ORIGINAL ARTICLE

Serum levels of IL-17, IL-4, and INFγ in Serbian patients with early rheumatoid arthritis

Voja Pavlovic, Aleksandar Dimic[1], Sasa Milenkovic[1], Dane Krtinic[2]
Institute of Physiology, Faculty of Medicine, University of Nis, [1]Institute for Treatment and Rehabilitation "NiskaBanja", NiskaBanja, [2]Faculty of Medicine, Department of Pharmacology, University of Nis, Serbia

Background: Rheumatoid arthritis (RA) is a chronic, systemic, inflammatory disease with autoimmune etiology, characterized by synovial inflammation and destruction of joint cartilage and bone. There are controversial data about the profile of interleukin-17 (IL-17A), interleukin-4 (IL-4), and interferon-gamma (INFγ), indicating in some studies the key role of IL-17, while in others the Th1 cytokines. **Materials and Methods:** Serum samples of 31 early RA patients were evaluated for erythrocytes sedimentation rate (ESR), rheumatoid factor (RF), C-reactive protein (CRP), anti-cyclic citrullinated peptide antibodies (anti-CCP), and for the tested cytokines (IL-17A, IL-4, and INFγ). Disease activity score (DAS28) calculation was done for all patients. Control serum samples were obtained from 29 healthy volunteers. **Results:** The levels of tested cytokines were significantly higher (IL-17A, $p < 0.001$; INFγ, $p < 0.001$; IL-4, $p < 0.01$) in patients with early RA, compared to the healthy controls. In early RA patients, a strong correlation of serum IL-17A was found with DAS28, ESR, and CRP. Also, significant negative correlation was found between serum INFγ levels and the DAS28 score, indicating that INFγ may play a key role in maintaining immune homeostasis in patients with RA. **Conclusion:** The mean serum IL-17A levels in patients with early RA, corresponded with the disease activity and severity. This might highlight the usefulness of the serum IL-17A level in defining the activity and predictive patterns, for aggressive disease therapy, and it might express specific therapeutically targets.

Key words: Early rheumatoid arthritis, IL-17A, IL-4, INFγ

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INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by synovial inflammation and destruction of the joint cartilage and bone, mediated mainly by the persistent synthesis of pro-inflammatory cytokines. A panel of cytokines are produced by different cells from the immune system and from the synovium, and become functionally active even in the early stages of RA.[1,2] For a long time, autoimmune diseases, such as RA, were thought to be a Th1- and not a Th2- associated disorder.[3] A new T-cell subset, termed 'Th17 cells', has been described in recent years and radically changed the pathophysiological concept of RA. Until now, it is unclear whether RA is a Th1- and / or a Th17-mediated disease.[4]

IL-17A, a main cytokine produced by TH17 cells, stimulated the production of TNF-α and IL-1β, and together with TNF-α, induced cartilage loss and osteoclastogenesis.[5] A recent report demonstrated that Th17 cells, but not Th1 cells, cooperated with the synovial fibroblasts in a pro-inflammatory feedback loop that drove the chronic destruction in RA.[6] These observation results indicated that Th17 cells and IL-17A critically contributed to the synovitis and bone destruction associated with RA. An increased number of Th17 cells was found in the peripheral blood mononuclear cells of RA patients compared to healthy controls.[7] Elevated expression of IL-17 was documented in the rheumatoid synovium[8] and synovial fluids of patients with early RA.[9] On the other hand, despite the light expression of signature cytokines for Th1- and Th2-cells, such as INFγ and IL-4,[10] huge amounts of IL-17A could be produced by the resident Th17 cells in the rheumatoid synovial tissues of patients with active RA.[11] Therefore, the current study was performed to evaluate the serum levels of cytokines representing the Th1 (INFγ), Th2 (IL-4) subpopulations, and IL-17A, in patients with early RA, and to analyze the cytokine profile contribution to disease activity and prediction, through correlation with different clinical and laboratory parameters.

