Levels of Transforming Growth Factor-ß Are Low in Systemic Lupus Erythematosus Patients with Active Disease

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ABSTRACT. ***Objective.*** Cytokines are central regulators of the immune response but the workings of this complex network in systemic lupus erythematosus (SLE) are not fully understood. We investigated a range of inflammatory and immune-modulating cytokines to determine their value as biomarkers for disease subsets in SLE.

Methods. This was a cross-sectional study in 102 patients with SLE (87% women, disease duration 10.6 yrs). Circulating concentrations of interleukin 1ß (IL-1ß), IL-4, IL-6, IL-10, IL-12, IL-17, monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory protein 1 (MIP-1α), MIP-1ß, interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and total transforming growth factor-ß1 (TGF-ß1) were related to disease activity (SLE Disease Activity Index; SLEDAI), lymphocyte subsets, autoantibody levels, accrued damage (Systemic Lupus International Collaborating Clinics/ACR Damage Index; SDI), and concomitant treatment.

Results. Patients with SLE had lower levels of TGF-ß1 ($p = 0.01$) and IL-1ß ($p = 0.0004$) compared to controls. TGF-ß1 levels were lower in patients with SLEDAI scores 1–10 and SDI > 3; and were correlated with CD4+, CD8+, and natural killer cell counts; and were independent of steroid or cytotoxic drug use. Treatment with cardiovascular drugs was associated with lower IL-12 levels. No consistent disease associations existed for the other cytokines investigated.

Conclusion. Lower TGF-ß1 was the most consistent cytokine abnormality in patients with SLE. The associations with disease activity, lymphocyte subsets, and damage suggest that TGF-ß1 may be a therapeutic target of interest in SLE. (First Release August 1 2010; J Rheumatol 2010;37:2039–45; doi:10.3899/jrheum.100180)

Key Indexing Terms:
CYTOKINES
DISEASE ACTIVITY
TRANSFORMING GROWTH FACTOR-ß
SYSTEMIC LUPUS ERYTHEMATOSUS

Systemic lupus erythematosus (SLE) is a complex chronic autoimmune inflammatory disease that runs an unpredictable course. Central irregularities are abnormal B and T lymphocyte functioning, the production of organ-specific and non-organ-specific autoantibodies, and defects in apoptosis with reduced clearance of immune complexes[1,2]. Cytokines are essential for immune cell interactions and are potential biomarkers and therapeutic targets in autoimmune diseases[3,4]. Increased concentrations of proinflammatory and antiinflammatory cytokines have been described in patients with SLE[5,6], while association studies linking cytokine profiles to specific disease manifestations have given conflicting results[7,8,9,10,11]. This may be due to methodological differences, but may also reflect the variable effects cytokines can exert depending on the local environment in which they operate[12,13,14,15,16]. The role of cytokines in inflammatory lesions may thus be different from their effects in organs where chronic damage has occurred[17,18,19]. Patients with SLE currently live longer, but with considerable damage in a range of organ systems[20]. Cytokine-blocking agents have been successful for inflammatory joint disease[21], but have not been as productive in SLE. This may be related to the presence of a more complex clinical and/or immunological profile in SLE.

We investigated concentrations of 12 cytokines involved in different aspects of autoimmunity to determine their potential role as biomarkers for various disease subsets in our SLE cohort.

MATERIALS AND METHODS

Patients and controls. The Tromsø Lupus cohort is a population-based observational longitudinal registry of all SLE patients in the northern-most counties in Norway[22,23]. Patients included in this cross-sectional study were registry participants who had provided written informed consent. The

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Supported by grants from Eimar Munthe's Minnefond (15.10.2007), Odd Fellows Medisinsk Vitenskapelig Forskningsfond (30.04.2008), and Kvinners Sanitetsforening (grant R3/03).

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Accepted for publication May 19, 2010.

control group consisted of 31 adult healthy volunteers (74% women, age 48.0 yrs).

Data collection. Patients underwent an extended outpatient visit, where current demographics, clinical findings, drug use, and routine hematology and biochemistry analyses were recorded on a predefined data-sheet. Disease activity was calculated according to the SLE Disease Activity Index (SLEDAI) score, while damage development was scored by Systemic Lupus International Collaborating Clinics/ACR Damage Index (SDI)[24,25]. Serum samples were stored at –70° Celsius for simultaneous cytokine determination at a later stage.

