Serum and *in vitro* production of IL-1 receptor antagonist correlate with C-reactive protein levels in newly diagnosed, untreated lupus patients

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**Abstract**

**Objective**

To examine the correlation between C-reactive protein (CRP) and CRP-inducing cytokines (IL-1, IL-6, TNF-α) and IL-1 receptor antagonist (IL-1ra), as well as to study their relationship with systemic lupus erythematosus disease activity (SLEDAI) in newly diagnosed, untreated lupus patients.

**Methods**

Sera from newly diagnosed untreated lupus and rheumatoid arthritis (RA) patients were examined for CRP and cytokines. Data were compared among patient groups and correlated individually among the lupus group. Lupus monocytes and neutrophils were cultured in vitro to produce IL-1ra and experimental results were related to CRP levels and SLEDAI.

**Results**

Within lupus, serum CRP, IL-6, IL-1 and TNF-α levels were significantly lower than those of RA (all p values were < 0.005) and generally higher than those in the controls (p = 0.002, < 0.001, > 0.2, and < 0.001, respectively). Except IL-1ra, which was correlated with CRP (p = 0.045), no substantial correlation was discovered between CRP and IL-1, IL-6 or TNF-α individually. Moreover, excluding IL-1ra (p = 0.024), there was no association between cytokines and SLEDAI. In vitro IL-1ra as secreted by monocytes correlated with serum CRP and SLEDAI.

**Conclusion**

In lupus patients, serum IL-1, IL-6 or TNF-α levels failed to correlate with low CRP levels. This indicates a complicated CRP production process, which can not be explained solely by single cytokines as reported previously. Both serum and in vitro produced IL-1ra may be applied clinically as a surrogate CRP marker in untreated lupus patients as they are both correlated with serum CRP.

**Key words**

Untreated lupus, IL-1ra, CRP, monocytes

IL-1α antagonist (IL-1ra) has been demonstrated to play an important role in immunoregulating IL-1 effects (17). Therefore, these three CRP-inducing cytokines as well as IL-1ra must be examined in newly diagnosed untreated patients (see Materials and Methods) to hopefully elucidate the true relationship between serum cytokines and CRP in SLE.

Obviously the cell type that is most closely related to the early initiated events in acute phase response is the blood monocyte and the tissue macrophage, which produce all three CRP-inducing cytokines (IL-1, TNFα, and IL-6) (18). Monocytes/macrophages are the only known cell types capable of synthesizing the above mentioned three CRP-inducing cytokines and IL-1ra. Hence, investigating their in vitro ability to generate these cytokines from SLE patients (particularly untreated) should be beneficial in solving the controversial relationship between significant lupus inflammation and low serum CRP responses.

Materials and methods

Collection of SLE and RA patients

Patients who fulfilled the 1982 American Rheumatism Association (ARA) criteria for systemic lupus erythematosus and the 1987 ARA criteria for rheumatoid arthritis, respectively, were enrolled in this study. Serum samples from 20 SLE, 18 RA patients and 14 healthy controls (see below for inclusion and exclusion criteria) were collected. These hospitalized lupus and RA patients were enrolled consecutively. Table I displays demographic, clinical and laboratory data of the two patient groups. All lupus patients had normal serum blood urea nitrogen and liver function. Fourteen healthy controls (female: male = 12: 2, mean age 44.9 yrs.) with no history of chronic hepatitis, sinusitis, allergic diseases (such as asthma or rhinitis) or acute infections with an age range of 22 to 65 years were recruited from the hospital staff or family members of the patients. Patients with SLE and RA were enrolled according to the same exclusion criteria. As corticosteroids can suppress production of cytokines (19) and immu-
munomodulating drugs have been known to suppress SLE and RA disease activity, only newly diagnosed, untreated patients were enrolled (from August 1995 through March 1999) to avoid possible manipulation of the disease process. In addition, patients who had been taking non-steroidal anti-inflammatory drugs (NSAIDs) were entered into the study. SLEDAI was employed to calculate the disease activity of lupus patients at the time of blood withdrawal (20). The additional 11 blood samples used for in vitro studies were derived from the same 20 SLE patients who were analyzed for cytokines in their sera. All subjects had provided informed consent prior to collection of a single blood sample.

