Interplay of Cytokines and Chemokines in the Pathogenesis of Systemic Lupus Erythematosus

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Abstract: Problem statement: Dysbalance of T-helper-cell (Th) cytokines and chemokines was suggested to contribute to the pathogenesis of Systemic Lupus Erythematosus (SLE). Recent reports suggest the involvement of cytokines and chemokines in the pathogenesis of SLE, but their relationship with each other and particularly in the severity of disease, was not yet understood. We analyzed the interaction between cytokines and chemokines and their relationship with severity of disease in SLE. Approach: Serum levels of cytokines and chemokines were determined by ELISA and severity of disease were measured by using SLE Disease Activity Index (SLEDAI) score. Results: The serum levels of cytokine (IL-2, IFN-γ, IL-6, IL-10 and IL-12) and chemokine (CCL2, CCL5 and CXCL10) were variably associated with disease activity in SLE patients. Strict interaction between cytokines and chemokines was observed in SLE patients. Interleukin-6 was positively correlated with CCL5 while IL-12 was also analogous correlated with CXCL10 in SLE patients. The kidney involvement in SLE patients was related with intense increased levels of cytokines (IFN-γ, IL-12) and chemokines (CCL2, CCL5 and CXCL10). Conclusion: These data suggest that interplay of cytokines and chemokines may be involved in the severity of disease. Also, a better understanding of the cytokines and chemokines interaction may likely to provide important clues to the pathogenic mechanism and pave the way toward more effective therapeutics.

Key words: Systemic Lupus Erythematosus (SLE), chronic autoimmune, population proliferates, chemoattractant molecules, American College of Rheumatology (ACR), levels upon correlating, cytokines present, secretes cytokines

INTRODUCTION

Systemic Lupus Erythematosus (SLE) is a chronic autoimmune disease characterized by the activation of T and polyclonal B lymphocytes, production of pathogenic autoantibodies and formation of immune complexes causing tissue and organ damage. The development of lupus and its manifestation involves several cell populations; however the T cells have been reported as a major contributor in the trigging and progression of disease (Cava, 2009). This heterogenous cell population proliferates differentially in response to antigen stimulation, secretes cytokines and triggers other cells to synthesize cytokines or chemokines (Kunz and Ibrahim, 2009), which are involved in the pathogenesis. The Interferon (IFN)-γ is known to induce synthesis of IL-12 by Antigen-Presenting Cell (APC) which generates IFN-γ and IL-2 secreting Th1 cells. However, IL-4 induces T cells to develop into IL-4, IL-5 secreting Th2 cells. Since the T cells with different cytokines profiles would lead to different subtypes, there has to be the right balance in their numbers. Hence, Th1/Th2 balance emerges as one of the known critical determinants for pathophysiology (Davis et al., 2011; Jacob and Stohl, 2011). There has been growing evidence suggesting that infiltration of T
lymphocytes and other leucocytes into the sites of inflammation plays a critical role in organ involvement (Enghard et al., 2006). Leucocyte migration is mediated by the interaction of a number of chemokines and their receptors (Sallusto and Lanzavecchia, 2000). These chemoattractant molecules have well defined roles in directing cell movements necessary for the initiation of T cell immune response, attraction of appropriate effector cells to sites of inflammation and regulation of differential recruitment of T helper (Th1 and Th2) lymphocytes (Premack and Schall, 1996; Sallusto, 1999). Chemokines are known to play an important role in the development and progression of SLE (Eriksson et al., 2003). Increased levels of inflammatory chemokines in serum (Vila et al., 2007), plasma (Lit et al., 2006) and urine (Barbado et al., 2010) have been reported in SLE patients. The chemokine CCL 2 (Monocyte chemoattractant protein-1/MCP-1) is known as a Th1 chemokine and has an important role in homing of lymphoid cells to the zone of inflammation (Shimizu et al., 2005). Another chemokine CXCL 10 (Interferon-inducible protein-10/IP-10) upon binding to its ligand CXCR 3 attracts Th1 cells and promotes its maturation (Kong et al., 2009).

Although a number a cytokines and chemokines has been investigated as possible peacekeepers for the pathogenesis of SLE, specific correlation between cytokines and chemokines with each other have not been resolute yet and interplay of cytokine and chemokines with severity of disease and organ involvement is far to be identified. This study was designed to investigate the interplay of T cell cytokines, associated chemokines and disease severity which is required to improve disease prevention and diagnosis.