Address for correspondence: Dr. Voja Pavlovic, Institute of Physiology, Medical Faculty University of Nis, Nikole Uzunovica 67, 18000 Nis, Serbia.
E-mail: vojapav@yahoo.com
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PATIENTS AND METHODS

Ethics statement

Enrollment took place between December, 2011 and December, 2012 at the Institute for Treatment and Rehabilitation, 'NiskaBanja', Niska Banja, Serbia. Our research had been approved by the Medical Ethical Committee of the Institute for Treatment and Rehabilitation 'NiskaBanja', Niska Banja, Serbia, and the Medical Ethical Committee of Blood Transfusion Institute in Nis, Serbia. A written informed consent document had been obtained from each participant.

Subjects and samples

Thirty-one patients (14 males and 17 females) with early RA, diagnosed at the Institute for Treatment and Rehabilitation 'NiskaBanja', Niska Banja, Serbia, were enrolled. All the patients fulfilled the American College of Rheumatology 1987 criteria for RA,[12] had a disease duration of less than one year, and had no prior use of any disease-modifying anti-rheumatic drugs (DMARDs) or corticosteroids. Another 33, age- and sex-matched, healthy volunteers obtained from the Blood Transfusion Institute in Nis, Serbia, served as the control group.

Blood samples from all subjects were obtained after overnight fasting (12 hours), immediately centrifuged, and the sera were collected and stored at –20°C, until analyzed. All the sera analysis was performed within seven days of blood collection and storage.

Clinical and laboratory data

The demographic characteristics of all subjects were determinated and presented in Table 1. All subjects underwent extensive medical examinations and serological evaluations, including measurements of rheumatoid (RF) factor (Human, Wiesbaden, Germany), Disease Activity Score,[13] based on the evaluation of 28 joints (DAS28) calculated with the number of tender and swollen joints, erythrocyte sedimentation rate (ESR), determined by the Westergren method, along with the patient's global assessment of disease activity on the visual analog scale (VAS) of 100 mm.[14] In addition, the anti-CCP antibody was tested by using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Euroimmun, Lubeck, Germany).

Cytokine measurements

The serum concentrations of INFγ and IL-4 were measured by using the ELISA kit (Invitrogen, Carlsbad, CA, USA), while the serum levels of IL-17A were evaluated by using the ELISA kit (BioSource, Nivelles, Belgium), according to the manufacturer's instructions.

Statistical analysis

The data were collected, tabulated, and analyzed. Descriptive statistical parameters were calculated via the database containing records for patients and control subjects. The distribution normality was analyzed using the Shapiro-Wilk test. Student's t test was used to compare the two means. Correlation between the variables was performed using the Pearson's correlation test. A values of $P < 0.05$ was considered significant.

RESULTS

Thirty-one patients with established RA and 29 healthy controls were recruited. There were no significant differences in age or sex between the groups. Demographic, clinical, and laboratory characteristics of patients and controls are shown in Tables 1 and 2.

Levels of cytokines representing the Th1 (INFγ), Th2 (IL-4) subpopulations, and IL-17A, were evaluated in the sera of patients with early RA and healthy controls, by using the commercially available ELISA assays. Patients with early RA showed significantly higher ($p < 0,001$) mean serum levels of IL-17A and INFγ and IL-4 ($p < 0.01$) compared to the healthy controls [Figure 1]. As shown in Figure 1, the most dramatic increase was observed for IL-17 in the sera of patients with early RA, while the RA / healthy ratio varied from approximately 2 for INFγ to 1.3 for IL-4. The mean serum levels of tested cytokines in RA patients with negative RF (IL-17: 14.72 ± 8.31; INFγ: 3.85 ± 1.67; IL-4: 6.09 ± 2.43) did

Table 1: Demographic characteristics of patients with early RA and healthy controls

		Early RA	Healthy controls
Demographic characteristics	Number of patients	31	29
	Number of females	17	13
	Number of males	14	16
	Age (years)	56.87±10.72	51.53±8.23

Age of patients with early RA and controls is presented as mean value ± standard deviation (SD)

