Elevated Serum Interleukin 33 Is Associated with Autoantibody Production in Patients with Rheumatoid Arthritis

RONG MU, HE-QING HUANG, YU-HUI LI, CHUN LI, HUA YE, and ZHAN-GUO LI

ABSTRACT. Objective. Interleukin 33 (IL-33) is a novel cytokine involved in joint inflammation in animal models. We analyzed the expression of IL-33 in the serum and synovial fluid of patients with rheumatoid arthritis (RA) and investigated its possible pathophysiological importance.

Methods. The concentration of IL-33 was measured by ELISA in the serum of 223 patients with RA and 159 controls. Anticyclic citrullinated peptide, rheumatoid factor (RF)-IgA, and RF-IgG were tested by ELISA. Antikeratin antibody and antiperinuclear factor were tested by indirect immunofluorescence assay. Erythrocyte sedimentation rate, C-reactive protein, and immunoglobulins were measured by standard laboratory techniques. The association of IL-33 level with clinical and serologic features of RA was analyzed. We tested the change of IL-33 level following tumor necrosis factor (TNF-α) blockade therapy in 40 patients with RA.

Results. In contrast to almost no detectable IL-33 in osteoarthritis and healthy serum, IL-33 could be detected in 94 out of the 223 RA cases (42.2%). Serum IL-33 concentration was significantly higher in patients with RA than in control groups. The level of serum IL-33 decreased after anti-TNF treatment. The level of serum IL-33 was correlated with the production of IgM and RA-related autoantibodies including RF and anticitrullinated protein antibodies. However, no correlation was found between IL-33 concentration and acute-phase inflammation reactant or the score of the Disease Activity Index, suggesting a complex or indirect character of the link between IL-33 and the inflammation in RA.

Conclusion. The level of IL-33 is abnormally elevated in RA serum. The elevation of serum IL-33 was at least partly attributed to excessive TNF-α in RA. IL-33 might be involved in the regulation of autoantibody production in RA. (First Release August 1 2010; J Rheumatol 2010;37:2006–13; doi:10.3899/jrheum.100184)

Key Indexing Terms:
SERUM INTERLEUKIN 33 RHEUMATOID ARTHRITIS AUTOANTIBODIES

Rheumatoid arthritis (RA) is a common autoimmune disease that frequently leads to severe joint damage, early disability, and premature death. Although the precise etiology is unknown, cytokines appear to play an important role in the development and course of the disease. RA is characterized by impaired function of T and B cells and abnormal production of cytokines and autoantibodies. Cytokines could have various important activities in the context of the pathogenesis of RA, for example, inducing antibody production by B cells. Biological agents directed to cytokines such as tumor necrosis factor (TNF-α) and interleukin 6 (IL-6) have demonstrated dramatic efficacy in the treatment of RA.

IL-33 is a newly described cytokine of the IL-1 family, which also includes IL-1α, IL-1β, and IL-18. It was identified as a ligand for the orphan receptor ST2. IL-33 locates mainly in the nucleus and may regulate the expression of some genes, but its biological function and mechanism are elusive. Most members of the IL-1 family are proinflammatory cytokines in RA. A previous study demonstrated that IL-33 could exacerbate collagen-induced arthritis (CIA) and elevate production of proinflammatory cytokines and anticollagen antibodies. In addition, an ST2 antibody that blocks IL-33 signaling could attenuate the severity of CIA. These studies suggest that IL-33 contributes to the pathogenesis of joint inflammation and destruction.

We speculate that IL-33 is associated with disease profiles and that serum IL-33 could be used as a marker of inflammation in RA. However, whether IL-33 is released...
into circulation in RA remains to be determined. Studies are needed on the relationship between serum IL-33 and the clinical profiles of RA. In our study, we compared the level of IL-33 in sera obtained from patients with RA to sera in patients with primary Sjögren’s syndrome (pSS), patients with osteoarthritis (OA), and healthy controls, and analyzed the association of the concentration with disease activity and serologic features in patients with RA.