Laboratory methods. Serum concentrations for interleukin 1ß (IL-1ß), IL-4, IL-6, IL-10, IL-12, IL-17, monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory protein 1 (MIP-1α), MIP-1ß, interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and total (latent + active) transforming growth factor-ß1 (TGF-ß1) were determined by a quantitative sandwich immunoassay technique (Single Analyte ELISArray™ kit; SuperArray Bioscience Corp., Frederick, MD, USA). All assays were run in duplicate using the same lot for each cytokine. The manufacturer's recommendations were followed throughout. For statistical purposes, values below the limit of detection (LOD) were replaced by the LOD value. The normal upper range for cytokines was defined as the upper quartile plus one interquartile range for controls (corresponding to cutoff > 96 percentile). Anti-dsDNA and other autoantibody assays were performed at our clinical immunology laboratory (EliA™ and VarelisA®; Phadia GmbH, Freiburg, Germany) with lupus anticoagulant tested in a phospholipid-dependent coagulation assay[26].

Statistics. As most data had a skewed distribution, numbers indicate median values with interquartile ranges (IQR) unless indicated otherwise and nonparametric methods were used in data analysis (chi-square or Fisher exact test for 2 by 2 tables, Mann-Whitney U test for numeric data). Dichotomized data are expressed as odds ratios with 95% confidence intervals. Analyses were performed with SPSS v.15.0 software (2006; SPSS Inc., Chicago, IL, USA). Resulting 2-sided p values are reported with cutoff for statistical significance at 0.05.

RESULTS

Half the patients with SLE had moderate disease activity despite long disease duration. Joint, skin, and renal disease and vasculitis were most frequent, together with serological disease activity (Table 1). Accrued organ damage was most frequent in the neuropsychiatric (20%), musculoskeletal (17%), cardiovascular (14.5%), and renal (11%) domains. Compared to controls, IL-1ß ($p = 0.0004$) and TGF-ß1 levels ($p = 0.01$) were significantly decreased in SLE patients (Figure 1A-1D). The IFN-γ/IL-4 ratio, which reflects Th1/Th2 balance, was comparable in patients and controls ($p = 0.7$, Figure 2). The Th17 profile correlated strongly with Th1 ($R > 0.34$; $p < 0.01$) and Th2 response ($R > 0.45$; $p < 0.001$) in SLE patients. Overall, all cytokine levels were within normal limits in 28% of patients, 1 or 2 cytokine levels were outside the normal range in 48% of patients and ≥ 3 cytokine levels outside the normal range in 24%. The total number of abnormal cytokines per patient did not correlate with SLEDAI, SDI, or lymphocyte counts.

Cytokine levels and disease activity. To determine if individual cytokines were related to disease activity, SLE patients were divided into 3 subgroups (Table 2). TGF-ß1 levels in patients with quiescent disease (SLEDAI 0) were comparable to controls and higher than in patients with moderate (SLEDAI 1–10; $p < 0.01$) and high disease activity (SLEDAI > 10; $p < 0.1$). In contrast, IL-1ß levels were not related to disease activity ($p > 0.2$). When analyzed by the presence of specific disease manifestations (skin lesions, vasculitis, proteinuria, etc.), no difference in cytokine levels was seen except for higher MIP-1ß levels in anti-dsDNA antibody-positive patients (196 vs 259 pg/ml; $p = 0.02$). Among standard laboratory findings (cell counts, creatinine, acute-phase reactants), only increased C-reactive protein (CRP) levels (> 5 mg/l) were associated with increased MCP-1 and IL-10 levels ($p < 0.02$) and lower TGF-ß1 levels ($p < 0.05$). TGF-ß1 levels correlated with total number of lymphocytes, CD4+ cells, CD4+/CD8+ ratio, and natural killer cells (Table 3). Inverse correlations existed between IL-6 and CD4+/CD8+ ratio and TNF-α with CD4+ cell counts. Responses in Th1, Th2, and Th17 were associated with a low CD4+/CD8+ ratio ($p < 0.03$).

