

BIOMARKERS

Induced sputum levels of IL-33 and soluble ST2 in young asthmatic children

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Abstract

Objective: Interleukin-33 is an IL-1 family cytokine which signals via its T1/ST2 receptor, and acts as a key regulator of inflammation, notably the type-2 response implicated in asthma. This study aims to measure the expression of soluble ST2 (sST2) and IL-33 in asthmatic children, depending on disease activity. **Methods:** Thirty-seven children with well-defined asthma (20 moderate and 17 mild asthmatics) were studied. IL-33 and sST2 were measured by ELISA in serum and induced sputum (IS) samples, and compared with 22 age- and sex-matched healthy controls. Real-time quantitative PCR was used to determine IL-33 and TNF- α mRNA expression in IS. **Results:** sST2 and IL-33 levels in IS and serum were significantly higher in patients compared with healthy controls ($p=0.0001$). The increase in sST2 and IL33 was significantly more important in moderate cases than in mild asthma. A significant correlation was observed between serum and IS IL-33 levels ($r=0.497$; $p=0.0018$). Higher levels of IL-33 mRNA were detected in IS from asthmatics than those observed in controls. A significant correlation was found between TNF- α and IL-33 mRNA expression in the asthmatic subjects ($r=0.772$, $p=0.0001$). **Conclusions:** Values of sST2 and IL-33 observed in IS were found to correlate with disease activity. Elevated IL-33 mRNA expression in IS and its correlation with TNF- α reflected the inflammatory process observed in the lung of young asthmatics.

Introduction

Asthma is a chronic inflammatory disease of the airways characterized mainly by Th2 lymphocyte-mediated immune responses. This Th2-biased inflammation is associated with leukocytes recruitment (mainly lymphocytes, eosinophils and mast cells) and type-2 cytokines production [1]. However, other inflammatory pathways have been identified in asthmatic patients, depending on the clinical phenotype. Neutrophils, Th1 and innate immune cells have been incriminated in severe asthma. Indeed, recent studies have reported increased levels of TNF- α specifically associated with severe and refractory phenotypes of the disease [2–4]. A skewed programming of CD4⁺ T cells toward Th2 or Th17 phenotype seems to be a primary cause of asthma and other immune dysfunctions of the airway. The most recently discovered member of the IL-1 family is IL-33 (IL-F11) [5]. IL-33 is emerging as a new regulator of immune responses and inflammatory vascular diseases. Recent studies emphasize the importance of IL-33 in amplifying innate immunity and possibly serving as an “alarmin” to activate the immune system following cell aggression and damage [6–8]. IL-33 has been identified

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History

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as a ligand for the orphan receptor, ST2 (IL-1RL1) [9]. Binding of IL33 to ST2 stimulates target cells, thereby activating nuclear factor- κ B and mitogen-activated protein kinase pathways [5].

Interest on IL33 in asthma derives from genetic studies using genome-wide association studies, describing strong associations between ST2 single nucleotide polymorphisms (SNPs), SNPs flanking IL33 gene and asthma [10–12]. Moreover, a major role of IL33 on Th2 allergic inflammation has been identified. ST2 receptor is considered as the best marker to characterize Th2 cells [13], mediating important effector Th2 functions [14]. It is expressed on numerous cells involved in Th2 pathways, such as eosinophils, mastocytes, T lymphocytes, NKT cells and ILC2 [type-2 innate lymphoid cells (ILC2s)] [12,15]. IL-33 is a chemo-attractant for Th2 cells, and its presence during Th2 cells activation greatly enhances their production of IL-5 and IL-13 [16]. Moreover, IL-33 promotes eosinophil survival and increases the cell surface expression of intracellular adhesion molecule-1 [13]. In addition, IL-33 may contribute to the development of irreversible structural changes in asthma by favoring the airway recruitment and profibrotic function of fibrocytes during episodes of allergen-induced asthma exacerbations [17]. Least but not last, IL33 is constitutively expressed on airway smooth muscle cells [18], endothelial cells and

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epithelium which is considered as a major actor of asthmatic inflammation [8,19].

