Soluble ST2 is increased in systemic lupus erythematous and is a potential marker of lupus nephritis

Running title: IL-33/ST2 axis in SLE and lupus nephritis

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Highlights

* IL-33 and sST2 levels are increased in the serum of SLE patients
* sST2 correlated with SLEDAI, while IL-33 did not
* sST2 levels were higher in patients with lupus nephritis than in SLE without renal involvement
* ST2L expression was significantly induced in the renal glomeruli of patients with lupus nephritis

Abstract (218 words)

Objective: To investigate the role of the interleukin IL-33/ST2 axis in systemic lupus erythematosus (SLE).

Methods: Serum concentrations of IL-33 and sST2 were measured by sandwich ELISA in SLE patients (n=111) compared to sex- and age-matched healthy controls (n=36). The serum concentrations of IL-33 and sST2 were correlated with various clinical and biological parameters. The expressions of IL-33 and ST2L were investigated in kidney sections by immunohistochemistry in lupus nephritis patients (n=23) and controls (n=10).

Results: Serum levels of IL-33 were significantly higher in SLE patients (11.64 ± 3.141 pg/mL) than in controls (1.043 ± 0.8526 pg/mL) ($p<0.0001$). Similarly, the serum concentrations of sST2 were significantly higher in SLE patients (34,013± 2,043 pg/mL) than in controls (25,278 ± 2,258 pg/mL) ($p=0.046$). sST2, but not IL-33, correlated significantly with disease activity index (SLEDAI). In addition, serum levels of sST2 were significantly higher in patients with lupus nephritis (45,438 ± 5,661 pg/mL) that in SLE patients without renal involvement (30,691 ± 1,941 pg/mL) ($p=0.016$). The immunoreactivity of IL-33 in renal biopsies of patients with lupus nephritis was not increased compared to controls, while the glomerular expression of ST2L was significantly higher in nephritis patients compared to controls.

Conclusions: Although IL-33 and sST2 levels are both increased in SLE, sST2 represents a surrogate marker of disease activity and complications of nephritis.

Key words: Systemic lupus erythematosus, soluble ST2, Interleukin-33, lupus nephritis

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the production of auto-antibodies, immune complex formation and the development of organ lesions, among which joints, skin, brain, lungs and kidneys are the most affected (1). The pathobiology of SLE is still not completely understood but several lines of evidence converge to underline that the disease occurs in genetically predisposed individuals exposed to environmental factors (2, 3). In SLE patients, innate and adaptive immune systems are inappropriately activated in an unbridled fashion entailing a breach of tolerance towards natural autoantigens. Apoptotic bodies that are secreted , are the principam source of these autoantigens; they are internalized by activated myeloid dendritic cells and presented directly to B cells with the help of CD4 T- cell lymphocytes. This results in the differentiation of B cells into autoantibodies-secreting plasma cells. Hence, circulating autoantibodies interact with autoantigens to form immune complexes. Some of the immune complexes stimulate plasmacytoid dendritic cells which, when activated, secrete interferon-α (2). This cytokine plays a pivotal role in the pathogenesis of SLE, since it promotes the activation of immature myeloid dendritic cells and their contribution to the capture of autoantigens (4). Many Th2 cytokines favor the vicious activation loop of B cells into auto-antibodies producing cells such as IL-4, IL-5 and IL-13, besides Th1 and Th17 cytokines (5-7). In more than 50% of cases, SLE patients develop or will develop kidney lesions, the culprit being the deposition of immune complexes into renal glomeruli (8, 9). This locally precludes the cascade of complement activation and an inflammatory response precipitating glomerulonephritis, and collectively referred as lupus nephritis (10). The overall 5-year survival rate of SLE patients is beyond 90% and is mostly driven by the occurrence of renal complications (11).

