Lenalidomide as a promising agent for managing autoimmune arthritis

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Received February 12, 2017; Accepted November 20, 2017; Epub March 15, 2018; Published March 30, 2018

Abstract: Cytokines, such as tumor necrosis factor (TNF), are crucial in the development of rheumatoid arthritis (RA). The thalidomide derivative lenalidomide (LD) is a potent inhibitor of TNF production and is used for treating multiple myeloma and myelodysplastic syndromes. However, the effects of LD on RA remain unclear. In the current study, we assessed the therapeutic potential of LD using collagen-induced arthritis (CIA) and RA fibroblast-like synoviocytes (RA-FLS). CIA was induced in DBA/1 male mice, and after being boosted on day 21, mice were treated with either vehicle (0.5% methocellulose) or LD (100 mg/kg) for 14 days. The arthritis score and joint pathology were assessed. Serum anti-collagen II antibody and expression of cytokines in the paws were determined by ELISA. Flow cytometry was used to detect the Th17 cells in the lymph nodes. Following TNF-α stimulation, RA-FLS were treated with LD for further investigation. We demonstrated that LD significantly reduced the arthritis score and joint destruction. LD treatment significantly reduced the concentrations of serum autoantibody, TNFα, IL-6 and IL-17 in the paws. Results demonstrated a marked decrease in Th17 cells and IL-17 in the draining inguinal lymph nodes (ILN). In vitro, LD suppressed the differentiation of CD4+ T cells into Th17 cells. In accordance, LD administration resulted in decreased secretion of IL-6 and IL-17 by RA-FLS dose-dependently. Our results show that LD attenuated autoimmune arthritis and identified LD as a promising agent in the treatment of human RA.

Keywords: Lenalidomide, collagen-induced arthritis, rheumatoid arthritis, fibroblast-like synoviocytes

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease, characterized by persistent synovitis, damage of joints and cartilage [1, 2]. Over 70 million cases of RA are estimated worldwide annually, and patients suffer from systemic complications, and even physical disability [3]. In the pathogenesis of RA, the activated fibroblast-like synoviocytes (FLS) prolong inflammatory duration and contributed greatly to the generation of inflammatory cytokines [4]. RA FLS are the main source of multiple inflammatory cytokines involved in the pathogenesis of arthritis, immunity, and metabolism of cartilage and bone [5].

The thalidomide derivative lenalidomide (LD) is used for the treatment of multiple myeloma and several myelodysplastic syndromes; it is a potent inhibitor of tumor necrosis factor (TNF) production [6]. LD also increases the production of interferon-γ, IL-10 and IL-2, and modulates natural killer cell and antibody-dependent cellular cytotoxicity [7, 8]. Cytokines-mediated pathways are central to the pathogenesis of RA [9]. Considering lenalidomide was reported to reduce inflammation, our study was designed to investigate the effects of LD on autoimmune arthritis and assess its feasibility as an agent for the treatment of RA.

Materials and methods

CIA induction

Six-week old male DBA/1 mice (22 g ± 3 g) were purchased from the Laboratory Animal Center of Nanjing University (Nanjing, China). Mice were housed in standardized condition. The mice were acclimatized for 1 week before the initiation of experiments. The study was approved by the Animal Care and Use Committee of Guangxi Medical University.

Collagen-induced arthritis (CIA) was induced according to previous publications [10]. Briefly, type II bovine collagen (CII; Chondex) was dissolved in 20 mM acetic acid with gentle stirring...
at 4°C overnight and then emulsified with complete Freund’s adjuvant (CFA) containing 4 mg/mL heat-killed Mycobacterium tuberculosis H37Ra (Chondex) (1:1 v/v). Each mouse was given a 100 μl aliquot of the emulsion containing 100 μg CII via subcutaneous injection into the tails. Three weeks after the primary immunization (on day 21), the mice received a boosted immunization with 100 μg CII emulsified with an equal volume of incomplete Freund’s adjuvant (IFA; Chondex).

Lenalidomide (LD) treatment and assessment of arthritis

After induction of arthritis (on day 21), mice were divided into vehicle (0.5% methocellulose)-treated or LD-treated group (n = 20 mice/group) for 2 weeks. The chosen dosage of LD (100 mg/kg in methocellulose) was based on the previously reported method [11]. On the final day of the experimental period, ethyl ether was used as an inhalant anaesthetic in all the mice. This method of anaesthesia had been approved by the IACUC of Guangxi Medical University. Scientific Justification was also addressed in the IACUC protocol. Blood was collected from each mouse and kept at -80°C until analyses.