Serum samples and reagents
Sera were obtained from non-heparinized blood samples after standing for 30 minutes at room temperature, and then centrifuged at 1,200 rpm for 5 minutes (Kubota centrifuge 2010, Tokyo, Japan). These sera were stored at -20°C until assayed for cytokines. Polyclonal goat and monoclonal mouse anti-TNF-, anti-IL-1, and anti-IL-6 antibodies with recombinant TNF-, IL-1, IL-6 and IL-1ra were purchased from R&D (Minneapolis, MN, USA). Goat and rabbit IgG anti-IL-1ra antibodies were also obtained from R&D. All these 8 antibodies are neutralizing antibodies, which should detect cytokines with active forms. RPMI-1640 medium and Hanks balanced salt solution (HBSS) were purchased from Gibco (Grand Island, NY, USA). Fetal calf serum (FCS) was obtained from Hyclone (Logan, UT, USA) and treated at 56°C for 30 minutes before use. Human serum albumin (HSA), rabbit anti-HSA antibodies (anti-HSA) and lipopolysaccharide (LPS) were obtained from Sigma (St. Louis, MO, USA); control rabbit IgG (Rab IgG) was purchased from Jackson ImmunoResearch (West Grove, PA, USA).

Cell separation
Heparinized blood was run on Ficoll-Hypaque as described, but with some modifications (21). Briefly, 0.3 ml of 3% methylcellulose was aspirated into 5 ml-syringes with number 18 needles. Then, 5 ml of blood was sucked into prepared syringes and placed upside down at room temperature for 30 minutes. The upper layer of cells was then collected in 50 ml plastic tubes and equal amounts of phosphate buffered saline (PBS) was added. Ficoll-Hypaque in 3 ml (sp. gr. 1.077, Pharmacia, Uppsala, Sweden) was added to the bottom of 15 ml tubes, followed slowly by the upper layer cell solution on top of Ficoll-Hypaque. This was then centrifuged at 4°C with 450 g force for 30 minutes (Beckman J-6B). Interface cells were obtained and washed three times with HBSS. These cells with an adjusted cell number (2 x 10^6 cells/ml) in RPMI-1640, containing 2 mM L-glutamate, 100 U/ml penicillin, 100 g/ml streptomycin, and 10 mM HEPES with 10% FCS, were then adhered in tissue culture dishes at 37°C for 2 hours (22). A rubber policeman was employed to rub off adherent cells. After washing and resuspension in RPMI-1640 medium with 2% FCS, adherent cells were examined for monocyte/macrophage purity via non-specific esterase staining and flow cytometric CD14 staining. The purity of monocytes was at least 90%. The non-adherent lymphocyte fraction contained less than 5% of monocytes based on Wright stain and non-specific esterase criterion. While the precipitated portion at the bottom, which contained red blood cells (RBCs) and polymorphonuclear cells (PMNs), was mixed and reacted with an equal volume of ddH_2O for 10 seconds. Mixing with the same amount of 2x HBSS solution followed this. After washing, RBCs were removed, leaving mainly PMNs. PMNs preparation purity was at least 95% by Wright stain.

Immune complex formation and in vitro monocyte culture
Immune complexes were generated herein as described (23). Briefly, 30 g of HSA in sterile saline was incubated in 24-well tissue culture plates (Costar, Cambridge, MA, USA) at 45°C for 2 hours. After washing with RPMI medium to remove unbound HSA, 30 g of rabbit polyclonal anti-HSA antibodies was added and incubated at 20°C for 1.5 hours. After three subsequent washings to remove unbound anti-HSA, adherent immune complexes were then incubated with 0.3 ml diluted normal human serum at room temperature for 1 hour. In three experiments, 15 g or 60 g of HSA with equal amounts of anti-HSA were also employed with results similar to those stimulated by 30 g of immune complexes. Prior to use, these immune complexes-adhered plates were washed with RPMI medium to accommodate the monocytes. Monocytes at 1 - 2 x 10^5 in 0.5 ml of RPMI-1640 with 2% FCS were then cultured in immune complexes-prepared plates with appropriate LPS (3 ng/ml) or HSA/anti-HSA complex concentrations for 2 days as described (24). After 2 and 12 days, culture supernatants were obtained and assayed for IL-1ra. In vitro high versus low IL-1ra producers were defined arbitrarily by the mean levels produced.
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below the 25th percentile value for serum IL-1ra of lupus patients, that is 109.83 pg/ml (see Results). LPS doses at 1, 3, 10, or 30 ng/ml were included when sufficient monocytes were present and came up with similar results. To serve as a control for HSA/anti-HSA immune complexes, adherence with rabbit IgG only was applied. The culture supernatants were obtained and stored at -20°C until cytokine assay by duplicate measurement was performed and mean values were taken.