MATERIALS AND METHODS

Patients and controls. Serum samples were obtained from 223 consecutive patients with RA (161 women, 62 men) admitted to the ward of the Department of Rheumatology and Immunology, Peking University People’s Hospital, from January 2005 to February 2008. All patients fulfilled the revised American College of Rheumatology criteria for RA20. Synovial fluids (SF) were obtained from 17 of the 223 patients at the same time.

Serum samples from 71 healthy blood donors, 48 patients with pSS, and 40 patients with OA were used as controls. Serum samples were stored at −80°C until used. No patient was pregnant or menopausal at the time of the study. The study protocol was approved by the ethics committee of Peking University People’s Hospital (FWA00001384).

TNF-α blockade therapy. We also collected serum from another 40 patients with RA who received infliximab therapy, 3 mg/kg, infused 4 times, at Weeks 0, 2, 8, and 16. Serum samples were collected before and after 4 dosings (Weeks 0 and 16) from January 2008 to June 2009. These patients also received traditional disease-modifying antirheumatic drugs (DMARD), including methotrexate, leflunomide, and hydroxychloroquine. The dosage and frequency of the DMARD remained constant throughout the TNF blockade therapy.

Assay for serum and synovial fluid IL-33. Serum and synovial fluid IL-33 concentration was determined with a quantitative sandwich ELISA kit (R&D Systems, Minneapolis, MN, USA). Detection limit was 23 pg/ml. Polystyrene microplates were coated overnight with goat anti-human IL-33 antibody (0.8 g/ml) and then blocked with 1% bovine serum albumin in phosphate buffered saline (PBS) for 2 h at room temperature. Serum samples diluted 1:4 in PBS were incubated 2 h at room temperature. Serum IL-33 was determined with biotinylated goat anti-human IL-33. The reaction was developed with streptavidin-horseradish peroxidase, and optical density was read at 450 nm.

Clinical data and inflammation marker analysis. All patients were followed up for complete clinical data: age, sex, disease duration, number of swollen joints, number of tender joints, global visual analog scale score, immunoglobulins (IgG, IgM, IgA), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), rheumatoid factor (RF), antikeratin antibodies (AKA), antiperinuclear factor (APF), anticitrullinated peptide antibodies, and anticyclic citrullinated peptide antibody (anti-CCP) class II HLA. ESR was evaluated by the Westergren method. Values ≤ 15 mm/h for men and ≤ 20 mm/h for women were considered normal. CRP and immunoglobulins were examined by immunonephelometry method. Values > 7.9 mg/l for CRP were considered positive. AKA and APF were tested by indirect immunofluorescence assay. Anti-citrullinated peptide antibody (anti-CCP) antibodies, RF, AKA, and anti-CCP were tested by ELISA, with normal ranges of 0–120 U/ml and 0–110 U/ml.

RESULTS

Patients. The mean age of the 223 patients with RA at the time of our study was 55.9 ± 13.9 years (range 15–93) and the mean disease duration was 61.8 ± 91.3 months (range 1–480). Major clinical and laboratory features are presented in Table 1. RF-IgM and anti-CCP antibodies were positive in 158 (70.9%) patients. RF-IgA, RF-IgG, AKA, and APF were detected in 12.6%, 33.6%, 35.4%, and 29.6% of the patients with RA, respectively. The presence of RF in RA serum was similar to that of patients with pSS (70.9% vs 68.8%).

IL-33 level was elevated in sera and SF of patients with RA. IL-33 was detectable in 94 of the 223 patients with RA (42.2%; Figure 1A), range 0–10068.16 pg/ml (median 0). IL-33 serum concentration was significantly higher in patients with RA than in age-matched healthy controls (392.0 ± 889.4 pg/ml, vs 7.0 ± 37.6 pg/ml; p < 0.001), patients with pSS (392.0 ± 889.4 pg/ml, vs 34.06 ± 45.51 pg/ml; p < 0.006), and patients with OA (392.0 ± 889.4 pg/ml vs 7.9 ± 34.9 pg/ml; p < 0.001) by the Mann-Whitney U test.

