Clinical significance of interleukin-32 expression in patients with rheumatoid arthritis

Ming Gui, Hao Zhang, Kuangbiao Zhong, Ying Li, Jian Sun and Li Wang

Summary

Objective **To explore the clinical significance of interleukin (IL)-32 in the treatment of rheumatoid arthritis (RA) patients and analyze the correlations of IL-32 with C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and rheumatoid factor (RF).**

Methods **A total of 97 patients with RA and 36 patients with non-RA connective tissue diseases (CTD) were included. The mRNA levels of IL-32 and TNF-α in peripheral blood mononuclear cells (PBMCs) were determined using a quantitative real-time polymerase chain reaction. The plasma concentrations of IL-32 and TNF-α were determined using enzyme-linked immunosorbent assays. Data were analyzed using one-way ANOVA and Spearman's method.**

Results **The mRNA levels of IL-32 and TNF-α in the active RA groups were significantly higher than those in the healthy control, stable RA, and non-RA CTD groups (P <0.01). There was no significant difference between the stable RA, healthy control, and non-RA CTD groups (P > 0.05). The concentrations of IL-32 and TNF-α in the active RA groups were significantly higher than those in the stable RA and control groups (P <0.01). IL-32 expression was positively correlated with TNF-α, ESR, CRP, RF, and DAS28 in patients with RA (P <0.01).**

Conclusion **The levels of IL-32 and TNF-α in the peripheral blood of patients with active RA are significantly higher than in that of patients with stable RA and healthy people. The IL-32 level in peripheral blood may provide a reference for the detection of RA activity.** ***(Asian Pac J Allergy Immunol 2012;31:73-8)***

Key words: *rheumatoid arthritis, interleukin-32, tumor necrosis factor-α, C-reactive protein, connective tissue diseases*

From Department of Rheumatology and Immunology, the Third Xiangya Hospital of Central South University, Changsha 410013, China
Corresponding author: Hao Zhang
E-mail: haozhangcn@126.com
Submitted date: 14/5/2012
Accepted date: 23/7/2012

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic articular inflammation.[1] Although the pathogenesis of RA remains unclear, T cells, B cells, macrophages, and synovial membrane fibroblasts are known to play a crucial roles in the development of articular inflammation and disease.[2-5] IL-32 is a relatively newly discovered cytokine and studies on it are still in their infancy. Studies on the correlation between IL-32 and RA are very limited. IL-32 induces the generation of pro-inflammatory factors (TNF-α, IL-1, and IL-6) and chemokines via the mitogen-activated protein kinases NF-κB and p38 in human and rat cells.[6-9] Recent studies have shown that IL-32 is closely correlated with the genesis of RA.[10,11] .IL-32 is highly expressed in RA synovial living tissues, but is poorly expressed in osteoarthritis (OA) synovial tissues.[11] There is no study on IL-32 expression in the serum of RA patients. IL-32 synovial staining is correlated with disease activity, synovitis indices, and other synovial inflammatory factors. IL-32 also has a close correlation with TNF-α, which plays an important role in the aggravation of TNF-α-related inflammatory arthritis and enteritis. These associations led us to question whether similar changes in IL-32 occur in the peripheral blood of patients with RA.

In the present study, the levels of IL-32 and TNF-α in the peripheral blood of patients with RA were determined. Correlation analyses of these data and clinical data were performed to explore the possible role of IL-32 in the genesis of RA.

Methods

Patients

A total of 97 patients in the Third Xiangya Hospital were diagnosed with RA between August 2010 and January 2011. All patients met the 1987 American College of Rheumatology diagnostic criteria for RA.[12] Among the patients, 23 were males and 74 were females with an approximate male to female ratio of 1:3. Their ages ranged from 23 years to 75 years with a mean age of 45 ± 13 years. Their duration of disease ranged from four months to twenty years. RA activity was evaluated according to the Disease Activity Score in 28 joints (DAS28). DAS28 > 2.6 was regarded as the threshold for RA in an active phase. Patients with DAS28 > 5.1 constituted the highly active RA group, and those with DAS28 between 2.6 and 5.1 were considerd to be the moderately active RA group. Patients with stable-phase RA must have DAS28 < 2.6, lasting at least two months according to the RA clinical remission criteria.[13] The steroid, NSAIDs and DMARDs taken by patients are shown in Table 1.

