TL1A increased the differentiation of peripheral Th17 in rheumatoid arthritis

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The interaction between TNF-like protein 1A (TL1A) and its receptors, death receptor-3 (DR3) may be involved in the pathogenesis of rheumatoid arthritis (RA) through the regulation of Th17. Our data here showed that TL1A could significantly promote Th17 differentiation and RORc mRNA expression from naïve T cells and enhance IL-17A level in cell supernatant in RA patients. Anti-TNF-α treatment had suppressive effects on TL1A-mediated Th17 differentiation and RORc mRNA expression. In addition, The percentage of peripheral CD4+DR3+T cells of RA was significantly higher than that of healthy controls (HC), and this increased percentage of CD4+DR3+T cells was obviously up-regulated when stimulated with anti-CD3 and anti-CD28 antibody in RA patients. However, anti-CD3 and anti-CD28 antibody stimulation did not increase the percentage of CD4+DR3+IL-17A+T cells in RA patients. These results suggested that TL1A could promote Th17 differentiation in RA via the activation of RORc, and this effect may be mediated by the binding of TL1A with DR3.

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1. Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by joint inflammation and synovial hyperplasia, frequently leading to cartilage and bone destruction [1]. Although the etiology of RA remains unclear, accumulating evidence has shown that CD4+ T cells, especially Interleukin (IL)-17-producing helper T cells (Th17), are pivotal in the pathogenesis of RA [2]. Th17 selectively produce some cytokines including IL-17A, IL-17F, IL-21 and IL-22, which greatly contribute to synovial inflammation and bone destruction by activating T cells, B cells and synovial-like fibroblasts [3]. It is well known that Th17 differentiation is initiated by transforming growth factor-β (TGF-β) and IL-6, and reinforced by IL-23 and IL-21 [4–6], in which signal transducer and activator of transcription 3 (STAT3) and two orphan nuclear receptors, RORγt (RORc in human) and RORα, are key transcription signal factors and mediate Th17 lineage specification [7,8]. However, whether other cytokine(s) is involved in Th17 differentiation and expansion is poorly understood.

TNF-like cytokine 1A (TL1A, TNFSF15) was recently identified as a member of tumor necrosis factor family and also recognized as a ligand of both death receptor 3 (DR3) and decoy receptor 3 (DcR3) [9–11]. Several lines of evidence point to a role for TL1A-DR3 binding in modulating CD4+ T cell activation [11–14], especially regulating Th1 and Th17 proliferation and differentiation [15–17], which suggests that the interaction of TL1A and DR3 is attributed to the development of diverse T cell-mediated autoimmune diseases. Our previous results showed that TL1A levels in RA serum and synovial fluid were increased and associated with autoantibody production [18], indicating that TL1A may contribute to RA development. However, the effect of TL1A on Th17 in RA is still elusive.

In this study, we found that TL1A could promote Th17 differentiation induced by TGF-β and IL-6 and increased the production of IL-17A, and this increased Th17 differentiation by TL1A may mechanically be caused through the up-regulation of RORc expression. Interestingly, anti-tumor necrosis factor-α (TNF-α) could
partly antagonize Th17 differentiation enhanced by TL1A in vitro. In addition, increased DR3 expression on CD4+ T cells suggests that TL1A contributes to Th17 differentiation in RA possibly through the interaction of TL1A and DR3.

2. Materials and methods

2.1. Patients and controls

Anticoagulated peripheral blood was collected from active RA patients (n = 42) admitted to the ward of the Affiliated Drum Tower Hospital of Nanjing University Medical School, from July 2012 to July 2013. All patients fulfilled the American College of Rheumatology criteria for the classification of RA and they had no other autoimmune or systemic diseases. None of these patients was pregnant at the time of the study. Age and sex matched healthy controls (HC, n = 17) were obtained from Medical Examination Center of the Affiliated Drum Tower Hospital. The study protocol was approved by the Ethics Committee of the Affiliated Drum Tower Hospital of Nanjing University Medical School. Detailed clinical characteristics and laboratory features of RA patients were shown in Table 1 and Supplementary Table 1. The background of HC was shown in Table 2.

