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SHORT COMMUNICATION

Sustained elevation of interleukin-33 in sera and synovial fluids from patients with rheumatoid arthritis non-responsive to anti-tumor necrosis factor: possible association with persistent IL-1β signaling and a poor clinical response

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Abstract Although TNF inhibitors have dramatically improved the outcome of patients with rheumatoid arthritis, 30–40% of patients do not respond well to them and treatment needs to be changed. In an effort to discriminate good and poor responders, we focused on the change in serum and synovial fluid levels of interleukin (IL-) 33 before and after treatment with TNF inhibitors. They were also measured in synovial fluids from 17 TNF inhibitor-naïve patients, and fibroblast-like synoviocytes (FLS) in-culture from 6 patients and correlated with various pro-inflammatory cytokines. Serum levels of IL-33 at 6 months after treatment decreased significantly in responders, while they did not change in non-responders. Synovial fluid levels of IL-33 in 6 patients under treatment with TNF inhibitors stayed high in 3 who were refractory and slightly elevated in 2 moderate responders, while they were undetectable in one patient under remission. Among inflammatory cytokines measured in 17 synovial fluids from TNF inhibitor-naïve patients, levels of IL-33 showed a significant positive correlation only to those of IL-1β. IL-1β increased IL-33 expression markedly in FLS in vitro, compared to TNF-α. IL-1β might be inducing RA inflammation through producing pro-inflammatory IL-33 in TNF inhibitor-hypo-responders. Sustained elevation of serum and/or synovial levels of IL-33 may account for a poor response to TNF inhibitors, although how TNF inhibitors affect the level of IL-33 remains to be elucidated.

Keywords Rheumatoid arthritis · Tumor necrosis factor · TNF inhibitor · Interleukin-33 · ST2 · Interleukin-1 family

Introduction

Tumor necrosis factor (TNF)-α plays a key role in inflammation of rheumatoid arthritis (RA) and has become a main therapeutic target. Infliximab and etanercept have shown good clinical efficacy and prevention of joint damage [1, 2]. However, 30–40% of patients do not respond well to these TNF inhibitors and novel therapeutic targets need to be found in non-responders.

Interleukin (IL-) 33 is a member of the IL-1 family and a ligand for ST2L [3]. When IL-33 binds to ST2L, it enhances inflammatory cytokines via the activation of NF-κB and MAP kinases. Although it was initially thought that IL-33 was crucial for Th2 cytokine-mediated host defense and allergic disorders, it is now known that the role of IL-33 is beyond Th2 immune responses [4] and the importance in RA has been vigorously pursued [5–8].

In murine arthritis models, the IL-33/ST2L axis enhances joint inflammation by inducing cytokines such as

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TNF-α, IL-1β, IL-6, IL-17, and interferon-γ [6, 7]. In human RA, IL-33 expression in fibroblast-like synoviocytes (FLS) increases after stimulation by TNF-α with or without IL-1β [6, 7]. We reported previously that IL-33 was elevated in sera and synovial fluids (SF) from RA patients and IL-33 was released from FLS after stimulation with TNF-α and IL-1β [8].

In the present study, serum and SF levels of IL-33 were measured and correlated with a clinical response to TNF inhibitors in RA patients. A possible mechanism of increased IL-33 levels was investigated using SF and FLS from RA patients.

Methods

Patients and response to TNF inhibitors

Forty-six RA patients who received TNF inhibitors (22 on infliximab and 24 on etanercept) at the Division of Rheumatology and Clinical Immunology, Jichi Medical University, from 2005 to 2010 were enrolled in serum analysis. Six RA patients treated with TNF inhibitors (3 on infliximab, 2 on etanercept, and 1 on intra-articular etanercept) and 21 TNF inhibitor-naïve patients were enrolled in SF analysis. Informed consent was obtained from the participants, and this study was approved by our institutional review board for human studies. The procedures followed were performed in accordance with the ethical standards laid down in an appropriate version of the 1964 Declaration of Helsinki.