A total of 36 patients constituted the non-RA CTD group. They received treatment in the Third Xiangya Hospital during the same period. There were 8 males and 28 females. Their ages ranged from 22 years to 55 years with a mean of 42 ± 7 years. There were 16 who had SLE and were taking prednisone (generic) (<20 mg/day) at the time ; 8 had AS and were taking methotrexate plus an anti-TNF agent; 5 had dermatomyositis and were taking prednisone (generic) (<15 mg/day) plus methotrexate, 3 had Sjgren's syndromes, 2 had systemic sclerosis but were not taking NSAIDs, steroid or DMARDs, and 2 had adult Still's disease and were taking prednisone (generic) (<10 mg/day) plus methotrexate. All cases met the corresponding diagnostic criteria and were in an active phase.

Table 1. The drugs used by patients in the RA group

Item	Highly active RA group (n=36)	Moderately active RA group (n=33)	RA remission group (n=28)
No	12	10	0
Only prednisone	3	4	0
Methotrexate	2	8	2
Leflunomide	0	1	1
NSAIDs	2	2	0
Methotrexate and prednisone	3	2	0
Methotrexate and leflunomide	9	4	4
Methotrexate and leflunomide and prednisone	3	1	0
Anti-TNF and Methotrexate	0	0	16
Anti-TNF and Methotrexate and leflunomide	2	0	5
NSAIDs and one or more DMARDs	14	6	0

A total of 30 healthy volunteers constituted the healthy control group. There were 8 males and 22 females. Their ages ranged from 23 years to 60 years with a mean of 48 ± 10 years. They were free of heart diseases, diabetes, tumors, chronic obstructive lung diseases, inflammatory bowel diseases, and recent infectious diseases.

This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of the Third Xiangya Hospital of Central South University. Written informed consent was obtained from all participants.

Real-time Polymerase Chain Reaction (PCR)

About 3 ml of venous blood was extracted from fasting subjects and was placed into a coagulation-promoting tube in the morning.

Total mRNA was extracted from the isolated PBMCs using an RNA extraction kit (Shanghai Biotechnology, China). cDNA was synthesized according to the instructions indicated in aReverTra Ace qPCR RT kit (TOYOBO, Japan). Based on the sequences in Genbank, target genes were designed using Primer 5.0 software. Syntheses were performed by Shanghai Biology Engineering. The sequences are listed in Table 2.

The previously prepared cDNA was used as the template. The optimal specific amplification conditions were as follows: the reaction system with a volume of 10 μl contained 5 μl of SYBR GreenER qPCRSuperMix, 0.6 μl of cDNA, 0.2 μl of the upstream primers, 0.2 μl of the downstream primers, and 4 μl of Rnase free water.The amplification conditions consisted of a pre-denaturation step at 94°C for 1 min, 40 cycles at 94°C for 15 s, 56°C for 15 s and 72°C for 45s, and a final extension step at 72°C for 7 min.

Target gene expression was determined using an Eppendorf quantitative PCR analyzer. The expression of IL-32, TNF-α, and β-actin (the internal standard) was determined using quantitative real-time (QRT)-PCR, and $2^{\Delta CT}$ values were calculated. The QRT-PCR products were analyzed

Table 2. PCR primer sequences, PCR product length of IL-32, TNF-α, β-actin

	Primer sequences	PCR product length (bp)
IL-32	Upstream: 5'-TGAGGAGCAGCACCCAGAGC-3'	310
	Downstream: 5'-CCGTAGGACTGGAAAGAGGA-3'	
TNF-α	Upstream: 5'-TC AGCCTCTTCTCCTTCCTG-3'	324
	Downstream: 5'-TGAAGAGGACCTGGGAGTAG-3'	
β-actin	Upstream: 5'-TGGACTTCGAGCAAGAGATGG-3'	298
	Downstream: 5'-ATCTCCTTCTGCATCCTGTCG-3'	

in an electrophoresis gel and then observed using an ultraviolet projectoscope. The mobility rates were compared with the marker, and their molecular weights were estimated. The products were found to be the fragments of the target genes.

