

Original Article

Pathologic finding of increased expression of interleukin-17 in the synovial tissue of rheumatoid arthritis patients

Ning Li^{1*}, Jun C Wang^{2*}, Toong H Liang^{3*}, Ming H Zhu⁴, Jia Y Wang¹, Xue L Fu³, Jie R Zhou¹, Song G Zheng⁵, Paul Chan⁶, Jie Han¹

¹Department of Rheumatology, Shanghai East Hospital, Tongji University, Shanghai, China; ²Department of Pathology, Shanghai East Hospital, Tongji University, Shanghai, China; ³Division of Rheumatology and Clinical Immunology, Department of Medicine, Taipei City Hospital-Heping Fuyoo Branch, Taipei, Taiwan; ⁴Department of Pathology, Shanghai Chang Hai Hospital, The Second Army Medical College, Shanghai, China; ⁵Division of Rheumatology & Immunology, Department of Medicine, University of Southern California, California, USA; ⁶Department of Medicine, Wan Fang Hospital, Taipei Medical University, Taipei City, Taiwan. *Equal contributors.

Received April 24, 2013; Accepted May 21, 2013; Epub June 15, 2013; Published July 1, 2013

Abstract: Rheumatoid arthritis (RA) is a common autoimmune disease of chronic systemic inflammatory disorder that will affect multiple tissues and organs such as skin, heart or lungs; but it principally attacks the joints, producing a nonsuppurative inflammatory and proliferative synovitis that often progresses to major damaging of articular cartilage and joint ankylosis. Although the definite etiology is still unknown, recent studies suggest that T-helper cells (Th17) may play a pivotal role in the pathogenesis of RA. And interleukin-17 (IL-17), which is a cytokine of Th17 cells, may be a key factor in the occurrence of RA. The binding of IL-17 to specific receptor results in the expression of fibroblasts, endothelial and epithelial cells and also synthesis of several major factors such as tumor necrosis factor alpha (TNF- α), IL-1 β that result in the structural damage of RA joints. Though some previous studies have shown that IL-17 exists in the synovium of RA, few has definite proof quantitatively by pathology about its existence in synovial membrane. This study comprised of 30 RA patients and 10 healthy control, pathologic study of the synovial membrane showed increased expression of IL-17 in the synovial tissue of RA patients, the intensity is compatible with clinical severity of disease as validated by DAS28 score and disease duration. Northern blot study also confirmed the increased expression of IL-17 in the synovial tissues. This study sheds further light that IL-17 may be a key factor in the pathogenesis of RA and a determinant of disease severity.

Keywords: Rheumatoid arthritis, interleukin-17, synovitis

Introduction

Rheumatoid arthritis (RA) is a complex, chronic autoimmune disease that associates with progressive disabling, major systemic complications, early death and increased socioeconomic costs [1]. It is characterized by synovial tissue inflammation and hyperplasia, autoantibody production, cartilage and bone destruction and systemic features such as joint deformities, pulmonary, cardiovascular and dermatological disorders.

RA involves a complex interplay among genotype, environmental factors and also by chance, twin studies showed that genetic factors carry

concordance rates of 15 to 30% among monozygotic twins and 5% among dizygotic twins [2]. The long established theory about the human leucocyte antigen (HLA)-DRB1 locus has been repeatedly confirmed in RA patients with positive rheumatoid factors (RF) [3].

RA causes a broad spectrum of morphological alterations which mainly locates at joints. Initially the synovial tissue becomes grossly edematous, which also has increased vascularity, intimal lining hyperplasia, accumulation of macrophages, plasma B cells, dendritic cells, natural killer cells and mast cells in the sublining [4].

Many previous studies have shown that multiple cytokines such as TNF- α and the interleukin family such as IL-1, IL-6, IL-8, IL-18, etc., [5]; has been found to be related to the pathogenesis or disease severity of RA, but IL-17 has more evidences to be one of the key factors [6-10]. In this report, we evaluated the pathology of the intensity of IL-17 expression in the synovial tissue at cellular level of RA patients, and compared with normal control. Although this present study is not the only innovative finding about IL-17, it can shed more light that the importance of this cytokine and tailor specific and effective therapy.

Materials and methods

This study was approved by the Shanghai Tongji University East Hospital institutional review board. All patients had given written informed consents. Four observers (N.L, J.Y.W, M.H.Z, and J.H.) evaluated the pathologic results.