IL-33 was also measured in 17 matched samples of both RA SF and sera. IL-33 in SF was detectable in 47.1% (8/17) of patients with RA. The level of IL-33 in SF was similar to the level in sera in these 17 patients (209.1 ± 495.7 pg/ml vs 199.7 ± 452.4 pg/ml; p = 0.889; Figure 1B).

The association between the clinical features and IL-33 levels is presented in Table 2. The serum IL-33 level was higher in patients with longer disease duration (r = 0.145, p = 0.03), and higher total IgM (r = 0.16, p = 0.017), RF-IgG, and CRP concentration.

Table 1. Clinical and laboratory features in 223 patients with rheumatoid arthritis. Clinical characteristics are presented as mean ± SD.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Values (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yrs</td>
<td>55.9 ± 13.9</td>
</tr>
<tr>
<td>Men/women</td>
<td>62/161</td>
</tr>
<tr>
<td>Disease duration, mo</td>
<td>61.8 ± 91.3</td>
</tr>
<tr>
<td>Tender joint count, 0–46 joints</td>
<td>7.8 ± 8.4</td>
</tr>
<tr>
<td>Swollen joint count, 0–48 joints</td>
<td>6.1 ± 7.2</td>
</tr>
<tr>
<td>DAS28</td>
<td>5.1 ± 1.4</td>
</tr>
<tr>
<td>ESR, mm/h</td>
<td>60.1 ± 34.0</td>
</tr>
<tr>
<td>CRP, mg/l</td>
<td>38.1 ± 52.8</td>
</tr>
<tr>
<td>RF-IgM-positive</td>
<td>158 (70.9)</td>
</tr>
<tr>
<td>RF-IgA-positive</td>
<td>28 (12.6)</td>
</tr>
<tr>
<td>RF-IgG-positive</td>
<td>75 (33.6)</td>
</tr>
<tr>
<td>AKA-positive</td>
<td>79 (35.4)</td>
</tr>
<tr>
<td>APF-positive</td>
<td>66 (29.6)</td>
</tr>
<tr>
<td>Anti-CCP-positive</td>
<td>158 (70.9)</td>
</tr>
</tbody>
</table>

The Mann-Whitney U test was used to analyze the serum IL-33 differences between each group. Student’s t test was applied to compare quantitative data between IL-33 undetectable and detectable groups. Qualitative data were compared by the chi-squared test. Potential correlation between IL-33 and clinical characteristics was examined using Spearman’s rank correlation test (for quantitative data) or the Wilcoxon signed-rank test (for qualitative data). P values < 0.05 were considered significant.

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(r = 0.347, p < 0.001), and anti-CCP antibody concentrations
(r = 0.253, p < 0.001; Table 2 and Figure 2). The duration of
disease in IL-33-positive patients ranged from 1 to 396
months (median 17 months). Nearly half (45/94) of the
patients’ disease duration was ≤ 12 months, suggesting that
IL-33 could become elevated at early stages of RA.

Serum IL-33 and disease activity in RA. A sample with
undetectable IL-33 (detection limit was 23 pg/ml) was arbi-
trarily defined as 0 pg/ml. The 223 patients were then cate-
gorized into IL-33-positive and IL-33-negative groups. The
characteristics of these 2 groups are shown in Table 3. The
2 groups did not differ significantly in the following fea-
tures: age (56.3 ± 14.3 vs 55.3 ± 13.2 yrs; p = 0.58), disease
duration (59.2 ± 96.6 vs 65.4 ± 84.0 mo; p = 0.62), and
female sex (69.8% vs 75.5%; p = 0.34).

The 2 groups had similar tender joint count (7.4 ± 7.8 vs
8.3 ± 9.1; p = 0.39), swollen joint count (5.7 ± 6.9 vs 6.6 ±
7.6; p = 0.36), and DAS28 score (5.0 ± 1.3 vs 5.1 ± 1.6; p =
0.59). Markers for inflammation (e.g., ESR and CRP) were
also comparable in the IL-33-positive and the IL-33-nega-
tive groups. Similarly, SF IL-33 levels were not correlated
with disease duration, inflammation markers, DAS28 score,
or radiographic stage (data not shown).

