RESEARCH ARTICLE

Prolonged standard treatment for systemic lupus erythematosus fails to normalize the secretion of innate immunity-related chemokines

Luisa Vega1, Julia Barbado1, Raquel Almansa1,2, Rocio González-Gallego1, Lucía Rico2, Antonio Jimeno1, Mercedes Nocito2, Raúl Ortiz de Lejarazu2, Jesus F. Bermejo-Martin2

1 Unidad de Enfermedades Autoinmunes, Servicio de Medicina Interna, Hospital Clínico Universitario, Valladolid, Spain
2 Unidad de Investigación en Infección e Inmunidad & Servicio de Microbiología e Inmunología, Hospital Clínico Universitario, IECSCYL, Valladolid, Spain

Correspondence: J. F. Bermejo-Martin, Unidad de Investigación en Infección e Inmunidad, Servicio de Microbiología, Hospital Clínico Universitario. Ramón y Cajal 3, 47005 Valladolid, Spain <jfbermejo@saludcastillayleon.es>

Accepted for publication December 7, 2009

ABSTRACT. The pathogenesis of systemic lupus erythematosus (SLE) is far from having been elucidated at the molecular level. Using a multiplex system, we profiled 18 immune mediators in the plasma from 57 patients with SLE. Thirteen of them showed mild to moderate disease activity, and 29 showed severe activity, based upon the SLEDAI score. Fifteen patients were in complete clinical remission. Those patients with active disease, and those in clinical remission had been undergoing immunomodulatory treatment for an average of 10.7 months and 19.2 months respectively at the time of the visit. Samples obtained from 10 healthy volunteers were used as control. Patients with active disease and those with inactive disease showed elevated levels of the chemotactic proteins MCP-1/CCL2, MIP1-β/CCL4 and IL-8/CXCL8 as compared to the control (p < 0.05). This pattern of increased mediator levels was observed regardless of the immunomodulatory drug regimen received (non-steroidal anti-inflammatory, steroids or immunosuppressants), and of the degree of tissue damage. Patients with anticardiolipin antibodies (ACAs) showed significantly higher levels of IL-8 and MIP-1β than those with no ACAs. Levels of MCP-1/CCL2, MIP1-β/CCL4 and IL-8/CXCL8 correlated significantly, indicating a coordinated regulation of their secretion. Conversely, levels of Th1, Th2, Th17 cytokines, IFN-γ and growth factors did not differ from those found in the healthy controls. IFN-α, IL-1β, IL-6, IL-7 and IL-13 were undetectable. In conclusion, long term treatment of SLE with standard immunomodulatory drug regimes fails to normalize levels of key chemotactic proteins linked to innate immunity. This might suggest the existence of a basal, pro-inflammatory state in patients with lupus, even in the absence of symptoms, which could serve as a “substratum” or initiator of the immunological events taking place during a flare-up of the disease.

Keywords: interferons, cytokines, chemokines, lupus, profiles

There is accumulative evidence for the key role of interferon α- and interferon-inducible chemokines and their signalling pathways in the pathogenesis of systemic lupus erythematosus (SLE) [1-4]. An association between transcription levels of interferon-inducible chemokines in peripheral blood leukocytes with disease activity, degree of organ damage, and specific autoantibody patterns in SLE has been recently described [1, 5]. With regard to the role of cytokines in SLE, one of the interleukins most intensively investigated in relation to lupus is TNF-α, given the fact that anti-TNF-α drugs are now widely used in the treatment of rheumatoid arthritis and Crohn’s disease. The role of TNF-α in lupus is controversial. Its levels are increased in this disease, but while there is evidence of disease improvement using short-term therapy with anti-TNF-α antibodies, long-term therapy with infliximab, has been associated with severe, adverse events [6, 7]. IL-10 levels are consistently high in the serum of patients with this condition, and anti-IL-10 antibodies have been found to improve disease in murine models of SLE [8, 9]. IL-10 induces polyclonal activation of B lymphocytes, potentially inducing the secretion of autoantibodies. Interferon-gamma-inducing monokines (IL-12, IL-18) are also increased in serum from patients with lupus [10]. Although IL-17 contributes to autoimmune disease in rheumatoid arthritis and Crohn’s disease, its role in systemic lupus erythematosus is far less clear [11-13]. In murine models of SLE, the development of autoantibodies is dependent on IL-6, a Th-17-related cytokine. As a consequence, a wide range of mediators play a role in lupus, including Th1, Th2, Th17 cytokines, type I and II interferons and chemokines [14]. Most of the work quantifying cytokines
and chemokines in lupus has been developed using ELISAs, which has resulted in the description of the role of a very limited number of mediators, making it difficult to obtain a full picture of their part in this disease. For the same reason, there is much left to understand concerning the regulatory mechanisms, the relationships between these mediators, and the influence of treatment on their secretion profiles. In the present work, we studied 18, soluble immune mediators in plasma samples from a cohort of patients diagnosed with SLE with different degrees of disease activity, and also in a cohort of patients in complete clinical remission, all receiving long-term, standard treatment with immunomodulators, and using a multiplex system. This approach allowed the simultaneous quantification of a large number of both innate- and adaptive immunity-related mediators which are present in very small amounts in plasma.