2.2. Th17 differentiation and maintenance

Peripheral blood mononuclear cells (PBMC) were isolated from RA patients by Ficoll–Hypaque (Axis-Shield Poc AS, Osloa, Norway) density gradient centrifugation. Naive CD4+ T cells were further purified using a human naive T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of naive CD4+ T cells was nearly 90%. For Th17 differentiation, naive T cells (1 × 10⁶/well) were stimulated with plate-bound anti-CD3 (eBioscience, 3 µg/ml) and soluble anti-CD28 antibody (eBioscience, 3 µg/ml in the presence of 50 ng/ml IL-6 (PeproTech, Rocky Hill, NJ, USA), 5 ng/ml TGF-β (Peprotech), 10 µg/ml anti-IFN-γ antibody (eBioscience), 10 µg/ml anti-IL-4 antibody (eBioscience) for 4 days. Th17 condition cultures were also treated with additional 100 ng/ml TL1A (R&D Systems) and/or 50 ng/ml anti-TNF-α (R&D Systems, Minneapolis, MN, USA) in some experiments. For Th17 maintenance, non-naive CD4+ T cells (1 × 10⁶/well) isolated from RA patients were stimulated with plate-bound anti-CD3 (5 µg/ml) and soluble anti-CD28 antibody (3 µg/ml) for 3 days in the presence or absence of TL1A (100 ng/ml).

2.3. Detection of DR3 expression

PBMC were isolated from 26 RA patients and 17 HC by ficoll–hypaque density gradient centrifugation. The cell suspension was washed three times with phosphate-buffered saline (PBS) for the detection of DR3 expression or resuspended in RPMI 1640 (Lidt Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and 1% penicillin–streptomycin, henceforth called “complete medium” for cell culture. PBMC (1 × 10⁶/well) were cultured in the presence or absence of plate-bound anti-CD3 (5 µg/ml) and soluble anti-CD28 antibody (3 µg/ml) for 3 days for the detection of DR3 expression.

2.4. Flow cytometric analysis

For the detection of DR3 expression, cells were washed with PBS and then labeled with the following monoclonal antibodies: PE-conjugated anti-human DR3 (Biolegend, San Diego, CA and USA), FITC-conjugated anti-human CD4 (eBioscience). Next, cells were maintained in the dark at 4 °C for 30 min and then washed twice with PBS.

For intracellular cytokine IL-17A detection, cells were stimulated with 50 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich, St. Louis, MO, USA) and 1 µg/ml ionomycin (Enzo Life Sciences, Boulevard Farmingdale, NY, USA) in the presence of 10 µg/ml brefeldin A (Enzo Life Sciences) for 6 h at 37 °C in a 5% CO2 incubator, then the cells were collected and washed with PBS. Cells were resuspended in 100 µl PBS and incubated in the dark at room temperature for 30 min with APC-conjugated anti-human CD4 (eBioscience) or PE-conjugated anti-human DR3 (Biolegend) and FITC-conjugated anti-human CD4 (eBioscience). The cells were then fixed and permeabilized with a cell permeabilization kit (ADG Bio Research GmbH, Gerichtsberg, Kaumberg, Austria) according to the manufacturer’s instructions. Subsequently, the cells were incubated in the dark at room temperature for 30 min with PE-conjugated anti-human IL-17A (eBioscience) or APC-conjugated anti-human IL-17A (eBioscience). The samples were analyzed using a flow cytometer (BD Biosciences, Franklin Lakes, NJ and USA).

2.5. Detection of IL-17A levels in supernatants

Cell-free culture supernatants were collected and stored at −80 °C. Quantitative analysis of IL-17A levels in supernatants was performed by enzyme-linked immunosorbent assay (ELISA) using a kit from Biolegend. The plate was read at 450 nm and sensitivity of the ELISA kits used in the experiment was 2 pg/ml.

2.6. Real-time quantitative PCR (RT-qPCR)

Total RNA was isolated from lymphocytes of 5 RA patients using TRIzol (Takara Biotechnology, Dalian, China). Reverse transcription of corresponding mRNA were performed as in conventional real-time PCR. PCR amplification and analysis were conducted using a Light-Cycler 2.0 instrument (Roche, Rotkreuz, Switzerland) with software version 4.0. All reactions were performed with the Light-Cycler FastStart DNA SYBR green I Master Mix (Roche). Amplification conditions comprised an initial preincubation at 95 °C for 10 min, followed by amplification of the target DNA for 45 cycles at 95 °C for 10 s, target annealing temperatures for 10 s, and 72 °C for 10 s. Melting curve analysis was performed immediately after amplification at a linear temperature transition rate of 0.1 °C from 65 °C to 95 °C. The results were systematically.

