

The correlation between IL-33 and inflammation factors in rheumatoid arthritis patients in vivo and in fibroblast-like synoviocytes in vitro

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Abstract

Background: Interleukin-33 (IL-33) is a member of the IL-1 family of cytokines whose role remains controversial in rheumatoid arthritis (RA), because no clear conclusion has been established regarding the relationship between IL-33 and other cytokines and chemokines. The present study was conducted to evaluate the correlation of IL-33 with other cytokines and chemokines in serum and the synovia, and to explore the nature of the relationship.

Results: IL-33 was found to exhibit an inverted-U-shaped correlation with multiple cytokines and chemokines in synovial fluid, including IL-6, IL-1 β , CXCL8 (IL-8), CXCL9 (MIG) and CXCL10 (IP-10), but not in serum. Moreover, in vitro experiments confirmed that IL-33 also exhibits U-type dose-dependent regulation of FLS function.

Conclusions: IL-33 exhibit an inverted-U-shaped correlation with multiple cytokines and chemokines in synovial fluid of RA patients. IL-33 affects the secretion of cytokines and chemokines in the synovium in a U-type dose-dependent relationship.

Background

Rheumatoid arthritis (RA) is a chronic and refractory autoimmune joint disease, characterized by the proliferation of synoviocytes in inflamed synovia and the expression of inflammatory cytokines and chemokines in synoviocytes [1]. Multiple pro-inflammatory and inflammatory cytokines have been thought to participate in the development of RA. A variety of cytokines and chemokines are present in the synovium of RA patients which play an important role in the maintenance of the inflammatory response [2, 3]. Important pro-inflammation cytokines, such as IL-33, IL-1 β , IL-6, IL-8 and TNF- α , and chemokines such as CXCL9 and CXCL10 are considered markers for the diagnosis of RA and represent therapeutic targets [3-9]. A complex mechanistic network exists between these cytokines and chemokines.

IL-33 is a member of the IL-1 family of cytokines [10]. Supporting evidence regarding the relationship between IL-33 and pathogenesis of RA has been provided by Matsuyama and colleagues, in which IL-33 levels have been found to be elevated in the serum and synovial fluid of RA-patients, demonstrating a positive correlation with disease activity[11]. IL-33 is expressed in human RA synovial fluid and released from FLS following stimulation with TNF- α and IL-1 β [11, 12]. IL-33 can activate human mast cells to release CXCL8 in RA patients[13]. One study has indicated that IL-33 is able to enhance the expression of CXCL9 and CXCL10 in wild-type mice with colitis [14]. IL-33 has an important role and a complicated relationship with cytokines and chemokines in RA [12, 15, 16]. However, the role of IL-33 remains controversial, a number of studies suggesting that it promotes inflammation[17], while others show that the response is inhibitory [18]. A confounding observation is that high levels of IL-33 can be detected in the serum or synovia fluid of just one third to one half RA patients, suggesting that IL-33 is widely variable in RA patients [11, 19, 20]. A significant number of patients with RA have low levels of IL-33 in serum but still exhibit high disease activity [19]. Since contrary results are increasingly observed in RA, we hypothesize that the concentrations of IL-33 and other cytokines and chemokines might not be linearly correlated, but a more complex polynomial correlation should be further investigated. The present research study aimed to establish a correlation curve model of cytokines (CXCL8, CXCL9, and CXCL10) and chemokines (IL-1 β , TNF- α , and IL-6) with IL-33 in serum and synovia, and to explore the nature of the relationship.

Results

Baseline clinical and demographics features

A total of 96 consecutive patients with RA were recruited, from which 40 serum samples (35 women and 5 men, mean age 51.80 \pm 13.37 years) and 56 synovial samples (43 women and 13 men, mean age 48.85 \pm 13.39 years) were obtained. Patients characteristics are displayed in Table 1, below.

Cytokines and chemokines levels in patients with RA

At baseline, there were no differences in IL-33, MIG and IL-6 concentrations between the synovial and serum samples of RA patients (Figure 1 A, B and D). IP-10 concentration was significantly higher in synovial fluid than in serum (Figure 1 C). IL-8, TNF- α and IL- β concentrations were significantly higher in serum than in synovial fluid (Figure 1 E, F and G).