Related Clinical Indexes

Serum IL-32 and TNF-α concentrations were determined using a human IL-32 (R&D, USA) and TNF-α (Neo Bioscience, China) ELISA kit. Rheumatoid factor (RF) and C-reactive protein (CRP) were detected by the nephrometry scoring system. The ESR was determined by the Westergren method.

Statistical Analysis

Data were analyzed using the SPSS17.0 software. Data with normal distribution were presented as means ± standard errors ($\bar{x} \pm s$). One-factor ANOVA was performed for comparison among groups and Spearman's analysis was performed for correlation analysis. P <0.05 was considered statistically significant.

Results

Patients

There was no significant differences between the gender, age, and body weights of the different groups (P >0.05) (Table 3).

PCR Amplification

PCR amplifications were performed using the previously mentioned cDNA as templates. The electrophoresis results are shown in Figure 1.

Expression of IL-32 and TNF-α

The mRNA expression of IL-32 and TNF-α in the active RA groups, which including highly active RA (n=36) and moderately active RA (n=33) was significantly higher than that in the stable RA (n=28) and healthy control groups (n=30) (P <0.05). No significant difference was observed between the stable RA and control groups (P >0.05). The results are shown in Table 4.

Correlation Analysis

In the active groups, the IL-32 mRNA expression in PBMCs was positively correlated with TNF-α mRNA expression (r = 0.587, P <0.01), ESR, RF, and DAS28, but had no correlation with age, disease course, or CRP (Table 5).

The protein concentrations of serum IL-32 and TNF-α in the active RA groups were noticeably higher than those in the other three groups (P <0.05) (Table 4). The protein concentrations were positively correlated with ESR, CRP, RF, and DAS28, but not with age or disease course (Table 6).

Table 3. General data for the different groups

Item	Highly active RA (n=36)	Moderately active RA (n=33)	Stable RA (n=28)	Healthy control (n=30)	Non-RA control(n=36)	
Age, years (mean ± SD)	44.25 ± 10.71	44.66 ± 10.40	41.21 ± 7.61	43.53 ± 8.89	42.16 ± 7.45	0.516
Sex						0.376
Male	9 (25)	8 (4.2)	6 (1.4)	8 (6.7)	8 (2.2)	
Female	27 (75)	25 (75.8)	22 (78.6)	22 (73.3)	28 (77.8)	
Weight, kg (mean ± SD)	53.2 ± 7.32	52.9 ± 10.21	54.1 ± 6.45	56.3 ± 5.76	55.45 ± 6.21	0.723

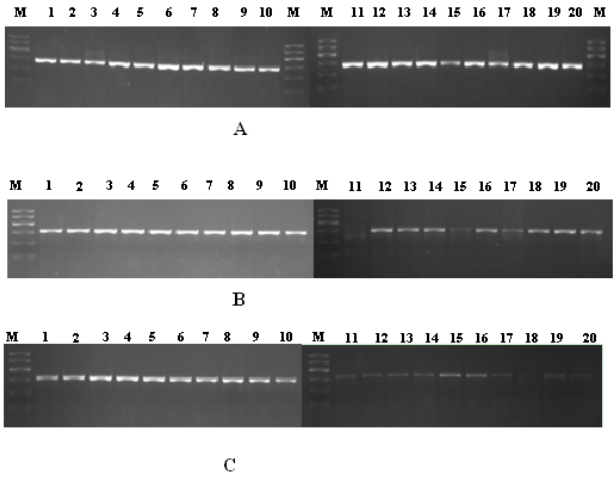

Figure 1. PCR products of the serum target genes (IL-32 and TNF-α) in the RA and control groups. A: β-actin corresponded by IL-32 and TNF-α in different groups; B and C: IL-32, in which 1 - 5 are for patients with highly active RA, 6 - 10 for those with moderately active RA, 11 - 13 for those with stable RA, 14 - 17 for the healthy control group, and 18 - 20 for the non-RA CTD group.