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**Table 1**

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<td>Disease duration (yrs)</td>
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<td>ESR (mm/h)</td>
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<tr>
<td>CRP (mg/l)</td>
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Clinical characteristics were presented as mean ± SEM. DAS28 score: 28-joint count disease activity score; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein.

**Table 2**

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<td>Age (yrs)</td>
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<td>Men/women</td>
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Clinical characteristics were presented as mean ± SEM.
normalized to the expression of the reference genes. Primers used in this study were: RORc: 5'-CTgCAaGACTCATgCAtCCAAG-3'(forward), 5'-TTTCCAATgCTgCtACAA-3'(reverse); GAPDH: 5'-CCAGGTCtACATGACAAC-3'(forward), 5'-AGGGCCATCCACCAGTCTTT-3'(reverse).

2.7. Statistical analysis

Data were summarized as means ± SEM. Statistical significance was performed by Student’s t test and the differences of DR3 expression between HC and RA patients were analyzed by Mann–Whitney U test. All statistical analyses were performed using GraphPad Prism software (Graph-Pad, San Diego, CA, USA). A p-value < 0.05 was considered statistically significant.

3. Results

3.1. TL1A enhanced Th17 differentiation not Th17 maintenance

Th17 have been identified as a major pathogenic T cell subset in some autoimmune diseases. To investigate the effect of TL1A on the developmental program of Th17, we first stimulated naive CD4+ T cells isolated from RA patients with TGF-β and IL-6 in the presence of anti-CD3, anti-CD28, anti-IFN-γ and anti-IL-4 antibody with or without TL1A. Four days later, we detected the percentage of Th17 in these cultures. As expected, we found that Th17 differentiation induced by TGF-β and IL-6 was effectively enhanced in the presence of TL1A (Fig. 1A). Consistent with the changes in Th17 percentage, IL-17A levels in supernatant by ELISA analysis were significantly increased in the culture condition of Th17-polarizing plus TL1A stimulation (Fig. 1B).

To further study the role of TL1A in Th17 maintenance, we cultured non-naive CD4+ T cells isolated from RA patients and stimulated with anti-CD3 and anti-CD28 antibody in the presence or absence of TL1A. The percentage of Th17 in these cultures was detected after 3 days. Unfortunately, TL1A did not significantly increase the percentage of Th17 when stimulated with anti-CD3 and anti-CD28 antibody (Fig. 1C).

Together, these findings suggest that TL1A could be involved in Th17 differentiation induced by TGF-β and IL-6, but had no effect on Th17 maintenance.

3.2. Increased DR3 expression on CD4+T cells in RA patients

The effect of TL1A on Th17 may be mediated by the binding of TL1A with DR3, so we detected DR3 expression on CD4+ T cells. The results showed that peripheral CD4+DR3+T cells in RA was significantly higher than that in HC (Fig. 2A). Moreover, when PBMC isolated from RA patients and HC were stimulated with anti-CD3 and anti-CD28 antibody for 3 days, the percentage of CD4+DR3+T cells was significantly increased in RA patients (Fig. 2B), but this effect was not shown in HC (Fig. 2C). However, anti-CD3 and anti-CD28 antibody stimulation did not increase the percentage of CD4+DR3+IL-17A+T cells compared to the cultures without anti-CD3 and anti-CD28 antibody in RA patients (Fig. 2D). These data indicates CD4+ T cells in RA may be more responsive for TL1A stimulation because of higher DR3 expression on these cells.

3.3. Anti-TNF-α treatment impaired Th17 differentiation up-regulation by TL1A

Recently, the use of “biological agents” such as anti-TNF-α is among the gold therapies in RA treatment. To determine the effect of anti-TNF-α therapy on TL1A-mediated Th17 differentiation, we performed an in vitro T-cell differentiation assay. We isolated naive CD4+ T cells from RA patients and stimulated with Th17-polarizing conditions and TL1A in the presence or absence of anti-TNF-α. The results showed that anti-TNF-α treatment could markedly decreased the frequency of CD4+IL-17A+T cells (Fig. 3A) and IL-17 levels (Fig. 3B) in supernatant, indicating an inhibitory effect of anti-TNF-α on TL1A-mediated Th17 differentiation in vitro.