Interleukin-33 (IL-33) is a member of the IL-1 cytokine family. IL-33 was identified as a ligand for the IL-1 receptor (IL-1R) family member ST2 (12) and is constitutively expressed in the nucleus of immune and non-immune cells including endothelial, epithelial and muscular cells (13). Acting

as a dual-function protein, similar to IL-1α and high-mobility group protein B1, IL-33 has both nuclear and extracellular effects (14, 15). The main function of IL-33 is believed to act as an alarmin, released upon cell damage (16). Of note, IL-33 expression is upregulated in epithelial cells stimulated with pro-inflammatory cytokines such as IL-1β and TNF-α or following activation of toll-like receptors -3, -4 or -9 pathways. Once extracellular, IL-33 binds to its receptor ST2 (ST2L), and to its coreceptor IL-1R accessory protein (IL-1RacP) ensuing activation of NFκB and MAPK pathways and leading to cell-specific downstream effects (12, 14, 15, 17, 18). Due to alternative splicing, the ST2 receptor exists as a soluble form (sST2) that acts as a decoy receptor (19, 20). The IL-33/ST2 axis has been involved in autoimmune diseases such as Sjögren's syndrome, systemic sclerosis and rheumatoid arthritis (21-24). The role of alarmins and their receptors such as IL-33/ST2 axis to the pathogenesis of SLE still remains uncompletely defined. Mok and coll. found a significant increase in sST2 levels in SLE patients that correlated with disease activity, without showing any changes in IL-33 levels (25). By contrast, another group reported a modest upregulation of serum IL-33 levels in SLE patients (26), that did not correlate with SLE disease activity. In the present study, we sought to investigate the involvement of the IL-33/ST2 axis in the pathogenesis of SLE and explore the use of either IL-33 or sST2 as surrogate markers of lupus nephritis.

Material and methods

Patients and controls

The study was approved by the local ethics committee of the Erasme University Hospital. Patients fulfilling the classification criteria of ACR 1997 for SLE were included in the study. Age- and sex-matched healthy individuals from the staff of Department of Rheumatology were included as healthy controls. All atopic and/or tobacco-smoking patients were excluded from the study. Informed consent was obtained from all recruited patients. Sera of SLE patients were collected from the Biobank of Clinical Biology Laboratory. Clinical and biological data including demographic, clinical and laboratory measurements (C-reactive protein, erythrocyte sedimentation rate, creatinine and urea levels, auto-antibody levels, anti-dsDNA, complement C3, C4, hematological count, liver function tests) were retrieved from medical records. Disease activity was assessed according to the SLEDAI and calculated via the medical record, using clinical data for the day ± 30 days of blood sampling. Active SLE was defined by SLEDAI > 4. SLE patients presenting with significant proteinuria > 0.5 g/day with active urinary sediments and/or renal biopsy-proven lupus nephritis at the time of the study were considered as having active renal disease. Renal biopsy-proven lupus nephritis was interpreted according to the International Society of Nephrology/Renal Pathology Society (ISN/RPS) classification criteria. Renal histological sections of patients with lupus nephritis (n=23, 2 sections for each patient) were provided by the Department of Pathology. As healthy controls, histologic sections from renal biopsies at day 0 post-renal transplantation were included (n=10). These renal biopsied showed no histological abnormalities.

ELISA

The concentrations of IL-33 and sST2 were measured in the sera of SLE patients (n = 111) and controls (n = 36) by sandwich ELISA according to manufacturer's instructions (IL-33 and ST2 human ELISA Quantikine® kits, R&D Systems, Minneapolis, MN, USA).

