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IL-37 Alleviates Rheumatoid Arthritis by Suppressing IL-17 and IL-17–Triggering Cytokine Production and Limiting Th17 Cell Proliferation

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IL-37 is a new member of the IL-1 cytokine family, which is a natural inhibitor of innate immunity associated with autoimmune diseases. This study was undertaken to evaluate whether IL-37 has antiarthritic effects in patients with rheumatoid arthritis (RA) and in mice with collagen-induced arthritis (CIA). In this study, we analyzed the expression of IL-37 in PBMCs, serum, and lymphocytes from RA patients as well as CD4+ T cells polarized under Th1/Th2/Th17 conditions. The role of IL-37 was assessed by investigating the effects of recombinant human (rh)IL-37 and an adenovirus encoding human IL-37 (Ad–IL-37) on Th17 cells and Th17-related cytokines in RA patients and CIA mice. We found that active RA patients showed higher IL-37 levels compared with patients with inactive RA and healthy controls. Upregulated IL-37 expression also was found in CD3+ T cells and CD4+ T cells from RA patients and in Th1/Th17-differentiation conditions. rhIL-37 markedly decreased IL-17 expression and Th17 cell frequency in PBMCs and CD4+ T cells from RA patients. Furthermore, IL-37 exerted a more suppressive effect on Th17 cell proliferation, whereas it had little or no effect on Th17 cell differentiation. IL-17 and IL-17–driving cytokine production were significantly reduced in synovium and joint cells from CIA mice receiving injections of Ad–IL-37. Our findings indicate that IL-37 plays a potent immunosuppressive role in the pathogenesis of human RA and CIA models via the downregulation of IL-17 and IL-17–triggering cytokine production and the curbing of Th17 cell proliferation. The Journal of Immunology, 2015, 194: 5110–5119.

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by the infiltration of activated immune cells and the production of inflammatory factors that lead to the formation of synovial hyperplasia and pannus, as well as the destruction of cartilage and joints (1, 2). Although the etiology and pathogenesis of RA are not fully understood, recent in vivo animal models and in vitro human studies demonstrated that IL-17 can be considered a decisive mediator in the pathogenesis of RA (3).

IL-17 (also known as IL-17A) is the signature cytokine of the newly discovered CD4+ Th17 subsets that are known to have potent proinflammatory functions (3, 4). Based on data from animal models of autoimmune arthritis, IL-17 is recognized as one of the important factors in the pathogenesis of RA (3). Recent studies revealed that the expression of IL-17 is increased in serum, synovial fluid, and synovium of patients with RA compared with healthy controls (HCs) (4). Moreover, IL-17–transgenic mice are prone to develop collagen-induced arthritis (CIA), whereas blocking of endogenous IL-17 with its receptor in CIA results in the suppression of arthritis and reduced joint destruction (4, 5).

IL-37, a new member of the IL-1 family of cytokines, is produced by various types of cells, including PBMCs, epithelial cells, and macrophages (6). IL-37 has five splice variants: IL-37a to IL-37e (6). Only IL-37b and IL-37c contain exons 1 and 2 and express an N-terminal prodomain that includes a potential caspase-1 cleavage site (7). IL-37b is the largest isoform, has significant sequence similarity with IL-18, and is the most studied. The IL-37b precursor is cleaved by caspase-1 into mature IL-37b (6, 7). IL-37b has been identified as a natural suppressor of innate immunity (8).

Recently, IL-37 was shown to be highly expressed in the serum and PBMCs of patients with systemic lupus erythematosus (9) and in the synovial tissues and plasma of patients with RA (8, 10). We recently revealed that recombinant human (rh)IL-37 suppresses the expression of proinflammatory cytokines IL-1β and IL-6 in PBMCs from patients with systemic lupus erythematosus in vitro (9). In vivo, mice transgenic for IL-37 exhibited markedly reduced production of IL-17 and other proinflammatory cytokines, including IL-1β and IL-6, following LPS stimulation (11). Other studies showed that IL-37 is strongly expressed by CD4+ T cells and macrophages in human psoriatic plaques, which may inhibit the excessive inflammation in the pathogenesis of psoriasis (12). Based on the data described above, it is postulated that IL-37, through the production of activated immune cells, may play a prominent role in the pathogenesis of RA by curbing IL-17 or other proinflammatory cytokines.

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In this study, we investigated the expression of IL-37 in patients with active and inactive RA. We also analyzed the expression pattern of IL-37 in cell populations isolated from the PBMCs of patients with RA, as well as CD4+ T cells under Th1/Th2/Th17-differentiation conditions. The effect of IL-37 on the modulation of IL-17 and IL-17–driving cytokines (IL-1β and IL-6) in vitro and in vivo was examined in this study. We further analyzed the effect of IL-37 on Th17 cell differentiation and proliferation.

To our knowledge, this is the first study to show the anti-inflammatory properties of IL-37 in both human RA and the CIA model through the inhibition of IL-17 and IL-17–triggering cytokine production and the suppression of Th17 cell proliferation, which may contribute to its antiarthritic effects.

Materials and Methods

Patients and HCs

A total of 49 patients with RA, from Peking University Shenzhen Hospital, was included in the current study. The classification of RA fulfilled the 1987 and 2010 American College of Rheumatology criteria (13, 14). RA patients were divided into active (n = 26) and inactive (n = 23) groups, according to their 28-joint disease activity score (DAS28); active disease is defined as DAS28 ≥ 3.2, based on the European League Against Rheumatism diagnostic criteria (15). At the time of sample acquisition, 38 patients were being treated with prednisone, and 11 patients were not. HC peripheral blood samples were obtained from 33 healthy volunteers. RA patients and HCs did not exhibit significant differences in terms of mean age or male/female distribution (Supplemental Table I). The study was approved by the Review Board for the Peking University Shenzhen Hospital. Informed consent was obtained from the recruited subjects.