Table 2: Clinical and laboratory characteristics of patients with early RA

	Parameters	Value
Clinical characteristics	Disease duration (months)	7.04±3.21
	DAS28 score	5.84±0.98
	VAS (mm)	50.8±12.11
Laboratory characteristics	ESR (mm/h)	48.25±26.96
	CRP (UI/L)	34.22±13.65
	Anti-CCP (U/ml)	69.01±39.47
	Number of RF positive patients (%)	17 (54.83%)
	RF (UI/ml)	76.23±23.62

Legend: DAS28 = Disease activity score in 28 joints with sedimentation; VAS = Visual analog scale; ESR = Erythrocyte sedimentation rate; CRP = C-reactive protein; RF = Rheumatoid factor; Anti-CCP-antibody to *citrullinated* polypeptide. Results are presented as mean value ± standard deviation (SD)

not significantly differ from those with positive RF (IL-17: 13.01 ± 6.28; INFγ: 4.48 ± 1.74; IL-4: 5.7 ± 1.81).

Next, we searched for correlations between the levels of cytokines in the sera of the patients with early RA and the diagnostic parameters. We found positive correlations between the sera levels of IL-17 and some inflammatory markers as ESR (r = 0.661, $p < 0.001$) and CRP (r = 0.510, $p < 0.01$) [Table 3]. Furthermore, results presented in Table 3 demonstrate a significant correlation between serum IL-17 levels in patients with early RA and DAS28 score (r = 0.612, $p < 0.001$). Also, the mean INFγ serum levels showed significant negative correlations (r = –0.534, $P < 0.01$) with a DAS28 score in patients with early RA [Table 3]. No significant correlations were found between serum levels of tested cytokines and patients' ages (IL-17: r = 0.131, $p = 0.481$; INFγ1: r = -0.308, $p = 0.091$; IL-4: r = 0.110, $p = 0.556$), disease duration (IL-17: r = –0.221, $p = 0.232$; INFγ: r = –0.105, $p = 0.573$; IL-4: r = –0.096, $p = 0.607$) and VAS (IL-17: r = 0.162, $p = 0.384$; INFγ: r = 0.254, $p = 0.169$; IL-4: r = 0.304, $p = 0.096$) [Table 3].

DISCUSSION

Rheumatoid arthritis is marked by the infiltration of macrophages and T cells into the joints, synovial hyperplasia, involvement of many cytokines, and progressive destruction of articular cartilage and bone.[15,16]

In the present study, we have evaluated type Th1 (INFγ), type Th2 (IL-4), and the IL-17A cytokine levels in the sera of patients with early RA and have searched for correlations between the cytokine levels and the clinical and laboratory parameters. The current study results demonstrate a markedly increased IL-17A mean serum level in patients with early RA compared to healthy controls. Significantly elevated IL-17A serum level in patients with early RA has been documented earlier,[17] and here we report, for the first time, an increased IL-17A level in patients with early RA in the Serbian population. Similar results have been obtained in earlier studies, demonstrating increased IL-17 serum levels in patients with well-established RA[18,19] and early RA.[20] IL-17A, the main cytokine of Th17 cells, enables the synthesis of several key factors, such as TNF-α, IL-1β, IL-6, IL-8, monocyte chemotactic protein-1 (MCP-1), prostaglandin E2, to promote neutrophil chemotaxis and granulocyte production.[21] Furthermore, this cytokine leads to the structural damage of RA joints by binding of the IL-17A specific receptor, expressed on fibroblasts, endothelial, and epithelial cells.[16] In line with those results, different studies have documented elevated IL-17A synovial fluid levels in patients with established or early RA,[17,18,22] as also increased Th17 frequency in the synovial tissue.[17,23] The presented results demonstrate significant correlation of the serum IL-17A levels with CRP and ESR. These findings are in accordance with the recent results,[17] suggesting that IL-17A is a potent inducer of CRP from human smooth muscle cells and hepatocytes.[24] On the other hand, other reports note that elevated IL-17A levels do not correlate with ERS and CRP, indicating that increased IL-17A blood levels from patients with established RA is of limited use as a biomarker to indicate disease activity.[23,25] The mean serum IL-17A levels have correlated positively with the DAS28 score. The given finding confirms the earlier report showing that IL-17A production strongly correlates with the magnitude of disease activity and systemic inflammation, in patients with early RA.[26] Taken together with our results it seems that the serum levels may represent possible important markers for the development and onset of RA.