Immunohistochemistry

We analyzed 46 slides of renal biopsies showing lupus nephritis and 10 controls. Immunostaining against human IL-33 and ST2L were performed as previously described using diaminobenzidine as chromogen (27). Briefly, five-microns thick kidney sections were deparaffinized and then placed in citrate buffer (pH 6.0) for 10 minutes at 800W in the microwave for a step of heat-induced epitope retrieval. For blocking aspecific antibody binding, we incubated slices with normal rabbit serum diluted 1:10 in TBS. The slices were immunostained overnight with goat anti-human IL-33 IgG (AF3625, R&D Systems) at a dilution of 1:200, or with goat anti-human ST2L IgG (AF523). The slices were then incubated in polyclonal biotinylated rabbit anti-goat IgG for 1 hour (BAF017, R&D Systems) at a dilution of 1:2000. Avidin-biotin complex method was used for amplifying antibody binding (ABC peroxidase kit, Vector Laboratories), followed by chromogenic revelation using diaminobenzidine. Finally, slices were counterstained with Mayer's haematoxylin, dehydrated and mounted in 1,3-diethyl-8-phenylxanthine. The semi-quantitative evaluation of ST2L immunostaining was blindly performed by one investigator (A. M.), including respectively 105 and 33 glomeruli in patients with lupus nephritis and in controls. Renal glomerulus was considered as immunoreactive when at least one glomerular cell displayed ST2L expression.

Statistical analysis

Data analysis was performed using GraphPad Prism 6.0. Data are represented as mean ± standard error of the mean (SEM) unless otherwise stated. We determined the normal distribution parameters using the Shapiro-Wilk test. According to Gaussian or non-Gaussian distributions, Student's t-test or Mann-Whitney test were used for group comparisons. The Spearman correlation coefficient was used to determine correlations between various clinical variables, biological and the concentrations of IL-33 or ST2. A p value <0.05 was considered statistically significant.

Results

Characteristics of SLE patients

One hundred and eleven patients (108 women and 3 men, ratio 36:1) were included in the study. The median age of lupus population at the time the blood sample is 42 years (P25 = 34, P75 = 53). Other characteristics of the study population are listed in Table 1. Twenty-five patients (22.5%) have depicted lupus nephritis proven on biopsy. The anatomo-pathological ISN/RPS classification of patients with lupus nephritis (SLE-LN) was available for 23 of them (Table 2).

Serum concentrations of IL-33 and sST2 are increased in SLE patients

The serum concentration of IL-33 was significantly higher in SLE patients (11.64 ± 3.141 pg/mL) than in controls (1.043 ± 0.8526 pg/mL; $p<0.0001$) (Fig. 1A). Similarly, the concentration of sST2 was significantly higher in the SLE patients (34,013 ± 2,043 pg/mL) than in controls (25,278 ± 2,258 pg/mL; $p=0.046$) (Fig. 1B). We next determined if serum concentrations of IL-33 and sST2 could correlate with SLEDAI and biological and clinical parameters. Serum IL-33 did not correlate significantly with SLEDAI or clinical parameters, apart weakly with C3 ($r=0.2348$; $p=0.0181$) and C4 levels ($r=0.2046$; $p=0.0382$) (Table 3). There was no significant correlation between serum IL-33 and sST2. On the other hand, we did observe a significant but weak correlation between sST2 and SLEDAI ($r=0.2699$, $p=0.0042$) (Fig. 1C). sST2 serum levels did not show any significant correlation with other clinical and biological parameters (anti-dsDNA, age, C3, C4, creatinine, proteinuria, GFR) (Table 3).

Serum concentrations of sST2 are increased in patients with lupus nephritis

A detailed subgroup analysis showed that the serum concentrations of sST2 are significantly higher in patients with lupus nephritis (45,438 ± 5,661 pg/mL) relative to SLE patients without lupus nephritis (30,691± 1,941 pg/mL; $p= 0.0158$) (Fig. 1D). On the other hand, serum concentrations of

IL-33 were not significantly enhanced in patients with lupus nephritis (14.64 ± 11.62 pg/mL) relative to SLE patients without renal involvement (10.77 ± 2.316 pg/mL; p=0.1235). We did not observe any statistical difference in the other subgroup analysis (articular, muscular, pulmonary, neurological, or cutaneous involvements) (data not shown).

