

RESEARCH ARTICLE

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

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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 chemoattractant 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-1 β 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-1 β , IL-6, IL-7 and IL-13 were undetectable. In conclusion, long term treatment of SLE with standard immunomodulatory drug regimens fails to normalize levels of key chemoattractant 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 leucocytes 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 simultaneously 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 Auto-immune 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; etericoxib, 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: hydroxycloquine, 350 mg/24 h; cloroquine, 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-1 β (2.54); IL-5(2.57); IL-7 (2.57); IL-12p70 (2.74); IL-13 (3.02); IL-17(4.89); GCSF (1.94); MCP-1 (2.23); MIP-1 β (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-1 β , 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-Kärber 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

Abbreviations

ACAs	anticardiolipin antibodies
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte macrophage colony-stimulating factor
IFN- γ	interferon γ
MCP-1	monocyte chemoattractant protein-1
MIP-1 α	macrophage inflammatory protein-1 α
MIP-1 β	macrophage inflammatory protein-1 β
PBMCs	peripheral blood mononuclear cells
SLE	systemic lupus erythematosus
TNF- α	tumor necrosis factor α

Table 1
Patient characteristics. Data are shown as mean (SD)

	Disease activity			Treatment		
	Complete remission (n = 15)	Mild-moderate (n = 13)	Severe (n = 29)	NSAID (n = 11)	Steroids (n = 19)	Immuno- suppressor (n = 12)
Sex (M/F)	5/10	3/10	4/25	1/12	5/20	6/13
Age	34.0 [12.7]	37.6 [11.9]	41.4 [13.3]	34.6 [8.5]	42.7 [14.2]	35.5 [12.7]
Age at diagnosis	22.3 [9.1]	29.9 [10, 6]	32.7 [12.4]	24.6 [7.9]	33.4 [13.8]	26.8 [9.7]
SLEDAI	0 [0]	4.6 [2.2]	16.6 [6.3]	10.6 [7.9]	8.8 [8.3]	9.2 [10.2]
SDI	2.7 [1.7]	2.5 [1.4]	3.1 [1.9]	2.3 [1.4]	2.9 [2.1]	3.1 [1.3]