**MATERIALS AND METHODS**

**Patients and controls:** Patients for the study were selected from individuals attending out-patient Department of Internal Medicine at Postgraduate Institute of Medical Education and Research, Chandigarh, India. The study included 30 patients with SLE (28 females, 2 males) and the control group consisted of 30 healthy volunteers (28 females, 2 males). The SLE was diagnosed using the American College of Rheumatology (ACR) 1997 revised criteria (Hochberg, 1997) and disease activity was determined by using SLE Disease Activity Index (SLEDAI) score (Bombardier et al., 1992). The study protocol was approved by the Institute Ethics Committee, Postgraduate Institute of Medical Education and Research, Chandigarh, India and informed consent was obtained from all the patients and controls (healthy subjects). The patients enrolled in the present study were non-smokers and non-alcoholics, not associated with any other autoimmune disease and were not undergoing any anti-inflammatory therapy.

**Blood samples:** Venous blood samples from patients and controls were collected into plain vacutainers (Becton Dickinson, USA), serum was separated, aliquoted and used for estimation of the cytokines (IL-2, IFN-γ, IL-4, IL-6, IL-10 and IL-12) and chemokines (CCL 2, CCL5 and CXCL 10).

**Cytokines assay:** The serum cytokine concentrations of SLE patients and control subjects were measured by Enzyme-Linked Immunosorbent Assay (ELISA) using OptEIA reagent kits (Pharmingen, BD Biosciences, San Diego, CA) according to the manufacturer’s instructions. For each cytokine the specific kit was used wherein briefly, diluents provided was added, followed by addition of the sample and incubation at 37°C for 2 h. The wells were washed 3-4 times, the working detector (Enzyme labeled conjugated antibody) was added and incubated for 1h at Room Temperature (RT). The substrate was added and kept in dark for 30 min at RT. Stop solution (50 µL) was added at the end of incubation and the plate was read at 450 nm.

**Chemokines assay:** The serum levels of CCL 2, CCL5 and CXCL 10 in all the samples were measured by Enzyme-Linked Immunosorbent Assay (ELISA) using OptEIA reagent sets (Pharmingen, BD Biosciences, San Diego, CA). Briefly, wells of the ELISA plate were coated with the capture antibody diluted in coating buffer and incubated at 4°C overnight. Plates were washed thrice with wash buffer and blocked with assay diluent for 1h at RT. The standards and sample were prepared in assay diluent, added accordingly and incubated at RT for 2 h. Washing thrice was carried out upon completion of this step and the biotinylated antibodies as well as the enzyme reagent (streptavidin-horse radish peroxidase) were added before incubating the plate for 1h at RT. The plates were washed seven times before incubating with Tetramethylbenzidine and hydrogen peroxide for 30 min at RT in dark. The reaction was stopped with 2N H₂SO₄ and read at 450 nm.

**Statistical analysis:** Statistically significant differences were determined using the Mann-Whitney U test. Correlation analyses were performed using two-tailed
Spearman’s rank correlation. Analyses were performed using GraphPad Prism v.5.00.288 for Windows (GraphPad Software, San Diego, CA) p values less than 0.05 were considered significant.

**RESULTS**

**Demographic profile of subjects:** The mean age of patients with SLE was 25.8±6.50 years and that of control group was 26.43±6.20 years. The demographic and clinical characteristics of SLE patients and healthy controls are summarized in the Table 1. Most of the patients had been suffering from the disease for a couple of years. The disease activity determined by SLE Disease Activity Index (SLEDAI) score (maximum score of 105: Mild score <10; Moderate score 10-20; Severe score >20) showed three patients with mild, five patients with moderate SLEDAI score while 22 patients were in the severe score category. The elevated level of laboratory parameters; sedimentation rate of the Erythrocytes (ESR), complement levels of C3 and C4 were measured in SLE patients which reflect severity of disease.