Preparation of human PBMCs

Peripheral blood samples were obtained from patients with RA and HCs. Serum samples were stored at −80°C until cytokines were determined. PBMCs from patients and HCs were isolated using lymphocyte separation medium (TBD Science), according to the manufacturer’s instructions. The collected cells were used for cell cultures or stored at −80°C until RNA extraction.

Cell sorting

Human PBMCs were stained using human-specific Abs, including FITC-conjugated anti-CD3, PE-conjugated anti-CD19, FITC-conjugated anti-CD4, and PE-conjugated anti-CD8 (all from BD Biosciences), and CD3+ T cells, CD19+ T cells, CD4+ T cells, and CD8+ T cells, respectively, were sorted using a FACS Aria II cell sorter (BD Biosciences).

T cell differentiation and proliferation in vitro

For human T cell differentiation, CD4+ T cells purified by flow cytometry were cultured in RPMI 1640 medium (HyClone, Thermo Fisher Scientific), supplemented with 10% FCS (Hangzhou Sijiqing Biological Engineering Materials) and 100 IU/ml penicillin and 100 μg/ml streptomycin (Beyotime), in the presence of anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml). The conditions for the different Th cell subsets are as follows: Th1 conditions: IL-12 (10 ng/ml), IL-2 (10 U/ml), and anti-IL-4 (10 μg/ml); Th2 conditions: IL-4 (20 ng/ml) and anti-IL-12 and anti–IFN-γ (10 μg/ml); and Th17 conditions: TGF-β (1 ng/ml), IL-6 (20 ng/ml), IL-1β (10 ng/ml), and anti–IFN-γ (1 μg/ml). Th17 cells were cultured from eBioscience. Cells were cultured for 5 d and then restimulated for 4 or 24 h with PMA (1 μg/ml) and ionomycin (1 μg/ml), or cells were treated for 4 or 24 h with LPS (1 μg/ml) for 24 h and then stimulated with LPS (1 μg/ml) for 6 h; culture supernatants were harvested and frozen at −80°C for later cytokine analysis by ELISA.

Flow cytometric analysis

Anti-human CD4-FITC and anti-human IL-17–PE were purchased from eBioscience. Anti-mouse CD4-allophycocyanin, anti-mouse IL-17–PE, and anti-mouse RORγt–PE were purchased from BD Biosciences. Human samples for intracellular staining of IL-17, PMA and Ion (500 ng/ml each), and brefeldin A (1 μg/ml) were added to cultures for the last 12 h before flow cytometric analysis. Mouse samples for staining of IL-17 and RORγt, PMA (50 ng/ml), Ion (1 μg/ml), and monensin (1000×) were added to cultures for the last 5 h before flow cytometric analysis, as previously described (16).

Construction of a recombinant adenovirus encoding human IL-37

Briefly, the human IL-37 gene (GenBank: AF167368) was amplified by PCR, using human PBMC CDNA as the template, and ligated to the pMd-1ST vector (Takara) for the construction of pMd-1ST–IL-37 plasmid. Subsequently, double digestion with EcoRV and Xhol (both from Takara) was conducted on plasmid pMd-1ST-IL-37 and shuttle vector pShuttle-IREs-hrGFP2 (Stratagene), respectively. Recovered products were connected with T4 DNA ligase (Takara), and the ligation mixture was transformed into DH5a bacteria (TransGen Biotech). Kana-resistant transformants were selected by plating the transformation mixture on Luria-Bertani (LB) agar plates supplemented with kanamycin (Sangon Biotech). After incubation overnight, the recombinant plasmid DNA was isolated and further confirmed via double digestion; the confirmed recombinant adenoviral vector was named pShuttle–IREs–hrGFP2–IL-37. Thereafter, pShuttle–IREs–hrGFP2–IL-37 was linearized by restriction endonuclease PmeI (Thermo Fisher Scientific) and subsequently cotransformed with adenoviral backbone vector pAdEasay-1 (Stratagene) in Escherichia coli DH1 cells (Stratagene) for homologous recombination. Then it was digested by Pac1 (Thermo Fisher Scientific) and transfected into HEK293 cells (Stratagene) with Lipofectamine 2000, according to the manufacturer’s instructions (Invitrogen), to generate an adenovirus encoding human IL-37 (Ad–IL-37). The empty adenovirus (Ad–EV) control used in this study was an empty pShuttle–IREs–hrGFP2 shuttle vector with no insert. Seven days later, green fluorescence could be seen under a fluorescence microscope. The concentration of the Ad–IL-37 was 3.5 × 10^10 PFU, as determined by plaque assay.

Adenovirus-mediated IL-37 gene expression

HEK293 cells were infected with Ad–IL-37 (0, 10^6, or 10^7 PFU) for 72 h, and cells were collected. Subsequently, Ad–IL-37 was detected in cell lysates by probing for Flag, and equal loading was determined by β-actin. The infection efficiency of Ad–EV and Ad–IL-37 in HEK293 cells was observed through GFP reporter fluorescence. The infection efficiency of Ad–EV and Ad–IL-37 in HEK293 cells was observed through GFP reporter fluorescence. The infection efficiency of Ad–EV and Ad–IL-37 in HEK293 cells was observed through GFP reporter fluorescence. The infection efficiency of Ad–EV and Ad–IL-37 in HEK293 cells was observed through GFP reporter fluorescence. The infection efficiency of Ad–EV and Ad–IL-37 in HEK293 cells was observed through GFP reporter fluorescence. The infection efficiency of Ad–EV and Ad–IL-37 in HEK293 cells was observed through GFP reporter fluorescence.
Preparation of synovial tissues and joint cells and collection of synovial fluids

Synovial tissues, joint cells, and synovial fluids were collected according to a previously described protocol (18). Synovial fluid samples were stored at −80°C prior to assay. Joint cells were cultured onto 24-well plates in RPMI 1640 medium (HyClone, Thermo Fisher Scientific) containing 10% FCS (Hangzhou Sijiqing Biological Engineering Materials) and incubated for 12 h in the presence of rhIL-37 (100 ng/ml).