Table 1. Data for the SLE cohort and controls; numbers are percentages or median values (interquartile range) unless otherwise indicated.

Feature	SLE	Controls
Female, %	87.3	74.2
Age, yrs	48.6 (24)	50.1
Disease duration, yrs	10.6 (12.0)	—
Arthritis, %	22	—
Inflammatory skin disease, %	16	—
Proteinuria/active urinary sediment, %	10	—
Vasculitis, %	8	—
SLEDAI score	5.5 (10.0)	—
SDI score	1.0 (2.0)	—
Anti-dsDNA antibody-positive, %	31.4	—
Hypocomplementemia, %	27.5	—
Antiphospholipid antibody-positive (any), %	17.6	—
Total lymphocytes, 10^9/l	1.3 (0.9)	2.0
B cells, 10^9/l	1.1 (0.20)	0.24
CD4+ cells, 10^9/l	0.58 (0.43)	0.99
CD8+ cells, 10^9/l	0.42 (0.30)	0.47
CD4+/CD8+ ratio	1.28 (0.3)	2.12
Natural killer cells, 10^9/l	0.10 (0.10)	0.25

Cytokine levels and organ damage. Severe organ damage (SDI > 3) was associated with higher MCP-1 and lower TGF-ß1 levels, while IFN-γ/IL-4 ratios did not differ (Table 4). No significant association existed between cytokine levels and damage in the renal, skin, or neuropsychiatric domains; patients with cardiac damage (n = 15) had reduced levels of IFN-γ, IL-12 and IL-17 (all $p = 0.02$), while patients with vascular damage (n = 7) had significantly increased levels of IL-1ß, IL-4, IL-6, IL-10, and MIP-1ß (all $p < 0.02$).

Cytokine levels and drug treatment. Cytokine levels did not differ between patients taking (n = 49) and those not taking corticosteroids, and no cytokine levels correlated with prednisone dose (all $p > 0.2$). Patients using hydroxychloroquine had higher TNF-α (54.4 vs 21.4 pg/ml; $p = 0.37$) and lower MIP-1α levels (15.0 vs 35.2 pg/ml; $p = 0.03$), while cytotoxic drug use (n = 36) was associated with slightly lower