**METHODS AND MATERIALS**

**Patients and control individuals**

SLE patients (n = 57) were recruited at the Unit of Autoimmune Diseases of our Hospital. A group of healthy volunteers of similar age and comparable sex distribution working at the University of Valladolid was recruited as the control (n = 10). The SLE patients fulfilled the classification criteria of the American College of Rheumatology for SLE [15]. For each patient, disease activity and disease-related damage were assessed at the time of blood sampling, using the SLE Disease Activity Index 2000 (SLEDAI-2K) [16] and the Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index (SDI) [17]. Fifteen patients were in complete clinical remission (SLEDAI-2K score = 0) and the remaining 42 showed different degrees of disease activity. Patients showing an SLEDAI index below 10 were classified as having mild/moderate disease (n = 13); patients with an index of 10 or higher were classified as having severe disease (n = 29), in accordance with the SLEDAI-2K flare system. Consequently, four comparison groups were considered in the analysis: 1) complete remission, 2) mild/moderate activity, 3) severe disease and 4) healthy controls. All of the patients (with active or no active disease) were receiving immunomodulatory therapy at the time of the sample collection. Patients were classified into three groups depending whether the treatment regimen included: a) non-steroidal anti-inflammatory drugs (NSAD) b) steroids alone or associated with chloroquine and c) immunosuppressors. Doses were as follows:

- **NSAID**: naproxen, 1,500 mg/24 h; indomethacin, 200 mg/24 h; celecoxib, 400 mg/24 h; etoricoxib, 120 mg/24 h;
- average steroid dose: prednisone, 10 mg/24 h; methylprednisolone, 10.6 mg/24 h; deflazacort, 7.5 mg/24 h;
- antimalarial: hydroxychloroquine, 350 mg/24 h; chloroquine, 150 mg/24 h;
- immunosuppressors: mycophenolate mofetil, 2 gr/24 h; azathioprine, 50 mg/24 h; cyclosporine, 100 mg/24 h; cyclophosphamide, 1,000 mg/month for six months; methotrexate, 15 mg/week.

**Sample collection and mediator profiling**

A blood sample was collected into a 10 ml EDTA tube. Plasma was obtained after proper centrifugation and was immediately frozen at - 80°C until evaluation. Mediators were analyzed in the Infection and Immunity Research Unit of our Hospital using two plates of a multiplex assay (Biorad TM, Hercules, CA, USA) on a Luminex TM platform (Austin, TX, USA). IFN-α was measured using two plates of an ELISA from Thermo TM. Detection limits (pg/mL) were as follows: IL-2 (2.68); IL-4 (0.37); IL-6 (2.93); IL-8 (1.77); IL-10 (2.17); GM-CSF (7.55); IFN-γ (6.0); TNF-α (6.62); IL-1β (2.54); IL-5 (2.57); IL-7 (2.57); IL-12 (2.74); IL-13 (3.02); IL-17 (4.89); GCSF (1.94); MCP-1 (2.23); MIP-1β (2.32); IFN-α (10.0). Measurements below the level of detection were reported equal to the level of detection. Levels of IFN-α, IL-1β, IL-6, IL-7 and IL-13 in plasma were consistently below the limit of detection, and were not included in further analyses.