| Table 1: Demographic characteristics of patients with Systemic Lupus Erythematosus (SLE) and healthy controls |
|---------------------------------------------------------------|
| **SLE patients** | **Controls** |
| Number (n) | 30 | 30 |
| Female/male | 28/2 | 28/2 |
| Age (yrs) | 25.8±6.50 | 26.43±6.20 |
| Duration of disease | 2.38±1.80 | NA |
| ESR (mm/hr) | 48.35±24.50 | NA |
| C3 (mg/dl) | 56.93±18.62 | NA |
| C4 (mg/dl) | 27.39±12.93 | NA |
| SLEDAI Score | 26.22±10.90 | NA |

Fig. 1: Th1/Th2 profile in healthy controls and SLE patients: a) Serum levels of IL-2 (pg/mL) determined by ELISA in samples from healthy controls and SLE patients. (b) serum levels of IFN-γ (pg/mL) in sera samples of controls and SLE patients. c) serum levels of IL=12 (pg/mL) in sera samples of controls and SLE patients. (d) serum levels of IL-4 (pg/mL) in healthy controls and SLE patients. (e) serum levels of IL-6 (pg/mL) in healthy controls and SLE patients. f) serum IL-10 levels (pg/mL) in healthy controls and SLE patients. Results are expressed as mean ± S.D.; *p<0.05, **p<0.01 and ***p<0.001 when compared to healthy controls.
**Th1/Th2 cytokine profile:** Serum levels of the Th1/Th2 cytokine are depicted in Fig. 1. The levels of IL-2 was significantly (p<0.001) decreased (9.67±2.36 pg mL\(^{-1}\)) in SLE patients as compared to healthy controls (13.03±3.69 pg mL\(^{-1}\)) (Fig. 1a). The levels of pro-inflammatory cytokine IFN-\(\gamma\) was notably (p<0.001) increased in SLE patients (60.86±9.48 pg mL\(^{-1}\)) as compared to healthy controls (5.42±1.86 pg mL\(^{-1}\)). A key cytokine for T cell differentiation; IL-12 has an important role in autoimmune disorders. The SLE patient samples exhibited substantially higher values of the cytokine (57.23±18.65 pg mL\(^{-1}\)) as compared to the healthy controls (145.9±34.0 pg mL\(^{-1}\)).

The order of significance found was that of p<0.001 (Fig. 1c). As compared to controls (10.19±3.20 pg mL\(^{-1}\)), a significant (p<0.05) but variable increase in IL-4 levels of serum from SLE patients (18.54±6.17 pg mL\(^{-1}\)) was observed (Fig. 1d). The levels of IL-6 (Fig. 1e) were markedly elevated (p<0.001) in SLE patients (26.52±1.89 pg mL\(^{-1}\)) as compared to controls (8.33±1.75 pg mL\(^{-1}\)). As shown in Fig. 1f, the levels of IL-10 were higher (p<0.001) in SLE patients (10.58±1.24 pg mL\(^{-1}\)) as compared to healthy individuals (5.72±1.86 pg mL\(^{-1}\)).

**Chemokine profile:** The levels of chemokine are portrayed in Fig. 2. The levels of chemotactic chemokine CCL2 was considerably (p<0.001) increased in SLE patients (552.0±55.27 pg mL\(^{-1}\)) as compared to healthy controls (226±28.08 pg mL\(^{-1}\)). As depicted in Fig. 2b, the levels of CCL5 in SLE (1742±563.4 pg mL\(^{-1}\)) was remarkably (p<0.001) elevated as compared to healthy controls (889.8±174.8 pg mL\(^{-1}\)). Similar pattern of increase (p<0.001) in the levels of inflammatory chemokine CXCL10 were obtained in SLE patients (113.4±15.50 pg mL\(^{-1}\)) and healthy controls (53.96±4.69 pg mL\(^{-1}\)).