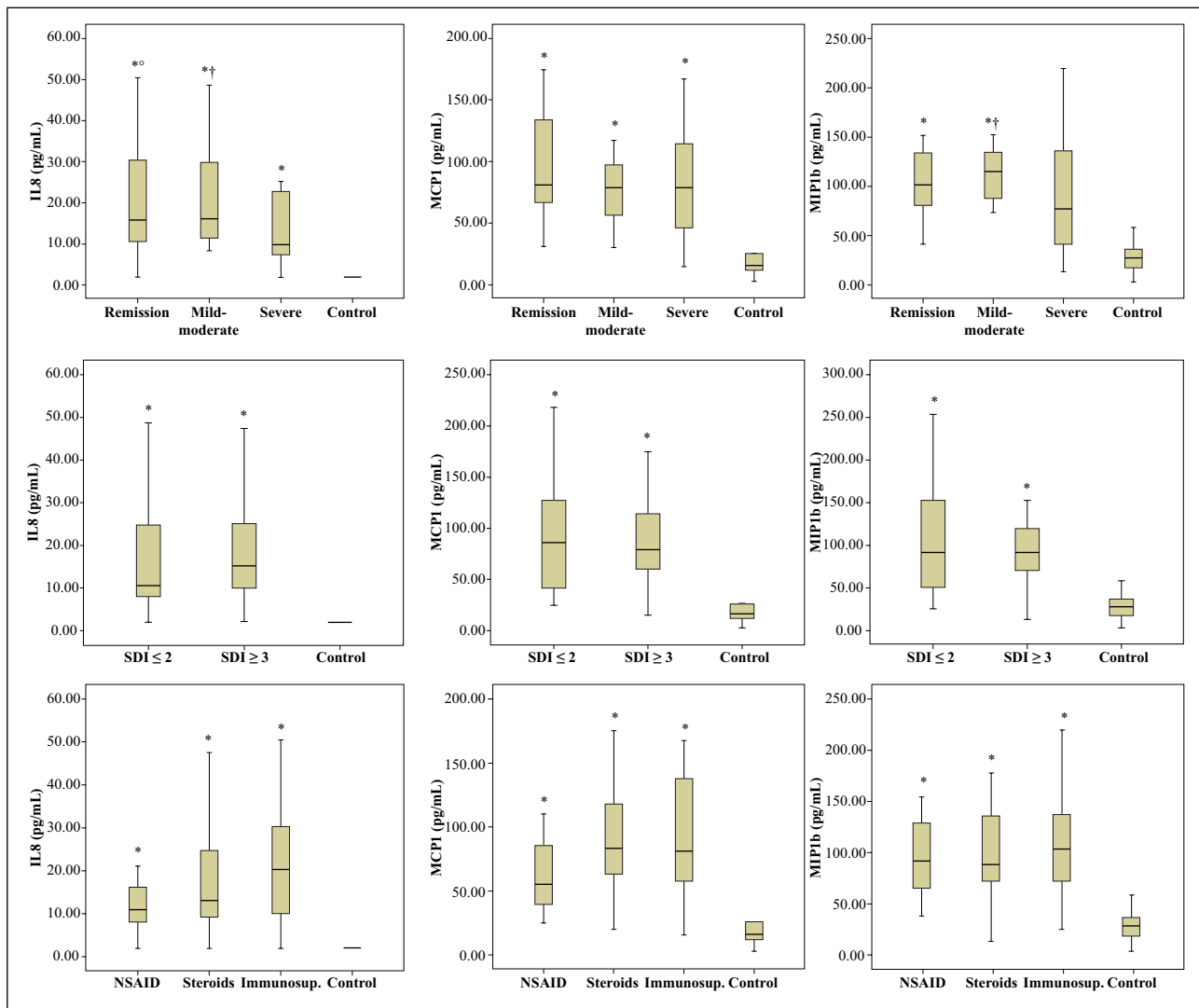


Figure 1

Comparison of mediator levels between groups according to the SLEDAI score (remission n = 15, mild-moderate n = 13, severe n = 29); treatment received (NSAID n = 13, steroids n = 25, immunosuppressors n = 19), and SDI score (≤ 2 n = 25, ≥ 3 n = 32) compared to control group (n = 10).

* 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-Jo antibodies (n = 1, 1.7%); anticardiolipin 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) did not differ from those of the control group. Interferon type I (IFN- α) 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 MIP1- β 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 MIP1- β .