IL-33 concentration follows an inverted-U-shaped curve in response to cytokines and chemokines

IL-33 concentration exhibited an inverted-U-shaped curve in response to IL-6, IL-1 β , CXCL8 (IL-8), CXCL9 (MIG) and CXCL10 (IP-10) but not TNF- α in synovial fluid of RA patients (Figure 2). The same IL-33 inverted-U-shaped correlation was observed after treatment with CXCL9

(MIG) but not CXCL10 (IP-10), TNF- α , IL-6, IL-1 β or CXCL8 (IL-8) in the serum of RA patients (Figure 3).

mRNA and protein expression levels of IL-6, CXCL 8, CXCL9, CXCL10, IL-1 β and TNF- α in FLS stimulated by IL-33

IL-33 affected the mRNA expression of IL-6, CXCL8, CXCL9, CXCL10, IL-1 β and TNF- α in FLS. However, only protein expression of IL-6, IL-8, CXCL10 and TNF- α was detectable in the supernatants of the FLS cell culture. CXCL9 and IL-1 β protein expression was not detected in the supernatants of FLSs, even at a low level. IL-33 affected both mRNA and proteins expression of IL-6 in FLS. A high response of FLS at a concentration of 50ng/ml IL-33 suggests that it has a narrow working concentration on FLSs (Figure 4 A). A greater response at 4h than 24h suggests that IL-33 is effective over a precise time frame in FLSs. Over time, the effect of IL-33 on IL-6 secretion by FLS changed (Figure 4 B). Different to IL-6, the expression of IL-8 reached a peak after 24h at a concentration of 50ng/ml of IL-33 (Figures 4 C, D). CXCL9 mRNA expression was up-regulated in 50ng and 100ng after 4h, but only 50ng after 24h, while CXCL9 protein could not be detected in the FLS culture supernatant (Figure 4 E). IL-6, IL-8, and IL-1 β mRNA were detected at high levels in both 50ng and 100ng concentrations of IL-33 at both time points, but protein expression (Figure 4 F). CXCL10 mRNA expression was down-regulated as the concentration of IL-33 increased. The ELISA results also demonstrated that CXCL10 protein expression decreased as the concentration of IL-33 increased after 24h. Conversely, TNF- α and IL-17 increased CXCL10 mRNA expression in FLS (Figure 4 H). TNF- α was not affected by different concentrations of IL-33, or by IL-17 (Figures 4 I, J).

Discussion

Previous studies have shown that IL-33 is expressed in human RA synovium, promoting articular inflammation [12, 15, 20], but the underlying mechanisms have not been fully elucidated and opposite experimental results have been observed on numerous occasions. In vivo, injection of IL-33 has been shown to exacerbate joint inflammation in a K/BxN serum-transfer mouse model of arthritis [17]. However, a separate study provided conflicting results, indicating that injection of IL-33 ameliorated joint inflammation in the same mouse model [18]. Interestingly, another two research groups observed similar contradictory results: Jérôme Biton found that IL-33 was able to suppress inflammation in CIA mice [21], but Xu demonstrated that IL-33 was a critical proinflammatory cytokine causing fibroblast activation in CIA mice [12]. We carefully analyzed two papers with contradictory findings and found that the different results may have been caused due to the different dosage of IL-33 injected. Therefore, we realized that whether the effect of IL-33 was proinflammatory or inhibitory may be related to its concentration.