Discussion

RA is a systemic inflammatory disease that affects multiple peripheral joints. The correlations of RA with numerous inflammatory cells (T, B, synovial, and antigen-presenting cells) and their produced inflammatory factors (TNF-α, IL-1, IL-6, IL-15, IL-17, and IL-18) have been previously demonstrated. However, the patho-mechanism of RA remains unclear. The pathology of RA synovial tissues is mainly characterized by the penetration of numerous macrophages and T-lymphocytes, synovial lining hyperplasia, angiogenesis, as well as pannus formation. TNF-α is a pro-inflammatory factor that plays a critical role in the pathogenesis of RA and is the most often studied cytokine. TNF-α can be produced by different types of inflammatory cells, such as macrophages, mononuclear cells, T-lymphocytes, and synovial membrane fibroblasts. TNF-α induces the production of other inflammatory factors and promotes the generation of osteoclasts that lead to bone destruction. Inflammatory arthropathy can develop spontaneously in TNF-α transgenic rats. By contrast, TNF-α inhibitors can mitigate the severity of arthritis and TNF-α monoclonal antibodies, as well as soluble TNF-α receptor analogs, have been applied in the treatment of RA and other types of inflammatory arthropathy.[16]

IL-32 is a recently discovered cytokine and performs an important role in the development of autoimmune diseases. It is highly expressed in chronic obstructive lung disease, Crohn's disease, psoriasis, and RA.[15-17] IL-32 is closely correlated with the development of these diseases. It is highly expressed in RA synovial tissues but weakly expressed in OA synovial tissues. It has noticeable correlations with the ESR, as well as local TNF-α, IL-1β, and IL-18 in RA synovial tissues. An animal experiment has revealed that recombinant human IL-32γ can stimulate the production of TNF-α, IL-1β, and macrophage inflammatory protein-2 to aggravate joint swelling, invasion of numerous inflammatory cells, and bone destruction in rats.[18] IL-32 γ can also promote the differentiation of osteoclast precursors into osteoclasts.[19] Therefore, IL-32 exhibits a pro-inflammatory feature and plays an important role in the pathogenesis of RA.

IL-32 is maintained at a stable level in human PBMCs when there is no stimulation and only low levels of IL-32 protein can be detected in supernatant liquid. However, the level increases significantly after lipopolysaccharide stimulation.

Table 4. Comparisons of the mRNA expression and protein concentrations of IL-32 and TNF-α in PBMCs and serum among different groups ($\bar{x} \pm s$)

		mRNA		Protein (pg/ml)	
Groups	Cases	IL-32	TNF-α	IL-32	TNF-α
Highly active RA	36	0.286 ± 0.072*#△	0.042 ± 0.022*#△	107 ± 42*#△	261 ± 77*#△
Moderately active RA	33	0.239 ± 0.0628*#△	0.012 ± 0.006*#△	66 ± 27*#△	186 ± 84*#△
Stable RA	28	0.136 ± 0.052	0.005 ± 0.003	21 ± 9	73 ± 24
Healthy control	30	0.112 ± 0.057	0.004 ± 0.002	20 ± 10	72 ± 20
Non-RA CTD	36	0.128 ± 0.058	0.007 ± 0.002	25 ± 9	92 ± 22
F value		53.707	71.319	75.488	77.949
P value		0.000	0.000	0.000	0.000

* P <0.01, compared with the stable RA group; # P < 0.01, compared with the healthy control group; and △ P < 0.01, compared with the non-RA CTD group.