The current study results showed significantly increased serum levels of INFγ in patients with early RA compared to the healthy controls. Furthermore, the mean serum INFγ

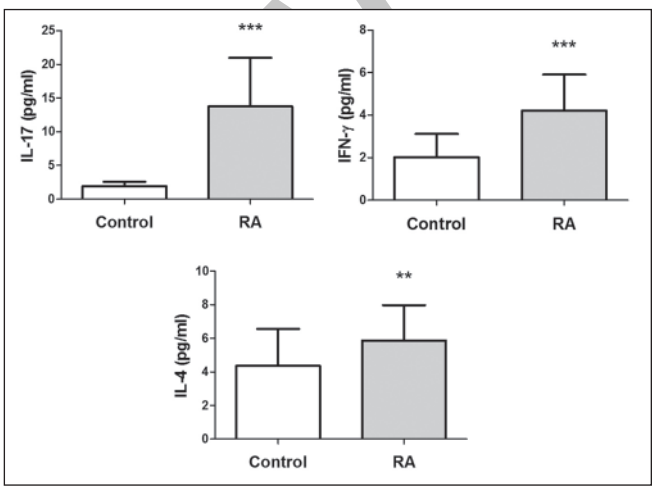

Figure 1: Serum levels of IL-17A, IL-4, and INFγ in patients with early RA and healthy controls, RA - patients with early rheumatoid arthritis; IL-17A-interleukin-17; IL-4-interleukin 4; INFγ-interferon gamma; ***$p < 0.001$; **$p < 0.01$, compared to the control samples

Table 3: Correlations of serum cytokine concentrations with clinical and diagnostic parameters of patients with early RA

Parameters	Correlation with serum cytokines		
	IL-17	INFγ	IL-4
DAS28	13.78±7.19[a]	4.2±1.71[b]	5.88±2.08[e]
VAS	13.78±7.19[e]	4.2±1.71[e]	5.88±2.08[e]
ESR	13.78±7.19[c]	4.2±1.71[e]	5.88±2.08[e]
CRP	13.78±7.19[d]	4.2±1.71[e]	5.88±2.08[e]
Anti-CCP	13.78±7.19[e]	4.2±1.71[e]	5.88±2.08[e]
Age of patient	13.78±7.19[e]	4.2±1.71[e]	5.88±2.08[e]
Disease duration	13.78±7.19[e]	4.2±1.71[e]	5.88±2.08[e]

Legend: DAS28 = Disease activity score in 28 joints with sedimentation; VAS=Visual analog scale; ESR = Erythrocyte sedimentation rate; CRP = C-reactive protein; RF = Rheumatoid factor; Anti-CCP-antibody to citrullinated polypeptide; a - r = 0.612, $p < 0.001$; b - r = –0.534, $p < 0.01$; c - r = 0.661, $p < 0.001$; d - r = 0.510, $p < 0.01$; e - n.s. - Not significant.

levels correlated negatively with the DAS28 score, which was in line with the previous report,[27] confirming the observations that treatment of patients with INFγ resulted in significant improvement of the clinical parameters,[28] indicating that INFγ might play a key role in maintaining the immune homeostasis in patients with RA.[27] Also, recent evidence from mouse models has suggested that IFNγ played a significant role in the resolution of synovial inflammation.[29] In established RA, the synovial T cells, after *in vitro* stimulation, increased the synthesis of mRNA for IFNγ and IFNγ production, compared to the T cells from early RA.[30] The discrepancy between the IFNγ levels in early RA and other early arthritis might be important to the pathology of the transition to persistent inflammation.[9]

The serum levels of IL-4 were increased in patients with early RA, but on the other hand, we were not able to find any correlation with some clinical and diagnostic parameters. Similar results were obtained from the *in vitro* study, where stimulated mononuclear cells from patients with early RA produced significantly lower amounts of IL-4, compared to the mononuclear cells from healthy controls.[7]

The role of IL-4 in early RA is largely unknown. It has been shown that IL-4 has had pro-inflammatory and anti-inflammatory effects on animal models with inflammatory arthritis.[9] Also, it is well documented that IL-4 prevents bone and cartilage erosion and enhances the synthesis of type I procollagen in patients with arthritis, suggesting that IL-4 promotes tissue repair.[31] Supplementation of IL-4 to synovial fibroblasts can dramatically modulate their gene expression profile, leading to the generation of a specific synovial environment in early RA, with a modulated fibroblast function.[32]

The major limitation of the study is the small number of patients included, but our results are consistent, therefore, we recommend conducting studies with a larger number of early RA patients.