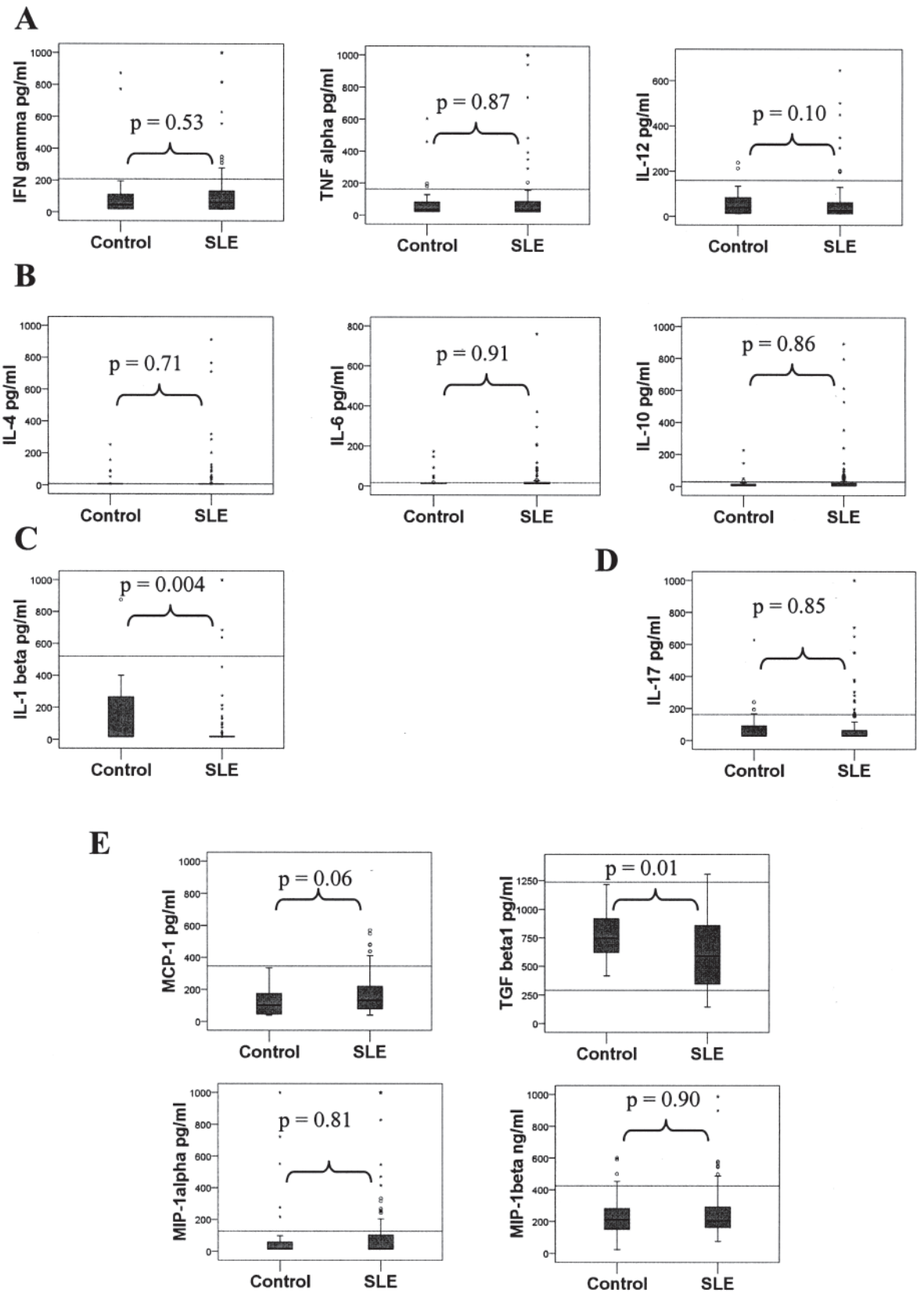

Figure 1. Circulating levels of cytokines in controls (n = 31) and SLE patients (n = 102). Horizontal lines indicate normal upper ranges for healthy controls. A. Cytokines involved in Th1 response (IFN-γ, TNF-α) and the Th1-inducing IL-12. B. Cytokines involved in Th2 response (IL-4, IL-6, IL-10). C. IL-1ß. D. IL-17. E. Chemokines (MCP-1, MIP-1α, MIP-1ß) and TGF-ß1. Asterisks indicate outliers.

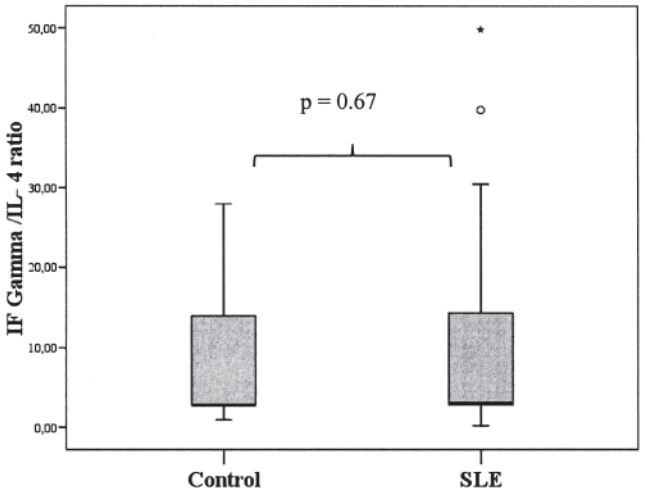

Figure 2. Comparison of the main Th1 and Th2 response cytokines between patients with SLE and controls. Outliers are indicated.

MCP-1 levels (35.1 vs 15.2 pg/ml; p = 0.07). Ratios for pro- and antiinflammatory cytokines (IFN-γ/IL-4, IFN-γ/IL-10, TNF-α/IL-4, TNF-α/IL-10) were not affected by corticosteroid, antimalarial, or cytotoxic drug use (all p > 0.3). Patients receiving vasoactive drugs (antihypertensives or statins; n = 38) had significantly lower IL-12 levels (12.6 vs 40.2 pg/ml; p = 0.009), while patients taking anticoagulants (n = 42) had lower levels of IL-12 (12.4 vs 37.3 pg/ml; p = 0.009) and TGF-ß1 (473 vs 713 pg/ml; p = 0.037).