Th1/Th2 cytokines and chemokines with each other and also with the severity of disease, correlations studied were accomplished. The values of spearman’s correlation coefficients were shown in Fig. 3 and 4. A negative correlation (r = -0.473, p<0.01) was observed between serum IL-2 and SLEDAI score (Fig. 3a). Notably a positive correlation (r = 0.532, p<0.01) was observed between IFN-\(\gamma\) and SLEDAI score in SLE patients (Fig. 3b). As shown in Fig. 3b, the levels IL-12 was substantially positively (r = 0.371, p<0.05) associated with the disease activity measure as SLEDAI score. An affirmative association (r = 0.532, p<0.01) was existed between IL-6 and SLEDAI score (Fig. 3d).
Fig. 3: Correlation amongst cytokines, SLEDAI score, Erythrocyte Sedimentation coefficient Rate (ESR) and complement proteins (C3 and C4) in SLE patients where \( r \) = Spearman’s rank correlation coefficient and \( p \) is level of significance. (a) relationship between IL-2 levels and SLEDAI score \( (r = -0.473, p<0.01) \). (b) relationship between IFN-\( \gamma \) levels and SLEDAI score \( (r = 0.533, p<0.01) \). (c) relationship between IL-12 level and SLEDAI score \( (r = 0.371, p<0.05) \). (d) relationship between IL-6 level and SLEDAI score \( (r = 0.532, p<0.01) \). (e) relationship between IL-10 level and SLEDAI score \( (r = 0.653, p<0.001) \). (f) relationship between IL-10 level and C3 \( (r = -0.388, p<0.05) \). (g) relationship between IL-10 level and C4 \( (r = -0.388, p<0.05) \). (h) relationship between IL-6 and ESR \( (r = -0.477, p<0.01) \).

**Correlation studies:** To evaluate the relationship of As shown in Fig. 3e, a clear-cut relation \( (r = 0.563, p<0.001) \) was found between IL-10 and SLEDAI score in SLE patients. A significant correlation was observed between IL-10 and clinical parameters; C3 \( (r = -0.388; p<0.05) \) and C4 \( (r = -0.388; p<0.05) \) as depicted in Fig. 3f and 3g. The IL-6 levels upon correlating with ESR revealed a considerable positive association \( (r=0.477, p<0.01) \) in SLE patients (Fig. 3h).

Furthermore, the serum chemokines CCL 2, CCL 5 and CXCL 10 showed a major positive correlation with disease activity measured as SLEDAI score in SLE patients \( (r = 0.488, p<0.01, \text{Fig. 4a}) \), \( (r = 0.557, p<0.01, \text{Fig. 4b}) \) and \( (r = 0.533, p<0.01, \text{Fig. 4c}) \) respectively. Interaction between cytokines and chemokines has been observed in SLE patients in the present study.

The levels of CCL 5 showed a remarkable positive correlation \( (r = 0.465, p<0.01, \text{Fig. 4d}) \) with serum IL-6 in SLE patients. Also, CXCL 10 levels correlated positively with IL-12 \( (r = 0.368, p<0.05, \text{Fig. 4e}) \) and IFN-\( \gamma \) \( (r = 0.533, p<0.01, \text{Fig. 4f}) \) in SLE patients.
Fig. 4: Correlation of chemokines with SLEDAI score, ESR and IL-12 in SLE patients revealed the following associations: (a) relationship between CCL 2 and SLEDAI score (r = 0.488, p<0.01). (b) relationship between CCL 5 and SLEDAI score (r = 0.557, p<0.01). (c) relationship between CXCL 10 and SLEDAI score (r = 0.533, p<0.01). (d) relationship between CCL 5 and IL-6 (r = 0.465, p<0.01). (e) relationship between CXCL 10 and IL-12 (r = 0.368, p<0.05). (f) relationship between CXCL 10 and IFN-γ (r = 0.533, p<0.01). (g) relationship between CXCL 10 and ESR (r = 0.519, p<0.01). (h) relationship between CXCL 10 and C4 (r = -0.393, p<0.05)

Moreover, the levels of CXCL 10 showed incredibly positive correlation with ESR (r = 0.519, p<0.01, Fig. 4g) and C4 (r = -0.393, p<0.05, Fig. 4h) in SLE patients. No such correlations were observed in healthy controls. In the present study, the major clinical problem had seen as obsessive association of nephritis with SLE patients. Out of 30 patients with SLE, 6 patients with increased levels of cytokines; IL-12 and IFN-γ and chemokines; CCL-2 and CCL5 had involvement of kidney (nephritis). These patients had also low level of laboratory parameter like C3 and C4.