Table 5. Correlation analyses of the mRNA expression of PBMC IL-32 and TNF-α and age, disease course, ESR, CRP, RF, and DAS28 in the active RA groups

Indexes	IL-32		TNF-α	
	r value	*P* value	r value	*P* value
Age (year)	0.173	0.073	0.129	0.315
Disease course (year)	0.081	0.405	0.039	0.692
ESR (mm/h)	0.321**	0.007	0.471**	0.000
CRP(mg/L)	0.185**	0.127	0.358**	0.003
RF (U/ml)	0.325**	0.000	0.453**	0.000
DAS28	0.303*	0.011	0.579**	0.000
TNF-α	0.568**	0.000		

** P <0.01, and * P <0.05.

Table 6. Correlation analyses of the mRNA expression of serum TNF-α and age, disease course, ESR, CRP, RF, and DAS28 in the active RA groups

Indexes	IL-32(pg/ml)		TNF-α(pg/ml)	
	r value	*P* value	r value	*P* value
Age (year)	0.173	0.073	0.129	0.315
Disease course (year)	0.081	0.405	0.039	0.692
ESR (mm/h)	0.401**	0.001	0.562**	0.000
CRP (mg/L)	0.334**	0.005	0.457**	0.000
RF (U/ml)	0.325**	0.000	0.453**	0.000
DAS28	0.535**	0.000	0.625**	0.000
TNF-α	0.527**	0.000		

** P < 0.01.

In TNF-α-free rats, joint swelling caused by IL-32 cannot be observed and cell invasion is greatly reduced. By contrast, such effects cannot be observed after proteoglycan loss.[16] These findings suggest that IL-32 activity partially depends on TNF-α.

To clarify whether IL-32 is highly expressed in the PBMCs of RA patients, whether IL-32 in peripheral blood is correlated with RA activity, and whether IL-32 expression is correlated with TNF-α expression, active RA, stable RA and control groups were compared in the present study. The results show that IL-32 mRNA expression in the PBMCs of patients with active RA is significantly higher than that in patients with stable RA and healthy people. Serum IL-32 protein expression in patients with active RA is also significantly higher. Serum IL-32 expression in patients with active RA is positively correlated with the RA activity indices ESR, CRP, RF, and DAS28. The mRNA and protein expression of IL-32 is significantly correlated with that of TNF-α in patients with active RA.

Interestingly, IL-32 mRNA expression is significantly higher than that of TNF-α mRNA expression. However, the serum IL-32 protein levels are two times lower than the TNF-α protein level in both the RA and healthy control groups. This result can be explained as follows.

First, an animal experiment has demonstrated an aggravation in collagen-induced arthritis after the transposition of IL-32β into CD4+ T cells, in contrast to the disappearance of the amplification effect of IL-32β after TNF-α removal. This finding suggests that the performance of IL-32 may be partially dependent on TNF-α.[18] Hence, IL-32 presumably regulates TNF-α expression upstream and then promotes the occurrence of inflammation.

Second, the present study detected IL-32 total mRNA in cells. However, there is a possibility that some IL-32 subtypes have been secreted out of the cells to perform external pro-inflammatory roles, leaving others inside. This possibility can be clarified by further exploring the intra- and extra-cellular subtypes of IL-32.

Intracellular IL-32 protein was not detected in this study due to the limited volume of each blood sample.

To the best of our knowledge, there has been no reported study on IL-32 expression in patients with non-RA CTDs, such as SLE, AS, dermatomyositis and systemic sclerosis. In this study, the expression of IL-32 and TNF-α in the peripheral blood of patients with active non-RA CTDs was also detected. No significant difference is found between the IL-32 expression of the non-RA CTD and healthy control groups. However, only a small sample size of each non-RA disease was included in this study. Thus, the possible correlations between IL-32 and the development of these diseases cannot be completely excluded.

In conclusion, IL-32 is highly expressed in patients with active RA and its expression is positively correlated with the RA activity indices ESR, CRP, RF, and DAS28. These results are consistent with high IL-32 expression in RA synovial tissues, which further demonstrates the possible important role of IL-32 in the pathogenesis of RA. Therefore, the detection of IL-32 and TNF-α expression in patients with RA greatly aids studies on the role of IL-32 in RA pathogenesis and provides new ideas for the targeted treatment of RA.

However, IL-32 has six subtypes. The subtype that performs a major role in the peripheral blood of patients with RA is still uncertain at present. IL-32-related receptors and their action mechanisms also remain to be explored.

Conflict of interest

The authors declare that they have no conflicts of interest concerning this article.