Expression of IL-33 and ST2L in the kidneys

We next studied the expression of IL-33 and its membrane receptor ST2L in renal biopsies of patients with lupus nephritis (n=23) and control patients (n=10). In both SLE-LN patients and controls, IL-33 immunoreactivity was detected as a weak and diffuse staining in the cytoplasmic compartment of tubular cells (Fig. 2A, left panel), as well as a more intense staining in the nuclei of intra-/extra-glomerular endothelial cells (not shown). Even in the SLE-LN kidneys with most chronic lesions, IL-33 immunoreactivity was barely detected inside the glomeruli. However, many tubulointerstitial cells expressed high levels of IL-33 in SLE-LN (arrows in Fig. 2A). In kidneys from controls, the pattern of ST2L expression was similar to the one described for IL-33. Remarkably, in SLE-LN patients, ST2L immunoreactivity was increased in the glomerular cells (arrowheads in Fig. 2A). Subsequently we performed a semi-quantitative analysis of all biopsies from SLE-LN patients and controls, respectively including 105 and 33 glomeruli. In SLE-LN patients, 33 glomeruli were highly immunoreactive (31.4%) against 2 (7.4%) in controls (p=0.0310) (Fig. 2B). Noteworthy, ST2L was also detected in the cytoplasm of tubulointerstitial or infiltrating cells (arrows in Fig. 2A).

Discussion

In this study, we showed that the serum levels of IL-33 and sST2 were significantly higher in SLE patients than in controls. This is in accordance with previous publications reporting either elevated IL-33 levels (26, 28, 29) or sST2 levels (25, 29-32) in SLE. In addition, our study confirmed previous findings about a positive, but weak, correlation existing between sST2 and SLEDAI (25, 29-31), although this correlation may be dependent on the origin of population included in the study (32). Interestingly, our data showed that serum levels of sST2 were significantly higher in the subset of patients that have complications of lupus nephritis compared to SLE patients without renal manifestations. In contrast, no significant difference in serum levels of IL-33 was observed between patients with lupus nephritis and patients without kidney involvement.

The increased serum concentration of IL-33 in SLE patients may be related to several mechanisms. In active SLE, complement consumption and the resulting deposition of immune complexes are partly responsible for cell and tissue damage in various organs, that could cause local and significant IL-33 release upon necrotic cell death. It has been speculated that IL-33 may be released following cell damage thereby acting as a DAMP (33). In the context of SLE, no correlations were found between IL-33 concentrations and C3 / C4 levels nor anti-dsDNA antibodies, classically used as prognostic markers of organ involvement. Another hypothesis could be that the increase in serum levels of IL-33 would be secondary to a chronic inflammatory state. In rheumatoid arthritis, it was described that inflammatory cytokines, such as IL-1β, increase tissue expression and release of IL-33. Many proinflammatory cytokines are upregulated during SLE (29) and we can therefore assume that they could stimulate the release of IL-33.

Although IL-33 immunostaining showed that it was expressed and upregulated in renal tissues from patients with lupus nephritis, the cellular source of IL-33 was not thoroughly investigated. In physiological conditions, a limited number of renal cells constitutively express IL-33. This includes tubulointerstitial cells co-expressing vimentin and α-smooth muscle actin, as well as vascular

endothelial cells from small and large vessels, but not glomerular capillaries (34, 35). In an experimental model of kidney injury following ureteral obstruction, the number of IL-33$^+$ vimentin$^+$ tubulointerstitial cells drastically increased, whereas vascular endothelial cells did not upregulate their IL-33 production (35). Similarly, our anatomo-pathological findings suggest that either tubulointerstitial cells or infiltrating immune cells (e.g. activated macrophages, dendritic cells or T cells) are sources of IL-33 in renal tissues from patients with lupus nephritis. Whether tubulointerstitial cells are more potent contributors than recruited immune cells remains to be demonstrated. Several authors suggested that, besides its role as as a DAMP, intra-tissular IL-33 may be involved in the progression towards renal fibrosis (10, 36). Interestingly, targeted inhibition of IL-33 release using neutralizing antibodies decreases renal damages, suppresses Th17 cells and pro-inflammatory responses in a mouse model of lupus, thereby suggesting that IL-33 blockade could have a protective effect on renal complications observed in SLE (37).