Patient population

Over 300 RA patients were treated at the Department of Rheumatology of the East Hospital. Synovial tissues of 30 RA patients (9 males and 21 females; mean age 50.2 ± 16.8 years; disease duration 73.8 ± 49.6 months; DAS28 score 4.89 ± 0.88 ($3.89 \sim 7.41$)) were obtained for pathologic studies when these patients received joint surgery. Control synovial tissue specimens were obtained during surgery for knee tear ligaments from 10 healthy patients. All the procedures were followed in accordance with the ethical standards of the Helsinki declaration of 1975, as revised in 2008.

Pathologic study of synovial tissue

Synovial membrane specimens were collected from 30 patients who had undergone joint surgery with confirmed pre-operative diagnosis. Two to three pieces of tissues from knee joints, around 1 cm in size, were collected from area with less hemorrhage, were soaked in 4% polyoxyethylene solution, and covered for 24 hours. Specimens were fixed with 10% buffered formalin and embedded in paraffin wax, then were stained with Hematoxylin and Eosin for examination by light microscope. Histopathological parameters of synovitis were evaluated in accordance with established criteria [11, 12],

and a histological score for each lesion was established according to previous studies [13, 14]. Paraffin sections were analyzed randomly and the histological features scored in blinded fashion. This included blinding to the sequence of biopsies as well as patient identification.

Immunohistochemical study

The procedure was referred from previous standard methods [14]. Thin sections from 30 RA patients and from 10 normal controls were analyzed for IL-17 synovium expression. Serial sections of 5- μ m thickness were obtained from each paraffin-embedded block previously prepared. The sections were dewaxed in xylene and rehydrated in graded alcohols. Heat induced epitope retrieval was performed by microwave at 650 W for 20 minutes and 30 minutes cooling. These sections were then treated with 5% hydrogen peroxide in PBS for 30 minutes and the nonspecific binding was blocked by incubating the slides for one hour with 5% BSA in PBS. As primary antibody was used rabbit polyclonal IL-17 in dilution 1:500 and 18 hours incubation at 40 °C [15]. The visualization system that we used was a Streptavidin-Biotinperoxidase complex kit [Splink HRP Broad Spectrum (Ms./Rb.) kit, GBI, INC, USA] in accordance with the protocols recommended by the manufacturer. The signal was detected with 3,3'-diaminobenzidine (DAB-GBI, INC, USA) and counterstaining was done with Hematoxylin. Quantitative immunohistochemical assessment was targeted, we were just especially interested in the presence of the IL-17 reactivity in the synovial tissue. Images acquisition was done with 40 \times and 200 \times objectives-Leica DM6000B microscope (Leica, Germany) equipped with a 5 megapixels CCDcamera and Nikon NIS-Elements software.

Statistical analysis

Data are presented as mean \pm SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) or the unpaired Student's *t*-test. *P* values less than 0.05 were considered to be statistically significant.

Results

Pathologic changes of synovium of RA patients

HE staining was performed on the synovial membrane tissues collected from RA patients

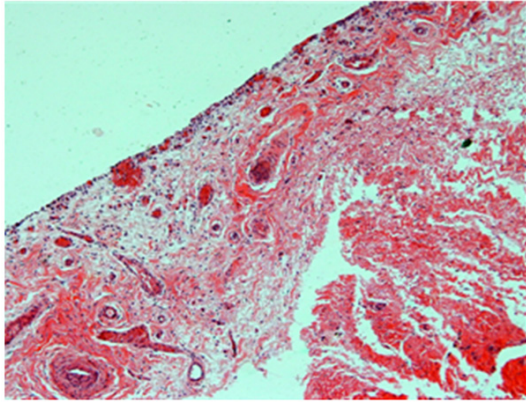


Figure 1. HE staining of synovial membrane of healthy people.

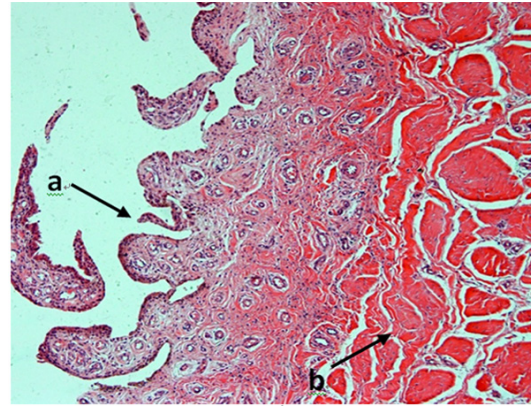


Figure 2. HE staining of synovial membrane of RA patients.