Arthritis was assessed using a scoring system referring to the reported protocol [12]. To avoid cage effects, treatment groups were randomized between cages. Mice were monitored every 2 days for arthritis scoring. Paw thickness was measured with calipers. Visual assessment of arthritis severity was performed in a blinded manner and scored on a scale of 0-3, where 0 = normal, 1 = slight swelling and/or erythema, 2 = pronounced swelling, and 3 = ankylosis. All 4 limbs were scored, and the results were summed, giving a maximum score of 12. Serum and paws were collected at the end of this study.

Histologic assessment of joints

At the end of this study, the first affected paws were harvested, fixed in 10% neutral buffered formalin, and then decalcified in 10% EDTA for 3 weeks before standard processing for paraffin embedding. Sections (5 μm) were cut and stained with hematoxylin and eosin (H&E) and toluidine blue in a blinded manner.

Synovial inflammation was scored as follows: 0, no inflammation; 1, slight thickening of the lining layer with some infiltrating cells in the sublining layer; 2, moderate thickening of the lining layer with a moderate number of infiltrating cells in the sublining layer; 3, extensive thickening of the lining layer with a moderate number of infiltrating cells in the sublining layer and the presence of inflammatory cells in the synovial space; 4, substantial influx of inflammatory cells into the synovium.

Bone erosion was defined as demarcated defects in cartilage or bone associated with pannus invasion and was scored visually on a scale of 0-4, where 0 = none, 1 = minimal (1-2 sites of surface erosion), 2 = mild (at least 3 sites of surface erosion), 3 = moderate (discrete foci of erosion), and 4 = marked (large erosions extending into the marrow space) [12, 13].

Detection of anti-mouse collagen II (anti-CII) IgG and cytokines

The ELISA kit (Chondrex, Redmond, WA) was used to detect anti-CII IgG. Sera were obtained at the end of this study, and the anti-CII IgG concentrations were measured according to the manufacturer’s instructions. Diluted serum samples were added to mouse CII-coated plates and incubated at 4°C overnight. Bound IgG was detected by incubation with HRP-conjugated anti-mouse IgG followed by OPD substrate. Plates were read at a wavelength absorbance of 450 nm (OD 450 nm) with ELISA reader (Tecan Sunrise).

Cytokines in the paws were quantified by specific enzyme-linked immunosorbent assay (ELISA) kits (R&D system, Minneapolis, MN, USA) according to the user’s manual.

Flow cytometry

The draining inguinal lymph nodes (ILN) were prepared for single cell suspensions after stimulation with PMA (20 ng/ml, Sigma-Aldrich, USA), 1 μg/ml ionomycin (Sigma-Aldrich, USA) and 10 μg/ml brefeldin A (Sigma-Aldrich, USA) for 4 hours at 37°C, 5% CO2. Cells were subjected to staining of anti-CD4 antibody (BD Biosciences, San Diego, CA, USA) and anti-IL-17 antibody (MiltenyiBiotec, Bergisch Gladbach, Germany), using 0.5% saponin to permeabilize cells%. Isotype controls were included in all experiments to adjust the background. Flow cytometry analysis was performed in a
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**Table 1. Features of rheumatoid arthritis patients in this study**

<table>
<thead>
<tr>
<th>Features</th>
<th>RA patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of women/men</td>
<td>14/20</td>
</tr>
<tr>
<td>Age at operation, years</td>
<td>67 (54-77)</td>
</tr>
<tr>
<td>Disease duration, years</td>
<td>10 (6-40)</td>
</tr>
<tr>
<td>Serum CRP, mg/dl</td>
<td>0.24 (0.03-4.13)</td>
</tr>
<tr>
<td>RF, units/ml</td>
<td>18 (0-80.2)</td>
</tr>
<tr>
<td>CCP, units/ml</td>
<td>106 (4.8-346)</td>
</tr>
<tr>
<td>Swollen joint count</td>
<td>4 (1-7)</td>
</tr>
<tr>
<td>Tender joint count</td>
<td>3 (1-13)</td>
</tr>
<tr>
<td>DAS28</td>
<td>4.3 (2.2-5.1)</td>
</tr>
<tr>
<td>Methotrexate use, no. (%)</td>
<td>11 (55)</td>
</tr>
<tr>
<td>Biologic agent use, no. (%)</td>
<td>8 (40)</td>
</tr>
</tbody>
</table>

FACSCalibur instrument and analyzed with Cell Quest Pro software (BD Biosciences, USA).