ST2 is known to exist in a transmembrane form (ST2L) and is also alternatively spliced to produce a secreted soluble form (sST2) [20]. The soluble form acts as a regulator of IL33 pathway, as it binds free IL33 and inhibits its activity [8]. Elevated serum sST2 levels have been reported in sepsis [21], acute myocardial infarction [22] and asthma exacerbations [23], correlating with disease severity. IL-33 mRNA expression appears likely to be influenced by the inflammatory milieu, especially after activation with TNF- α and IL-1 β [5]. Indeed, TNF- α increases the expression of IL33 mRNA on bronchial muscle cells [18]. Of note, production of TNF α has been reported during the early phase of acute asthma models [24].

We therefore decided to analyze the level of IL-33 and the soluble form of its receptor ST2 in young patients with asthma in serum and induced sputum (IS). Furthermore, we assessed IL-33 and TNF- α mRNA expressions in IS cells.

Materials and methods

Patients

Thirty-seven children with well-defined asthma (20 with moderate asthma and 17 with mild asthma) were recruited from the Department of Paediatrics and Respiratory Disease, Homeostasis and Cell Dysfunction Unit Research, Abderrahman Mami Hospital (Ariana, Tunisia), using the criteria set by the Global Initiative for Asthma guidelines [25]. Detailed definitions of the inclusion and exclusion criteria for the enrolment of asthmatic subjects were reported previously [26,27]. The protocols for the study were reviewed and approved by the ethics committees of the Abderrahman Mami Hospital, and informed consent was obtained from all participating subjects. Children with mild and moderate asthma were treated with regular inhaled glucocorticoids (ICS), but variable daily doses were required to control the symptoms (at the time of evaluation daily ICS dose ranged 200–800 μ g/day). Nine children with exacerbation were excluded from the study. Only patients whose asthma was controlled were retained. Table 1 describes the characteristics of the asthmatic patients in the study: 54.05% had moderate asthma and 45.95% had mild asthma. Twenty two healthy control children (female/male: 10/12) were recruited (aged

4–12 years, means 6.5) with no respiratory nor allergic manifestations.

Sputum induction

Sputum was induced as described previously [26,27]. Patients inhaled hypertonic saline solution (3–5% NaCl) briefly after a premedication with 200 μ g of inhaled salbutamol. The collected sputum volume was measured, mixed with an equal volume of 0.1% dithiotreitol and then rocked at room temperature for 15 min. The resulting samples were subsequently filtered through a 0.42- μ m Millipore filter (Billerica, MA) and centrifuged at 1500 *g* for 10 min. The supernatants were immediately aliquoted and frozen at –70 °C until further analysis for IL-33 and sST2. Sputum cells were transferred to RNA extraction buffer.

Assay of human IL-33, sST2

Patients and healthy controls donated 10 mL of blood, which was collected in heparinized tubes. Peripheral blood mononuclear cells were isolated using Percoll separation (Amersham, Castle Hill, NSW, Australia). IL-33 and sST2 levels were measured in serum and IS supernatant by commercial sandwich ELISA (GenWay Biotech, San Diego, CA). In summary, an ELISA plate was coated with capture antibody (affinity-purified chicken anti-human IL-33 mAb) in 0.05 M carbonate–bicarbonate followed by blocking as we have recently reported [28]. Serum samples were then added followed by horseradish peroxidase (HRP)-conjugated secondary mAb. Tetramethyl benzidine was subsequently added to the reaction which was stopped by applying 2 M H₂SO₄. Optical density was measured by microtiter plate reader at 450 nm. Serum level of IL-33 was read off from a standard curve according to the manufacturer's instruction. Serum level of sST2 was measured by commercial sandwich ELISA (Mannan-binding lectin, BioPorto Diagnostic. Grusbakken; Gentofte, Denmark). The capture antibody and the HRP-conjugated detection antibody involve two anti-human ST2 mAbs that recognize two different epitopes. The sensitivity of the immunoassays for IL-33 and sST2 are 0.7 and 0.032 ng/ml, respectively.

Determination of IL-33 and TNF- α mRNA levels in IS

Total cellular RNA from IS cells was isolated by using RNeasy micro kit extraction columns (Qiagen, Chatsworth, CA, USA). RNA was reverse-transcribed using oligo(dT)12–18 primer in the presence of RNAGuard (both from GE Healthcare, Velizy Villacoublay, France) and Superscript II reverse transcriptase (Invitrogen, Life Technologies Products HTDS. 2035 Carthage Aeroport, Tunis; Tunisia). Quantification of mRNA levels was performed by real-time PCR using the LightCycler PCR (Roche Diagnostics, Indianapolis, Ind.) and QuantitTect SYBR Green PCR master mix (Qiagen, Chatsworth, CA, USA). Relative quantification of the PCR products was achieved using a standard curve, which was obtained by simultaneously amplifying samples with serial dilutions of the amplicon. The results were analyzed with LightCycler software, version 3.5.3 (Roche Diagnostics). Both melting curve analysis and agarose gel electrophoresis were used to assess the specificity of the

Table 1. Patients' characteristics.