To confirm this hypothesis, we conducted an additional series of experiments. Firstly, the concentrations of a variety of cytokines and chemokines were measured in synovia and serum. Of note, the level of IP-10 was considerably higher in synovial fluid than in serum, indicating that inflamed joints constitute the primary source of circulating IP-10 in arthritis. Conversely, IL-8, TNF- α and IL-1 β levels were substantially higher in serum than in synovial fluid. Secondly, we investigated the association of IL-33 with other cytokines and chemokines in RA patients. As expected, IL-33 was not linearly correlated with other cytokines and chemokines but exhibited an inverted-U-shaped relationship. In the present study, we found that IL-33 exhibited an inverted-U-shaped correlation with IL-6, IL-8, IL-1 β , CXCL9 and CXCL10 but not TNF- α in the synovia of RA patients, although other studies have demonstrated that TNF- α was able to stimulate IL-33 release in mouse experiments and in cell culture. [22]. Our research suggests that IL-33 has a more specific role in synovia than in serum, since this U-shaped relationship occurs only in synovia. Synovia is principally produced by synovial cells, and so we aimed to explore the role of IL-33 in synovial cells. The experiment was designed with IL-33 concentrations that stimulated FLSs. As expected, the most exciting result was that specific concentrations of IL-33 were important in FLS cultures, 10 ng/ml being too low to cause an impact on FLS, but 150 ng/ml IL-33 too high. This response was consistent with the U-type correlation between IL-33 and other cytokines observed in synovial fluid. The most appropriate concentration of IL-33 was 50 ng/ml for 20000 FLSs, although its influence was not comparable with IL-17 and TNF- α , which strongly influenced FLSs. We considered other studies that demonstrated the negative results [23] might be because IL-33 operated within a narrow range of working concentrations, and the most appropriate ratio of IL-33 concentration/cell number was important in FLS cultures. Time points were an additional factor that could affect FLS, culturing for 4 h being more effective than for 24 h regarding IL-6 release, suggesting that IL-33 affect FLS over a precise time frame. IL-6 was released earlier than IL-8, suggesting that different cytokines released by FLSs were controlled by different mechanisms. We found a significant inverted-U-shaped influence on IL-6 and IL-8 but not CXCL10 and TNF- α , so the results imply that different cytokines released by FLSs were controlled by different mechanisms. IL-33 regulated the production of IL-1 β in FLS in an inverted-U-shaped manner, and combined with other research findings established that IL-1 β regulated the production of IL-33 in FLS [22], demonstrating that IL-33 plays a key role in the positive and negative feedback mechanisms with IL-1 β . In this study, we were concerned about the relationship between IL-33 and other cytokines and chemokines. We initially discovered the inverted-U-shaped relationship between IL-33 and the chemokines CXCL8, CXCL9 and CXCL10 in synovia. These results very important in RA, suggesting that IL-33 has the potential capability to recruit and regulate inflammatory cells.

Conclusions

In the present study, we reported that IL-33, a recently-described member of the IL-1 family of cytokines, exhibits an inverted-U-shaped correlation with multiple cytokines and chemokines in synovial fluid, including IL-6, IL-1 β , CXCL8 (IL-8), CXCL9 (MIG) and CXCL10 (IP-10) but not in serum. IL-33 also triggered the expression of the pro-inflammatory cytokines IL-6 and IL-1 β , and chemokines CXCL8 and CXCL9 in a U-shaped dose-dependent manner in RA-FLS.

Methods

Patient enrollment

A total of 96 Patients with RA who had presented at the rheumatology clinic of ZhuJiang Hospital of Southern Medical University were recruited to the study. All patients fulfilled the 2010 rheumatoid arthritis classification criteria [24]. Samples of serums and synovial fluid, as appropriate were collected from RA patients after obtaining signed informed consent.

Human FLS culture

Synovial tissue samples were obtained by knee joint arthroscopy from the knees of five active RA patients, DAS28>5.1. All RA patients fulfilled the 2010 rheumatoid arthritis classification criteria. Synovial tissues were diced into 1-2 mm³ pieces, cultured with Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum, 100U/ml penicillin and 100ug/ml streptomycin, then incubated in a humidified atmosphere containing 5% CO₂. The culture medium was refreshed every 3-4 days. Cells at passage 3 to 6 were used for experiments.

Synovial cell activation

RA-FLSs were seeded in the wells of 24-well plates at a density of 2×10^4 cells/well for 24h, then 10ng, 50ng, 100ng or 150ng/ml human IL-33 (R&D USA) were added and incubated for 4h and 24h. Human IL-17 (100ng/ml, R&D USA) and TNF- α (100ng/ml, R&D USA), two cytokines known to activate synoviocytes were performed as positive controls.

Quantitative PCR

Total RNA was isolated from RA-FLSs using Trizol reagent (Invitrogen, USA) in accordance with the manufacturer's protocols. Reverse transcription was conducted using first-strand cDNA synthesis (TaKaRa, China). Real-time PCR was performed using a SYBR Premix ExTaq kit (TaKaRa, China) to assess the expression of IL-6, CXCL8, CXCL9, CXCL10, IL-1 β and TNF- α .