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Fig. 1. TL1A increased Th17 differentiation, but not Th17 maintenance in RA patients. (A) Naive CD4+ T cells isolated from RA patients (n=5, patients No. 1–5) were stimulated with plate-bound anti-CD3 (5 μg/ml) and soluble anti-CD28 antibody (3 μg/ml) in the presence of 50 ng/ml IL-6, 5 ng/ml TGF-β, 10 μg/ml anti-IFN-γ antibody, 10 μg/ml anti-IL-4 antibody with or without 100 ng/ml TL1A. Four days later, CD4+IL-17A+T cells were analyzed by flow cytometric analysis. Data represents the mean ± SEM. (B) Naive CD4+ T cells isolated from RA patients (n=5, patients No. 1–5) were stimulated with plate-bound anti-CD3 (5 μg/ml) and soluble anti-CD28 antibody (3 μg/ml) for 3 days in the presence or absence of TL1A (100 ng/ml). CD4+ T percentages were examined. Data represents the mean ± SEM. Statistical analysis was performed using the Student’s t-test (*p < 0.01).
Fig. 2. Increased DR3 expression on CD4+ T cells in RA patients. (A) DR3 expression on CD4+ T cells was substantially up-regulated in RA patients (n = 13, patients No. 10–22) than in HC (n = 11, HC No. 1–11). Data represents the mean ± SEM. Statistical analysis was performed using Mann–Whitney U test. (B, C) PBMC from either RA patients (n = 7, patients No. 21–27) or HC (n = 6, HC No. 12–17) were cultured with or without TL1A (100 ng/ml) and DR3 expression on CD4+ T cells was analyzed after 72 h. Data represents the mean ± SEM. Statistical analysis was performed using the Student’s t-test. (D) PBMC from either RA patients (n = 8, patients No. 28–35) were cultured with or without TL1A (100 ng/ml) and DR3 expression on CD4+IL-17A+T cells was analyzed after 72 h. Data represents the mean ± SEM. Statistical analysis was performed using the Student’s t-test (**p < 0.01; *p < 0.05).

Fig. 3. Anti-TNF-α impaired Th17 differentiation up-regulated by TL1A. (A) Naive CD4+ T cells isolated from RA patients (n = 5, patients No. 1–5) were cultured under Th17 polarizing conditions in the presence of 100 ng/ml TL1A with or without 50 ng/ml anti-TNF-α. After 4 days, Th17 percentages were examined. Data represents the mean ± SEM. (B) Naive CD4+ T cells isolated from RA patients (n = 5, patients No. 1–5) were cultured under Th17 polarizing conditions in the presence of 100 ng/ml TL1A with or without 50 ng/ml anti-TNF-α. After 4 days, quantitative analysis of IL-17A levels in supernatants was performed by ELISA. Data represents the mean ± SEM. Statistical analysis was performed using the Student’s t-test (**p < 0.01; *p < 0.05).

Fig. 4. The effect of TL1A on the Th17 lineage-specific transcription factor RORc. (A) Non-naive CD4+ T cells (1 × 10⁶/well) isolated from RA patients (n = 7, patients No. 36–42) were stimulated with plate-bound anti-CD3 (5 μg/ml) and soluble anti-CD28 antibody (3 μg/ml) in the presence or absence of TL1A (100 ng/ml). After 3 days, RORc mRNA relative expression was detected by quantitative real-time PCR. Data represents the mean ± SEM. (B) Naive CD4+ T cells isolated from RA patients (n = 5, patients No. 36–40) were cultured under Th17 polarizing conditions with or without 100 ng/ml TL1A or 50 ng/ml anti-TNF-α. After 4 days, quantitative analysis of IL-17A levels in supernatants was performed by ELISA. Data represents the mean ± SEM. Statistical analysis was performed using the Student’s t-test (**p < 0.01; *p < 0.05).
3.4. TL1A promoted the expression of Th17 lineage-specific transcription factor RORc

We detected Th17 lineage-specific transcription factor RORc mRNA expression on CD4+ T cells in cultures aforementioned. The results showed RORc mRNA expression did not change on non-naive CD4+ T cells in the presence or absence of TL1A (Fig. 4A). However, TL1A promoted RORc mRNA expression on naive CD4+ T cells in the presence of TGF-β and IL-6 (Fig. 4B). In addition, RORc mRNA expression was significantly attenuated when anti-TNF-α was added to the cultures (Fig. 4B). These results implicated that TL1A increased Th17 differentiation possibly by promoting the Th17 transcription factor RORc mRNA expression, and anti-TNF-α weakened the potential of TL1A in a RORc-dependent manner.