Cytokine and CRP assay
Using specific anti-cytokine antibodies, IL-1α, IL-6, TNF-α, and IL-1ra in the sera and culture supernatants were measured by ELISA (25, 26). Nephelometry detected serum CRP levels and values less than the detection sensitivity of 5 mg/L were assigned arbitrarily as 0 mg/L for easy comparison. The procedures employed herein followed those described for TNF-α measurement by ELISA assay with modifications (25). Briefly, as suggested by the manufacturer, 96-well plates (Costar, Cambridge, MA) were coated with polyclonal anti-TNF-α, IL-1α, IL-1ra or IL-6 antibodies in coating buffer (carbonate-bicarbonate buffer, pH 9.6) and incubated overnight. Sera and culture supernatants were added at four-fold dilution and reading results at the end were multiplied by 4 to obtain original concentrations. Recombinant TNF-α, IL-1α, IL-1ra or IL-6 was included individually at various concentrations as standards to calculate cytokine concentrations. To capture cytokines in the sera or culture supernatants, individual monoclonal anti-cytokine antibodies (except polyclonal rabbit anti-IL-1ra antibody) were added, followed by secondary antibody conjugated alkaline phosphatase. P-nitrophenol phosphate, which was used as the substrate, was detected at 405 nm. The assay sensitivity for TNF-α, IL-1α, IL-1ra and IL-6 herein was 2 pg/ml, 5 pg/ml, 10 pg/ml, and 10 to 20 pg/ml, respectively. Each serum was assayed in duplicate and the mean value gathered for group computation.

All sera were tested for cytokine concentrations without knowledge of the clinical parameters of the subjects or related laboratory data status. Furthermore, according to the manufacturer, there has been no cross-reactivity between these products and other cytokines.

Statistical analysis
Generally, sera data are expressed as the median as well as the whole range, except where indicated. However, to ease visualization and comparison, some (Fig. 1) are presented as mean ± standard deviation (SD). Due to the non-Gaussian (non-normal) data result distribution, a non-parametric method, Mann-Whitney U test, was employed to calculate the statistical significance of most serum data among the groups. Figures 3, 4 and 5 depict individual correlations between CRP, cytokines and lupus disease activity (SLEDAI) as an X-Y correlation pattern. P value was calculated by both linear correlation coefficient and the Spearman rank correlation coefficient. In addition, Macintosh Staview II software was employed to compute all statistical data analyses. P < 0.05 was considered significant, however p < 0.025 was used for multiple cytokine data comparisons (Bonferroni method for correction, see reference 27).

Results
Serum cytokines and CRP in disease and control groups
As indicated previously (1, 2), serum CRP levels in SLE sera (median 6.81 mg/L and 25th to 75th percentile range of 0 to 10.89 mg/L) were much lower than those in RA sera (61.55 mg/L and 19.60 to 97.50 mg/L) (Fig. 1, p = 0.0001 by the Mann-Whitney U test, same below). However, lupus CRP levels were significantly higher than controls were (median 0 mg/L and 25th to 75th percentile range, 0 to 0 mg/L, p = 0.002, with the same trend but different expressions as in Fig. 1). To explore the correlation between CRP-inducing cytokines and serum CRP in newly diagnosed untreated lupus patients, the same sera were assayed for IL-6, IL-1 and TNF-α production. Excluding IL-1α, the IL-6, IL-1α and TNF-α serum levels of patients with SLE (median 17.51 pg/ml; 0.35 pg/ml; 2.90 pg/ml, respectively) were significantly higher

Table II. Comparison of serum cytokine levels in the three groups.