In renal biopsies from patients with lupus nephritis, we have also demonstrated that glomeruli expressed significantly ST2L, unlike healthy controls where no immunostaining was observed on glomerular capillaries. In SLE patients, such a local ST2L expression supports the IL-33 responsiveness of glomerular cells towards increased intra-tissular IL-33 levels, which might be involved in the worsening of glomerular lesions towards more advanced-stage lupus nephritis. We also observed that, in many inflammatory infiltrates, some immune cells were intensely immunostained for ST2L. While the expression of ST2L by tubular epithelial cells or glomerular cells remains poorly described in the literature, it is well described that endothelial cells (38), cardiomyocytes (39), T cells (36) or neutrophils (40) express ST2L, and respond to IL-33 stimulation. A fundamental concept of the IL-33–ST2 axis is the balance between cytokine and the competing receptor antagonist influencing cytokine bioavailability. The sST2 protein is reported to possess anti-inflammatory properties such as the blockade of IL-1R, but more importantly serves as a decoy receptor in forestalling the effects of IL-33 signaling through the ST2L. The significantly

increased serum levels of sST2 in SLE group relative to control groups is expected as a counter-regulatory measure to dampen the effects of IL-33.

Our study confirmed that serum levels of the sST2 were increased in SLE patients and correlated with SLEDAI. Furthermore, sST2 were significantly higher in patients with lupus nephritis compared to patients without renal involvement. This suggests that sST2 may serve as a surrogate marker of SLE disease activity as stated previously by others (25, 29), but would also help at identifying patients at risk of - or developing complications of lupus nephritis. Accordingly, Mok et coll. reported that serum sST2 levels were higher among Class IV lupus nephritis compared to other ISN/RPS classes (25). Unfortunately, we were not able to perform a subclass analysis due to the limited number of patients for which we had access to ISN-RPS staging. The use of sST2 as a specific marker of lupus nephritis should still be considered with caution. First, because in a large cohort of SLE patients, Mok and coll. were not able to discriminate patients who had active lupus nephritis and those who had non-renal active disease based on sST2 levels only. Second, sST2 release does not seem to be organ- or disease- specific. Indeed, increased sST2 levels are commonly found in septic shock (41), asthma (42), myocardial infarction (43), or even in SLE associated with subclinical myocardial dysfunction (30).

In conclusion, this study highlights the involvement of the IL-33/ST2 axis during SLE and also during its exacerbation into lupus nephritis. While serum IL33 concentrations were not correlated to SLEDAI, a greater clinical interest relies in sST2, whose serum concentrations were correlated with SLEDAI and furthermore boosted in patients with lupus nephritis. Of note, the levels of IL-33 and sST2 are already implemented in routine to establish prognosis of renal diseases such as AKI, CKD, diabetic nephropathy or renal cell carcinoma (44). The present data illustrate the clinical association between serum sST2 levels and lupus nephritis, without demonstrating any causation. Further studies are needed to use sST2 as a specific serum biomarker of renal complications during SLE and determine its active contribution to disease pathogenesis.