Disease activity was assessed with the Disease Activity Score 28 based on the C-reactive protein level (DAS28-CRP) at baseline (0 M) and 6 months (6 M) after treatment [9]. Patients with good and moderate responses, and no response were grouped as responder and non-responder, respectively.

Measurement of IL-33, ST2, and other cytokines

To avoid the interference of rheumatoid factor (RF) on measurement of IL-33, ST2, and other cytokines by ELISA, all samples were pre-cleared by protein A-Sepharose beads as described previously [8]. Samples being made RF-negative, which were confirmed by Rheumatoid Arthritis Particle Agglutination (RAPA) test (SERODIA® RA; Fujirebio Inc., Tokyo, Japan), were included and analyzed.

Measurement of IL-33 by ELISA was performed as described previously [8]. Measurement of TNF-α, and IL-1β, IL-6, IL-8, and IL-17 (R&D, Minneapolis, MN), and ST2 (MBL, Nagoya, Japan) were performed by commercial ELISA kits. The minimum detection level for IL-33 by this ELISA was determined to be 3.4 pg/mL.

Assessment of IL-33 expression in fibroblast-like synoviocytes by real-time PCR

FLS from 6 RA patients were purchased from Cell Applications, Inc. (San Diego, CA) and used at passages 3–4; clinical data on these patients were not available. Recombinant human IL-1β (Peprotech, Rocky Hill, NJ), TNF-α (Peprotech), IL-2, IL-4, IL-6, IL-12, IL-17 (R&D), and IL-33 (MBL) were adjusted to 1 nM or 10 nM with phosphate-buffered saline and were added to FLS in-culture. Total RNA was isolated from the cells with TRI reagent (Sigma–Aldrich, St Louis, MO). Reverse transcription reactions were performed with a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Real-time PCR was conducted by the TaqMan Gene Expression Assay kit (Applied Biosystems) and primer probe sets for human IL-33 (Hs00369211_m1) and human β-actin (4333762F).

Statistics

Continuous variables were analyzed by the Mann–Whitney U test or the Wilcoxon signed-rank test. Categorical data were compared with the chi-squared test or Fisher's exact probability test. Correlations between IL-33 and other cytokines or ST2 in SF were determined by Spearman's rank correlation coefficient (R^s) analysis. $P < 0.05$ was considered significant.

Results

Clinical profiles and IL-33 at baseline and after treatment with TNF inhibitors

Sera from 12 patients and SF from 4 patients with residual RAPA titer of 1:40 or more after preclearance were excluded from ELISA analysis. Finally, sera from 34 patients (18 on infliximab and 16 on etanercept), SF from 6 patients receiving TNF inhibitors, and SF from 17 TNF-inhibitor-naïve patients were analyzed.

Changes in the median DAS28-CRP (range) at 0 M and 6 M were 5.2 (3.8–7.5) and 2.1 (1.3–4.4) among responders, and 5.3 (2.7–7.1) and 5.0 (3.0–6.8) among non-responders, respectively (Table 1). Serum levels of IL-33 among responders and non-responders were not different at 0 M. They decreased significantly in responders at 6 M, while they remained high in non-responders (Table 1). Among 6 patients treated with TNF inhibitors in whom IL-33 levels were determined in SF, they stayed markedly elevated in 3 patients at 1,997, 451, and 167 pg/ml after treatment. Articular symptoms of these patients were poorly controlled and the DAS28-CRP were 4.4 and 3.9 after 15- and 32-month

Table 1 Clinical profiles and interleukin 33 at baseline (0 M) and 6 months after treatment with tumor necrosis factor inhibitors (6 M)