Characteristics	Data
Number of subjects	37
Gender (female/male)	12/25
Age (range), years	9.8 (5–16)
Positive skin test results (%)	67.58
FVC (%)	85.42 (58–112)
FEV ₁ (%)	92.64 (53–122)
FEV ₁ /FVC	0.9 (0.79–1)
Passive smoking	47.5%
Severity (%)	
Mild	45.95
Moderate	54.05

FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity.

amplification products as well as primer-dimer formation. Quantification of mRNA encoding endogenous 40S ribosomal protein S9 was performed as a housekeeping gene and used to correct for variations in cDNA content among samples. The ribosomal protein S9 recently ranked in the top 100 best housekeeping genes. Primers were designed to span an intron and the sequences were as follows (5'-3'): CAAAGAAGTTTGCCCCATGT (IL-33 sense) and AAGG CAAAGCACTCCACAGT (IL-33 antisense); TGCTGACGC TTGATGAGAAG (S9 sense) and CGCAGAGAGAAG TCGATGTG (S9 antisense); and TCAGCCTCTTCTCC (TNF- α sense) and TCAGCTTGAGGGTT (TNF- α antisense; Invitrogen). PCR amplicons were 180, 307 and 199 bp in length for IL-33, S9 and TNF- α , respectively. Unless specified, data were expressed as the calculated ratio of the values obtained for IL-33 versus the values of the housekeeping gene S9. Data were expressed as a fold increase compared with the ratio (IL-33/S9) obtained for the vehicle-treated or TNF- α -treated cells.

Statistical analysis

The significance of differences was assessed by ANOVA test, followed by Bonferroni's multiple comparisons test for normally distributed values. Data with non Gaussian distribution were analyzed by Kruskal-Wallis test, followed by Dunn's multiple comparisons test. Paired samples were analyzed using the Wilcoxon matched pairs test. A difference between groups was considered to be significant if $p < 0.05$. We used Spearman's rank correlation coefficient to assess relationships between two variables. Correlation was considered to be significant if $p < 0.05$.

Results

Serum and IS levels of IL-33 and sST2

IL-33 and sST2 were measured in IS of young asthmatic patients (20 with moderate asthma and 17 with mild asthma)

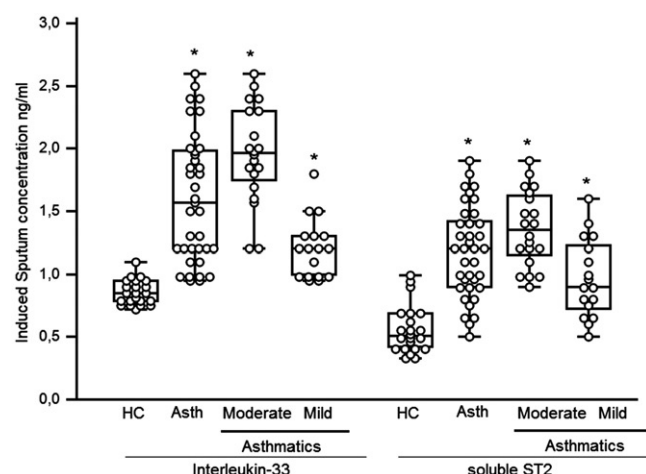


Figure 1. IL-33 and soluble ST2 (sST2) levels in induced sputum (IS) from young asthmatic patients (Asth) and age- and sex-matched normal controls. In the box whisker plots, the line inside each box is the median, upper box border represents the 75th quartile, lower box border represents the 25th quartile and whiskers represent the range. Data for individual value are presented. (*) $p = 0.0001$, significantly different from healthy controls.

and age- and sex-matched healthy controls (Figure 1). IL-33 level in IS was found to be higher in asthmatics than in healthy controls (1.60 ± 0.50 versus 0.86 ± 0.09 ng/ml; $p = 0.0001$). IL-33 was increased (1.96 ± 0.39 ng/ml) in patients with moderate asthma compared with patients with mild asthma (1.20 ± 0.23 ng/ml; $p = 0.0001$; Figure 1).