ELISA assays

Concentrations of cytokines and chemokines in serum and synovial fluid samples were measured using ELISA assays, including IL-33 (R&D, USA), IL-1 β (R&D USA), TNF- α (Thermo Fisher Scientific, USA), IL-6 (Thermo Fisher Scientific, USA), IL-8 (Thermo Fisher Scientific, USA) MIG (Invitrogen, USA) and IP-10 (Invitrogen, USA). After stimulation of 2×10^4 RA-FLSs with human IL-33 as described above, the supernatants of the FLS cell cultures were collected and IL-6, IL-8, MIG, IP-10, TNF- α and IL-1 β concentrations were quantified. The optical density (OD) values of each sample were measured at 450 nm.

Statistical analysis

Statistical analyses were performed using IBM SPSS 24.0 and GraphPad Prism 5.0. Data are presented as means \pm standard deviation (SD) or medians (QR). Differences between groups were analyzed using Kruskal-Wallis test (more than two groups), or a Mann-Whitney U test (two groups). A second-order polynomial regression analyses were performed using SPSS 24.0 to quantify the associations among the variables. Other statistical tests were conducted by ANOVA. All statistical tests and confidence intervals were two-sided, with $P < 0.05$ considered statistically significant.

Declarations

Ethics approval and consent to participate

The study is approved by Ethics Committee of ZhuJiang Hospital of Southern Medical University. This is to certify that the research design and methods are in accordance with the requirements of the ethical standards in the 2013 Declaration of Helsinki regulations and procedures regarding to human subject protection in laws. And written informed consent was obtained from all study participants.

Consent for publication

Not applicable.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Availability of data and materials

The datasets used and analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

JW, QL, JX D and J S performed experiments. JW and QL conceived the study and analyzed the results. JJZ and QH Y supervised the study and prepared the manuscript. All authors read and approved the final manuscript.

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None.

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Tables

Table 1. RA Patients characteristics

n	Gender (M/F)	Age(y)	IL-33 (pg/ml)	MIG (pg/ml)	IP-10 (pg/ml)	IL-6 (pg/ml)	IL-8 (pg/ml)	TNF- α (pg/ml)	IL-1 β (pg/ml)
40	5/35	51.80 \pm 13.37	36.46(16.05, 191.92)	1081.88(460.67, 1384.15)	143.40(90.50, 316.40)	94.95(3.33,172.46)	675.23(117.55, 1042.17)	20.17(12.02, 32.53)	12.85(10.21, 22.73)
56	13/43	48.85 \pm 13.39	40.45(22.40,80.36)	563.59(404.68,1176.68)	201.12 (269.35,606.46)	34.13(9.53,222.03)	14.13(6.74,44.44)	9.68(3.38,16.22)	8.56(1.74, 9.45)

Data are presented as mean \pm standard deviation (SD) and Median (QR). IL: Interleukin; MIG: Monokine induced by gamma interferon; IP-10: Interferon gamma-induced protein 10.