	Responders (n = 16)	Non-responders (n = 18)
Type of TNF-inhibitors		
Infliximab	7	11
Etanercept	9	7
Age, years, median (range)	55 (18–70)	55.5 (26–71)
Female/male	13:3	16:2
RF^+, n (%)	9 (56.2)	12 (66.7)
CCP^+, n (%)	16 (100.0)	16 (88.9)
Disease duration, years, median (range)	3.3 (0.5–33.0)	5.9 (0.8–18.0)
Prevalence of allergy, n (%)	6 (37.5)	7 (38.9)
Other treatments at baseline		
Prednisolone, n (mean mg/day)	13 (5.5)	12 (5.4)
Methotrexate, n (mean mg/week)	14 (7.0)	15 (7.1)
DMARDs, abbreviations (n)	SASP (2), ACT (1), BUC (1), GST (1), INF (1), MZB (1)	BUC (2), ETN (1), LEF (1), TAC (1)
Serum interleukin 33 levels, pg/ml, median (range)		
0 M	83.8 (0.0–1473.0)	74.3 (0.0–3824.0)
6 M	0.0# (0.0–529.0)	54.5 (0.0–2212.0)
DAS28-CRP, points, median (range)		
0 M	5.2 (3.8–7.5)	5.3 (2.7–7.1)
6 M	2.1# (1.3–4.4)	5.0 (3.0–6.8)
Serum CRP levels, mg/dl, median (range)		
0 M	2.3 (0.2–6.1)	2.6 (0.1–8.0)
6 M	0.1# (0.0–2.4)	3.0 (0.1–8.5)

RF IgM-rheumatoid factor, *CCP* anti-cyclic citrullinated peptide antibodies, *DMARDs* disease modifying anti-rheumatic drugs, *SASP* sulphasalazine, *ACT* actarit, *BUC* bucillamine, *GST* gold sodium thiomalate, *INF* infliximab, *MZB* mizoribine, *ETN* etanercept, *LEF* leflunomide, *TAC* tacrolimus, *DAS28-CRP* the Disease Activity Score 28 based on the C-reactive protein level

# $P < 0.05$ compared to 0 M

treatment with etanercept, and 4.2 after 22-month treatment with infliximab, respectively. Two patients with a moderate response to infliximab, levels of IL-33 after treatment were measured intermediate at 54 and 45 pg/ml with DAS28-CRP of 2.9 and 3.1, respectively. The last patient on intra-articular etanercept with a DAS28-CRP of 2.2 after treatment showed an undetectable level of IL-33. SF levels of soluble ST2 were undetectable in all 6 samples.

IL-33 and other cytokines in SF from TNF inhibitor-naïve patients

In 17 TNF inhibitor-naïve patients, SF levels of IL-33 showed a significant correlation only to those of IL-1β ($R^s = 0.551$, $P = 0.035$); they did not show any significant correlations to those of TNF-α or other cytokines (Fig. 1).

IL-33 expression in FLS after cytokine stimulation

IL-33 expression was induced in FLS by TNF-α or IL-1β, and not by IL-2, IL-4, IL-6, IL-12, IL-17, or IL-33 (Fig. 2a). IL-33 expression was enhanced synergistically by co-stimulation with IL-1β and TNF-α (Fig. 2b). IL-1β showed a higher enhancement in IL-33 expression than TNF-α at a molar basis, and except for FLS from a 72-year-old woman, the level of IL-33 induction by 1 nM of IL-1β was higher than that by 10 nM of TNF-α. In a case of FLS from a 69-year-old woman, IL-33 expression was increased strongly only by IL-1β; TNF-α alone did not show any effects.

Discussion

Serum levels of IL-33 decreased significantly in responders to TNF inhibitors after treatment, while non-responders did not show such changes. IL-33 levels in SF taken before treatment with TNF inhibitors were correlated only with IL-1β. IL-1β at 1 nM increased IL-33 expression in FLS from RA patients much more than TNF-α at 1 nM or 10 nM, or other cytokines, in vitro, although synergy of IL-1β and TNF-α was observed to some extent. It is also of note that there is an FLS in which IL-33 expression was markedly increased by IL-1β alone (69-year-old woman). Although we have not proven directly in vivo, it seems highly probable that an increased IL-33 expression in inflamed joints could be induced, at least in part, by the over-expression of IL-1β.