In summary, this study finds that, out of the tested cytokines, increased serum IL-17A levels correspond simultaneously to the degree of disease activity and severity, in patients with early RA. Also, further and larger analysis should be conducted in order to consider IL-17A as a possible marker in the early course of disease or in response to targeted therapies.

Authors' contributions

VP carried out the design, participated in most of the experiments and prepared the manuscript. AD provide assistance in the design of the study, coordinated all the experiments and participated in manuscript preparation. SM provided assistance for all experiments and participated in manuscript preparation. DK participated in manuscript preparation. All authors have read and approved the content of the manuscript.

REFERENCES

1. Lubberts E. Th17 cytokines and arthritis. Semin Immunopathol 2010;32:43-53.
2. McInnes IB, Schett G. Cytokines in the pathogenesis of rheumatoid arthritis. Nat Rev Immunol 2007;7:429-42.
3. Firestein GS. Evolving concepts of rheumatoid arthritis. Nature 2003;423:356-61.
4. Lubberts E. IL-17 / Th17 targeting: On the road to prevent chronic destructive arthritis? Cytokine 2008;41:84-91.
5. Van Bezooijen RL, Van Der Wee-Pals L, Papapoulos SE, Lowik CW. Interleukin 17 synergies with tumor necrosis factor alpha to induce cartilage destruction in vitro. Ann Rheum Dis 2002;61:870-6.
6. Van Hamburg JP, Asmawidjaja PS, Davelaar N, Mus AM, Colin EM, Hazes JM, *et al*. Th17 cells, but not Th1 cells, from patients with early rheumatoid arthritis are potent inducers of matrix metalloproteinases and proinflammatory cytokines upon synovial fibroblast interaction, including autocrine interleukin-17A production. Arthritis Rheum 2011;63:73-83.
7. Colin EM, Asmawidjaja PS, van Hamburg JP, Mus AM, van Driel M, Hazes JM, *et al*. 1,25-dihydroxy vitamin D3 modulates Th17 polarization and interleukin-22 expression by memory T cells from patients with early rheumatoid arthritis. Arthritis Rheum 2010;62:132-42.
8. Chabaud M, Lubberts E, Joosten L, van Der Berg W, Miossec P. IL-17 derived from juxta-articular bone and synovium contributes to joint degradation in rheumatoid arthritis. Arthritis Res Ther 2001;3:168-77.
9. Raza K, Falciani F, Curnow SJ, Rosa EJ, Lee CY, Akbar AN, *et al*. Earlyrheumatoid arthritis is characterized by a distinct and transient synovial fluid cytokine profile of T cell and stromal cell origin. Arthritis Res Ther 2005;7:R784-95.
10. Dong W, Zhu P. Functionalniche of inflamedsynovium for Th17-cellexpansion and activation in rheumatoid arthritis: Implication to clinical therapeutics. Autoimmun Rev 2012;11:844-51.
11. Shahrara S, Huang Q, Mandelin II AM, Pope RM. TH-17 cells in rheumatoid arthritis. Arthritis Res Ther 2008;10:R93.
12. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fires JF, Cooper NS, *et al*. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315-24.
13. Prevoo ML, van't Hof MA, Kuper HH, van Leeuwen MA, van de Putte LB, van Riel PL. Modified disease activity scores that include twenty-eight-joint counts. Development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. Arthritis Rheum 1995;38:44-8.
14. Van der Heijde DM, van 't Hof MA, van Riel PL, Theunisse LA, Lubberts EW, van Leeuwen MA, *et al*. Judging disease activity in clinical practice in rheumatoid arthritis: First step in the development of a disease activity score. Ann Rheum Dis 1990;49:916-20.
15. Christodoulou C, Choy EH. Joint inflammation and cytokine inhibition in rheumatoid arthritis. Clin Exp Med 2006;6:13-9.
16. 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.
17. Rosu A, Margaritescu C, 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.