RNA extraction and real-time PCR

RNA samples were extracted by TRizol reagent (Invitrogen), according to the manufacturer’s instructions. cDNA was prepared using the iScript cDNA Synthesis Kit (Bio-Rad). The primer sequences are summarized in Supplemental Table II. Real-time PCR amplification reactions were prepared with the SYBR Green PCR Kit (Bio-Rad) and performed using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems). PCR products were verified by melting curve analysis. Relative expression levels of target genes were calculated by normalization to β-actin values using the 2−ΔΔCt method.

ELISA

Human and mouse cytokine levels were measured by ELISA, according to the manufacturer’s instructions (eBioscience). Concentrations of CHI-specific IgG1 and IgG2a Abs (eBioscience) in the serum and synovial fluid of mice were measured by ELISA, as described previously (18).

Histopathologic analysis

Mice were anesthetized and killed on day 32, and knee joints were removed for histopathologic examination. Tissue sections were prepared and stained with H&E. Histopathologic scoring of joint damage was performed under blinded conditions, according to a widely used scoring system for evaluating synovitis, cartilage degradation, and bone erosion (18).

Statistical analysis

Data are expressed as mean (± SEM) or median (range) and were analyzed using GraphPad Prism v5.00 software (GraphPad). A two-tailed Student t test was used for the statistical comparison of two groups. Where appropriate, two-way ANOVA, followed by the Bonferroni post test for multiple comparisons, and a Mann–Whitney U test for nonparametric data were used. The Spearman correlation test was used to assess the association between serum IL-37 levels and different variables. The p values ≤ 0.05 were considered significant.

Results

Increased IL-37 mRNA and serum protein levels in patients with active RA and their correlation with the inflammation response

To investigate the potential role of IL-37 in patients with RA, 49 RA patients and 33 age- and gender-matched HC were recruited (Supplemental Table I). Distinct differences in IL-37 mRNA and serum protein levels were present between active RA patients and inactive RA patients (Fig. 1A, 1B). Patients with active RA showed higher IL-37 mRNA (Fig. 1A) and serum IL-37 (Fig. 1B) protein levels than did HCs. However, we did not observe differences in IL-37 mRNA and protein levels between inactive RA patients and HCs (Fig. 1A, 1B). To observe the effect of prednisolone on IL-37 expression, RA patients were divided into treated and untreated groups. We found that prednisolone-treated RA patients showed lower IL-37 levels than did RA patients before treatment (Fig. 1C). However, there was no difference in the levels of IL-37 in RA patients not treated with prednisolone compared with RA patients before treatment (Fig. 1C). At the time of the highest inflammatory activity during the observation period, as determined by maximal C-reactive protein (CRP) and/or erythrocyte sedimentation rate (ESR) values, serum levels of IL-37 correlated with CRP (Supplemental Fig. 1A, R = 0.4141, p = 0.0031) and ESR (Supplemental Fig. 1B, R = 0.3973, p = 0.0047). Similarly, there was a positive correlation between serum IL-37 levels and DAS28 (Supplemental Fig. 1C, R = 0.4172, p = 0.0029). No significant correlation was observed between serum levels of IL-37 and rheumatoid factor (RF) or anti-cyclic citrullinated peptide Abs (anti-CCPs) (Supplemental Fig. 1D, 1E).

IL-17 and IL-17–driving cytokines (IL-1β and IL-6) play an important role in the initiation and progression of CIA. We next assessed whether serum IL-37 levels correlated with these cytokines. As seen in Supplemental Fig. 1F–H, serum IL-37 concentrations correlated positively with the levels of serum IL-17 (R = 0.3388, p = 0.0173), IL-1β (R = 0.3554, p = 0.0122), and IL-6 (R = 0.3215, p = 0.0243) in RA patients.

Upregulated IL-37 mRNA expression in peripheral CD3+ T cells and CD4+ T cells from patients with RA

To further investigate whether CD4+ Th cell (Th1, Th2, and Th17) differentiation regulates the expression of IL-37, CD4+ T cells from PBMCs of HCs were primed in vitro for 5 d under Th1-, Th2-, or Th17-polarizing conditions. Cells were then restimulated with PMA/Ion or LPS and examined for IL-37 expression using real-time PCR and ELISA. The results showed that both PMA/Ion and LPS clearly induced IL-37 and IFN-γ mRNA expression in CD4+ T cells under Th1-polarizing conditions (Fig. 2A), consistent with elevated IL-37 and IL-17 mRNA levels in CD4+ T cells under Th17-polarizing conditions (Fig. 2B). Although IL-4 mRNA expression was highly increased in CD4+ T cells polarized under Th2 conditions, the expression of IL-37 mRNA in CD4+ T cells polarized under Th2 conditions was unaffected by treatment with PMA/Ion or LPS (Fig. 2C). Notably, IL-37 protein also was stably expressed in CD4+ T cells under Th1- and Th17-polarizing conditions via stimulation of PMA/Ion or LPS (Fig. 2D). Taken together, these results indicate that IL-37 is consistently elevated during CD4+ T cell activation, especially activated Th1 and Th17 cells.