Figures

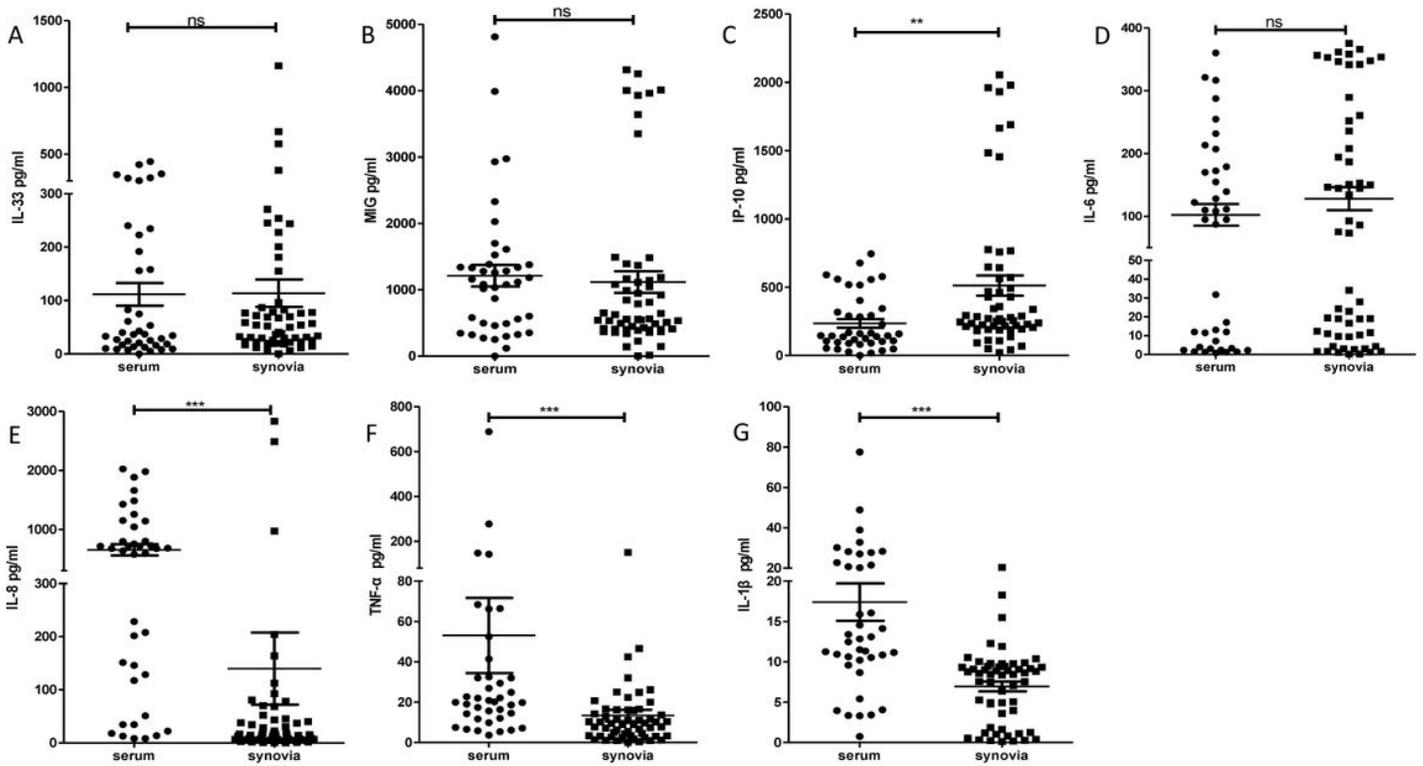


Figure 1

Serum and synovia cytokines and chemokines concentration, measured by ELISA, were obtained from 96 patients with RA, 40 serum, 56 synovia. A. IL-33 was detected in RA synovia compared with serum ($p=0.640$); B: MIG was detected in RA synovia compared with serum ($p=0.183$). C: IP-10 was detected in RA synovia compared with serum ($p=0.001$). D: IL-6 was detected in RA synovia compared with serum ($p=0.376$). E: IL-8 was detected in RA synovia compared with serum ($p=0.000$). F: TNF- α was detected in RA synovia compared with serum ($p=0.000$). G: IL-1 β was detected in RA synovia compared with serum ($p=0.000$) ** $P<0.01$, *** $P<0.001$ as assessed by Mann–Whitney U test, as appropriate.