18. Metawi SA, Abbas D, Kamal MM, Ibrahim MK. Serum and synovial fluidlevels of interleukin-17 in correlation with disease activity in patients with RA. Clin Rheumatol 2011;30:1201-7.
19. Melis L, Vandooren B, Kruithof E, Jacques P, De Vos M, Mielants H, *et al*. Systemic levels of IL-23 are strongly associated with disease activity in rheumatoid arthritis but not spondyloarthritis. Ann Rheum Dis 2010;69:618-23.
20. Kokkonen H, Söderström I, Rocklöv J, Hallmans G, Lejon K, Rantapaa-Dahlqvist S. Up-regulation of cytokines and chemokines predates the onset of rheumatoid arthritis. Arthritis Rheum 2010;62:383-91.
21. Shahrara S, Pickens SR, Dorfleutner A, Pope RM. IL-17 induces monocyte migration in rheumatoid arthritis. J Immunol 2009;182:3884-91.
22. Moran EM, Mullan R, McCormick J, Coonolly M, Sullivan O, Fitzgerald O, *et al*. 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.
23. Gullick NJ, Evans HG, Church LD, Jayaraj DM, Filer A, Kirkham BW, *et al*. Linking power Doppler ultrasound to the presence of Th17 cells in the rheumatoid arthritis joint. PLoS One 2010;5:e12516.
24. Patel DN, King CA, Bailey SR, Holt JW, Venkatachalam K, Agrawal A, *et al*. Interleukin-17 stimulates C-reactive protein expression in hepatocytes and smooth muscle cells via p38 MAPK and ERK1 / 2-dependent NF-kappaB and C / EBPbeta activation. J Biol Chem 2007;282:27229-38.
25. Shen H, Goodall JC, Hill Gaston JS. Frequency and phenotype of peripheral blood Th17 cells in ankylosing spondylitis and rheumatoid arthritis. Arthritis Rheum 2009;60:1647-56.
26. Leipe J, Grunke M, Dechant C, Reindl C, Kerzendorf U, Schulze-Koops H, *et al*. Role of Th17 cells in human autoimmune arthritis. Arthritis Rheum 2010;62:2876-85.
27. Tukaj S, Kotlarz A, Jóźwik A, Smolenska Z, Bryl E, Witkovski JM, *et al*. Cytokines of the Th1 and Th2 type in sera of rheumatoid arthritis patients; correlations with anti-Hsp40 immune response and diagnostic markers. Acta Biochim Pol 2010;57:327-32.
28. Machold KP, Neumann K, Smolen JS. Recombinant human interferon gamma in the treatment of rheumatoid arthritis: Double blind placebo controlled study. Ann Rheum Dis 1992;51:1039-43.
29. Seo SK, Choi JH, Kim YH, Kang WJ, Park HY, Suh JH, *et al*. 4-1BB-mediated immunotherapy of rheumatoid arthritis. Nat Med 2004;10:1088-94.
30. Morita Y, Yamamura M, Kawashima M, Harada S, Tsuji H, Shibuya H, *et al*. Flow cytometric single-cell analysis of cytokine production by CD4+ T cells in synovial tissue and peripheral blood from patients with rheumatoid arthritis. Arthritis Rheum 1998;41:1669-76.
31. Lubberts E, Joosten LA, Chabaud M, van Den Bersselaar L, Oppers B, Cooenen-De Roo CJ, *et al*. IL-4 gene therapy for collagen arthritis suppresses synovial IL-17 and osteoprotegerin ligand and prevents bone erosion. J Clin Invest 2000;105:1697-710.
32. Parsonage G, Falciani F, Burman A, Filer A, Ross E, Boffill M, *et al*. Global gene expression profiles in fibroblasts from synovial, skin and lymphoid tissue reveals distinct cytokine and chemokine expression patterns. Thromb Haemost 2003;90:688-97.

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