IL-37 suppresses proinflammatory cytokine expression in PBMCs from patients with RA

To determine whether IL-37 inhibits proinflammatory cytokine production in RA, we expressed and purified rhIL-37 protein (9). Our results revealed that rhIL-37 downregulated proinflammatory cytokine expression in PBMCs from HCs in a dose-dependent manner (optimum concentration of rhIL-37 was 100 and 200 ng/ml) (data not shown). To further address the effects of IL-37 on proinflammatory cytokines in PBMCs from RA patients, PBMCs from 49 RA patients and 33 HCs were either left untreated or treated with rhIL-37 (100 ng/ml) for 6 or 24 h, and cells and culture supernatants were harvested for real-time PCR and ELISA analysis, respectively. We found that the transcriptional levels of proinflammatory cytokines IL-17 (Fig. 3A), IL-1β (Fig. 3B), and IL-6 (Fig. 3C) in PBMCs of RA patients were significantly suppressed by rhIL-37. rhIL-37 also markedly reduced the secretion of IL-17 (Fig. 3D), IL-1β (Fig. 3E), and IL-6 (Fig. 3F) in PBMCs of RA patients. However, the cytokine mRNA (Fig. 3A–C) and protein (data not shown) levels in PBMCs of HCs were not noticeably changed by treatment with rhIL-37.
IL-37 inhibits IL-17 expression in CD4+ T cells from patients with RA and decreases RA Th17 cells

To further investigate the effects of IL-37 on IL-17 expression from CD4+ T cells in patients with RA, CD4+ T cells from PBMCs of RA patients and HCs were cultured or not with rhIL-37. We demonstrated that the expression of IL-17 mRNA in CD4+ T cells from patients with RA was markedly higher than that in cells from HCs (Fig. 4A). More importantly, rhIL-37 significantly reduced the expression of IL-17 mRNA in CD4+ T cells from patients with RA, as determined by real-time PCR, but IL-17 mRNA expression in HCs was not inhibited by treatment with rhIL-37 (Fig. 4A). To further analyze the levels of IL-17–driving cytokines IL-1β and IL-6, we next examined the levels of these cytokines in CD4+ T cells of RA patients. Consistent with the above findings, IL-1β (Fig. 4B) and IL-6 (Fig. 4C) mRNA levels were significantly higher in CD4+ T cells of RA patients. Notably, rhIL-37 also significantly downregulated the expression of IL-1β (Fig. 4B) and IL-6 (Fig. 4C) mRNA in CD4+ T cells from patients with RA. We further observed that the mean percentage of Th17 cells was significantly reduced by treatment with rhIL-37 (4.2% without rhIL-37 versus 2.0% with rhIL-37) (Fig. 4D). However, IL-1β is not required for IL-37–mediated suppression of IL-17 expression in CD4+ T cells from RA patients (Supplemental Fig. 2). Collectively, these data indicated that IL-37 suppresses IL-17 and IL-17–triggering cytokine expression and reduces the proportion of Th17 cells; the suppressive effect of IL-37 was independent of IL-1β.

IL-37 limits Th17 cell proliferation from naive CD4+ T cells in culture but not Th17 cell differentiation

To evaluate whether IL-37 exerts any direct effects on Th17 cell differentiation and proliferation in vitro, naive CD4+ T cells purified from the spleens of normal mice were cultured with IL-37 under Th17-polarizing conditions and the frequency of IL-17+CD4+ T cells was analyzed by flow cytometry. As shown in Fig. 5A, IL-37 did not significantly change the percentage of IL-17+CD4+ T cells, it only slightly reduced Th17 cell differentiation. Interestingly, upon treatment with IL-37 (100 ng/ml), the number of Th17 cells in the total CD4+ T cell population was markedly reduced (Fig. 5B). Because the differentiation of Th17 cells is governed by the transcript factor RORγt (5), we investigated whether IL-37 affects the expression of RORγt in naive T cells at the stage of Th17 cell differentiation. As shown in Fig. 5C, the expression of RORγt showed no obvious changes upon IL-37 treatment, indicating the minor effect of IL-37 on Th17 cell differentiation. To further investigate whether IL-37 can suppress Th17 cell proliferation, CFSE-labeled naive CD4+ T cells were cultured with IL-37 and then restimulated for 4 h with PMA (500 ng/ml) and Ion (500 ng/ml); or the cells were treated for 4 h with LPS (1 μg/ml). The mRNA levels of IL-37 and IFN-γ (Th1 polarization) (A), IL-37 and IL-17 (Th17 polarization) (B), and IL-37 and IL-4 (Th2 polarization) (C) were assessed by real-time PCR. (D) CD4+ T cells from PBMCs of HCs were prepared in Th1- and Th17-polarizing conditions. The cells were cultured for 5 d and then restimulated for 4 h with PMA (500 ng/ml) and Ion (500 ng/ml), or cells were treated for 4 h with LPS (1 μg/ml). The mRNA levels of IL-37 and IFN-γ (Th1 polarization) (A), IL-37 and IL-17 (Th17 polarization) (B), and IL-37 and IL-4 (Th2 polarization) (C) were assessed by real-time PCR. (D) CD4+ T cells from PBMCs of HCs were prepared in Th1- and Th17-polarizing conditions. The cells were cultured with PMA (500 ng/ml) and LPS (1 μg/ml).
cultured with IL-37 (50 and 100 ng/ml) under Th17-differentiation conditions. Flow cytometric analysis revealed that Th17 cell proliferation was significantly suppressed by 100 ng/ml IL-37 (Fig. 5D). Taken together, these data suggest that although IL-37 efficaciously inhibits the proliferation of Th17 cells, it does not readily suppresses Th17 cell differentiation.