DISCUSSION

Despite a considerable body of literature on the role of cytokines in experimental lupus, there is limited information on human lupus. The current data obtained in a patient cohort with few remarkable characteristics[7,27,28,29,30] imply downregulation of TGF-ß1 as the most consistent cytokine abnormality in SLE, with associations to disease susceptibility, activity, and damage.

TGF-ß1 is the main immune-modulating member of the TGF-ß protein family and maintains homeostasis by promoting and safeguarding formation of regulatory T cells (Treg) among CD4+ T cells[31,32]. TGF-ß1 deficiency leads to multiorgan autoimmune disease in gene-knockout mice and treatment of lupus-prone mice that includes a pro-form of TGF-ß1 reduces renal pathology and mortality in lupus-prone mice[33,34]. The finding of lower levels of total (latent + active) TGF-ß1 in patients with active disease in our study supports earlier findings. Even though TGF-ß1 levels were not lowest in patients with SLEDAI > 10, they were still well below levels in controls and in patients in remission. There are several possible explanations for this inconsistency: while our SLEDAI cutoff levels clearly distinguish patients with high disease activity, this assignment is arbitrary, as various cutoff levels for active disease are in use. Also, as the number of TGF-ß-producing Treg increases with corticosteroid treatment of active lupus[35], we found in a post-hoc analysis that patients with SLEDAI > 10 were more likely to

Table 2. Comparison of cytokine levels, median values (IQR), pg/ml, between controls and categories of disease activity by SLE Disease Activity Index (SLEDAI) in SLE patients.

Cytokine	Controls, n = 31	SLEDAI = 0, n = 22	SLEDAI = 1–10, n = 49	SLEDAI > 10, n = 29
IFN-γ	47.3 (94.3)	48.2 (107.4)	46.0 (102.0)	96.2 (128.1)
IL-12	40.4 (73.6)	27.7 (53.2)	32.6 (57.9)	12.6 (42.3)†
TNF-α	35.9 (70.9)	26.0 (51.5)	30.9 (54.6)	61.8 (91.8)
IL-4	7.0 (0)	7.0 (0)	7.0 (2.6)	7.0 (0.5)
IL-6	21.5 (1.7)	14.0 (0.5)	14.0 (10.5)	14.0 (8.7)
IL-10	5.9 (11.7)	5.9 (1.2)	5.9 (22.8)	5.9 (19.6)
IL-1ß	17.9 (251.1)	17.9 (0)**	17.9 (0)**	17.9 (0)*
IL-17	28.4 (66.5)	28.4 (0.5)	28.4 (127.0)	28.4 (30.9)
MCP-1	102.4 (150.3)	141.6 (131.7)	134.8 (174.4)	122.8 (129.1)†
TGF-ß1	746.7 (314.9)	705.5 (480.8)	473.3 (488.8)**	598.8 (502.5)†
MIP-1α	15.0 (55.3)	18.5 (186.6)	15.0 (67.2)	15.0 (44.8)
MIP-1ß	212.6 (137.7)	204.3 (132.5)	217.9 (121.1)	194.2 (202.0)

** p < 0.01; * p < 0.05; † p < 0.1 (borderline significance).

Table 3. Spearman rank coefficients for correlations between circulating levels of cytokines and immune cells.