**Statistical analysis**

The Mann-Whitney U test was used to assess the significance of differences in the levels of mediators between groups. Associations between mediator levels, laboratory parameters and severity scores were studied calculating the Spearman-Karber correlation coefficients.

**Ethics**

This study was approved by the Review Board of the Hospital Clínico Universitario de Valladolid, Spain. Informed consent was obtained from all study participants.

**RESULTS**

Patients’ general characteristics are detailed in **table 1**. Disease duration in years (mean; min; max) was (9.9; 0.2; 42.5) for those with active disease and (11.6; 2.0; 35.0) for those in complete remission. Treatment duration in months was (mean; SD) (10.7; 3.7) at the time of sample collection for those with active disease, and (19.2; 13.2) for those with “0” points according to the SLEDAI score. All of the patients except one showed positive antinuclear antibodies. The percentage of patients showing positive autoantibodies were as follows: anti-DNA antibodies (n = 34, 59.6%); Anti-Ro antibodies (n = 30, 52.6%); anti-La antibodies (n = 7, 12.2%); anti-histone antibodies (n = 7, 12.2%); antiribosomal antibodies
Table 1

Patient characteristics. Data are shown as mean (SD)

<table>
<thead>
<tr>
<th>Disease activity</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n = 15)</td>
<td>(n = 13)</td>
</tr>
<tr>
<td>(n = 29)</td>
<td>(n = 11)</td>
</tr>
<tr>
<td>(n = 19)</td>
<td>(n = 12)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Complete remission</th>
<th>Mild-moderate</th>
<th>Severe</th>
<th>NSAI</th>
<th>Steroids</th>
<th>Immuno-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M/F)</td>
<td>5/10</td>
<td>3/10</td>
<td>4/25</td>
<td>1/12</td>
<td>5/20</td>
<td>6/13</td>
</tr>
<tr>
<td>Age</td>
<td>34.0[12.7]</td>
<td>37.6[11.9]</td>
<td>41.4[13.3]</td>
<td>34.6</td>
<td>42.7[8.5]</td>
<td>35.5[14.2]</td>
</tr>
<tr>
<td>SLEDAI</td>
<td>0[0]</td>
<td>4.6[2.2]</td>
<td>16.6[6.3]</td>
<td>10.6</td>
<td>8.8[3.3]</td>
<td>9.2[10.2]</td>
</tr>
<tr>
<td>SDI</td>
<td>2.7[1.7]</td>
<td>2.5[1.4]</td>
<td>3.1[1.9]</td>
<td>2.3</td>
<td>2.9[2.1]</td>
<td>3.1[1.3]</td>
</tr>
</tbody>
</table>

* Significant differences with control at the level $p < 0.05$.
† Significant differences between mild-moderate and severe patients at the level $p < 0.05$.
° Significant differences between patients in clinical remission and severe patients at the level $p < 0.05$.
(n = 4, 7.0%); anti-Sm antibodies (n = 5, 8.7%); anti-RNP antibodies (n = 5, 8.7%); anti-Ig antibodies (n = 1, 1.7%); anticytodioplin antibodies, ACAs (n = 18, 31.5%). Six patients (10.5%) had positive anti-lupic coagulant.

When mediator levels were compared between patients and controls on the basis of their SLEDAI score, the three groups of patients (complete remission, mild-moderate and severe) showed higher levels of the chemoattractant proteins MCP-1/CCL2, MIP1-β/CCL4, IL-8/CXCL8 than the control (p < 0.05) (figure 1). Levels of GM-CSF were increased compared to the control in the two groups of patients with active disease (data not shown). Levels of MIP1-β and IL-8/CXCL8 were higher in the mild/moderate group compared to the severe group (figure 1). Conversely, levels of Th1, Th2, Th17 cytokines, and type II interferon (IFN-γ) could not be detected in those patients with active disease, those in clinical remission or in the controls.