4. Discussion

In this study, we showed that TL1A could enhance Th17 differentiation induced by TGF-β and IL-6, maybe contributing to RA development. In addition, the up-regulation of DR3 expression in RA may substantially implicate the enhancement of Th17 differentiation mediated by TL1A via TL1A-DR3 pathway in a RORc-dependent manner. More importantly, our data discovered that anti-TNF-α could impair Th17 differentiation, which may be involved in the potential mechanism of TNF-α blocking in RA treatment.

TL1A is expressed by monocytes, macrophages, dendritic cells (DC), synovial fibroblasts, and endothelial cells. TL1A exerts its effects by binding and signaling with its receptor DR3, and the interaction of TL1A and DR3 has been shown to enhance CD4+ T cell proliferation and induce the secretion of IFN-γ by activated CD4+ T cells in synergy with IL-12 and IL-18, and bias the immune response to Th1 [11,19]. More importantly, TL1A-DR3 signaling also promotes Th17 functions and responses [20]. Here we found that TL1A could enhance Th17 differentiation from naive CD4+ T cells through the up-regulation of RORc expression. This may confirm other findings that TL1A-DR3 interaction up-regulated Th17 and was involved in IL-17-mediated autoimmune diseases including inflammatory bowel disease [19], experimental autoimmune encephalomyelitis [21], collagen induced arthritis (CIA) [22] and antigen induced arthritis [23].

DR3 is expressed predominately by CD4+ T cells and essential for the development of diverse T cell–mediated inflammatory diseases. In this study, we observed higher percentage of CD4+DR3+ T cells in PBMC of RA. More importantly, we found this increased DR3 expression on CD4+ T cells was more obviously especially after activation in RA patients. Higher DR3 expression may be more responsive for TL1A stimulation and greatly enhanced Th17 differentiation induced by TGF-β and IL-6. However, no change of DR3 expression on Th17 after activation in RA patients probably accounts for TL1A inability to expand Th17.

In recent years, TNF has arisen as a pivotal proinflammatory cytokine with a prominent pathogenetic role in RA [24–26]. Accordingly, biological therapy with TNF-blocking agents has become a mainstay of the current management of RA [27–29]. Apart from its direct TNF neutralizing activity, the anti-inflammatory mechanism of TNF blockade is not completely understood, but accumulating evidences have suggested that its anti-inflammatory mechanisms may be related to the inhibition of both proinflammatory cytokine production and T cell infiltration in intestinal mucosa [30]. It has been reported that anti-TNF therapy in RA patients decreased Th1 and Th17 populations, and decreased Th17 response may be ascribed to a rise in IFN-γ-producing NK cell and regulatory T cell subsets. Here, we revealed a novel anti-inflammatory activity of TNF blockade by decreasing TL1A-induced Th17 differentiation via the inhibition of RORc expression. It has been reported that TNF-α and IL-1α could stimulate TL1A expression and TNF neutralization leads to suppression of TL1A expression, which may impair TL1A-induced Th17 differentiation [9]. One study showed that TL1A, together with IL-12, IL-15 and IL-18, directly induced the production of IL-6 and TNF-α from leukocytes [31]. Their results indicate that TL1A represents an interesting target which seems to be the upstream of both IL-6 and TNF-α. However, our experiment suffers from some limitation because we also have not revealed whether anti-TNF-α treatment directly suppressed TL1A expression or partly antagonized TL1A downstream.

5. Conclusion

Taken together, we uncover that TL1A-DR3 pathway promote Th17 differentiation in RA via the activation of RORc. More significantly, the suppression of TL1A-mediated Th17 differentiation by anti-TNF-α may serve as a potential therapy mechanism in autoimmune diseases associated with Th17 activation, such as RA. However, the exact mechanism about how anti-TNF-α inhibits TL1A-mediated-Th17 differentiation will need further exploration in vivo and in vitro.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cyto.2014.04.007.

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