In the same way, sST2 levels in IS were significantly different between asthmatics (1.17 ± 0.36 ng/ml) and healthy controls (0.56 ± 0.19 ng/ml; $p = 0.0001$). Higher levels were observed in moderate cases (1.36 ± 0.29 ng/ml) than in mild asthma cases (0.96 ± 0.31 ng/ml; $p = 0.003$) (Figure 1). The difference between the three groups was significant ($p = 0.0001$).

Serum from young asthmatics expressed high levels of IL-33 (moderate: 1.87 ± 0.71 ng/ml; mild: 1.12 ± 0.29 ng/ml) and sST2 (moderate: 1.91 ± 0.79 ng/ml; mild: 1.45 ± 0.49 ng/ml) when compared with healthy controls (IL-33: 0.92 ± 0.14 ng/ml; sST2: 0.51 ± 0.20 ng/ml; Figure 2).

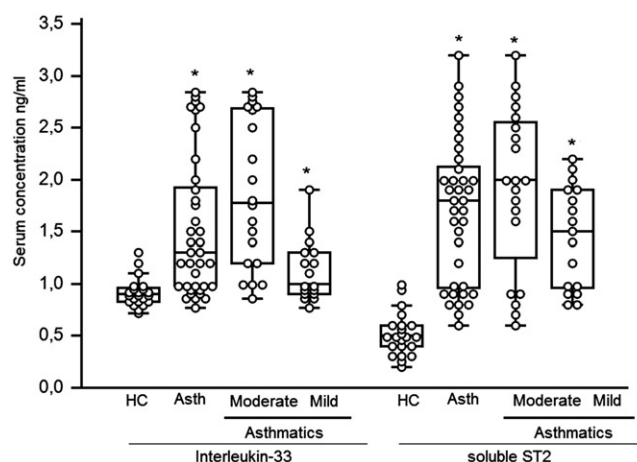


Figure 2. Soluble ST2 (sST2) and IL-33 levels in serum from young asthmatic patients (Asth) and age- and sex-matched healthy controls (HC). In the box whisker plots, the line inside each box is the median, upper box border represents the 75th quartile, lower box border represents the 25th quartile and whiskers represent the range. Data for individual value are presented. (*) $p = 0.0001$, significantly different from healthy controls. Low significant difference for sST2 was observed between mild and moderate asthmatics ($p = 0.044$).

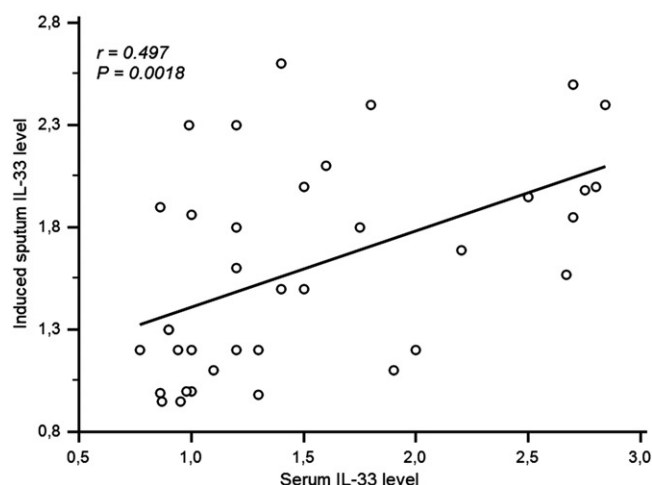


Figure 3. Correlation between serum and induced sputum (IS) IL-33 levels. Pearson correlation test showed an association between IL-33 levels in serum and IS ($r = 0.497$; $p = 0.0018$).