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

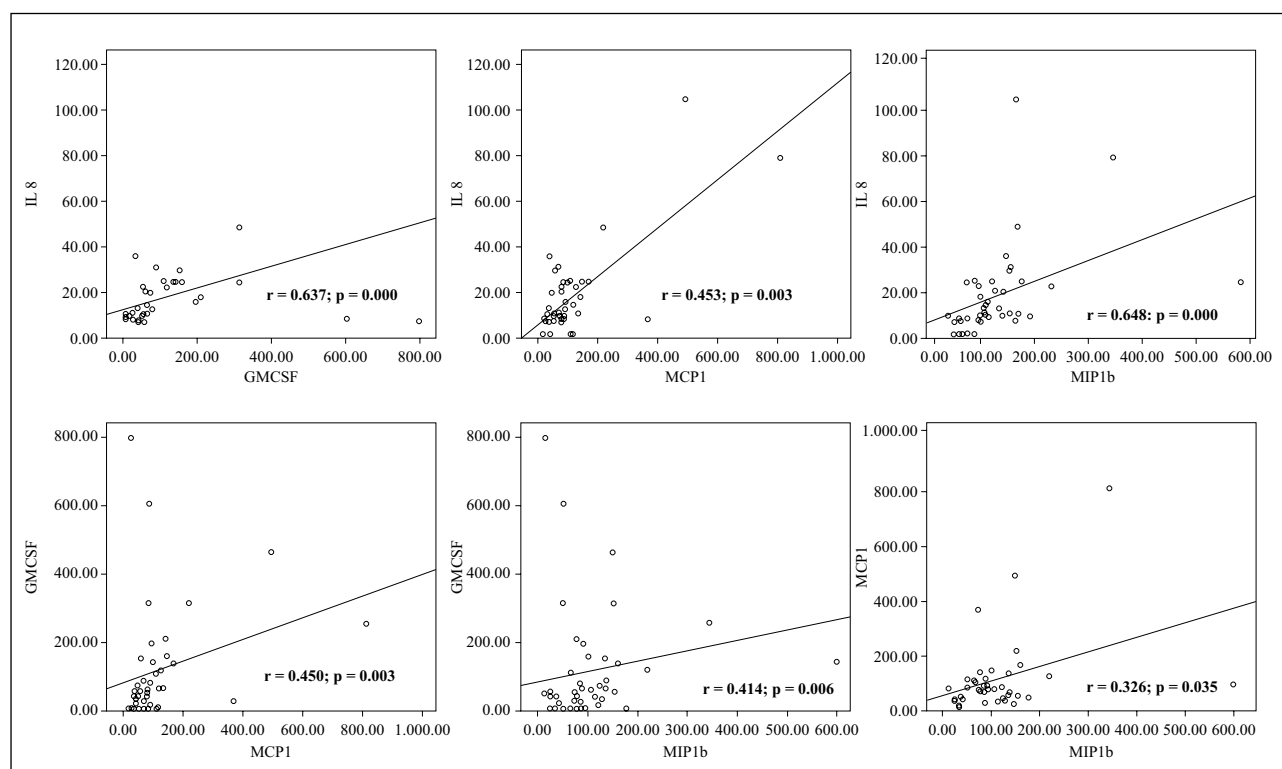


Figure 2

Dot plot showing the linear correlations between mediators in those patients with active disease. The Spearman correlation coefficient and p value are provided for each plot.

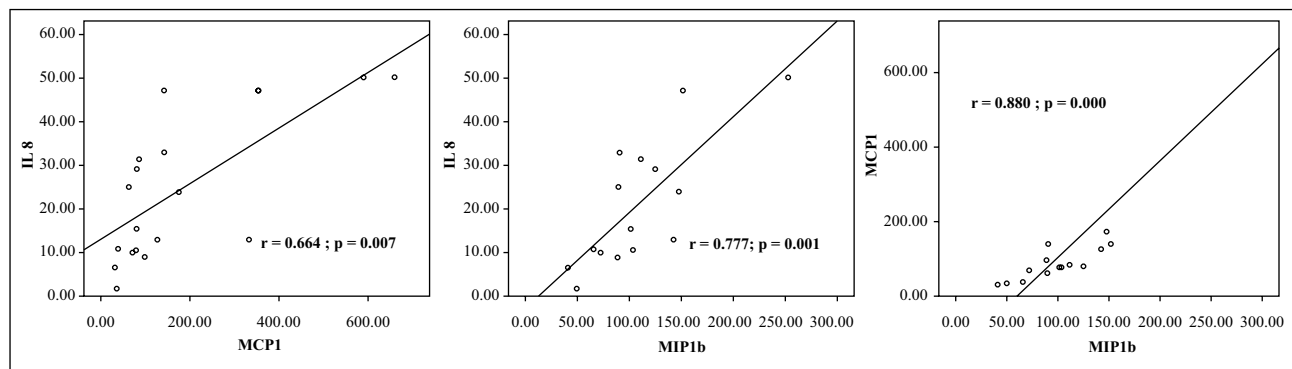


Figure 3

Dot plot showing the linear correlations between mediators in those patients in complete clinical remission. The Spearman correlation coefficient and p value are provided for each plot.

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, regardless 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 participates 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 mediators measured (Th1, Th2, Th17 cytokines, IFN- γ) differed 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 suggested 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- κ B, the secretion of MIP-1 β [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-1 β . 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 clinical 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 timing 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 secretion in this disease. This coordinated secretion might indicate a synergistic role in the pathogenesis of the disease, 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 analysis 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, even in 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].

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