References

1. Andersson AK, Li C, Brennan FM. Recent developments in the immunobiology of rheumatoid arthritis. Arthritis Res Ther. 2008;10:204.
2. Singh K, Deshpande P, Pryshchep S, Colmegna I, Liarski V, Weyand CM, et al. ERK-dependent T-cell receptor threshold calibration in rheumatoid arthritis. J Immunol. 2009;183:8258-67.
3. Gottenberg JE, Miceli-Richard C, Ducot B, Goupille P, Combe B, Mariette X. Markers of B-lymphocyte activation are elevated in patients with early rheumatoid arthritis and correlated with disease activity in the ESPOIR cohort. Arthritis Res Ther. 2009;11:R114.
4. Tanaka M, Nagai T, Tsuneyoshi Y, Sunahara N, Matsuda T, Nakamura T, et al. Expansion of a unique macrophage subset in rheumatoid arthritis synovial lining layer. Clin Exp Immunol. 2008;154:38-47.
5. Bartok B, Firestein GS. Fibroblast-like synoviocytes: key effector cells in rheumatoid arthritis. Immunol Rev. 2010; 233:233-55.
6. Li W, Liu Y, Mukhtar MM, Gong R, Pan Y, Rasool ST, et al. Activation of Interleukin-32 Pro-Inflammatory Pathway in Response to Influenza A Virus Infection. PLoS One. 2008;3:e1985.
7. Marcondes AM, Ramakrishnan A, Deeg HJ. Myeloid Malignancies and the Marrow Microenvironment: Some Recent Studies in Patients with MDS. Curr Cancer Ther Rev. 2009;5:310-4.
8. Kobayashi H, Yazlovitskaya EM, Lin PC. Interleukin-32 positively regulates radiation-induced vascular inflammation. Int J Radiat Oncol Biol Phys. 2009;74:1573-9.
9. Gabay C, McInnes IB. The biological and clinical importance of the 'new generation' cytokines in rheumatic diseases. Arthritis Res Ther. 2009;11:230.
10. Cagnard N, Letourneur F, Essabbani A, Devauchelle V, Mistou S, Rapinat A, et al. Interleukin-32, CCL2, PF4F1 and GFD10 are the only cytokine/chemokine genes differentially expressed by in vitro cultured rheumatoid and osteoarthritis fibroblast-like synoviocytes. Eur Cytokine Netw. 2005;16:289-92.
11. Joosten LA, Netea MG, Kim SH, Yoon DY, Oppers-Walgreen B, Radstake TR, et al. IL-32, a proinflammatory cytokine in rheumatoid arthritis. Proc Natl Acad Sci USA. 2006;103:3298-303.
12. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries 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, et al. 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. Lipsky PE, van der Heijde DM, St Clair EW, Furst DE, Breedveld FC, Kalden JR, et al. Infliximab and methotrexate in the treatment of rheumatoid arthritis. Anti-Tumor Necrosis Factor Trial in Rheumatoid Arthritis with Concomitant Therapy Study Group. N Engl J Med. 2000; 343:1594-602.
15. Choi JD, Bae SY, Hong JW, Azam T, Dinarello CA, Her E, et al. Identification of the most active interleukin-32isoform. Immunol. 2009;126:535-42.
16. Carter KL, Cahir-McFarland E, Kieff E. Epstein-Barr virus-induced changes n B-lymphocyte gene expression. J Virol. 2002; 76:10427-36.
17. Park GH, Choe J, Choo HJ, Park YG, Sohn J, Kim MK. Genome-wide expression profiling of 8-chloroadenosine-and 8-chloro-cAMP-treated human neuroblastoma cells using radioactive human cDNA microarray. Exp Mol Med. 2002;34:184-93.
18. Kim SH, Han SY, Azam T, Yoon DY, Dinarello CA. Interleukin-32: a cytokine and inducer of TNFalpha. Immunity. 2005:22:131-42.
19. Brown J, Matutes E, Singleton A, Price C, Molgaard H, Buttle D, et al. Lymphopain a cytotoxic T and naturalkiller cell-associated cysteine proteinase. Leukemia. 1998;12:1771-81.