EXTENDED REPORT

Ex vivo interleukin 1 receptor antagonist production on lipopolysaccharide stimulation is associated with rheumatoid arthritis and with joint damage

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Objectives: (1) To assess innate ex vivo production of interleukin 1β (IL1β) and interleukin 1 receptor antagonist (IL1Ra) in patients with recent-onset rheumatoid arthritis (RA) as compared with healthy controls; (2) to assess the association of ex vivo IL1β and IL1Ra production with progression of joint damage in RA; (3) to determine whether differences in ex vivo IL1β production are explained by distribution of the IL1β single nucleotide polymorphism C-511T.

Methods: Levels of IL1β and IL1Ra (measured by ELISA after whole-blood stimulation with lipopolysaccharide) and distribution of IL1β C-511T were compared in 76 patients with recent-onset RA who had received no disease-modifying antirheumatic drugs (DMARDs), and 63 healthy controls. ORs for RA based on ex vivo IL1β and IL1Ra production were calculated. Association of ex vivo IL1β and IL1Ra production with progression of joint damage (Sharp–van der Heijde score over 2 years) was determined by linear regression with correction for baseline characteristics.

Results: Patients with recent-onset RA showed lower ex vivo IL1β and higher ex vivo IL1Ra production than healthy controls ($p<0.001$), with ORs for RA of 2.4 (95% CI 1.2 to 4.9) for low IL1β-producers and 7.6 (95% CI 3.2 to 18.0) for high IL1Ra-producers. High ex vivo IL1Ra production was associated with progression of joint damage ($p=0.01$). The IL1β C-511T genotype distribution was not significantly different between patients and controls.

Conclusions: Patients with recent-onset RA had decreased ex vivo IL1β production and increased ex vivo IL1Ra production compared with controls. Ex vivo IL1Ra production is an independent predictor of progression of joint damage in recent-onset RA.

Rheumatoid arthritis (RA) is a complex disease in which both genetic and environmental factors play important aetiological roles.[1] Cytokines play a pivotal role in the inflammatory process observed in RA, and interindividual differences in the capacity to produce cytokines may be associated with susceptibility to and/or severity of RA.[1 2] Studies of twins have shown that the heritability of ex vivo production of cytokines in whole blood induced by lipopolysaccharide (LPS) varies from 50% to 80%.[3 4] The interindividual differences in the capacity to produce cytokines can be used as an endophenotype to enhance the elucidation of the genetic risk factors for RA.[5–7]

There is ample evidence for the key role of the proinflammatory cytokine interleukin 1 beta (IL1β) in the pathogenesis of RA.[8 9] Enhanced levels have been measured in synovial fluid and in plasma of patients with RA, and increased plasma levels of IL1β have shown a positive correlation with disease progression.[10–12] Even low concentrations of IL1β can result in destruction of cartilage and bone.[10] Interleukin 1 receptor antagonist (IL1Ra), a member of the IL1 family that binds to the IL1 receptors but does not induce an intracellular response, is the most important physiological regulator of IL1β activity.[8 13] In vivo, the balance between IL1β and IL1Ra is important and a considerable excess of IL1Ra is needed to inhibit IL1β.[8 9] Therefore, it is relevant to know whether the capacity to produce IL1Ra is different in patients as compared with controls.

The IL1 gene family, comprising IL1β, IL1α and IL1RN, has been located on the long arm of human chromosome 2 (band 2q13). Two polymorphisms in the IL1Ra gene (IL1RN) and three polymorphisms in the IL1β gene have been described, which are all in linkage disequilibrium.[14] One polymorphism on the IL1β gene is located in the exon region (C+3954T) and two are located in the promoter region (C-511 T and T-31C).[14 15] The polymorphism C→T at position -511 in the IL1β gene has been suggested to be the tagging single nucleotide polymorphism (SNP) of a haplotype associated with a 2–3-fold increase in LPS-induced ex vivo production of IL1β.[15]

We designed a study to investigate whether differences in innate ex vivo production of IL1β and IL1Ra contribute to susceptibility and severity of RA. We determined whether patients with recent-onset RA have different IL1β and IL1Ra production on in vitro stimulation with LPS as compared with healthy controls. We investigated whether, in recent-onset RA, innate ex vivo production of IL1β and IL1Ra was associated with radiographic progression. In addition, we investigated whether the haplotype tagged by C-511T was differently distributed in RA versus healthy controls and associated with different levels of ex vivo IL1β production.