**Preparation of synovial tissue and RA-FLS**

Synovial tissues were obtained during synovectomy or joint replacement surgery from 10 patients with RA after written informed consent. The patients’ characteristics and clinical features are summarized in Table 1. This study was approved by the Guangxi Medical University. All subjects satisfied the American College of Rheumatology/European League Against Rheumatism 2010 classification criteria for RA [14]. Synovial tissues were harvested and collected in phosphate buffered saline (PBS). The obtained synovial tissue was used for generating the FLS that were investigated in this study.

As previously reported [15, 16], the synovium was removed, minced, and placed in 10 ml Hanks’ balanced salt solution containing type I collagenase (Sigma). After a 2-hour digestion at 37°C, each digest was sequentially passed through a metal mesh and then a nylon mesh with 100 μm pores. The liberated cells were centrifuged and collected in a 75 cm² culture flask containing 15 ml Iscove's modified Dulbecco's medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 IU/ml), and streptomycin (100 μg/ml) (both from Sigma). The liberated cells were then cultured at 37°C in a humidified atmosphere of 5% CO₂. FLS at passages 3-6 were used for subsequent experiments. As the previous data showed that FLS expressed Hsp47/serpin H1 [17], >97% of our cells expressed this marker.

**MTT assay**

To measure cell viability, FLS were trypsinized, washed, and then seeded at 2 × 10⁴ cells/well in 96-well plates, incubated overnight, and then exposed to LD at concentrations of 0 to 20 nM for 24 h and then stimulated with TNFα (10 ng/ml, R&D Systems, USA) for 12 h. The working solution was 0.5 mg/ml MTT in PBS for 4 h at 37°C, and then replaced by DMSO. The final optical density was measured at 570 nm.

**In vitro differentiation of Th17 cells from CD4⁺ T cells**

Positive selection using anti-CD4 microbeads (Miltenyi Biotec) was used to purify CD4⁺ T cells from spleens of normal DBA/1 mice according to the users protocols. After that, the cells were cultured as described previously [18]. Purified naïve CD4⁺ T cells were seeded at 2 × 10⁶ per well into 96 well U-bottom microplates with RPMI 1640 medium (Hyclone) supplemented with 10% fetal bovine serum (Hyclone, CA), 100 units/ml penicillin, and 100 μg of streptomycin (all from Invitrogen-Gibco). Th17 differentiation was promoted by the stimulation of naïve CD4⁺ T cells with 1 μg/ml plate-bound anti-CD3 (BD Pharmagen), 1 μg/ml soluble anti-CD28 (BD Pharmagen), 50 ng/ml IL-6 (Pepro Tech), 10 ng/ml TGF-β1 (Pepro Tech) 10 μg/ml anti-interferon-γ (IFN-γ) antibody (R&D Systems) and 10 μg/ml anti-IL-4 antibody (R&D Systems). LD was used at the dose indicated at the beginning of the induction. For flow cytometry and ELISA, the cells were cultured for 4 days.

**Statistical analysis**

Data are presented as the mean ± SD. Depending on the data type, the Student’s t test or one way ANOVA followed by Dunnet’s post hoc test for parametric data, or the Kruskal-Wallis test followed by Mann-Whitney U test for nonparametric data was used for statistical analysis (GraphPad Prism 6.0 software, USA). P<0.05 was considered significantly different.

**Results**

**Attenuation of CIA by LD treatment**

Mice were treated with oral LD (100 mg/kg) or vehicle control for 2 weeks. LD significantly reduced arthritis score from day 8 after arthritis onset, in comparison with the vehicle-treat-
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Antennation of collagen-induced arthritis (CIA) following lenalidomide (LD) treatment. Mice with CIA were treated with oral 100 mg/kg LD or vehicle. A. The arthritis score in each treatment group was determined every two days. B. Paw swelling was measured daily using microcalipers, and the mean width of the hind paw in which arthritis developed in each treatment group was determined. Values are the mean ± SD of 20 mice in each group. *P<0.05, LD-treated versus vehicle-treated group. Student’s t test was used for statistical evaluation of the data.