**DISCUSSION**

There is growing evidence that cytokines and chemokines playing vital roles in the initiation and continuation of autoimmune disorders such as SLE. A defective regulation of Th1 and Th2 cytokines has been found typically in SLE and a Th1/Th2 imbalance appears to be an important factor responsible for the pathophysiology in lupus nephritis (Cava, 2009). The cytokine imbalance can thereby lead to alter immune responses which might influence the chemokines and subsequent severity of the disease. The present study evaluates the relationship between Th1- Th2 cytokine levels with the Th1 or Th2 chemoattractants chemokines and SLEDAI score of SLE patients. IL-2, a key molecule, known to maintain a link between autoimmunity & tolerance is a signature cytokine of Th1 cells. Deficient IL-2 synthesis has been suggested to have a physiopathogenic role in autoimmunity (Gomez-
The other Th1 cytokine IFN-γ was higher, which can be attributed to elevated IL-12 levels as shown by (Tokano et al., 1999). Santer et al. (2009) demonstrated that enhanced IFN-γ production leads to the tissue damage responsible for clinical symptoms in patients with SLE (Santer et al., 2009). In the previous study, we have showed that elevate levels of IFN-γ in the culture supernatant from SLE patients were associated with nephritis (Shah et al., 2010). In the current study, a positive correlation between serum IFN-γ and SLEDAI score suggests that IFN-γ is actively involved in the pathogenesis of the disease. Promotion of IFN-γ production by IL-12 has been reported to lead to polarization of peripheral cells to Th1 phenotype in lupus nephritis (Tucci et al., 2008). They have reported that intrarenal inflammation and cytokine imbalance of peripheral cells toward a Th1 phenotype in lupus nephritis. The renal damage has been suggested to be promoted by the imbalance of the peripheral IFN-γ; IL-4. It was shown that T cells from lupus patients tilted to a Th1 phenotype with low IL-2 and a high IFN-γ expression toning the severity of lupus. Also, the IFN-γ correlated positively with the SLEDAI score.

The elevated level of cytokine IL-4 observed in patients in our study was contrary to the previous reports of Funauchi et al. (1991) and Shiroiwa et al. (2007). However, our results are similar to the study of Wong et al. (2000). Shiroiwa group has suggested that the role of IL-4 in the pathogenesis may differ between certain subsets of SLE, even if they show similar disease phenotypes. Several lines of evidence suggest that IL-6 plays a central role in the pathogenesis of SLE; including regulation of growth and differentiation of B cells and auto-antibody production and that it may have a direct role in mediating tissue damage (Younou and Jamin, 2009). In our study, serum IL-6 was found to be higher in lupus nephritis patients (n = 6) and it also positively correlated with ESR and SLEDAI score. The result is similar to the study of (Chun et al., 2007) and (Esposito et al., 2009) who advocate that this cytokine is like a biomarker which moves up and down with inflammation and treatment respectively. Reports from other laboratories suggest its role in manifestation of neuropsychiatric SLE along with other cytokines such as IL-2, IFN-γ and IL-10 (Eftimiu and Blanco, 2009).

Interleukin-10, a B-cell stimulatory cytokine is known to inhibit type 1 cytokine response and has a critical role in the immunopathogenesis of SLE (Rouset et al., 1992). Spontaneous and mitogen-induced IL-10 levels have been reported to be higher in SLE patients (Llorente et al., 1994). The levels of IL-10 were remarkably higher in SLE patients as compared to healthy controls. Furthermore, a positive correlation of serum IL-10 levels with disease activity and a negative correlation of this cytokine with complement proteins C3 and C4 have been observed in SLE patients which is very similar to other studies (Chun et al., 2007; Park et al., 1998). It has been reported that monocytes and CD4+ as well as CD8+ T lymphocytes contribute to overproduction of IL-6 and IL-10 in SLE (Mellor-Pita et al., 2009) which correlated with the disease activity but was independent of the treatment the patients received. The role of cytokine balances in classification of renal pathology has been shown to involve in laser micro dissection of renal biopsy (Wong et al., 2010). Interleukin-12, a proinflammatory cytokine induces the production of IFN-γ, favors the differentiation of Th1 cells and links innate resistance and adaptive immunity (Bertagnolli et al., 1992). There are conflicting differences in the reports on IL-12 activity in SLE: It was reported that IL-12 production by PBMC was lower in SLE patients than in healthy controls (Liu and Jones 1998), whereas plasma level of IL-12 in active SLE patients was found to be higher than in the controls (Wong et al., 2000). The present study, levels of serum IL-12 were high and positive correlation with SLEDAI score in SLE patients. Higher serum IL-12 levels have been found to be indicative of nephritis (Tucci et al., 2008) in the present study. This discrepancy in the result may be due to diverse manifestation of SLE.