Cell Type	IFN-γ	IL-1ß	IL-4	IL-6	IL-10	IL-12	IL-17	MCP-1	MIP-1α	MIP-1ß	TNF-α	TGF-ß1
Lymphocytes	–0.05	0.02	–0.13	–0.13	–0.07	0.06	0.003	–0.06	0.03	0.07	–0.12	0.22*
CD4+ cells	–0.06	0.01	–0.17†	–0.18*	–0.16	0.09	–0.02	0.01	0.04	0.06	–0.20*	0.27**
CD8+ cells	–0.08	–0.08	–0.16	–0.23*	–0.15	0.02	–0.08	0.06	0.039	0.13	–0.09	0.28**
B cells	–0.03	–0.04	0.1	–0.06	0.03	0.01	0.04	–0.06	0.19*	–0.04	–0.02	0.11
Natural killer cells	–0.05	0.06	0.01	–0.09	0.04	0.14	0.06	0.08	0.20*	0.05	–0.04	0.25**

** p < 0.01; * p < 0.05; † p < 0.1 (borderline significance).

Table 4. Comparison of cytokine levels, median values (IQR), pg/ml, between controls and categories of organ damage by SLICC/ACR Damage Index (SDI) in SLE patients.

Cytokine	Controls, n = 31	SDI = 0, n = 41	SDI = 1–2, n = 37	SDI > 3, n = 22
IFN-γ	47.3 (94.3)	74.9 (135.2)	45.7 (88.0)	107.1 (244.2)
IL-12	40.4 (73.6)	26.0 (59.1)	33.0 (45.0)†	17.1 (74.2)
TNF-α	35.9 (70.9)	41.3 (58.9)	29.5 (68.5)	35.1 (111.0)
IL-4	7.0 (0)	7.0 (0)	7.0 (0)	7.0 (46.7)†
IL-6	21.5 (1.7)	14.0 (9.1)	14.0 (0)	14.8 (27.6)
IL-10	5.9 (11.7)	5.9 (15.4)	5.9 (5.5)	11.2 (68.0)
IL-1ß	17.9 (251.1)	17.9 (0)**	17.9 (0)**	17.9 (116.2)
IL-17	28.4 (66.5)	28.4 (56.1)	28.4 (11.5)	28.4 (130.2)
MCP-1	102.4 (150.3)	135.7 (133.4)	113.8 (132.2)	191.3 (235.3)*
TGF-ß1	746.7 (314.9)	587.4 (535.5)**	697.6 (535.0)	567.6 (566.4)*
MIP-1α	15.0 (55.3)	15.0 (137.7)	15.0 (61.7)	15.0 (546.5)
MIP-1ß	212.6 (137.7)	191.8 (123.2)	221.2 (137.3)	248.2 (218.6)

** $p < 0.01$; * $p < 0.05$; † $p < 0.1$ (borderline significance).

be treated with methylprednisone pulse therapy close to the time of investigation (OR 1.07, p = 0.06), while the use of other immunosuppressive therapies did not differ among the groups.

Further evidence for a central role of TGF-ß1 in human SLE comes from a pilot study, where treatment with a tolerogenic peptide reduced disease activity with downregulation of TNF-α and IFN-γ and upregulation of TGF-ß1 and forkhead box P3 (FoxP3) expression in lupus patients[36,37]. Also in the number of Treg increased despite the continued presence of anti-dsDNA antibody. Treg are characterized by CD25 (IL-2 receptor alpha chain) and FoxP3 protein surface expression after stimulation by TGF-ß1 and IL-2. IL-2 is a key element in immune homeostasis and has both stimulatory and suppressive effects on T cells. Reduced IL-2 production is a central finding in SLE and represents intrinsic T cell defects[38]; low IL-2 may thus have contributed to the low CD4+ and CD8+ cell counts observed in our study that suggest a Treg deficit[32,38,39]. TGF-ß1 was the only cytokine that clearly correlated with the overall number of lymphocytes as well as the number of CD4+ T cells in the circulation, which included Treg. Low numbers and defective functioning of Treg have been described in patients with SLE[40,41] and correction of Treg efficacy in SLE leads to B cell suppression[42,43]. While more study is needed, our finding of reduced levels of TGF-ß1, which has a documented effect on Treg expansion, indicates a potential pathway for biological intervention in human SLE[34].