Histologic analysis of ankle joints in mice with collagen-induced arthritis (CIA). At the termination of this experiment on day 14 (n = 20 per group), paws in the Lenalidomide (LD) or vehicle-treated groups were harvested and prepared for histologic analysis. A. Representative paraffin-embedded ankle joints were stained with hematoxylin and eosin (H&E) and toluidine blue. B. The histological scores for synovial inflammation and bone erosion were evaluated by a blinded independent observer. Bar = 100 µm. Data represent the mean ± SD; *P<0.05, versus vehicle-treated. Mann-Whitney U or Student’s t test was used for statistical evaluation of the data.

Anti-CII Abs is crucial in the pathogenesis of CIA. We found that anti-CII IgG was significantly decreased by LD treatment, relative to vehicle-treated mice (Figure 3A, P<0.05). In addition, the proinflammatory cytokines, including TNFα, IL-6 and IL-17 were detected. In contrast to vehicle-treated mice, the production of TNFα, IL-6 and IL-17 were significantly decreased by LD (Figure 3B, P<0.05).

Reduced frequency of Th17 cells in ILN by LD treatment

To explore the potential mechanism of the protective role of LD in CIA, we evaluated the frequency of Th17 cells in the draining ILN. Significant reduction of Th17 cells (CD4+ IL17+) was found in the LD-treated mice (Figure 4A, 4B). Accordingly, IL-17 in the ILN was also significantly lower in the draining ILN of LD-treated mice than that of the controls (Figure 4C).

Histologically, the ankle joints were analyzed using hematoxylin and eosin (H&E) and toluidine blue. Control mice showed severe synovial inflammation and bone erosion, along with large amounts of inflammatory cells. In contrast, the joints of LD-treated group were much less severe (Figure 2). Representative images of ankle joints from both groups are shown in Figure 2A. LD inhibited joint inflammation and bone damage in CIA.

Decreased anti-CII antibody and cytokines by LD treatment

Reduced frequency of Th17 cells in ILN by LD treatment

To explore the potential mechanism of the protective role of LD in CIA, we evaluated the frequency of Th17 cells in the draining ILN. Significant reduction of Th17 cells (CD4+IL17+) was found in the LD-treated mice (Figure 4A, 4B). Accordingly, IL-17 in the ILN was also significantly lower in the draining ILN of LD-treated mice than that of the controls (Figure 4C).
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To begin with, we assessed whether LD was cytotoxic to the cells in vitro culture. MTT assay suggested that 24 h treatment of LD at various concentrations (0, 5, 10 and 20 nM) had no cytotoxicity to RA FLS (Figure 5). Thus, we chose this concentration range for the following in vitro studies.

**LD suppressed CD4+ T cell differentiation into Th17 cells**

It is well accepted that Th17 cells contributes greatly to RA pathogenesis. To further investigate whether the effects of LD were associated with Th17 differentiation, splenic naïve CD4+ T cells purified from normal DBA/1 mice were treated with 0, 5, 10, 20 nM LD or vehicle under Th17-differentiation conditions for 4 days. IL-17A expression from CD4+ T cells was analyzed using ELISA. As shown in Figure 6, LD suppressed IL-17A production in a dose-dependent manner. These data suggest that LD can inhibit Th17 cell differentiation from CD4+ T cells in vivo.

**LD suppresses pro-inflammatory cytokines in RA FLS**

IL-6 and IL-17 are implicated in the pathogenesis of RA. To investigate the possible effects of LD on TNFα-induced proinflammatory cytokines, RA FLS were pretreated with or without different concentrations of LD ranging from 0 to 20 nM for 24 h and then exposed to TNFα (10 ng/ml) for 12 h. As shown in Figure 7, LD markedly reduced the production of IL-6 and IL-17 in TNFα-stimulated RA FLS dose dependently.

**Discussion**

In the present study, we aimed to test our hypothesis that LD can attenuate autoimmune arthritis. First of all, CIA was induced according to previous reports using type II bovine collagen and Freund’s adjuvant in DBA/1 mouse, which is the most commonly studied autoimmune model of RA. It is reported that the CIA model

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**Figure 3.** Decreased levels of serum anti-mouse collagen II (anti-CII) IgG and cytokines in the paws in mice with collagen-induced arthritis (CIA) treated with lenalidomide (LD). At the termination of this experiment, mice were bled, and serum anti-CII IgG (A) and proinflammatory cytokines in the paws (B) were measured by ELISA. Values are the mean ± SD of 20 mice in each group. *P<0.05, LD-treated versus vehicle-treated group. Student’s t test was used for statistical evaluation of the data.