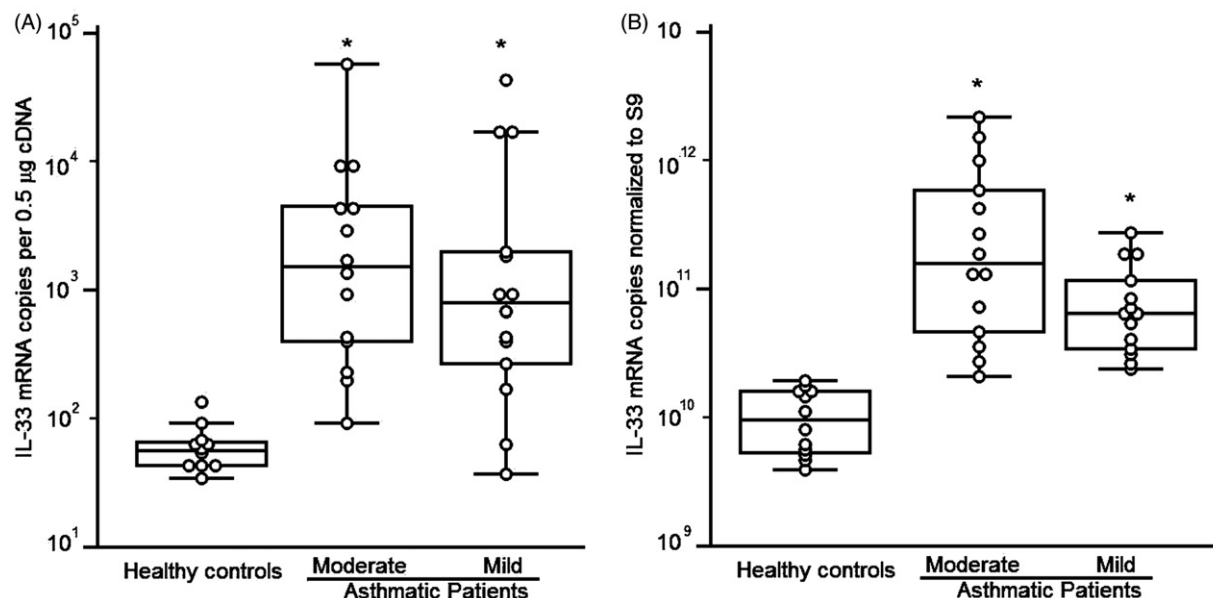


Figure 4. IL-33 gene expression in induced sputum (IS) from young patients with asthma. IS from a cohort of subjects with asthma and healthy controls were processed for RT-PCR gene expression profiling as described in the “Materials and methods” section. (A) Raw IL-33 transcript levels are presented for moderate ($n = 14$) and mild ($n = 14$) asthma compared with healthy subjects ($n = 12$). (B) IL-33 mRNA data are normalized to ribosomal protein S9 mRNA and expressed as relative IL-33/S9 mRNA ratios; moderate and mild asthmatics versus healthy controls are shown. (A and B) In the box whisker plots, the line inside each box is the median, upper box border represents the 75th quartile, the lower box border represents the 25th quartile and whiskers represent the range. Data for individual value are presented. (*) $p = 0.0001$ compared with healthy controls.

A significant correlation was observed between serum and IS IL-33 levels ($r = 0.497$; $p = 0.0018$) in asthmatic patients (Figure 3). An equally significant correlation was found between sST2 levels in serum and IS ($r = 0.430$; $p = 0.0078$). No correlations were found in healthy controls ($r = 0.216$; $p = 0.334$).

Increase in IL-33 gene expression from asthmatics compared with healthy subjects

IS cells from asthmatic subjects (14 moderate and 14 mild asthmatics) and from 12 healthy control subjects were used to assess IL-33 mRNA expression by quantitative PCR. As shown in Figure 4(A), increased levels of IL-33 mRNA transcripts were detected in patients compared with healthy controls ($p = 0.0001$). When these IL-33 transcript data were normalized to the expression of 40S ribosomal protein S9 mRNA (Figure 4B), IS from asthmatics still exhibited increased IL-33/S9 mRNA ratios compared with those from healthy controls ($p = 0.0022$). There were no significant differences in IL-33 expression ($p = 0.493$) or IL-33/S9 expression ($p = 0.446$) between the two groups of asthmatics based on the severity.

Increased IL-33 expression in asthmatics correlates with TNF- α mRNA levels

There is now evidence for an increased expression of TNF- α in asthma [2]. IS mRNA used to determine whether TNF- α mRNA, along with IL-33 mRNA, was expressed in moderate and mild asthmatics compared with healthy controls. Samples from patients with moderate and mild asthma displayed significantly higher TNF- α /S9 mRNA levels compared with healthy controls ($p = 0.001$) (Figure 5). Interestingly, a decrease in the TNF- α /S9 mRNA ratio was found between patients with moderate and mild asthma ($p = 0.002$).