Serum IL-33 and autoantibody production in RA. The
IL-33-positive group had higher levels of RF than the
IL-33-negative group (521.6 ± 764.3 vs 223.8 ± 514.0
pg/ml; p = 0.001). Anti-CCP was also significantly higher in
the IL-33-positive group compared with the IL-33-negative
group (336.5 ± 358.4 vs 159.0 ± 279.0 pg/ml; p < 0.001).
Similarly, the frequency of several other autoantibodies,
including RF-IgG (52.1% vs 20.2%; p < 0.001), AKA
(46.8% vs 27.1%; p = 0.002), and APF (42.6% vs 20.2%;
p < 0.001) was also higher in the IL-33-positive group com-

Table 2. Correlation analysis for serum IL-33 and clinical data.

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Spearman Rank Correlation Coefficient (r)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>−0.5</td>
<td>0.461</td>
</tr>
<tr>
<td>Disease duration</td>
<td>0.145</td>
<td>0.03*</td>
</tr>
<tr>
<td>Tender joint count, 0–46 joints</td>
<td>0.013</td>
<td>0.846</td>
</tr>
<tr>
<td>Swollen joint count, 0–48 joints</td>
<td>0.053</td>
<td>0.431</td>
</tr>
<tr>
<td>ESR</td>
<td>−0.008</td>
<td>0.906</td>
</tr>
<tr>
<td>CRP</td>
<td>−0.033</td>
<td>0.619</td>
</tr>
<tr>
<td>DAS28</td>
<td>0.027</td>
<td>0.693</td>
</tr>
<tr>
<td>IgA</td>
<td>0.078</td>
<td>0.249</td>
</tr>
<tr>
<td>IgG</td>
<td>−0.08</td>
<td>0.235</td>
</tr>
<tr>
<td>IgM</td>
<td>0.16</td>
<td>0.017*</td>
</tr>
<tr>
<td>Rheumatoid factor</td>
<td>0.347</td>
<td>&lt; 0.001**</td>
</tr>
<tr>
<td>Anti-CCP</td>
<td>0.253</td>
<td>&lt; 0.001**</td>
</tr>
</tbody>
</table>

* p < 0.01. ** p < 0.05. ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; DAS: 28-joint count Disease Activity Score; anti-CCP: anti-citrullinated peptide antibodies.
Figure 2. Correlation between serum IL-33 and concentrations of IgM, rheumatoid factor (RF), and anticitrullinated peptide antibodies (anti-CCP). Spearman’s rank correlation test was used to assess correlations. Association of IL-33 to antikeratin antibodies (AKA) and antiperinuclear factor (APF) was analyzed using Wilcoxon signed-rank test.
pared with the IL-33-negative group. A slightly higher level of RF-IgA in the former group did not reach statistical significance (p = 0.086). Together, RF (IgM and IgG), AKA, APF, and anti-CCP levels were significantly higher in the IL-33-positive group than in the IL-33-negative group, as shown in Table 3.

A correlation analysis showed that in patients with RA, IL-33 concentration was positively correlated with the anti-CCP level (Figure 2), RF-IgM, and IgM. IL-33 concentration in AKA-positive patients was significantly higher than that in AKA-negative patients. A similar trend was identified in APF-positive patients (Figure 2).

Serum IL-33 level and previous treatment. DMARD were used in 69.1% (65/94) of the IL-33-positive group and 38.0% (80/129) of the IL-33-negative group of patients. The IL-33-positive group had a higher frequency of DMARD usage than the IL-33-negative group (p = 0.002, Table 4).

Table 4. Previous therapies in IL-33-positive and IL-33-negative patients.