Overexpression of Ad–IL-37 in HEK293 cells and mice joints

Because the mouse IL-37 sequence has not been reported, we constructed recombinant Ad–IL-37 to further investigate the anti-inflammatory properties of IL-37. To verify that Ad–IL-37 was capable of expressing IL-37, HEK293 cells were infected with 0, 10⁵, or 10⁷ PFU of Ad–IL-37 for 72 h, and IL-37 protein expression was determined by Western blot. We observed adenovirus-mediated IL-37 expression (10⁵ or 10⁷ PFU) from HEK293 cell lysates (Fig. 6A). Fluorescence microscopy further confirmed that the infection efficiency of 10⁷ PFU Ad-EV or Ad–IL-37 was near 100% in HEK293 cells (Fig. 6B). In vivo measurement of GFP reporter fluorescence did not differ between Ad–IL-37–expressing mice and Ad-EV control mice showing high expression in the joints (Fig. 6C). Subsequently, we used real-time PCR to assess IL-37 expression in the joint, spleen, liver, and kidney of mice with intra-articular injection of 10⁷ PFU of Ad-EV and Ad–IL-37. We found that Ad–IL-37 strongly expressed IL-37 only in the joints of mice, with no change in spleen, liver, or kidney (Fig. 6D). Thus, we used 10⁷ PFU of Ad–IL-37 and Ad-EV for subsequent experiments.

Local intra-articular injection of Ad–IL-37 attenuates the development of CIA

To confirm whether locally elevated IL-37 concentrations can alleviate CIA progression, we injected Ad–IL-37 and Ad-EV

FIGURE 3. IL-37 inhibited the expression of IL-17, IL-1β, and IL-6 in PBMCs from patients with RA. PBMCs from RA patients (n = 49) and HCs (n = 33) were stimulated or not with rhIL-37 (100 ng/ml) for 6 h, and the mRNA levels of IL-17 (A), IL-1β (B), and IL-6 (C) were detected by real-time PCR. PBMCs from RA patients (n = 49) were stimulated or not with rhIL-37 (100 ng/ml) for 24 h and then incubated with LPS (1 μg/ml) for 6 h, and IL-17 (D), IL-1β (E), and IL-6 (F) levels in supernatant were assessed by ELISA. *p < 0.05, **p < 0.01.

FIGURE 4. IL-37 downregulated Th17-related cytokine expression in CD4⁺ T cells from patients with RA and reduced Th17 cells. CD4⁺ T cells from PBMCs of RA patients and HCs were cultured in the absence or presence of rhIL-37 (100 ng/ml) for 24 h and then stimulated with LPS (1 μg/ml) for 6 h. The mRNA levels of IL-17 (A), IL-1β (B), and IL-6 (C) were measured by real-time PCR. (D) Frequencies of IL-17⁺CD4⁺ cells (Th17 cells) were quantified in RA peripheral blood CD4⁺ T cells treated with PMA, Ion, and brefeldin A in the absence or presence of rhIL-37 (100 ng/ml). Data are integrated from three independent experiments. All data are mean ± SEM. *p < 0.05, **p < 0.01.
(10^7 PFU each) intra-articularly into the knee joint of DBA/1J mice on days 17 and 23 after the first immunization (Fig. 7A). The results showed that the incidence and symptoms of arthritis in Ad–IL-37–treated mice were significantly reduced compared with Ad-EV–treated mice (Fig. 7B, 7C). Histopathologic examination showed that, compared with Ad-EV–treated mice with CIA, the knee joints of Ad–IL-37–treated mice with CIA exhibited a significant reduction in synovial hyperplasia, pannus formation, cartilage damage, and bone erosion (Fig. 7D, 7E). To ascertain whether the CII-specific humoral immune response was modulated by Ad–IL-37 treatment, serum and synovial fluid from Ad–IL-37–treated and Ad-EV–treated mice were analyzed for the presence of CII-specific IgG1 and IgG2a Abs. Interestingly, no obvious changes in the levels of serum and synovial fluid CII-specific IgG1 Abs were observed in either group (Fig. 7F). In contrast, synovial fluid levels of CII-specific IgG2a Abs were dramatically lower in Ad-IL-37–treated mice compared with Ad-EV–treated mice, whereas serum CII–specific IgG2a Ab levels were not affected (Fig. 7G). Taken together, these results indicate that local expression of IL-37 contributes directly to the suppression of joint inflammation and pathologic changes in CIA.

**FIGURE 5.** IL-37 inhibited murine Th17 cell proliferation but not Th17 cell differentiation. (A) Naïve CD4^+ T cells from normal mice were cultured under Th17-polarizing conditions in the presence of IL-37 (0, 50, and 100 ng/ml) for 72 h, and then the percentage of CD4^+IL-17^+ cells was determined by flow cytometry. The flow cytometric profiles are representative of four independent experiments with similar results. (B) Total numbers of IL-17^+ CD4^+ cells in each treatment described in (A) were enumerated by cell counting. Data are mean ± SEM (n = 4). (C) The expression of RORγt in naïve CD4^+ T cells under Th17-polarizing conditions in the presence of IL-37 (0, 50, and 100 ng/ml) was determined by flow cytometry. The shaded line graph represents isotype staining. Results represent four independent experiments. (D) CFSE-labeled purified naïve splenic CD4^+ T cells were cultured with IL-37 (0, 50, and 100 ng/ml) in Th17-polarizing medium for 3 d, and cells were stimulated with PMA, Ion, and monensin for 5 h. Th17 cells were analyzed by flow cytometry; each peak of Th17 cells represents their division in different culture conditions. Results are representative of four independent experiments. *p < 0.05.