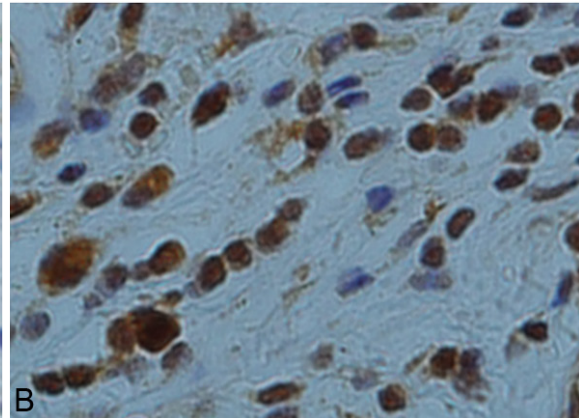
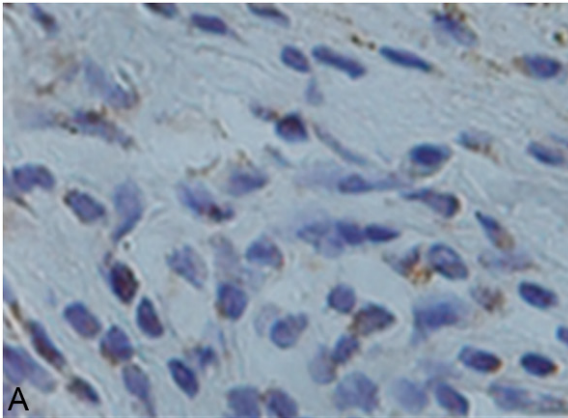


Figure 3. A, B. Synovial IL-17 immunohistochemical staining of healthy people and RA patients (brownish yellow color).

and healthy people. Observation under 40× light microscope showed more neutrophil granulocytes infiltration in the synovial tissues of RA patients as compared to healthy control (**Figure 1**) with concomitant local fibrous tissue proliferation (arrows) (**Figure 2**).

Synovial IL-17 immunohistochemical staining

IL-17 immunohistochemical staining was performed. The expression of IL-17 in the synovial membranes of RA patients and healthy people was observed. IL-17 was primarily expressed in the cytoplasm and appeared to be brownish yellow under the light microscope after immunohistochemical staining. The result was positive. The observation first took place under a low power (×40) light microscope on the area with a higher density of cells and then a high power (×200) light microscope. For each slide,

five fields of view were selected and the numbers of stained positive cells in each field were counted. Results showed that there were 63.8 ± 23.5 cells expressing IL-17 in the synovial membranes of healthy control (**Figure 3A**) and there were 175.6 ± 104.3 IL-17 stained positive cells in the synovial membranes of RA patients (**Figure 3B**). The IL-17 level was significantly higher in the synovial membranes of RA patients than healthy people ($P < 0.05$). RA synovial IL-17 immunohistochemical staining showed more brownish yellow positively stained particles. The area pointed to by the arrow in the **Figure 3B** is IL-17 stained positive.

Discussion

Rheumatoid arthritis (RA) has been known to be a chronic inflammatory disease characterized by the destruction of articular cartilage

and bone [15, 16]. The levels of monocyte-macrophage-derived cytokines such as IL-1, IL-6, and soluble IL-6 receptor and IL-17 are all elevated in the synovial fluids of RA patients.

In this study, we find that the expression of IL-17 in synovial tissue is significantly higher in those active RA patients when comparing with the normal controls. These results are consistent with previous studies [17, 18]. There is other report suggested that IL-17 is also higher in osteoarthritis patients (OA) when comparing with normal people [19], but IL-17 is even higher in RA patients when comparing with OA patients. From the present data, we again find that the expression of IL-17 is significantly higher than OA patients (data not shown); that means, IL-17 expression is RA>OA>normal.