<table>
<thead>
<tr>
<th></th>
<th>IL-6 (pg/ml)</th>
<th>IL-1 (pg/ml)</th>
<th>TNF-α (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.00 *</td>
<td>0.00 *</td>
<td>0.00 *</td>
</tr>
<tr>
<td>SLE</td>
<td>17.51 **</td>
<td>0.35 **</td>
<td>2.90 @</td>
</tr>
<tr>
<td>RA</td>
<td>1014.30 @</td>
<td>1425.00 @</td>
<td>230.65 @</td>
</tr>
</tbody>
</table>

Control: healthy controls, SLE: systemic lupus erythematosus, and RA: rheumatoid arthritis.

Data are presented as medium with range in parentheses.

IL-6 levels compared between groups: *denotes p < 0.001, # denotes p < 0.005; IL-1 levels compared between groups: + denotes p >0.2, @ denotes p < 0.005; TNF-α levels compared between groups: = denotes p < 0.001, $ denotes p < 0.005.

Fig. 1. Serum CRP levels in SLE patients (n = 20), compared to both control (n = 14) levels and RA patients (n = 18). Data are mean ± standard deviation (SD). Mean CRP concentration was 10.51 ± 14.27 mg/L (range 0 - 47.94) in SLE sera, 63.42 ± 44.18 mg/L (range 7.62 - 168.00) in RA sera.
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than the control ones (Table II). However, cytokine levels in lupus sera were lower than corresponding cytokines in RA sera (p < 0.005, Table II). IL-1 has been demonstrated to be an important cytokine, which induces CRP synthesis and inhibits fibrinogen and haptoglobin production that are acute phase reactants stimulated mainly by IL-6 (8). Therefore, IL-1ra as an IL-1 antagonist was examined further. Although serum IL-1ra levels in lupus patients (median 254.10 pg/ml) were lower, they did not differ significantly from RA (412.2 pg/ml, p = 0.267, Fig. 2). However, serum IL-1ra levels of lupus patients were much higher than those of the controls were (median 37.49 pg/ml, p = 0.0002, Fig. 2). The trend of low levels of serum CRP-inducing cytokines in SLE was compatible with the faint CRP levels. However, whether this trend conferred an individual correlation between cytokines and CRP was examined further and the experimental results are presented below.

Inter-related association between serum cytokines, CRP and SLE disease activity

Interesting results occurred when individual cytokines were correlated to serum CRP levels in lupus patients. Neither IL-6, IL-1 nor TNF- level was associated with CRP significantly (Table III). These experimental results indicate that no linear or proportional relationship between IL-6, IL-1 or TNF- and CRP exists. Alternately, when the IL-1 receptor antagonist with CRP was examined further, a virtuous correlation occurred between IL-1ra and CRP (p < 0.001 and p = 0.045 by linear and Spearman rank correlation coefficients, respectively) (Fig. 3). These findings indicate that, although individual CRP-inducing cytokines induce CRP production by hepatocytes in vitro alone or in synergy (reviewed in ref. 8), they fail to elicit CRP in vivo production proportionally. Moreover, serum CRP levels correlated significantly with SLEDAI (p = 0.04). As no studies on the relationship between these cytokines and SLE disease activity have been reported for untreated
lupus patients, such a possible association is investigated herein. Also, there was no individual correlation between IL-6, IL-1 or TNF- levels and SLEDAI (Fig. 4). A significant relationship between IL-1ra and SLEDAI was discovered (Fig. 5, p = 0.024). Therefore, it is suggested herein that IL-1ra and CRP are both reliable indicators of disease activity in lupus patients. It is well known that mononuclear phagocytes, such as monocytes, and neutrophils are the major blood cells that produce IL-1ra (28). Consequently, the correlation between IL-1ra production by monocytes or neutrophils and SLE disease activity or CRP in untreated patients was investigated.