Recent studies have shown that chemokines and their receptors are intimately involved in regulating organ specific leucocyte trafficking and inflammation, suggesting their important roles in the pathophysiology of SLE (Vielhauer et al., 2007). These observation added the association between cytokines and chemokines in the leucocyte trafficking and inflammation in SLE. The chemokine monocyte chemotactic protein 1 (CCL2) is mainly expressed by activated monocyte/macrophages, helper T1 (Th1) cells and natural killer cells and it attracts leucocytes and other mediators into sites of inflammation (Lit et al., 2006). The chemotactants for Th1 lymphocytes; CCL5 and CXCL10 are synthesis and expression from neutrophils, macrophages and other immune cells is induced by IFN-γ and is suppressed by IL-10 and IL-4 in autoimmune disease (Zlotnik and Yoshie, 2000). In the present study, the levels of inflammatory chemotactic chemokines for Th1 and Th2 cells (CCL2, CCL5 and CXCL 10) were significantly higher in SLE patients and positively correlated with disease activity.
Patients (n=6) with increased levels of cytokines; IL-12 and IFN-γ and chemokines; CCL-2 and CCL5 had involvement of kidney (nephritis). Our result is restricted with the study of (Lit et al., 2006) and (Bauer et al., 2009). They have showed that CCL-2 and CXCL 10 were higher in SLE patients and plasma IL-18 concentration showed strong positive correlation with inflammatory chemokines; CXCL 10 and may be better indicators to assess the current disease activity, prediction of future disease flares and overall clinical decision-making. Our report shows elevated CXCL 10 levels in sera from SLE patients who could serve as good indicators of severity and activity of disease, as suggested by studies conducted at cellular level (Kong et al., 2009). Also, a good association between CXCL 10 and ESR in SLE may suggest Th1 chemokine activity in the disease which may have important roles in leukocyte homing, in the perpetuation of inflammation and concomitant tissue damage (Lee et al., 2009). Parallel to the positive correlation of plasma IL-18 with IP-10 (Bauer et al., 2009), in the present study, strong positive correlations were obtained between some of the cytokines and chemokines; the level of IFN-γ was positively correlated with CXCL 10, the level of IL-6 was positively associated with CCL 5 and the level of IL-12 was also positively linked with the level of CXCL 10 in SLE patients. These correlation may suggests that increase level of IFN-γ may be due to excessive accumulation of neutrophils at the inflammatory site and increased production of inflammatory chemokines CXCL 10 in SLE patients (Gasperini et al., 1999). Also, a good association of CXCL 10 with C4 and ESR in SLE patients in the present study may suggest that Th1 chemokine activity in the disease which may have important roles in leukocyte homing, in the perpetuation of inflammation and concomitant tissue damage (Lee et al., 2009).

The leucocytes at the sites of acute inflammation depend on the repertoire of inducible chemokines synthesized locally, which are, in part, influenced by pro-inflammatory cytokines present in the local environment of the cells at the time of stimulation (Lit et al., 2006). The synthesis and expression of Th1 chemoattractants, CCL5 and CXCL10 from neutrophils, macrophages and other immune cells is induced by IFNy and this response is suppressed by IL10 and IL4 (Gasperini et al., 1999). These reports may support our finding that cytokine and chemokine are interdepending and involved in the pathogenesis of SLE. Additional, the inflammatory cytokines may even enhance the Th1 mediated inflammatory process, the activation and migration of leukocytes and macrophages for initiation and perpetuating the inflammatory chemokines production.

**CONCLUSION**

In conclusion, present study depicts that imbalance Th1-Th2 cytokines and chemokines may be involved in the auto-inflammatory process of SLE. Taken together with present finding on the association of cytokines (IFN-γ, IL-6, IL-10 and IL-12) and inflammatory chemokine (CCL5 and CXCL 10), the result may provide new clue for their potential roles in the exacerbation of SLE disease and may act as a new reliable marker for SLE. However, more intensive studied are required to clarify the possible role of inflammatory chemokines and their association with cytokines involved in the inflammatory mechanism-including cell activation, tissue destruction and other process underlying the immuneopathogenesis of SLE.

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