Synovial levels of IL-33 after treatment with TNF inhibitors were correlated positively with the levels of DAS-28. Thus, 3 patients without favorable response to TNF inhibitors

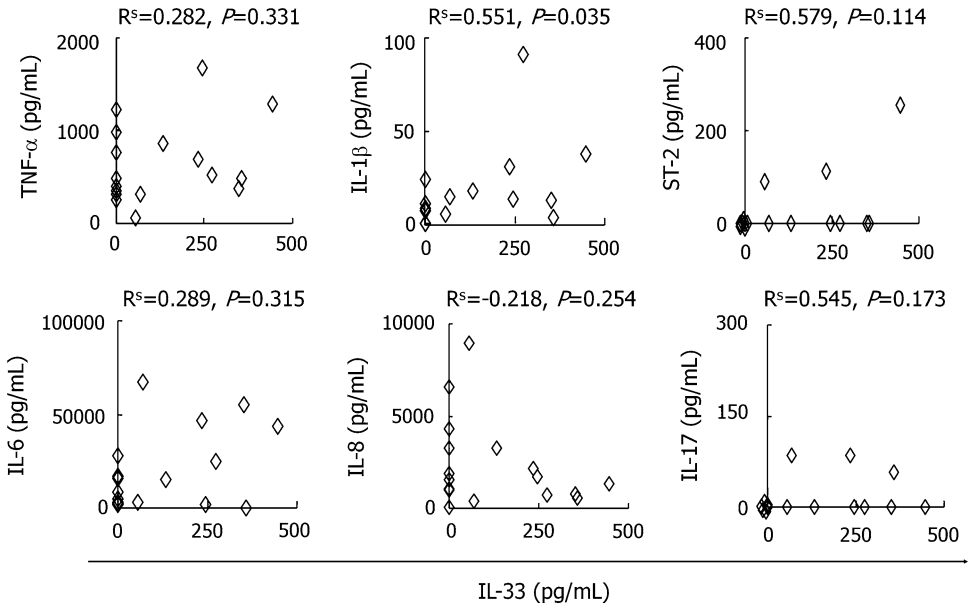

Fig. 1 Correlation between the levels of interleukin-33 and ST2 (a soluble decoy receptor) or other cytokines in synovial fluids from TNF inhibitor-naïve patients with rheumatoid arthritis. *Open diamonds* represent individual samples. R^s represents Spearman's rank correlation coefficient. $P < 0.05$ was considered significant