<table>
<thead>
<tr>
<th>Therapy</th>
<th>IL-33– Group, n = 129 (%)</th>
<th>IL-33+ Group, n = 94 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMARD</td>
<td>49 (38.0)</td>
<td>65 (69.1)</td>
</tr>
<tr>
<td>MTX</td>
<td>17 (13.2)</td>
<td>45 (47.9)</td>
</tr>
<tr>
<td>LEF</td>
<td>15 (11.6)</td>
<td>25 (26.6)</td>
</tr>
<tr>
<td>SSZ</td>
<td>7 (5.4)</td>
<td>16 (17.0)</td>
</tr>
<tr>
<td>HCQ</td>
<td>21 (16.3)</td>
<td>20 (21.3)</td>
</tr>
<tr>
<td>Combination therapy</td>
<td>11 (8.5)</td>
<td>31 (33.0)</td>
</tr>
</tbody>
</table>

DMARD: disease-modifying antirheumatic drugs; MTX: methotrexate; LEF: leflunomide; SSZ: sulfasalazine; HCQ: hydroxychloroquine.

This suggests that the IL-33 serum level was affected by previous DMARD treatment, or that patients in the IL-33-positive group had more severe disease.

Decreased serum IL-33 with anti-TNF treatment. During the course of infliximab treatment, another 40 patients experienced partial remission. Prior to the treatment, serum IL-33 levels were below the detection limit in 22 out of the 40 patients. In the remaining 18 patients with detectable serum IL-33 prior to the treatment, IL-33 concentration decreased in 15 patients (83.3%) after the treatment (Figure 3). Overall, serum IL-33 concentration tended to be lower after anti-TNF treatment (587.7 ± 300.8 pg/ml vs 331.5 ± 151.5 pg/ml; p = 0.002).

DISCUSSION

Our study demonstrated that roughly 40% of patients with RA had detectable levels of IL-33 in serum, supporting the idea that IL-33 is implicated in the pathogenesis of RA. To our surprise, serum IL-33 concentration was not correlated to inflammation markers or disease activity, but to the level of RA-associated autoantibodies, suggesting that IL-33 may be involved in autoantibody production in RA.

IL-33 has been implicated in joint inflammation and destruction in animal models. This cytokine was initially described as “nuclear factor from high endothelial venules” due to the presence in the nucleus of high endothelial cells. It remains an interesting issue whether IL-33 is released in vivo, especially under disease conditions such as RA. Findings from our study demonstrate that IL-33 can be released into circulation in patients with RA even at an early stage.
IL-33 and RA

stage of the disease, although the molecular size of serum IL-33 has not yet been determined. Matsuyama, et al also showed that circulating levels of IL-33 were elevated in 30 of 59 patients with RA and revealed a 30-kDa protein in RA serum by immunoprecipitation, although it could be detected in only 1 patient.

The molecular mechanism for IL-33 release is not fully understood as this IL-1 family member lacks an apparent secretory signal peptide. Because of its homology to IL-1β and IL-18, IL-33 was believed to be synthesized as an inactive 31-kDa precursor in the cell and liberated by caspase-1 in response to inflammatory stimuli. Lüthi, et al recently showed that human and mouse full-length IL-33 possesses potent biological activities regulating transcription through its DNA-binding motif in vitro. In response to a variety of DNA-damaging agents and when treated with anti-Fas, TNF-related apoptosis-inducing ligand, or TNF-α plus cycloheximide, IL-33 was processed during apoptosis that involved caspase 3 and 7. Importantly, this process led to inactivation of IL-33 rather than its activation. However, processed IL-33 is largely retained inside apoptotic bodies. Extracellular IL-33 is believed to be released from necrotic cells without caspase processing, rather than from apoptotic cells, which inactivate IL-33. Consistent with this notion, it seems that a substantial portion of the circulating serum IL-33 is full-length pro-IL-33, as shown by immunoprecipitation in RA serum.

Synovial fibroblasts are believed to be one of the main sources of IL-33 in RA, producing huge amounts of IL-33 in the presence of TNF-α stimulation in vitro. According to this concept, we evaluated the role of anti-TNF treatment on serum IL-33 concentrations before and after infliximab treatment. Results from our study showed a trend of decreasing serum IL-33 concentration after anti-TNF treatment, suggesting that TNF-α is at least partly responsible for the overproduction of IL-33 in patients with RA.