Similar results were observed when the levels of mediators were compared as regards the degree of chronic and irreversible tissue damage, and as assessed by the SDI score (figure 1). Both groups of patients (those with SDI scores of 1 to 2 and those with scores above 2), showed significantly higher values for MCP-1/CCL2, MIP1-β/CCL4, IL-8/CXCL8 and GM-CSF than the control (p < 0.05), but with no significant differences between them (figure 1). Since the different immunomodulatory drug regimens could theoretically influence the patterns of mediators observed, patients were re-classified on the basis of treatment received. Similarly to the results obtained regarding degree of severity, increases in MCP-1/CCL2, MIP1-β/CCL4, IL-8/CXCL8, GM-CSF over control levels were observed in the three treated groups, with no significant differences between them (figure 1). No differences were found between the three treatment groups for the SLEDAI score.

Patients with active disease and ACAs showed significantly higher levels of IL-8 and MIP-1β than those with no ACAs. Median levels of these mediators were respectively 2.3-fold and 1.7-fold the median of those with no ACAs: 21.6 pg/mL versus 9.3 pg/mL in the case of IL-8 and 131.3 pg/mL versus 74.9 pg/mL in the case of MIP-1β.

Interestingly, when associations between MCP-1/CCL2, MIP1-β/CCL4, IL-8/CXCL8, GM-CSF were studied on the basis of 2-by-2 comparisons in the group of patients with active disease, significantly positive associations were observed between all of them (figure 2). MCP-1/CCL2, MIP1-β/CCL4, IL-8/CXCL8 showed also positive correlations in the group of patients with non-active disease (figure 3). On the other hand, none of these mediator levels correlated with the SLEDAI, the SDI scores or with disease duration.

DISCUSSION

The roles of both innate and adaptive immunity mediators have been extensively demonstrated in the pathogenesis of lupus. Currently available multiplex systems allow the simultaneous detection of a large number of immune mediators. This technology, when applied to the study of a complex disease such as SLE, provides us with a broad picture of the ongoing immune response taking place at a given moment. The patients studied in our work suffered
form from different degrees of disease severity, requiring long-
term treatment with standard immunomodulatory drugs
commonly employed in the treatment of lupus (NSAIDs, 
steroids, chloroquine and immunosuppressors). We also
included a group of patients showing complete clinical 
remission for comparison purposes. Interestingly, regard-
less of the degree of disease activity, none of the drug
combinations employed was able to normalize the plasma
levels of a group of chemotactic factors which partici-
pates in the innate response to pathogens: MCP-1/CCL2, 
MIP1-β/CCL4 and IL-8/CXCL8. Conversely, none of the levels of the adaptive immunity-related medi-
ators measured (Th1, Th2, Th17 cytokines, IFN-α) dif-
fered from those found in the healthy controls. Similarly,
IFN-α, a key initiator of innate immunity, was not
detected in the plasma of the patients. It has been sug-
gested that IFN-α plays a central role in SLE. IFN-α
stimulates the production of chemokines such as 
MCP-1 and IL-8 [5]. TNF-α, in turn, is able to induce,
via NF-κB, the secretion of MIP-1β [18]. The absence of
increased levels of both IFN-α and TNF-α in the plasma
of patients with lupus suggests that other factors are
inducing the secretion of MCP-1, IL-8 and MIP-1β.
Alternatively, IFN-α and TNF-α could be playing a role
in this disease, but at levels below the limits of detection
of the measurement methods employed here. The third
possibility is that both cytokines could be increased at
the local rather than systemic level.

MCP-1/CCL2, MIP1-β/CCL4 and IL-8/CXCL8 can be
induced by a wide variety of stimuli, including growth
factors, bacterial and viral products [3]. The increased
levels of GM-CSF found in the plasma of patients with
active lupus is consistent with the findings of Willeke
et al., who described an increased frequency of GM-
CSF-secreting PBMC in these patients [19]. The absence
of increased levels of GM-CSF in those patients in clin-
im remission could reflect the success of treatment in
controlling the secretion of this mediator.

