to integrin receptors could activate an Akt/NF-kB signaling pathway (20, 42, 43). On the basis of these results, we propose that Cyr61 induces IL-6 production in FLS via the cy5.5/Akt/NF-kB signaling pathway.

An interesting observation in OJ93G9-treated mice was that the expression of IL-6R on CD4+ T cells was decreased when these mice were treated with the anti-Cyr61 Ab. Similarly, the same finding was observed on human CD4+ T cells cocultured with FLS in which the endogenous Cyr61 was knockdown (data not shown). It is well known that a signaling cascade is triggered when IL-6 binds its receptor and gp130 homodimerization occurs (12, 44). Our observation is consistent with the finding by others that IL-6R signaling is responsible for the IL-6 effect on activation of its own downstream transcription factors. Therapeutic benefit of blocking IL-6 activity with an anti–IL-6 receptor mAb in RA treatment has been proved effectively (45). Thus, by blocking Cyr61-induced IL-6 production from FLS, the anti-Cyr61 Ab treatment could serve to reduce both IL-6 production (in FLS) and IL-6 receptor expression on T cells, resulting in effective inhibition of the IL-6 signaling pathway. Given the known critical role of IL-6 signaling in Th17 differentiation, we suggest that the reduced IL-6 signaling is likely an important mechanism underlying the attenuated Th17 response in anti-Cyr61–treated mice.

In summary, our results show that Cyr61 produced by FLS, which act as a key part of the local immune system in RA, is not only involved in hyperplasia of synovial lining cells but also acts as a novel proinflammatory factor contributing to the inflammation of RA via stimulation of Th17 differentiation. Notably, a recent study showed that Cyr61 induces macrophage differentiation into the M1 type macrophage in vitro (46), and recent reviews synthesize the idea that CCN proteins (including Cyr61) are novel inflammatory modulators (47–49), thus also supporting the role of Cyr61 as a proinflammatory factor. Given the critical role of Cyr61 in mediating the malignant cycle of mutual stimulation between FLS and Th17 cells (Fig. 7), we suggest that targeting of Cyr61 might represent a useful therapeutic strategy for the treatment of RA.

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Disclosures
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References