METHODS

Patients and controls

In all, 76 patients with recent-onset RA (American College of Rheumatology 1987 criteria, symptom duration <2 years)

Abbreviations: DMARD, disease-modifying antirheumatic drug; HAQ, health assessment questionnaire; IL1 interleukin 1, ; IL1β, interleukin 1 beta; IL1Ra, interleukin 1 receptor antagonist; LPS, lipopolysaccharide; RA, rheumatoid arthritis; SHS, Sharp–van der Heijde score

Table 1 Baseline characteristics of patients with rheumatoid arthritis (n = 76)

Age (years)*	55.5 (15.3)
Females, n (%)	51 (67)
Duration of symptoms (weeks)†	20.9 (13.5–33.9)
Time between diagnosis and inclusion (weeks)†	1.4 (0.5–3.9)
IgM rheumatoid factor positive, n (%)	48 (63.2)
ESR*	41 (29)
Ritchie articular index*	16 (8)
Swollen joints, n*	14 (7)
HAQ*	1.3 (0.7)
SHS†‡	3.0 (1.0–8.1)
Patients with erosive disease, n (%)‡	53 (72)
Progression of SHS over 2 years†‡	1.0 (0.0–3.8)

ESR, erythrocyte sedimentation rate; HAQ, health assessment questionnaire; SHS, Sharp–van der Heijde score.
*Mean (SD).
†Median (IQR).
‡Radiographs at baseline and after 2 years of follow-up available for 70 patients.

participating in the BeSt Study and 63 healthy controls were included.

Details of the BeSt Study have been published previously.[16] Briefly, this randomised multicentre clinical trial compared four treatment strategies in 508 patients with recent-onset RA: (1) sequential monotherapy; (2) step-up combination therapy; (3) initial combination therapy with prednisone; and (4) initial combination therapy with infliximab. Patients had received no prior treatment with disease-modifying antirheumatic drugs (DMARDs), with the exception of antimalarials. In the present analysis, 76 patients, who were consecutively enrolled in the study at the Leiden University Medical Center, Leiden, The Netherlands (group 1, n = 18; group 2, n = 20; group 3, n = 19; and group 4, n = 19) were included.

The 63 healthy controls were recruited from 54 families who had participated in a previous study on multiple sclerosis and systemic lupus erythematosus. The control individuals were spouses and first-degree relatives of the patients with systemic lupus erythematosus or multiple sclerosis. For the current analysis, all unrelated individuals (n = 63), preferably women aged >40 years, were recruited.[17] The medical ethics committee of the Leiden University Medical Center approved the protocols of both the studies. Patients and controls gave written informed consent for the current research.

Measurement of IL1β and IL1Ra

Peripheral blood samples were taken at the first visit to the outpatient clinic, before initiation of treatment with DMARDs. In the controls, the peripheral blood samples were taken after a physical examination by a physician to confirm healthy state. Blood samples were collected in pyrogen-free heparinised tubes between 8:00 and 11:00. The samples were cultured within 2 h after collection, with and without (negative control) LPS 10 ng/ml, in 4 ml tubes for 24 h, after which the supernatant was collected and stored at −70℃. IL1β and IL1Ra were measured by ELISA (IL1β: Sanquin, Amsterdam, The Netherlands; IL1Ra: Biosource, Camarillo, California, USA) at the same time in all samples in one batch. At the time of sample collection the patients had normal white cell counts.

Genotyping

The polymorphism C→T at position 511 (rs 16944) in the promoter region of the IL1β gene was typed in all patients and controls with DNA available (n = 72 patients; n = 61 controls). Primer sequences and PCR conditions were: forward primer 5′ GGT AAC AGC ACC TGG TCT TGC-3′; reverse primer 5′ GCA CAT ACT TTT CTT CAT TCA CTT C-3′; PCR cycles: 95℃ for 5 min, followed by 35 cycles of 95℃ for 30 s, 55℃ for 1 min and 72℃ for 30 s, followed by 10 min at 72℃. PCR products were digested with AvaI at 37℃ for 90 min and digests were resolved on 2.5% agarose gels. Samples were typed by visual examination of the present size fragments; 10% of all typings were repeated. The error rate was <0.5%.