**FIGURE 6.** Adenovirus-mediated overexpression of IL-37 in HEK293 cells and in mice joints. (A) HEK293 cells were infected with 0, 10^5, or 10^7 PFU of Ad–IL-37 for 72 h, and IL-37 protein expression was determined by Western blot. (B) Infection efficiency of Ad-EV or Ad–IL-37 was near 100% in HEK293 cells, as indicated by GFP reporter fluorescence. Original magnification ×200. (C) The joints of mice were injected with 10^7 PFU of Ad-EV and Ad–IL-37, and GFP reporter fluorescence was measured in vivo using the IVIS imaging system. (D) IL-37 mRNA expression was determined by real-time PCR in the indicated tissues of mice with intra-articular injection of 10^7 PFU of Ad-EV or Ad–IL-37. Data are integrated from three independent experiments (mean ± SEM). **p < 0.01.
FIGURE 7. Local overexpression of IL-37 ameliorates the pathology of CIA. (A) Protocol for CIA induction and IL-37 administration in CII-immunized DBA/1J mice. Mice (age 8–12 wk) were immunized intradermally at the base of tail with 200 μl CII emulsified in CFA. On day 21, mice were given a boost immunization of 80 μl CII dissolved in CFA. Ad–IL-37 (10⁷ PFU) or Ad-EV (10⁷ PFU) was injected intrarticularly into the knee joint on days 17 and 23 after the first immunization. Mice were killed on day 32 or later for experimental analysis. Incidence (B) and mean clinical scores (C) of CIA in mice treated with Ad–IL-37 or Ad-EV. Data are from five independent experiments (n = 3/group in each experiment). (D) Representative H&E staining of knee joints of Ad–IL-37– or Ad-EV–treated mice with CIA. The images on the right are enlargements of the boxed areas in the images on the left. (E) Evaluation of synovitis, pannus, and erosion of bone and cartilage in the knee joint sections of Ad–IL-37– or Ad-EV–treated mice with CIA. Levels of CII-specific IgG1 (G) and IgG2a (G) Abs in serum and synovial fluid from CIA mice treated with Ad-EV or Ad–IL-37 were assessed by ELISA (n = 5). Data are mean ± SEM. *p < 0.05, **p < 0.01.

The anti-inflammatory effects of Ad–IL-37 are associated with downregulated IL-17 and IL-17–triggering cytokines in the joints of mice with CIA

Proinflammatory cytokines (IL-17, IL-6, and IL-1β) are implicated in the pathogenesis of RA (2). Therefore, we asked whether IL-37 inhibits the expression of these proinflammatory factors in vivo. Compared with Ad-EV–treated mice with CIA, IL-17 levels were significantly decreased in the synovial fluid of Ad–IL-37–treated mice with CIA (Fig. 8A). IL-17–triggering cytokines (IL-6 and IL-1β) also were significantly reduced in the synovial fluid of Ad–IL-37–treated mice with CIA (Fig. 8A). Consistent with the above findings, the expression of IL-17 (Fig. 8B), IL-1β (Fig. 8C), and IL-6 (Fig. 8D) was dramatically downregulated in synovium and joint cells from Ad–IL-37–treated mice with CIA. Importantly, our results also revealed that rhIL-37 could significantly decrease IL-17 and IL-17–triggering cytokine mRNA expression in joint cells from CIA mice in vitro (Fig. 8E). These results suggest that local overexpression of IL-37 could curb IL-17 and IL-17–triggering cytokine (IL-1β and IL-6) production in joints of mice with CIA.

Discussion

IL-37 was shown to be involved in autoimmune disease as an anti-inflammatory cytokine and an inhibitor of both innate and acquired immune responses (6, 8). However, the source of IL-37–expressing cells in RA is unclear, and the effect of IL-37 in the pathogenesis of RA remains unknown. In this study, we provide a detailed analysis of IL-37 expression in various immune cells from patients with active/inactive RA and HCs. More importantly, we show for the first time, to our knowledge, that IL-37 markedly suppressed the production of IL-17 and IL-17–triggering cytokines and the frequency of Th17 cells in CD4⁺ T cells and PBMCs in RA patients; these immunosuppressive effects of IL-37 appear to be independent of IL-1β. Additionally, injection of Ad–IL-37 effectively reduced the clinical and histologic scores in CIA mice by downregulating IL-17 and IL-17–triggering cytokine production. The antiarthritic effects of IL-37 on the production of IL-17 can be attributed, in part, to its inhibitory effects on Th17 cell proliferation but not Th17 cell differentiation.

Recent data demonstrated that IL-37 expression was significantly elevated in the plasma and synovium of patients with RA (8, 10). Consistent with these findings, our results also revealed higher levels of IL-37 in PBMCs and serum from RA patients compared with HCs. Additionally, we clearly observed that IL-37 mRNA and protein levels were significantly higher in 26 patients with active disease than in 23 patients with inactive disease and 33 HCs. To the best of our knowledge, the monitoring of RA disease activity is traditionally based on clinical observations and laboratory values, such as DAS28, CRP, ESR, RF, and anti-CCP. Although CRP concentrations and ESR are reliable biochemical indicators of the acute-phase response in RA, neither is sufficient for distinguishing among RA patients with active disease. Consistent with previous results (10), serum IL-37 correlated closely with DAS28 and CRP. It is important to mention that serum IL-37 level also correlated with ESR, but it lacked an association with other laboratory values (RF and anti-CCP). Given that IL-17 and IL-17–triggering cytokines (IL-1β and IL-6) are implicated in RA inflammatory responses (2), a significantly positive correlation was observed between serum IL-37 levels and these three cytokines. It is worth noting that IL-1β and IL-6 are the main stim-
ulators of the acute phase response in RA (19), and IL-17 had a significant correlation with the inflammation marker CRP and ESR (20). Hence, these data suggest that IL-17 and IL-17–driving cytokines enhance the acute-phase response, as well as stimulate anti-inflammatory cytokine IL-37 expression to downregulate excessive inflammation during the pathogenic process of RA.