IL-17 is a recently cloned cytokine, and it is secreted by activated memory CD4+ T cells and modulates the early stage of immune responses [20]. Rouvier et al. have cloned cytotoxic T lymphocyte-associated antigen-8 (rat IL-17) from a T-cell subtraction library [21].

In this study, we examined the expression of IL-17 in synovium from active RA patients, at the same time with serum and synovial fluid levels of IL-17. We demonstrated that IL-17 expression was markedly higher in RA synovial tissue when comparing with normal controls, these results were consistent with previous studies [17, 18]. There is other report demonstrated that IL-17 levels in synovial homogenates were different in OA and normal synovial tissues [19]. There have been evidences also suggested correlation may be existing between IL-17 synovial tissue immunoreactivity and histochemical-pathologic score, which was previously demonstrated as a predictor for disease activity and progression [13, 22]. Findings that are compatible with previous report that have already revealed that synovial tissue IL-17 mRNA expression have been independently associated with progressive joint damage in RA [23]. Our study has also revealed increased mRNA expression of IL-17 in the synovial tissue of these Chinese patients (data not shown). Another former report has shown significant elevations of IL-17+ T-cells in synovial tissue, but only in patients with active RA, suggesting that IL-17+ T-cell numbers may vary with disease activity. The present study uniquely shows the elevation of cell numbers quantitatively in

these RA cases. There is still lack of strong evidence that IL-17 is the key determinant of RA etiology since studies regarding IL-17 in RA synovium are so varied, but this may be due in part to different demographics and treatment regimes in the RA patients. Actually, most of the studies regarding the etiologic role of these cytokines have been focused mainly on established RA, these patients have been treated with a wide variety of DMARDs regimens, which, in combination with their impact on disease activity, could influence both IL-17 behavior and the correlations with other disease activity markers.

This study has a major limitation is that the number of RA patients receiving tissue study is relatively small, but our results are consistent; which can shed more light in the field of IL-17 as a causative role in the pathogenesis of RA.

In conclusion, thru histopathological investigation, this study again finds that the increased expression of IL-17 in the synovium of RA patients, which suggest that IL-17 may be one of the key factors in the etiology of RA.

Acknowledgements

This work was partly funded by a Research Grant for Health Science and Technology development and innovation of Pudong New Area of Shanghai (Grant No. PKJ2011-Y06 and PKJ2011-Y31).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jie Han, Department of Rheumatology, Shanghai East Hospital, Tongji University, 150, Ji Mo Road, Shanghai, China 200120. E-mail: hjgj85528@163.com; Dr. Paul Chan, Department of Medicine, Wan Fang Hospital, Taipei Medical University, 111, Hsing-Lung Road, Section 3, Wen-Shan District, Taipei City, Taiwan 116. E-mail: chanpaul@w.tmu.edu.tw

References

- [1] Firestein GS. Evolving concepts of rheumatoid arthritis. *Nature* 2003; 423: 356-361.
- [2] MacGregor AJ, Snieder H, Rigby AS, Koskenvuo M, Kaprio J, Aho K, Silman AJ. Characterizing the quantitative genetic contribution to rheumatoid arthritis using data from twins. *Arthritis Rheum* 2000; 43: 30-37.