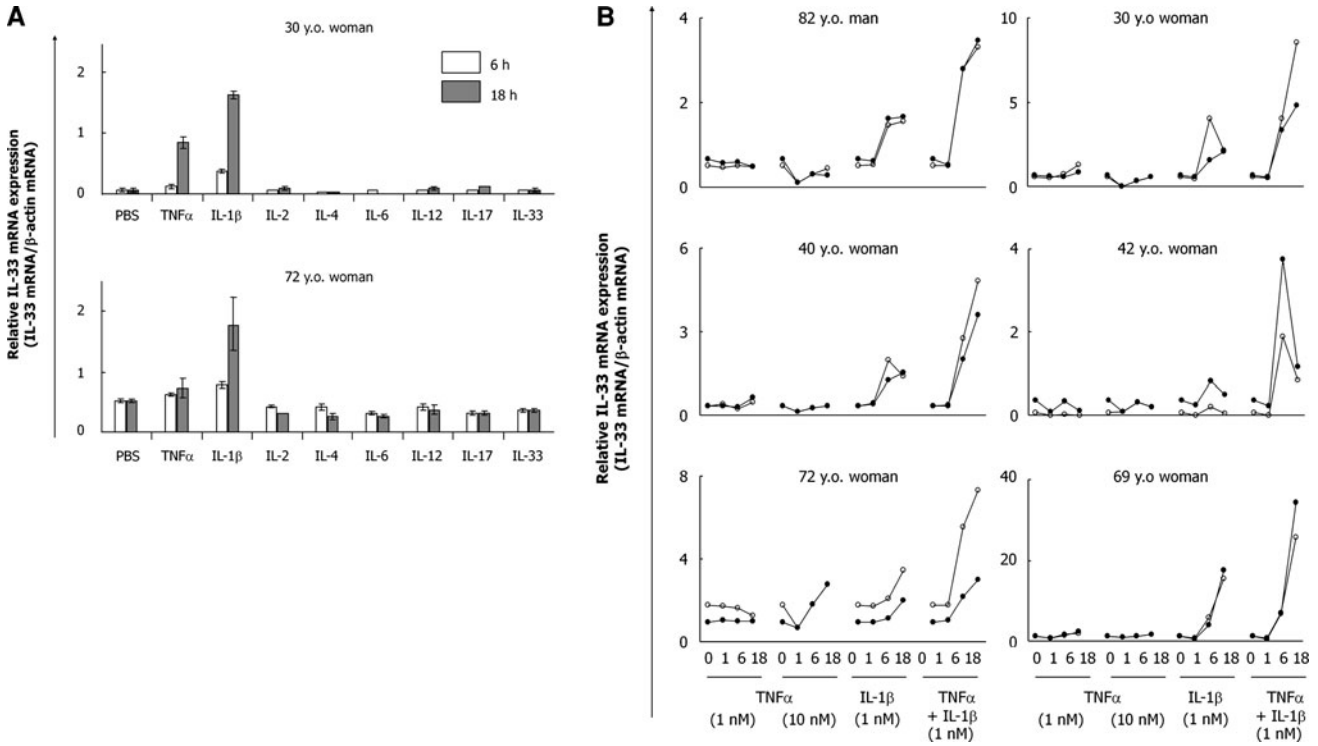

Fig. 2 Interleukin (IL-) 33 expression in fibroblast-like synoviocytes (FLS) from patients with rheumatoid arthritis. **a** FLS from 2 patients were stimulated for 6 h (*white bar*) or 18 h (*gray bar*) with 1 nM tumor necrosis factor (TNF)-α, IL-1*β*, IL-2, IL-4, IL-6, IL-12, IL-17, IL-33, or PBS. IL-33 mRNA levels were analyzed by real-time PCR. IL-33 expression was normalized based upon the level of *β*-actin. Values are the mean and SD from triplicate determinations. **b** FLS from 6 patients were stimulated for 1, 6, or 18 h with 1 nM TNF-α or 1 nM IL-1*β*, or combination thereof. *Open* and *closed circles* represent the mean from triplicate determinations in two cultures performed separately

had high levels of synovial IL-33, while the amount of synovial IL-33 from a patient with a good response was less than the lower detection limit of the ELISA. Two patients in-between had intermediate levels. Regret to say that synovial fluids from these 6 patients before treatment with TNF inhibitors were not available; whether synovial levels of IL-33 change after treatment needs to await future study.

The level of soluble ST2, which is a splice variant of ST2L and blocks IL-33 [10], was elevated in only 3 of 23 SF samples. This is discordant with a previous report by

Fraser et al. [11]; RF interference could be one of the reasons which influenced ST2 measurement by ELISA. The concentration of endogenous ST2 in SF was low compared with that of IL-33. IL-33 might, therefore, be uncoupled from ST2 and could bind to ST2L to provoke inflammation in RA joints. In murine arthritis models, administration of anti-ST2L antibody [7] or soluble ST2-Fc fusion protein [12] improves joint inflammation. Thus, blocking IL-33/ST2L axis by monoclonal antibody or soluble ST2 protein could be a novel therapy in human RA, and the clinical trials are aspired to.

The combination therapy with anti-IL-1β and anti-TNF agents might block IL-33 expression more strongly than each alone. Genovese et al. reported that a combination with etanercept and anakinra provides no added benefit and increases the incidence of infectious diseases compared with etanercept alone in patients' refractory to methotrexate [13]. Levels of IL-33 were not measured in this report, however, the combination of anti-IL-1β and TNF inhibitors might have broader effects than blocking IL-33 only. The main origin of IL-33 seems to be FLS in patients with RA, and targeting IL-33 directly could avoid such deleterious effects.

Whether sustained levels of IL-33 in sera and/or synovial fluids are merely a reflection of continued RA-inflammation or high levels of IL-33 per se are important in continuum of RA-inflammation needs to await clinical studies using anti-IL-33 antibodies. Judging from the experiments in mice, the therapeutic potential of anti-IL-33 antibodies might be promising.

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