Radiographs

Radiographs of hands and feet at baseline and after 2 years of follow-up were available for 70 of 76 patients. Radiographic progression was determined (Sharp–van der Heijde score, SHS) by using the mean score of two physicians, who scored the radiographs paired, independently, in random order, blinded for clinical data.[18] Median (interquartile range, IQR) progression was 1.0 (0.0–3.8), mean (SD) 4.7 (11.7), which was comparable with the progression observed in the other patients participating in the BeSt Study (p = 0.474). Three groups of patients were defined (tertiles): (1) non-progressive RA, progression score ⩽0; (2) mildly progressive RA, progression score >0 and ⩽2; and (3) severely progressive RA, progression score >2.

Statistical analysis

For comparing the means, Student's *t* test and the Mann–Whitney U/Kruskal–Wallis tests were used where appropriate. Proportions were compared with the χ^2 test. Genotype frequencies were tested for the Hardy–Weinberg equilibrium.

To compare ex vivo IL1β and IL1Ra production levels between patients and controls with correction for age and gender, logistic regression was performed. To this end, patients were identified as high or low IL1β- and IL1Ra-producers with

Table 3 OR (95% CI) for rheumatoid arthritis*

Baseline variable	OR (95% CI)
IL1β†	2.4 (1.2 to 4.9)
IL1Ra ‡	7.6 (3.2 to 18.0)

IL1β, interleukin 1 beta; IL1Ra, interleukin 1 receptor antagonist.
* Given low ex vivo IL1β production and high ex vivo IL1Ra production, with correction for age and gender.
†Level lower than median in controls.
‡Level higher than median in controls.

Table 2 Ex vivo interleukin 1β and interleukin 1 receptor antagonist production*

Cytokine	Patients with RA (n = 76)	Controls (n = 63)	p Value
IL1β	1536 (606–2854) †	2773 (1487–4901)	<0.001
IL1Ra	29 010 (20 459–41 195)	18 234 (14 387–22 293)	<0.001

IL1β, interleukin 1 beta; IL1Ra, interleukin 1 receptor antagonist; RA, rheumatoid arthritis.
Of patients with recent-onset RA and healthy controls, on stimulation with lipopolysaccharide; pg/ml; median (interquartile range).
†n = 75 patients.

cut-off levels based on the median production levels in healthy controls.

In patients, univariate and multivariate linear regression analyses were performed to describe the relation between radiographic progression and ex vivo IL1β and IL1Ra production with correction for baseline characteristics associated with more severe progression (age, gender, duration of symptoms, C reactive protein, rheumatoid factor positivity, anti-cyclic-citrullinated peptide positivity (n = 68), number of painful joints (Ritchie articular index), swollen joint count, health assessment questionnaire (HAQ) score, visual analogue scale for morning stiffness and total SHS[19 20]), and for treatment group (categorical). IL1β and IL1Ra were added to a multivariate regression model including significant variables resulting from a backward selection procedure (stepwise removal of variables with p>0.10).

RESULTS

Baseline characteristics

Table 1 shows the baseline characteristics of the patients. At baseline, this subgroup of patients from the BeSt Study did not differ from the other 432 patients with the exception of the number of painful joints (mean 16 for 76 patients vs 14 for the other 432 patients (p = 0.014)) and the period between diagnosis and inclusion (median 1.4 weeks for the 76 included patients vs 2.6 weeks for the other 432 patients (p = 0.001; other data not shown).

Among the 63 controls, 34 were women (54%; p = 0.114 compared with patients) and the mean (SD) age was 54.6 (11.9) years (p = 0.685 compared with patients).

Ex vivo IL1β and IL1Ra production

The ex vivo production of IL1β was significantly lower and that of IL1Ra was significantly higher in patients than in healthy controls (table 2).

Subjects characterised by low ex vivo IL1β production or high ex vivo IL1Ra production had increased risk for diagnosis of RA (table 3). A multivariate model including both IL1β production and IL1Ra production showed consistent associations of ex vivo IL1β production and IL1Ra production with the diagnosis of RA (data not shown).

To rule out possible effects of prescribed medication on the ex vivo production of IL1β and IL1Ra, it was tested whether in patients who had received no DMARDs, the ex vivo IL1β and IL1Ra production levels were associated with the use of non-steroidal anti-inflammatory drugs, paracetamol, opioids or other concomitant medication used at the time of blood sample collection. None of the medications were associated with ex vivo IL1β or IL1Ra production (data not shown).