Interleukin-17 in synovium of RA

- [3] Gregersen PK, Silver J, Winchester RJ. The shared epitope hypothesis: an approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. *Arthritis Rheum* 1987; 30: 1205-1213.
- [4] Hitchon CA, El-Gabalawy HS. The synovium in rheumatoid arthritis. *Open Rheumatol J* 2011; 5: 107-114.
- [5] McInnes IB, Schett G. The pathogenesis of rheumatoid arthritis. *N Engl J Med* 2011; 365: 2205-2219.
- [6] Lubberts E, Koenders MI, van den Berg WB. The role of T-cell interleukin-17 in conducting destructive arthritis: lessons from animal models. *Arthritis Res Ther* 2005; 7: 29-37.
- [7] Chabaud M, Fossiez F, Taupin JL, Miossec P. Enhancing effect of IL-17 on IL-1-induced IL-6 and leukemia inhibitory factor production by rheumatoid arthritis synoviocytes and its regulation by Th2 cytokines. *J Immunol* 1998; 161: 409-414.
- [8] Chabaud M, Garnero P, Dayer JM, Guerne PA, Fossiez F, Miossec P. Contribution of interleukin-17 to synovium matrix destruction in rheumatoid arthritis. *Cytokine* 2000; 12: 1092-1099.
- [9] Lubberts E, Koenders MI, van den Berg WB. The role of T cell interleukin-17 in conducting destructive arthritis: lessons from animal models. *Arthritis Res Ther* 2005; 7: 29-37.
- [10] Van Bezooijen RL, Van Der Wee-Pals L, Pappalopoulos SE, Löwik CW. Interleukin 17 synergises with tumour necrosis factor alpha to induce cartilage destruction in vitro. *Ann Rheum Dis* 2002; 61: 870-876.
- [11] Rosu A, Margaritescu CL, Stepan A, Musetescu A, Ene M. IL-17 patterns in synovium, serum and synovial fluid from treatment-naïve, early rheumatoid arthritis patients. *Rom J Morphol Embryol* 2012; 53: 73-80.
- [12] Koizumi F, Matsuno H, Wakaki K, Ishii Y, Kurashige Y, Nakamura H. Synovitis in rheumatoid arthritis: scoring of characteristic histopathological features. *Pathol Int* 1999; 49: 298-304.
- [13] Rosu A, Ciurea P, Simionescu C, Margaritescu C, Musetescu AE, Ciurea R, Vreju AF. The study of angiogenesis in early rheumatoid arthritis—clinical, immunohistochemical and immunological correlations. *J Med Life* 2008; 1: 287-294.
- [14] Tsubaki T, Arita N, Kawakami T, Shiratsuchi T, Yamamoto H, Takubo N, Yamada K, Nakata S, Yamamoto S, Nose M. Characterization of histopathology and gene-expression profiles of synovitis in early rheumatoid arthritis using targeted biopsy specimens. *Arthritis Res Ther* 2005; 7: R825-R836.
- [15] Lutzky V, Hannawi S, Thomas R. Cells of the synovium in rheumatoid arthritis. *Dendritic Cells. Arthritis Res Ther* 2007; 9: 219.
- [16] Andreas K, Lübke C, Häupl T, Dehne T, Morawietz L, Ringe J, Kaps C, Sittlinger M. Key regulatory molecules of cartilage destruction in rheumatoid arthritis: an in vitro study. *Arthritis Res Ther* 2008; 10: R9.
- [17] Chabaud M, Durand JM, Buchs N, Fossiez F, Page G, Frappart L, Miossec P. Human interleukin-17: a T cell-derived proinflammatory cytokine produced by the rheumatoid synovium. *Arthritis Rheum* 1999; 42: 963-970.
- [18] Moran EM, Mullan R, McCormick J, Connolly M, Sullivan O, Fitzgerald O, Bresnihan B, Veale DJ, Fearon U. Human rheumatoid arthritis tissue production of IL-17A drives matrix and cartilage degradation: synergy with tumour necrosis factor-alpha, Oncostatin M and response to biologic therapies. *Arthritis Res Ther* 2009; 11: R113.
- [19] Shahrara S, Huang Q, Mandelin AM 2nd, Pope RM. TH-17 cells in rheumatoid arthritis. *Arthritis Res Ther* 2008; 10: R93.
- [20] Broxmeyer HE. Is interleukin-17, an inducible cytokine that stimulates production of other cytokines, merely a redundant player in a sea of other biomolecules? *J Exp Med* 1996; 183: 2411-2415.
- [21] Rouvier E, Luciani MF, Mattéi MG, Denizot F, Golstein P. CTLA-8, cloned from an activated T cell, bearing AU-rich messenger RNA instability sequences, and homologous to a Herpesvirus saiminri gene. *J Immunol* 1993; 150: 5445-5456.
- [22] Vreju F, Ciurea M, Roşu A, Muşetescu A, Grecu D, Ciurea P. Power Doppler sonography, a non-invasive method of assessment of the synovial inflammation in patients with early rheumatoid arthritis. *Rom J Morphol Embryol* 2011; 52: 637-643.
- [23] Kirkham BW, Lassere MN, Edmonds JP, Juhasz KM, Bird PA, Lee CS, Shnier R, Portek IJ. Synovial membrane cytokine expression is predictive of joint damage progression in rheumatoid arthritis: a two-year prospective study (the DAMAGE study cohort). *Arthritis Rheum* 2006; 54: 1122-1131.