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Table 1. Clinical features of SLE patients

Number of patients	**n = 111**
Sex (F/M)	103/8
Age (years) at blood sampling	42 (34-53) **
Age (years) at the moment of diagnosis	32 (25-45) **
SLEDAI at blood sampling	3.68
Clinical (n/111):	
- neurological involvement	2
- Vasculitis	2
- Arthritis	15
- Pleurisy / pericarditis	0 / 2
- Thrombopenia / Leucopenia	2 / 3
- Lupic nephritis proven on biopsy	25
- Pulmonary involvement	10
- Antiphospholipid syndrome	9
- Alopecia / skin rashes	2 / 9
Auto-immunity:	
- ANA	111/111
- Anti-dsDNA (UI/mL)	9,5 (1,3-42) **
- C3 (mg/dL)	92,75 (± 24,6) *
- C4 (mg/dL)	16 (11-22) **
Biology	
- Haemoglobin (g/dL)	12,79 (± 1.708) *
- Platelets (x103/mm3)	228 (183-279) **
- White blood cells (x103/mm3)	5,7 (4,5-7,8) **
- Urea (mg/dL)	32 (23-44) **
- Creatinine (mg/dL)	0,8 (0,7-0,9) **
- GFR (ml/min/1.73m2)	96 (60-108) **
Urine :	
- Proteinuria (g/24h)	0,13 (0,09-0,19) **
Treatment:	
- Corticosteroids	67
- Hydroxychloroquine	75
- Azathioprine	12
- Mycophenolate Mofétil (MMF)	10
- Methotrexate	15
- Nivaquine	1
- Cyclophosphamide	4
- Belimumab	2
- Cyclosporine	3
- Rituximab	1
- Anti-CD22	1

* Mean (± SEM). Gaussian distribution

** Median (P25-P75). Non-Gaussian distribution

Table 2. Staging of SLE-LN patients according to ISN/RPS

Anatomo-pathological diagnosis	**Number of patients**
Class I : minimal mesangial glomerulonephritis	0
Class II : mesangial proliferative lupus nephritis	0
Class III : focal proliferative nephritis - Active lesions - Active lesions/chronic - Chronic lesions	 4 1 0
Class IV : diffuse proliferative nephritis - Global - Active lesions - Active lesions/chronic - Chronic lesions	 100% 5 2 1
Class V : membranous nephritis	10
Class VI : sclerosing lupus nephritis	0

Table 3. Spearman's rank correlation coefficient between IL33, sST2 and biological parameters

	IL-33		sST2	
	Spearman *r*	*p* value	Spearman *r*	*p* value
IL-33	n.a.	n.a.	n.a.	n.a.
sST2	0,106	0,2683	n.a.	n.a.
Age	0,1141	0,233	-0,03029	0,7523
SLEDAI	0,05611	0,5586	0,2699	0,0042
GFR	-0,04814	0,6158	-0,1037	0,2786
Creatinine	-0,02339	0,8075	0,1598	0,0939
Proteinuria	-0,02873	0,8093	0,1783	0,1312
C3	0,2348	0,0181	-0,008671	0,9314
C4	0,2046	0,0382	-0,03374	0,7351
Anti-dsDNA	-0,0424	0,6586	0,1462	0,1259

Figure legends

Figure 1. Serum levels of IL-33 and sST2 in SLE patients related to controls. (A) Serum IL-33 concentration is significantly increased in SLE patients compared to age- and sex- matched healthy controls. (B) Serum sST2 concentration is significantly increased in SLE patients compared to age- and sex- matched healthy controls. (C) A significant positive, but weak, correlation is demonstrated between SLEDAI and sST2 levels. (D) SLE patients with lupus nephritis had higher levels of sST2 than patients without lupus nephritis. Abbreviations: (non)-LN, (non)-lupus nephritis.

Figure 2. IL-33 and ST2L immunolabelling in renal biopsies from controls and SLE-LN patients (A). Semi-quantification of ST2L immunoreactivity shows a significantly higher proportion of glomeruli immunostained for ST2L in SLE-LN compared to controls (B). Results are expressed as mean ± standard deviation. ST2L immunoreactivity was semi-quantified in 105 and 33 glomeruli respectively for controls and SLE-LN patients. Scale bar represents 50 μm.

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