**Figure 4.** Lenalidomide (LD) reduced Th17 cells (CD4+ IL-17+ T cells) in the draining inguinal lymph nodes (ILN). Single cell suspensions were isolated from draining ILN, followed by stimulation with 20 ng/ml PMA and 1 μg/ml ionomycin in the presence of 10 μg/ml brefeldin A for 4 hours. Isotype controls were included in all experiments to adjust the background. T cell subsets were detected by flow cytometry. A. Representative results in each group are shown. The number in the upper right represents the percentage of positive cells. B. The percentage of Th17 cells in each group. C. IL-17 in the ILN by ELISA. Data are expressed as mean ± SD (n = 20). *P<0.05, LD-treated versus vehicle-treated group. Student’s t test was used for statistical evaluation of the data.

**Cytotoxicity of LD in RA FLS**

To begin with, we assessed whether LD was cytotoxic to the cells in vitro culture. MTT assay suggested that 24 h treatment of LD at various concentrations (0, 5, 10 and 20 nM) had no cytotoxicity to RA FLS (Figure 5). Thus, we
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has many similar characteristics with the human RA in the joint pathologies as well as the cellular and humoral immunities [19]. Results showed that LD significantly attenuated the arthritis and histological damage. Serum anti-CII Ab, TNFα, IL-6 and IL-17 in the paws were significantly diminished by LD treatment. LD treatment reduced Th17 cell response. In vitro, LD suppressed the differentiation of CD4+ T cells into Th17 cells. In TNFα-stimulated human RA-FLS, the secretion of IL-6 and IL-17 was inhibited in a dose-dependent manner. Our results indicate that LD protects against autoimmune arthritis by inhibiting Th17 response and identified LD as a potential agent in the treatment of human RA.

The cytokines, such as TNFα, IL-6 and IL-17, play pivotal roles in the pathogenesis of RA. A novel finding of our study is the inhibitory of LD on Th17 cell differentiation. Pathogenic Th17 cells mediate pannus growth, osteoclastogenesis, and synovial neoangiogenesis and anti-IL-17 antibodies or anti-TNF/IL-17 bispecific antibodies are currently under investigation in RA, indicating Th17 cells are key players in the development of the disease [20]. While a therapeutic effect of LD in RA model has not been reported previously, LD treatment has been shown to delay the onset of experimental auto-

Figure 5. Lenalidomide (LD) had no effect on RA FLS cell viability. 24-h treatment of LD at various concentrations (0, 5, 10 and 20 nM) exhibited no cytotoxicity in RA FLS cells. P>0.05 versus control group. Analysis of variance (ANOVA) was used for statistical evaluation of the data.

Figure 6. Lenalidomide (LD) inhibited Th17 cell development. Purified CD4+ T cells from naive DBA/1 mice were cultured under Th17 polarizing conditions with water or betahistine (0, 5, 10 and 20 nM) for 4 days and then analyzed for IL-17A by ELISA. IL-17A expression in CD4+ T cells was shown. The values shown are the mean ± SD. Results are representative of 3 independent experiments. *P<0.05, **P<0.01, versus control group. Analysis of variance (ANOVA) was used for statistical evaluation of the data.

Figure 7. Effect of Lenalidomide (LD) on TNF-α-induced production of cytokines in RA FLS. After treatment with or without LD for 24 h, RA FLS were stimulated with 10 ng/ml TNF-α for 12 h. The levels of IL-6 and IL-17 in cultured cell supernatants were measured by ELISA. Data are representative of 3 independent experiments (mean ± SD) from different patients. *P<0.05, **P<0.01, vs treatment with TNF-α alone. Analysis of variance (ANOVA) was used for statistical evaluation of the data.
immune encephalomyelitis (EAE) [21]. Initially LD was shown to be a TNFα inhibitor, but it has more recently been found to possess additional immune modulatory actions [22]. Clinical evidence has demonstrated that targeting TNFα or IL-6 significantly halts RA development [23]. In the present study, LD significantly inhibited the production of TNFα, IL-6 and IL-17. Thus, LD may be a promising agent for human RA.

Taken together, our results demonstrated that LD has potent anti-inflammatory activity in CIA and human RA FLS. The protective role of LD underlying its anti-arthritic effect may be attributed to its modulation of inflammation and Th17 response. As a whole, these findings indicate that LD is a potential candidate for treating arthritis and bone destruction in human RA.

Disclosure of conflict of interest

None.

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