Furthermore, our findings found that there was no association between IL-37 and RF/anti-CCP, indicating the preponderant role of T cells in the pathogenesis of RA, because RF and anti-CCP Ab production were mainly derived from plasma cells and B lymphocytes (21).

The cumulative evidence suggested that the inflammatory response induced the upregulation of IL-37, which exerts an immunosuppressive role in autoimmune diseases (6, 8). An interesting question that emerges from these findings is “What is the source of IL-37 expression in the process of RA inflammatory response?” Considering the crucial role of T and B cells in the pathogenesis of RA (1), we attempted to investigate whether T and B cells are critical for IL-37 expression in RA patients. Our results indicate that IL-37 is predominantly detected in CD3+ and CD4+ T cells, but not in CD8+ T cells or CD19+ B cells, from patients with RA. Thus, increased IL-37 expression in CD3+ and CD4+ T cells might be due to the activation of T cells in RA patients. Meanwhile, the expression of IL-37 in activated CD3+ and CD4+ T cells could be involved in the adaptive immune response in RA. Of note, numerous CD4+ T cells, but not CD8+ T cells, accumulate mainly in RA periphery and joint and may be involved in the pathogenesis of RA (1, 22). These data probably reflect the fact that, in RA periphery–activated immune cells, CD3+ and CD4+ T cells are the mostly like source of IL-37. Therefore, IL-37–mediated anti-inflammatory effects could be mediated by CD3+ and CD4+ T cells. Despite all of this, the presence of immune cells and the expression of IL-37 may be affected by RA disease activity. Further research is necessary to determine the expression of IL-37 immune cells during the different stages of RA disease activity.

Many studies demonstrated that the balance of CD4+ T cell subsets (Th1, Th2, and Th17) is involved in the pathogenesis of RA via secretion of IFN-γ, IL-4, and IL-17 (2, 5). In this study, we showed that Th1- and Th17-polarizing conditions induced stable IL-37 expression, but the expression of IL-37 in CD4+ T cells polarized under Th2 conditions was unaffected. Furthermore, Th1-secreted cytokine IFN-γ and Th17-secreted cytokine IL-17 were increased in CD4+ T cells polarized under Th2 conditions was unaffected. Furthermore, Th1-secreted cytokine IFN-γ was highly effective in inducing IL-37, whereas Th2-secreted cytokine IL-4 plus GM-CSF inhibited constitutive IL-37 expression (8). Actually, as mentioned above (Supplemental Fig. 1F), there was an obvious correlation between IL-37 and Th17-secreted cytokine IL-17. These observations suggest that Th1/Th17 cytokines (IFN-γ and IL-17) may play a part or a key role in inducing IL-37 production in CD4+ T cells. In contrast, Th2 cytokine IL-4 may provide an inhibitor signal for IL-37 production in CD4+ T cells.

The observations accumulated thus far indicate that Th17 cells and their signature cytokine IL-17 are involved in the development, as well as the progression, of RA (3, 4). Increased IL-17 expression in serum and synovial tissue from patients with RA was demon-

FIGURE 8. IL-37 suppressed the expression of inflammatory factors in joints of CIA mice. (A) Concentrations of synovial fluid cytokines (IL-17, IL-1β, and IL-6) from CIA mice treated with Ad-EV or Ad–IL-37 were measured by ELISA. Expression of IL-17 (B), IL-1β (C), and IL-6 (D) in synovial tissues and joint cells of CIA mice treated with Ad-EV or Ad–IL-37 was determined by real-time PCR. (E) Joint cells from mice with CIA were cultured or not with rhIL-37 (100 ng/ml) for 12 h. The expression of IL-17, IL-6, and IL-1β mRNA was determined by real-time PCR. All data are mean ± SEM (n = 3). *p < 0.05, **p < 0.01.
neutralizing Ab did not affect the frequency of IL-17+CD4+
PBMCs from RA patients, IL-37 did not appear to exert its anti-
inflammatory properties (30). In addition, IL-37 fails to inhibit IL-17–triggering cytokine (IL-1β and IL-6) production and the MAPK signaling pathway in DCs from IL-1R8–deficient cells (31). A recent study showed that IL-17 expression was dramatically enhanced in IL-1R8–deficient cells (31). These results suggest that IL-37 may suppress IL-17 and IL-17–triggering cytokine production in the development of RA via binding IL-1R8 and curbing the MAPK signaling pathway in DCs. Nevertheless, further investigations and manipulations of IL-37 signaling may improve therapeutic options for RA patients.

In summary, we demonstrate for the first time, to our knowledge, that IL-37 levels were elevated in active RA and were associated particularly with Th17 cytokines and with Th17/Th1 differentiation. More importantly, the results reveal an anti-inflammatory effect of IL-37 in human RA and the CIA model. IL-37 may mediate its antiarthritic effects through inhibition of IL-17 and IL-17–triggering cytokines and via suppression of Th17 cell proliferation. These data suggest a possible therapeutic significance for IL-37 in RA.

Acknowledgments
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Disclosures
The authors have no financial conflicts of interest.