In vitro IL-1ra production by lupus monocytes or neutrophils and correlation with SLEDAI or serum CRP levels

To examine the correlation between cellular IL-1ra synthesis and SLE disease activity further, healthy control and lupus monocytes were cultured in vitro with LPS, adherent IgG and adherent HSA/anti-HSA immune complexes. Figure 6A depicts that lupus monocytes produced spontaneous IL-1ra concentrations, which decreased over time. Control monocytes generated moderate levels of IL-1ra spontaneously and increased much more IL-1ra production after stimulation via LPS, rabbit IgG (as a control for rabbit IgG anti-HSA) or HSA/anti-HSA immune complexes (Fig. 6B). However, experimental results for lupus patients differed. Monocytes from a subset of lupus patients (high spontaneous subset) secreted high IL-1ra levels spontaneously and reacted to LPS and rabbit IgG stimulation with limited or low response (Fig. 6B). In contrast, another subset of lupus monocytes (low spontaneous subset) produced low spontaneous levels of IL-1ra in vitro (about 40 pg/ml in Fig. 6B), and similarly, had a limited response to both rabbit IgG and LPS stimulation (Fig. 6B). In general, HSA/anti-HSA immune complexes elicited higher IL-1ra synthesis in both high and low producer subsets than adherent IgG alone did (Fig. 6B). Interestingly, the high spontaneous subset (IL-1ra ranged from 223.00 ± 40.04 pg/ml to 1098.00 ± 3.20 pg/ml) had higher serum CRP levels and SLEDAI scores (n = 7, average CRP concentration = 8.47 mg/L, mean score = 11). Conversely, the CRP levels and SLEDAI scores (n = 4, mean CRP = 0 mg/L, average score = 4.5) were lower in the low spontaneous subset (IL-1ra ranged from 10.99 ± 2.36 mg/L to 99.8 ± 7.78 mg/ml which was lower than the 25th interquartile serum IL-1ra level of
Table III. Relationship between serum cytokines and CRP levels.

<table>
<thead>
<tr>
<th></th>
<th>Correlation coefficient</th>
<th>P-value</th>
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<tbody>
<tr>
<td>Interleukin-6</td>
<td>Rho=0.328</td>
<td>0.153</td>
</tr>
<tr>
<td>Interleukin-1</td>
<td>Rho=0.154</td>
<td>0.501</td>
</tr>
<tr>
<td>Tumor necrosis factor</td>
<td>Rho=0.121</td>
<td>0.597</td>
</tr>
</tbody>
</table>

Serum cytokines and CRP levels in untreated lupus patients were correlated by the Spearman rank correlation coefficient method.

109.83 pg/ml in lupus, see the first paragraph of the Result section). Moreover, lymphocytes and polymorphonuclear cells (PMNs) from lupus (two experiments) secreted IL-1ra at levels that were the same as the blank wells on ELISA plates (as zero pg/ml) either spontaneously or by LPS stimulation at 1, 3, 10, and 30 ng/ml. After culturing with HSA/anti-HSA complexes at 15, 30, and 60 g, when compared to lupus monocytes, both lymphocytes and PMNs still continued to produce very low IL-1ra (10–20 pg/ml) levels. These experimental results indicate that lupus monocytes differ from controls in terms of in vitro IL-1ra synthesis by monocytes. Furthermore, in untreated lupus, monocytes are the primary producers of IL-1ra. Therefore, it is inferred that IL-1ra production by lupus monocytes in vitro is compatible with serum CRP levels as well as lupus disease activity.

Discussion

It has been reported that CRP has a positive relationship with IL-6 in RA or SLE (13, 29, 30). However, this relationship is inconsistent (9-11). Moreover, IL-1ra at 10- to 100-fold concentrations may inhibit the IL-1 effect by competitive binding to IL-1 receptor (31). Hence, the correlation between serum IL-6, IL-1, TNF- and IL-1ra and CRP in recently diagnosed untreated lupus patients was investigated here.