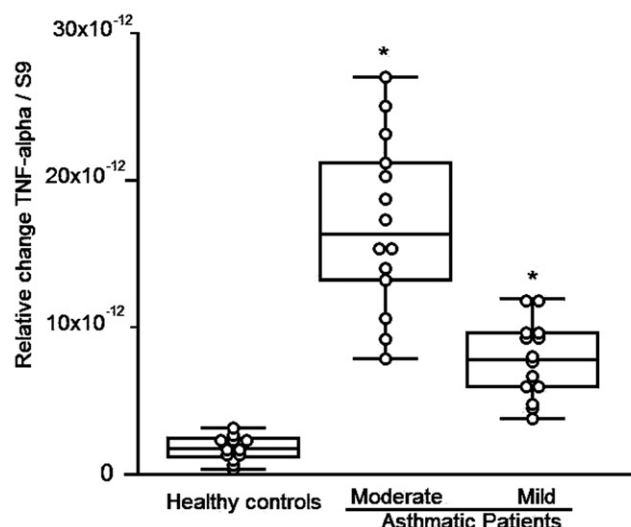


Figure 5. TNF- α gene expression in induced sputum processed for RT-PCR. TNF- α /S9 mRNA ratios obtained from 14 patients with moderate and 14 patients with mild asthma were compared with 12 healthy subjects. In the box whisker plots, the line inside each box is the median, upper box border represents the 75th quartile, the lower box border represents the 25th quartile and whiskers represent the range. Data for individual value are presented. (*) $p = 0.001$ compared with healthy subjects.

A significant correlation ($r = 0.772$, $p = 0.0001$) was found between TNF- α /S9 and IL-33/S9 mRNA ratios detected in all asthmatic patients (Figure 6).

Discussion

The initial description of IL-33 as an IL-1-type cytokine associated with Th2 inflammation, both *in vitro* and *in vivo* [5], raises questions about its possible involvement in Th2-mediated airway diseases, such as asthma. Our patients

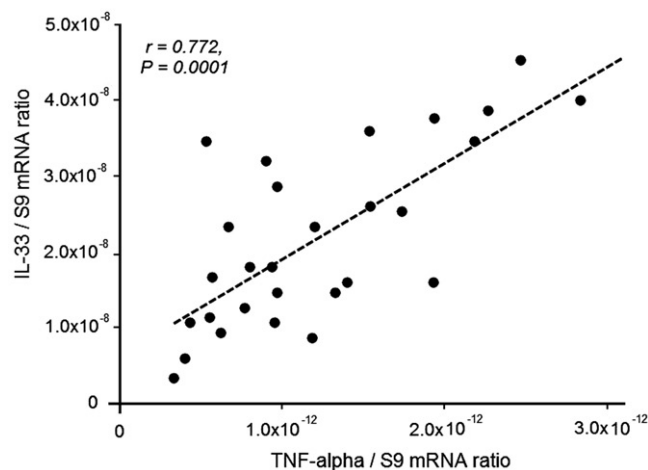


Figure 6. TNF- α /S9 and IL-33/S9 mRNA ratios from induced sputum from all asthmatic subjects are analyzed for correlation ($r=0.772$; $p=0.0001$). Data from control subjects are excluded from the Spearman correlation analysis.

were studied at distance from any exacerbation. Our study reveals elevated levels of IL-33 and sST2 in IS and serum of asthmatic children. Moreover, IL33 gene was highly expressed in patients IS, along with TNF- α gene. These data confirm the involvement of IL33 pathways in pediatric asthma. Genetic variation in the IL33 and ST2 genes had identified IL-33-induced signaling through ST2 as one of the central pathways in asthmatic patients [10]. SNPs in the ST2 locus have been associated with childhood asthma and airway function [8].

IL33 is produced early during the allergic inflammatory process and induces Th2 activation through ILC2s, which express IL-13 and IL-4 and promote type-2 immune activity [16,29,30]. These ILC2s are present in the lung and contribute to airway inflammation and airway hyper-responsiveness in response to IL-33 [16]. IL-33 enhances type-2 responses after allergenic but also non-allergenic stimulation suggesting a role of this cytokine in non-atopic asthma [31]. In this way, we found elevated levels of IL-33 whatever the atopic status of our patients.