References


Supplementary material

Interleukin-37 alleviates rheumatoid arthritis via suppressing IL-17 and IL-17 triggering cytokines production and limiting Th17 cell proliferation

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Running head: IL-37 suppresses Th17 response in arthritis

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Supplemental Table 1.

Clinical and laboratory characteristics in patients with rheumatoid arthritis (RA) and healthy control (HC) subjects.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>RA patients</th>
<th>Healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO. of cases</td>
<td>49</td>
<td>33</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>42(85.71%)</td>
<td>28(84.85%)</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>7(14.29%)</td>
<td>5(15.15%)</td>
</tr>
<tr>
<td>Age, years (range)</td>
<td>45.6(22-73)</td>
<td>43.2(20-74)</td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>3.4±2.1</td>
<td>-</td>
</tr>
<tr>
<td>ESR (mm/h)(mean±SD)(%)</td>
<td>27.6±20.6(59.18%)</td>
<td>-</td>
</tr>
<tr>
<td>RF concentration (IU/mL)(mean±SD)(%)</td>
<td>127.9±101.0(91.84%)</td>
<td>-</td>
</tr>
<tr>
<td>CRP (mg/L)(mean±SD)(%)</td>
<td>8.6±8.9(36.73%)</td>
<td>-</td>
</tr>
<tr>
<td>Anti-CCP (U/mL) (mean±SD)(%)</td>
<td>63.6±53.0(89.80%)</td>
<td>-</td>
</tr>
<tr>
<td>DAS28 (mean±SD)</td>
<td>4.6±2.2</td>
<td>-</td>
</tr>
<tr>
<td>PSL drug responders, n(%)</td>
<td>38(77.55%)</td>
<td>-</td>
</tr>
<tr>
<td>PSL drug non-responder, n(%)</td>
<td>11(22.45%)</td>
<td>-</td>
</tr>
</tbody>
</table>

Healthy controls did not have inflammatory or autoimmune disorders, cancer or a history of cancer, or bacterial, viral, or fungal infectious diseases and were not receiving any immunosuppressive or immunomodulating agents. Except where otherwise indicated, values are expressed as mean±standard deviation. There were no significant differences between patients with RA and healthy controls in terms of age and sex. ESR, erythrocyte sedimentation rate; RF, rheumatoid factor; CRP, C-reactive protein; anti-CCP, anti-cyclic citrullinated peptide antibodies; DAS28, 28-joint Disease Activity Score; PSL, prednisolone.
### Supplemental Table 2.

List of the sequence of gene primers.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward (5’ to 3’)</th>
<th>Reverse (5’ to 3’)</th>
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<tr>
<td>Human IL-37</td>
<td>AGTGCTGCTTAGAAGACCCGG</td>
<td>AGAGTCCAGGACCAGTACTTTTGTGA</td>
</tr>
<tr>
<td>Human IL-17</td>
<td>ACCTCATTGGTGCTACTGCTAC</td>
<td>GTTCAGGTTGACCATCACAAGTC</td>
</tr>
<tr>
<td>Human IL-1β</td>
<td>CCACAGACCTTCAGGAGAAT</td>
<td>GTGCACATAAGCCTCAGTATCC</td>
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<tr>
<td>Human IL-6</td>
<td>AGCCACTCACCCTCTCAGAAC</td>
<td>ACATGTCCTCCTTTCACAGGCC</td>
</tr>
<tr>
<td>Human IL-4</td>
<td>ACGGACACAAAGTGCGATACGCA</td>
<td>ATGTCTGTGTTACGGTCAACTCGG</td>
</tr>
<tr>
<td>Human IFN-γ</td>
<td>TTCTCTTGGCTTGTTACTGCCAG</td>
<td>CGCTACATCTGAATGACCTGCA</td>
</tr>
<tr>
<td>Human β-actin</td>
<td>CCTGACTGACTCCTCATGAAAG</td>
<td>GACGTAGACAGCCTTCTCCTTA</td>
</tr>
<tr>
<td>Mouse IL-17</td>
<td>CAGCAGCGATCATCCTCAAAGG</td>
<td>CAGGACCAGGATCTCTTGGCTG</td>
</tr>
<tr>
<td>Mouse IL-1β</td>
<td>CCTTCCAGGAGGAGGACATGA</td>
<td>TGAAGTACAGAGGATGGGCTC</td>
</tr>
<tr>
<td>Mouse IL-6</td>
<td>ATGGATCTACCAAACCTGGAAT</td>
<td>TGAAGGACTCTGGCTTTGTCT</td>
</tr>
<tr>
<td>Mouse β-actin</td>
<td>GTGACGTTGACATCCGTAAGAGA</td>
<td>GCCGGACTCAGTCTCC</td>
</tr>
</tbody>
</table>
Supplemental Figure 1. Correlation analysis between RA serum IL-37 levels and laboratory values as well as cytokines. DAS28, 28-joint Disease Activity Score; ESR, erythrocyte sedimentation rate; RF, rheumatoid factor; CRP, C-reactive protein; anti-CCP, anti-cyclic citrullinated peptide antibodies. Spearman’s correlation test was used.
Supplemental Figure 2. IL-1β is not required for IL-37 mediated suppression of IL-17 expression in CD4⁺T cells from RA patients. CD4⁺T cells from PBMCs of RA patients were cultured with or without IL-37 (100 ng/ml) or IL-37 (100 ng/ml) plus anti-IL-1β neutralizing antibody for 24 hours, then stimulated with PMA and Ion for 4 hours, IL-17⁺CD4⁺T cells were performed by flow cytometric analysis. Values are the mean ± SEM (n=4). * = P < 0.05. ns = no significant