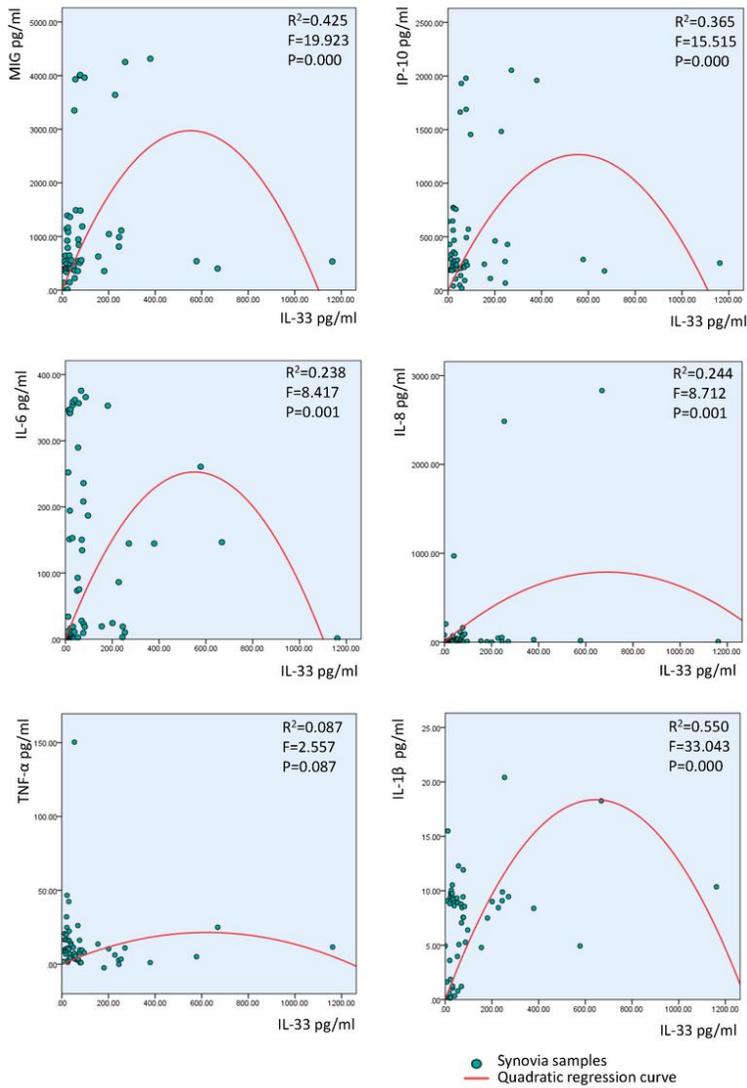


Figure 2

The Inverted-U-shaped correlations showed between synovial IL-33 and CXCL9 (MIG), CXCL10 (IP-10), IL-6, CXCL8 (IL-8), TNF- α and IL-1 β . Second-order polynomial regression curves were made by SPSS 24.0. $P < 0.05$ as assessed by ANOVA test.

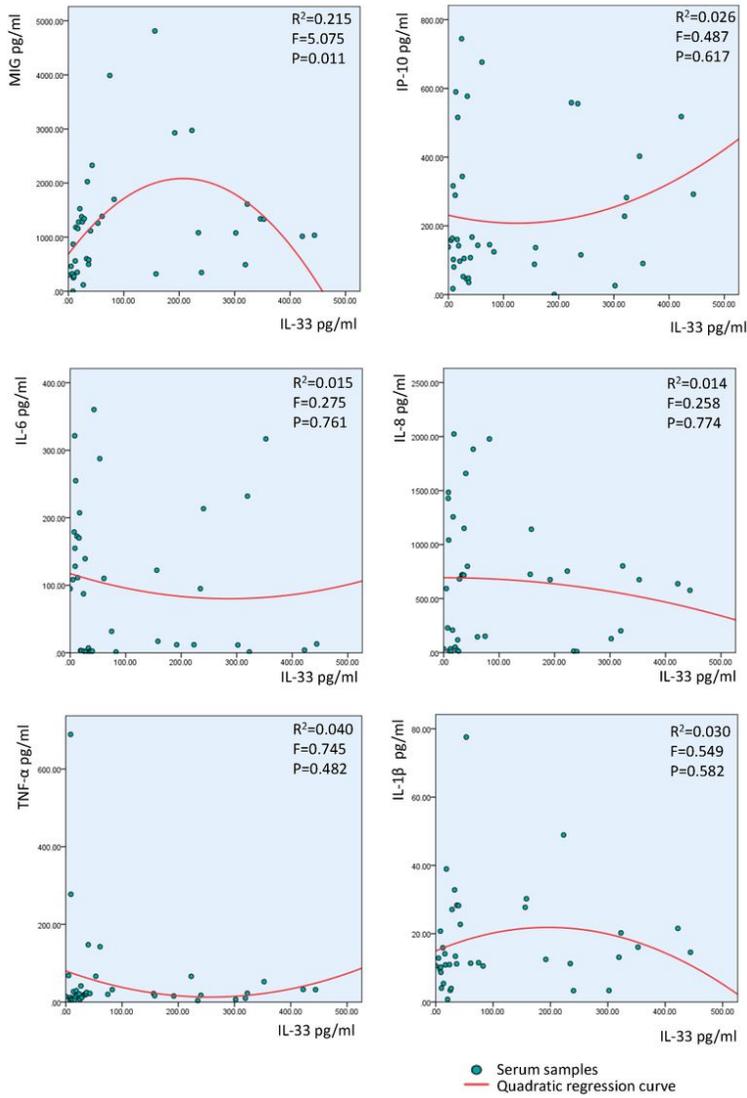


Figure 3

The Inverted-U-shaped correlations showed between serum IL-33 and CXCL9 (MIG), CXCL10 (IP-10), IL-6, CXCL8 (IL-8), TNF- α and IL-1 β . Second-order polynomial regression curve were made by SPSS 24.0. $P < 0.05$ as assessed by ANOVA test.