Serum sST2 level has previously been demonstrated to be elevated in adult asthmatic patients [32]. It was substantially higher during acute asthma compared with levels after the attack and was inversely related to eosinophil counts [32]. Such a result was in accordance with our finding of increased presence of sST2 in asthmatic children, although our patients were not in exacerbation. sST2 acts as a negative regulator of Th2 cytokine production by the IL-33 signaling [33]. Delivery of sST2 depresses the allergic responses, and its over-expression suppresses allergic-induced bronchial inflammation [34]. Our data confirm sST2 involvement in the asthmatic airways, as it was increased both in serum and IS. These results suggest a local production of sST2 in the airways, acting probably as an inhibitor of IL33-induced inflammation.

Furthermore, IL33 and sST2 were associated with severity. Indeed, we observed higher levels of IL-33 and sST2 in IS in moderate cases compared with mild asthmatics. Similarly, data from adult patients showed that release of IL33 in the airway lumen increased along with asthma severity [18].

Data from Préfontaine et al. proposed IL-33 as a novel inflammatory marker of asthma [18,19]. Our study provides evidence that IL-33 gene expression is increased in IS from asthmatic subjects. In contrast to the current view that inflammatory mediators arise mainly from hematopoietic cells (lymphocytes and granulocytes), findings from Préfontaine et al. [19] on IL-33 in human asthma support the increasing role of structural cells in the production of pro-Th2 cytokines. Our data, reporting high levels of IL33, together with IL-33 mRNA expression in IS suggest its production from cells localized in the bronchial mucosa. A significant correlation was observed between IL-33 levels in the peripheral circulation and IS. In adults, IL-33 was highly expressed in bronchoalveolar lavage (BAL) fluid and in endobronchial bronchial biopsies [1,19]. Significant levels of IL-33 were measured in BAL from moderate, but not mild, asthmatics compared with normal controls. IL-33 was highly expressed on airway epithelial cells and smooth muscle cells compared with those of healthy control subjects [18,19]. Primary production of IL-33 by epithelial cells suggests a mechanism whereby the respiratory tract can generate a “danger signal” that drives a subsequent Th2 immune response. This “alarmin” effect would be the initial trigger of asthma in response to an insult from an infectious or environmental agent like allergen that results in necrosis of the epithelial cell layer [8,13]. Recently, Hardman et al. [30] reported that type-2 pneumocytes constitute the major source of IL-33 upon allergic lung inflammation following exposure to ovalbumin or fungal extract. Binding of IL-33 to the membrane-bound IL-1 type receptor ST2 (ST2L) on immune cells of the lung parenchyma triggers the production of Th2 cytokines and mediators that are described as important contributors to the clinical features of asthma [35,36]. IL-33 could act as a feedback cytokine released by structural cells under certain inflammatory conditions (including TNF- α), activating and recruiting inflammatory cells in the asthmatic airways.

IL-33 can enhance both Th1-type and Th-2-type responses, a feature of severe asthma [8]. During asthmatic exacerbations, IL-33 release may interfere with several inflammatory pathways, through Th2 response activation with type-2 cytokines production together with enhancing IL-6, IL-8 and IFN- γ production enhancement [8,16,31]. Our report depicted an increased expression of the cytokine IL-33 in sera and IS from young human asthma, along with TNF- α expression. Samples from our patients with moderate and even mild asthma displayed significantly higher TNF- α /S9 mRNA levels compared with healthy controls. Genetic studies have demonstrated some relationship between TNF- α gene polymorphisms and asthma [37]. Biopsy-derived cDNA samples from subjects with moderate and severe asthma displayed significantly higher TNF- α levels compared with healthy controls [19,24]. Up-regulated TNF- α expression has been detected in alveolar macrophages in asthma [24], particularly in overweight/obese patients who are difficult to control [38]. After activation with TNF- α and IL-1, IL-33 is expressed at higher levels in human endobronchial biopsies from asthmatic subjects compared with healthy controls [19]. Both TNF- α and IFN- γ up-regulated significantly IL-33 gene expression in ASMC, in a synergistic way [19]. A significant

correlation was found between both TNF- α /S9 and IL-33/S9 mRNA ratios detected in all asthmatic patients. No correlation was found in healthy controls. In bronchial biopsies of asthmatic subjects, a significant correlation was found between TNF- α and IL-33/S9 mRNA ratios.

Our data point at bronchial cells as a source of IL-33 in asthma. Considering the biological functions of IL-33, this study supports the potential role of this cytokine in childhood asthma. Its early involvement during Th2 responses together with its association with severity suggests new treatment possibility [39].

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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