IL-1ß levels also differed between SLE patients and controls. IL-1 is considered a "master" cytokine in the network of proinflammatory cytokines[44]; its family consists of 2 agonistic proteins (IL-1α and IL-1ß) and the natural inhibitor IL-1 receptor antagonist (IL-1Ra). While IL-1ß is the secreted active form produced mainly by macrophages, few studies have investigated IL-1ß in SLE[6,45]. We found lower levels of IL-1ß in all SLE patients but no relation with disease activity, which is not in line with the experimental data that IL-1ß deficiency protects against autoimmunity[44]. The good correlation of IL-1ß with almost all other inflammatory cytokines does confirm a master position for IL-1 in SLE. In addition, our data show no relation between IL-1ß and TNF-α levels *in vivo*, supporting divergent roles for TNF-α and IL-1ß in SLE[7,46].

Th1 cells (IFN-γ production, phagocyte activation, and production of complement-fixing antibodies) and Th2 cells (IL-4, IL-5, IL-9, and IL-13 production) are central agents in autoimmunity[47,48,49]. However, our data do not support the concept of an imbalance in Th1 and Th2 responses in SLE compared to controls or in relation to specific disease manifestations, which is in agreement with most other studies[7,12,14,50]. The central Th1-Th2 paradigm was recently expanded by the identification of a third T helper cell subset, Th17 lymphocytes[51,52]. While their role is not clearly defined, Th17 cells are codependent on TGF-ß1 for differentiation and function[51,53,54]. While TGF-ß1 provides a link between Th17 cells and induced Treg, the effect of TGF-ß1 in humans differs from that in experimental models[52,55]. As IL-17 levels were comparable in SLE patients and controls and had no clear association with disease activity, lymphocyte counts, or TGF-ß1, our data could not confirm the role of IL-17 in SLE[56].

While the remaining cytokines did not discriminate SLE patients from controls, this does not preclude a possible association with specific disease features within the SLE cohort. Although we performed numerous subgroup analyses for each cytokine in the current dataset, only increased CRP levels (> 5 mg/l) were found to be associated with higher MCP-1 and IL-10 and lower TGF-ß1 levels. Thus, an important, although negative finding from our study is that single inflammatory cytokines cannot be readily related to overall disease activity or specific manifestations. This clearly limits the potential for cytokines to serve as biomarkers for disease subsets in SLE. A likely reason for this is the strong interdependency of cytokines, as illustrated by their multiple correlations with only moderate coefficients; this indicates a

state of relative balance between pro- and antiinflammatory cytokine profiles in our patients, as in other cohorts[7].

The high rate of cardiovascular drug use illustrates that prophylaxis and treatment for comorbidity have become increasingly important in management of SLE[57]. While cytokine levels were unrelated to immunosuppressive therapies, IL-12 was significantly lower in patients receiving vasoactive and anticoagulant therapy. IL-12 is produced by dendritic cells and B cells after bacterial antigenic stimulation, and induces a Th1 response with subsequent IFN-γ production. While it is implicated in psoriatic arthritis and Crohn's disease, there are no data to support a role for IL-12 in the pathogenesis of SLE[58]. These preliminary observations suggest that the beneficial effect of cardiovascular drugs in SLE may also be through reducing IL-12 and TGF-ß1; this is supported by recent experimental studies in which antihypertensive treatment reduced IL-12 levels and renal damage in patients with anti-glomerular basement membrane disease, while increased circulating TGF-ß1 promoted thrombosis[59,60].

There are apparent limitations to these results. Single-center data in a homogenous cohort of patients with longstanding disease do not necessarily reflect data in other settings. Cross-sectional analysis may also be insufficient to determine the dynamics of relatively short-lived cytokine biomarkers in a chronic disease. Although the cytokine panel we studied represents a significant portion of the inflammatory process in SLE, we realize the limitations imposed by our selection of cytokines, which are elements of a much larger network. Studying a larger number of cytokines and in a longitudinal fashion will require considerable resources and will challenge our ability to analyze such complex data.

In summary, in a study of a large number of cytokines in a large cohort of patients with SLE, lower levels of TGF-ß1 were most consistently associated with disease susceptibility, disease activity, lymphocyte subsets, and damage. TGF-ß1 might thus be an interesting target for the reconstitution of immune balance in SLE. While immunosuppressive therapy was not associated with specific cytokine changes, concentrations of IL-12 and TGF-ß1 were reduced in patients receiving cardiovascular drugs.

ACKNOWLEDGMENT

The technical assistance provided by Kirsten Nilsen is greatly appreciated.

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