Table II displays that SLE sera contained much less IL-6, IL-1 and TNF- than RA sera did. Although these low cytokine levels in SLE were compatible with low serum CRP level trend (Fig. 1, Table II), cytokines and CRP in lupus were not correlated directly nor proportionally (Table III). Therefore, it is indicated that serum IL-6, IL-1 and TNF- levels are not proportionally responsible for CRP synthesis, as they are major CRP inducers in vitro (7). Alternately, IL-1ra levels present a proportional relationship with CRP levels (Fig. 3) and correlate with SLE disease activity (SLEDAI) (Fig. 5). This later result is consistent with reports on steroid-treated lupus patients (32). Intriguingly, in contrast to the report of low serum IL-1ra levels in treated lupus patients with kidney involvement (33), 7 of our untreated patients with significant proteinuria (Table I) had higher serum IL-1ra levels (mean 485.35 pg/ml) than the other 13 did without kidney involvement (mean 372.02 pg/ml). This implies that after kidney damage, IL-1ra increases as a protective reaction in untreated lupus. Although corticosteroids and immunosuppressive agents may explain such a difference, whether higher IL-1ra response offers a protective role for renal involvement in lupus is still unresolved. All SLE patients herein had an active disease by two different criteria (Fig. 5) (32, 34). However, the experimental results presented in Figure 2 disagree with the findings which state that serum IL-1ra levels in active, prednisolone-treated lupus patients were higher than those of rheumatoid arthritis ones (32). The difference might be due to that the patients in the other study with rheumatoid arthritis (32) were receiving prednisolone 30 mg/day, which suppresses IL-1ra synthesis (15). Moreover, a subset of our untreated lupus patients had high spontaneous IL-1ra monocyte secretion, which was in contrast to the very low IL-1ra production in medium alone by treated patients (32). Again, this discrepancy cannot be explained. However, prednisolone treatment could explain such a conflicting result (15). Thus, to resolve such conflicting findings, a larger study, which includes treated and untreated lupus patients, is required.

The drawback in this study potentially resides in that the mean age and age range of the lupus patients is less than those of RA and healthy controls (see Materials and Methods, and Table I). Moreover, it has been reported that there is a positive correlation between mean serum IL-6 levels and age, that is, these levels are higher in older people (35). However, in this study, serum cytokine levels in lupus patients (younger in the mean age) are significantly higher than healthy control ones (older in the mean age) (Fig. 2 and Table II). Hence, it seems unlikely that age effects cytokine between separate groups significantly. Most of our RA patients receive corticosteroids and/or disease modifying anti-rheumatic drugs (DMARDS) within 2 months of their diagnosis. Therefore, our RA patients differ substantially from RA patients with very mild symptoms (a RA subgroup) which do not require corticosteroids and/or DMARDS therapy for an average of 9.14 months (36). This could partly explain our high serum cytokine levels, which have yet to be obtained by others. Nevertheless, our controls revealed very low serum cytokine levels, similar to those shown by others (37, 38). These data indicate that our assay system is accurate.

IL-1ra correlation with CRP may be explained as follows; once IL-6, IL-1 and TNF- are produced outside hepatocytes, they in turn act on hepatocytes for CRP production (the periphery to the liver) (7). As all three cytokines can directly or indirectly induce CRP synthesis (8), the net effect may be not proportional to the individual cytokines (Table III). However, IL-1ra has been shown not to inhibit CRP production by primary human hepatocytes (39). Therefore, the one-way blocking effect (the periphery to the liver) is not valid for IL-1ra. In contrast, CRP has been demonstrated to induce IL-1ra preferentially (5- to 10-fold greater) over IL-1 by human peripheral blood mononuclear cells (40). This reverse induction (the liver to the periphery) of CRP on
IL-1ra synthesis might produce the correlation between serum IL-1ra and CRP (Fig. 3). Moreover, it has been demonstrated that soluble IL-1ra, which is produced by human hepatocytes, can be regulated by pro-inflammatory cytokines (IL-1 or combined IL-1 and IL-6) as an acute phase protein, similar to C-reactive protein production (41). Since both IL-1ra and CRP are produced by CRP-inducing cytokines stimulation (41), this phenomenon supports a correlation between them. Therefore, whether IL-1ra has any therapeutic benefit to the affected organ in lupus patients and whether it can be applied as a surrogate marker for CRP in corticosteroid- or immunosuppressant-treated SLE patients remains unknown. Hence, future studies are required to investigate these possibilities.

In summary, it has been demonstrated herein for the first time that serum IL-6, IL-1 and TNF- levels do not correlate proportionally with serum CRP levels in newly diagnosed untreated lupus patients. Conversely, serum IL-1ra levels and in vitro monocyte IL-1ra production correlate significantly with serum CRP and SLE disease activity as SLEDAI. Furthermore, monocytes, but not lymphocytes and PMNs, are the major blood cells that generate IL-1ra in untreated lupus patients within this in vitro system. Therefore, this suggests that IL-1ra may be used as a substitute CRP marker in lupus patients.

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