The absence of increased plasma levels of IFN-α and
adaptive immunity mediators in patients with active
lupus and in those in remission could also be due to a
modulatory effect exerted by the treatment administered
to these patients. Further studies collecting sequential
samples are necessary to clarify this particular aspect,
however previous reports demonstrating increases in
IFN-α, IL-6, TNF-α, IL-10, IL-17 and IL-12 [5, 8-14,
20-22] in the plasma of patients with lupus supports a
down-modulatory effect exerted by the treatment, at
least on these cytokines. On the other hand, our results,
which contradict previous reports involving Th1, Th2
and IL-10 levels, could be explained on the basis of the tim-
ing of the sample collection, which used to be early in the
course of a flare. The samples in our study were collected
well after treatment instigation, by which time, cytokine
levels in our patients might have been greatly modified.
The increase in MCP-1/CCL2, MIP1-β/CCL4 and
IL-8/CXCL8 levels over control values was observed in
those patients in clinical remission, in those with mild/
moderate disease, as well as in the severely affected
ones (based in the SLEDAI score). Increases in these
chemokines were also observed in those patients with
and without chronic tissue damage (based in the SDI
score), indicating the active role of these mediators in
all the stages of the disease. This is reinforced by the
absence of any association between chemokine levels,
SLEDAI/SDI scores and duration of the disease.
A major finding of this study is the evidence of a positive
 correlation between MCP-1/CCL2, MIP1-β/CCL4 and
IL-8/CXCL8 in both active and inactive lupus, pointing
somehow towards a coordinated regulation of their secre-
tion in this disease. This coordinated secretion might
indicate a synergistic role in the pathogenesis of the dis-
ease, since these four molecules mediate a chemotactic
activity inducing cell migration towards inflammatory
sites. The significance of the markedly higher levels of
MIP1-β and IL-8 in the mild/moderate patients compared
to severe patients, and of the increase in IL-8 in those
patients in remission compared to patients with severe
disease, is difficult to interpret, since these chemokines
were increased, compared to control values, in all the
patient groups. Studies involving a larger number of
patients would help to clarify this particular issue.

An association of anti-Sm or anti-RNP autoantibodies
with chemokine mRNA levels has previously been described
[5]. It was not possible to consider this variable in our anal-
ysis given the low number of patients positive for these
autoantibodies in our study population. However, we were
able to compare the levels of mediators in those patients
with and without anticardiolipin antibodies. Here we
describe, for the first time, significantly higher levels of
IL-8 and MIP-1b in patients with positive ACAs. The relationship between ACAs and chemokines has not been studied in depth. The presence of ACAs is known to be linked to thrombosis and endothelial damage. Whether chemokines such as IL-8 are at the origin of the vascular damage seen in patients with ACAs or, conversely, whether their levels rise as a consequence of it, remains to be elucidated. In conclusion, a coordinated secretion of both CC chemokines (MCP-1/CCL2, MIP1-β/CCL4) and CXC chemokines (IL-8/CXCL8) was observed in SLE patients receiving long-term treatment with immunomodulators. This was seen in absence of detectable levels of IFN-α in plasma, and with normal levels of TNF-α and adaptive-immunity mediators, regardless of disease severity. These results indicate the existence of a basal, pro-inflammatory state in patients with lupus, regardless of the absence of symptoms. This might serve as a “substratum” or initiator of the immunological events taking place during a flare of the disease. Our results also highlight a failure of the standard drug regimens employed in the treatment of lupus in targeting these innate immunity pro-inflammatory molecules. Addition of specific chemokine inhibitors to the classical drug regimens might contribute to improve the clinical response to treatment [23].

Acknowledgments. The authors thank the Nursing Team of the Internal Medicine Service of our Hospital, who kindly collected the samples. The authors would also like to thank Epifanio Ramos, Ana Loma and Concha Nieto for performing autoantibody detection. This work was possible thanks to a grant obtained from “Caja de Burgos” (“Premios de Investigacion Biomédica”), Jesus F Bermeno-Martin, R. Almansa and L. Rico are supported by “Fondo de Investigaciones Sanitarias”, FIS, Ministry of Science and Innovation, Spain, EMER07/050, “Programa para favorecer la incorporacion de grupos de investigacion en las Instituciones del Sistema Nacional de Salud, EMER07/050” and “proyectos de investigacion en salud” PI081236.

Disclosure. None of the authors has any conflict of interest to disclose.

REFERENCES