Association of ex vivo IL1β and IL1Ra production with progression of joint damage

Patients with non-progressive RA (n = 26) had the lowest baseline ex vivo IL1β production (median (IQR) 1369 pg/ml (605–2804 pg/ml)), followed by patients with mildly progressive RA (n = 22; 1611 pg/ml (636–2176 pg/ml)) and then by patients with severely progressive RA (n = 22; 1881 pg/ml (422–4000 pg/ml); p = 0.811 for comparison among the three groups).

The patients with severely progressive RA had the highest baseline ex vivo IL1Ra production of 31 516 pg/ml (27 677–46 098 pg/ml), which was significantly higher than in patients with non-progressive (22 158 pg/ml (14 863–35 289 pg/ml)) and mildly progressive RA (30 208 pg/ml (23 042–43 365 pg/ml); p = 0.03 for comparison among the three groups).

In the univariate linear regression analysis, treatment group and total SHS at baseline were significantly associated with radiographic progression. Following these, ex vivo IL1Ra production had the highest explained variance ($R^2 = 4.4\%$). The backward selection procedure identified the following clinical variables as significantly contributing to radiographic progression: treatment group, C reactive protein, swollen joint count, HAQ, visual analogue scale for morning stiffness and total SHS. Adding ex vivo IL1β and/or IL1Ra production to the multivariate model including these variables showed that IL1Ra was significantly associated with radiographic progression after correction for these variables (table 4).

Table 4 Association of ex vivo IL1β and IL1Ra production and baseline disease characteristics with progression of joint damage*

	Univariate analysis			Multivariate analysis†	
Baseline variables	Standardised coefficient	R^2†	p Value	Standardised coefficient	p Value
Age	−0.163	0.026	0.179	–	–
Gender	−0.187	0.035	0.121	–	–
IL1β	−0.046	0.002	0.710	−0.05	0.715
IL1Ra	0.209	0.044	0.082	0.274	0.036
Treatment group‡	−0.382	0.146	0.001	−0.399	0.002
Duration of complaints	0.055	0.003	0.651	–	–
CRP	0.059	0.003	0.648	0.151	0.276
Anti-CCP	0.154	0.024	0.229	–	–
Rheumatoid factor	0.125	0.016	0.304	–	–
Ritchie articular index	−0.078	0.006	0.520	–	–
Total swollen joint count	−0.142	0.02	0.242	−0.042	0.747
HAQ	0.165	0.027	0.174	0.087	0.517
VAS morning stiffness	0.032	0.001	0.794	−0.101	0.459
SHS	0.327	0.107	0.007	0.151	0.118

CCP, cyclic-citrullinated peptide; CRP, C reactive protein; HAQ, health assessment questionnaire; IL1β, interleukin 1 beta; IL1Ra, interleukin 1 receptor antagonist; SHS, Sharp–van der Heijde Score; VAS, visual analogue scale–baseline variables not selected for multivariate analysis after backward selection procedure.
*Univariate and multivariate linear regression; regression coefficients, R^2 and p values.
†R^2 = explained variance; †multivariate linear regression analysis including the variables selected by backward selection procedure and IL1β and IL1Ra.
‡Treatment groups are: sequential monotherapy, step-up combination therapy, initial combination therapy with prednisone and initial combination therapy with infliximab.

Table 5 Genotype and allele frequencies in patients and controls

	IL1B C-511T				
	Genotype			Allele frequency	
	CC	CT	TT	C	T
Patients (n = 72)	31 (43.1%)	30 (41.7%)	11 (15.3%)	0.64	0.36
Controls (n = 61)	30 (49.2%)	27 (44.3%)	4 (6.6%)	0.71	0.29

IL1β, interleukin 1 beta.
Genotype values are n (%).

IL1β C-511T in patients and controls

Genotype frequencies showed a distribution in accordance with the Hardy–Weinberg equilibrium for both patients and controls. Although the frequency of the T-allele was slightly higher in the patients, no significant differences were observed in the genotype or allele distribution between patients and controls (table 5).

Comparisons of genotype distribution between patients and controls were as follows: for CC, CT, TT, $p = 0.28$; for CC and CT versus TT, $p = 0.11$; for CC versus CT and TT, $p = 0.48$, and for allele frequency, $p = 0.29$.