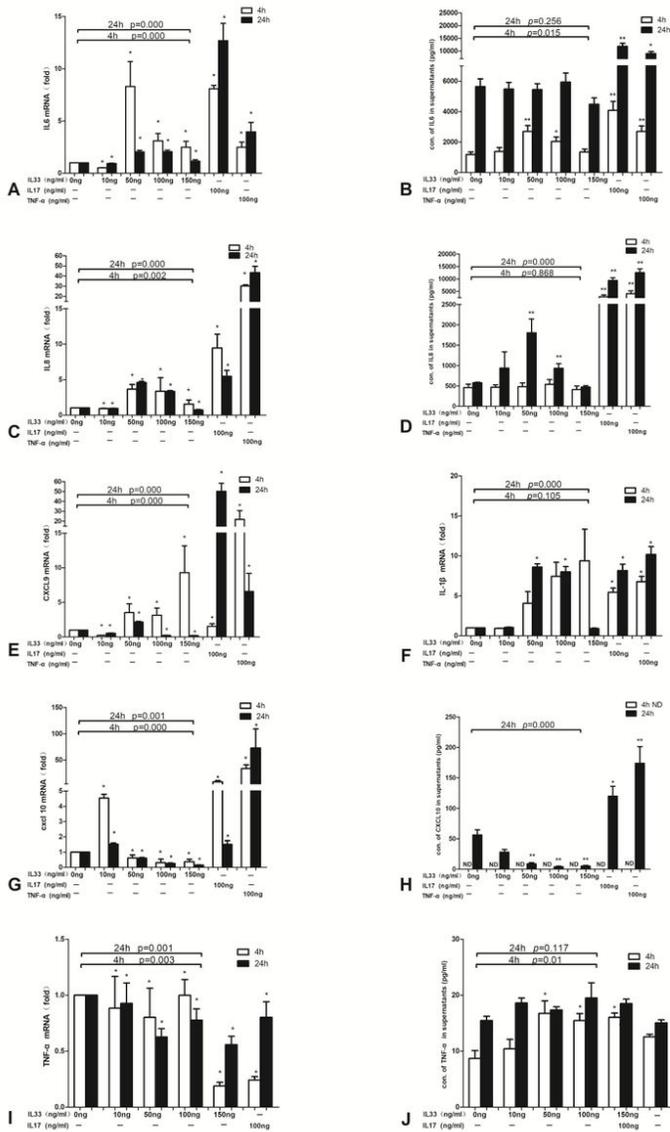


Figure 4

Effects of IL-33 on stimulated RA FLS. $n=5$ The expression levels of cytokines and chemokines of FLS, stimulated by IL-33 in different concentrations and time points, were analyzed using RT-PCR, the amount of cytokines and chemokines released into the cell culture supernatant was determined using ELISA. (ND: not determined) A: IL-6 mRNA expression increased: 50ng/ml of IL-33 is more powerful on RA-FLS. B: IL-6 concentration increased: 50ng/ml of IL-33 is more powerful on RA-FLS after 4h, no difference after 24h. C: IL-8 mRNA expression increased: 50ng/ml of IL-33 is more powerful on RA-FLS. D: IL-8 concentration increased: 50ng/ml of IL-33 is more powerful on RA-FLS after 24h, no difference after 4h. E: CXCL9 mRNA expression increased: 50ng/ml of IL-33 is more powerful on RA-FLS after 24h, in contrast, 150ng/ml of IL-33 is more powerful on RA-FLS after 4h. F: IL-1 β mRNA expression increased: 50ng/ml of IL-33 is more powerful on RA-FLS after 24h, in contrast, 150ng/ml of IL-33 is more powerful on RA-FLS after 4h. G: CXCL10 mRNA expression decreased: 150ng/ml of IL-33 is more powerful on RA-FLS. H: CXCL10 concentration decreased: 150ng/ml of IL-33 is more powerful on RA-FLS after 24h. I: TNF- α mRNA expression decreased: IL-33 is useful, but no difference between different concentrations of IL-33. J: TNF- α concentration increased in IL-33 stimulated groups in 4h not in 24h. *P<0.05, **P<0.01 as assessed by Kruskal-Wallis test and Mann-Whitney U test.