Evidence suggests that IL-33 may function as a cytokine through receptors (e.g., ST2) on effector cells, and stimulate the secretion of proinflammatory and Th2 cell-associated cytokines via the nuclear factor-κB-mediated pathways from mast cells and other immune cells. Although caspase-mediated processing inactivates IL-33 and prevents accidentally released IL-33 from stimulating ST2, cell death by necrosis could release significant amounts of IL-33 because unprogrammed cell death caused by ischemia or activated complement is a prominent feature of RA. In vivo data showed that administration of IL-33 exacerbates experimental arthritis. Therefore, it seems reasonable to assume that serum IL-33 is related to disease activity. However, in our study no correlation was found between serum IL-33 concentration and the disease activity or markers for inflammation (e.g., the acute-phase inflammation reactant in RA).

Matsuyama, et al reported that serum IL-33 was related to RA disease activity. As we did not find the association between IL-33 level and DAS28 score in our study, this discrepancy may be explained by several factors. First, patients recruited for our study showed higher disease activity than the patients described by Matsuyama, et al. Second, the demographic characteristics of the patients may differ in these 2 studies. Third, previous DMARD treatment may be different and may affect serum IL-33 levels. Fourth, more subjects were recruited in our study. However, the CRP level was not significantly different between the IL-33 detectable and undetectable groups in Matasuyama’s report, suggesting that the serum IL-33 level may not be related to the intensity of the inflammation.

That serum IL-33 concentration is not related to the disease activity suggests the complex or indirect character of the link between IL-33 and RA. It is still early to conclude that IL-33 is not associated with RA severity. More detailed investigations are required to further understand the precise role of IL-33 in RA pathogenesis in different patients with RA who have different disease categories, durations, and treatments. However, from a practical point of view, our data imply that neutralization of circulating IL-33, for exam-
ple using soluble ST2, may not control the inflammation and disease activity in RA directly.

Interestingly, we found serum IL-33 concentration correlated with the concentrations of RF and anticitrullinated protein/peptide antibodies (including AKA, APF, and anti-CCP), 2 recognized risk factors for poor prognosis in RA.\textsuperscript{41,42,43,44,45} Whether IL-33 could be used as a prognosis marker is of great interest. Perhaps more importantly, our findings raised the possibility that IL-33 may contribute to abnormal B cell autoimmunity, although a direct pathogenic role of IL-33 in the generation of autoantibodies could not be substantiated.

Although different cell types may play pathogenic roles in RA, a prominent participation of the B cell has been recently highlighted. B cells have emerged as a new target in RA. IL-33 drives production of Th2-associated cytokines including IL-5 and IL-13, which can promote B cell function such as autoantibody production.\textsuperscript{15} Thus IL-33 may be indirectly involved in B cell-mediated pathology in RA.

IL-33 may also contribute to the antibody overproduction by inducing mast cell activation. IL-33 is one of the strongest stimuli for mast cells that reside in the synovial tissue.\textsuperscript{30} Both human and animal studies have shown that extracellular IL-33 could stimulate the maturation and activation of mast cells.\textsuperscript{30,32,35} Mast cells have been confirmed to be a cellular link between autoantibodies and inflammatory arthritis.\textsuperscript{46,47} This might be the mechanism underlying our data that IL-33 level is associated with RA-related autoantibodies. In support of this hypothesis, IL-33 contributes to the antibody response and the severity of inflammation in a serum-induced arthritis mouse model that is mast cell-dependent.\textsuperscript{48} Our data presented new evidence on the involvement of the new cytokine IL-33 in abnormal humoral immunity. This may ultimately help in understanding the complex issue of autoimmunity in RA.

The limitation of this study is that the commercial IL-33 ELISA reagents have been generated and tested using an Escherichia coli-produced portion of hIL-33 (amino acids 112-270). IL-33 released from necrotic cells is full-length pro-IL-33. Thus, an independent ELISA assay using full-length pro-IL-33 to verify our results would be of great help.

We found that serum IL-33 is significantly and selectively increased in RA. It is not a sensitive marker of disease activity, but rather a part of the complicated autoimmune abnormality that is characteristic of RA.

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