For controls with the T-allele, a trend was observed for higher ex vivo production of IL1β (median IL1β production 2666 pg/ml for CC, 2801 pg/ml for CT and 3670 pg/ml for TT; $p = 0.078$); in patients this correlation was not observed. There was no difference in the distribution of the IL1β genotype among the subgroups of patients with non-progressive, mildly progressive and severely progressive RA.

DISCUSSION

The ex vivo LPS-induced production of IL1β and IL1Ra was observed to be significantly different between patients with recent-onset RA and healthy controls. Intriguingly, the patients with RA, produced less IL1β and more IL1Ra than controls in this assay. For the first time, this study showed that higher ex vivo IL1Ra production at baseline was associated with higher radiographic progression.

The current assay demonstrates a specific ex vivo cytokine production profile that is associated with diagnosis and severity of RA indicating a specific endophenotype of recent-onset RA. Determination of endophenotypes is a useful and novel method to target further research regarding the genetic background of multifactorial diseases.[6] In line with the current observations, a recently published paper on gene expression profiles in RA identified IL1Ra as one of the top discriminators between peripheral blood mononuclear cells of healthy controls and patients with RA.[21] Hence, the importance of IL1Ra in RA has been underscored by two completely different approaches.

The observation that patients with RA have increased ex vivo IL1Ra production and decreased ex vivo IL1β production seems paradoxical. The following considerations have to be made. Firstly, IL1β and IL1Ra production were determined in vitro using a powerful stimulator to maximise cytokine production. It is unclear how the measured maximum production capacity in vitro is related to actual (circulating) levels in patients with RA. Several reports in the field of cytokines have shown that observations made in vitro may not mimic the in vivo situation, as the multiple microenvironmental factors are important for cytokine production.[22] However, as clear differences are observed between healthy controls and patients with RA, these results point at crucial alterations in the regulation of IL1β and IL1Ra production in RA, which can be caused by a polymorphism in a transcription factor or in of one of the LPS receptors.

Secondly, the biological relevance of high IL1Ra levels in vivo is unclear, because a 10–500-fold excess of IL1Ra over IL1β is needed to decrease the stimulation of target cells.[23 24] Thus, the elevated levels of IL1Ra may not be sufficient to change the effects of IL1β.[25] It is probable that IL1Ra production increases in response to IL1β production to counterbalance the proinflammatory effects of IL1β.[25] If so, IL1Ra seems to be a more reliable indicator of disease activity than IL1β itself, possibly because of the strong signal peptide and the caspase-1-independent production.[26]

The reliability of the assay used to determine interindividual differences in cytokine production has been extensively demonstrated.[7] Regression analysis was performed to correct for age and gender,[27] and a possible effect of prescribed medication in patients on cytokine production was ruled out. Therefore, the described method is likely to be reliable in describing cytokine production profiles in recent-onset RA.

The heritability of ex vivo cytokine production was underscored in a twin study; estimates of heritability of IL1β and IL1Ra production were 86% and 53%, respectively.[3] Nevertheless, the difference in IL1β production between patients with RA and healthy controls could not be explained by the distribution of C-511T. This is in contrast to a previous study which found a specific haplotype tagged by C-511T to be associated with higher IL1β production in patients ($n = 25$) and in controls ($n = 31$).[15] Compared with the mentioned study, the results in the healthy control group ($n = 61$) seem to confirm these data as there was a trend for higher IL1β production with carriership of the T-allele. As no association was observed in the patients with RA ($n = 72$), we do not know whether the difference in innate IL1β production is caused by a mutation in the IL1 genes. In general, studies concerning the association of IL1 gene polymorphisms with IL1β production have shown different and conflicting results.[14 28–31] Probably, other related polymorphisms and/or epigenetic factors like methylation of genes, contribute to different levels of innate IL1β production.

A strength of the current study is that blood samples of 76 patients with severe RA were obtained very early in the disease course.[16] Remarkably, ex vivo IL1Ra production was a better predictor of radiographic progression than several well-known predictors of destructive RA.[19 20] The association of IL1Ra production with joint damage despite aggressive treatment and low overall progression scores in the cohort under study points to a key role for IL1β and IL1Ra in the pathogenesis of RA.

In conclusion, the results of this study show that ex vivo LPS-induced IL1β and IL1Ra production levels indicate a RA-specific endophenotype with high ex vivo production of IL1Ra being independently and strongly associated with radiographic progression. Further studies addressing the pathogenetic background of high ex vivo IL1Ra production in recent-onset RA are needed and are useful to unravel the genetic background of RA.

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