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ORIGINAL ARTICLE

Pleural Effusion IL-33/sST2 Levels and Effects of Low and High IL-33/sST2 Levels on Human Mesothelial Cell Adhesion and Migration

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Abstract— Interleukin 33 (IL-33) is an alarmin with multiple roles in immunity and cell homeostasis, highly expressed in barrier sites, acting *via* the suppression of tumorigenicity 2 receptor (ST2). Production of IL-33 and soluble ST2 (sST2), a decoy receptor for IL-33, has been implicated in several pulmonary diseases, but both have been scarcely investigated in pleural diseases. The aim of this study was to determine the levels of IL-33 and sST2 in transudative (TrPEs), malignant (MPEs), and parapneumonic (PPEs) pleural effusions (PEs) and investigate the effect of PE fluids from each group with low and high IL-33/sST2 levels on MeT-5A cell adhesion and migration. IL-33 and sST2 pleural fluid levels were similar among TrPEs, MPEs, and PPEs. However, a significant correlation was found between IL-33 and LDH and in sST2 levels with lymphocyte counts in TrPEs. Additionally, in MPEs the levels of IL-33 correlated with the levels of sST2 and with the red blood cell counts. Furthermore, incubation of MeT-5A cells with MPEs and PPEs bearing low or high levels of IL-33/sST2 yielded significant differential effects on MeT-5A cell adhesion and migration. In MPEs, high IL-33/sST2 levels led to increased adhesion and migration of MeT-5A cells, while in PPEs the effect was the opposite, while no effect in both cell phenotypes was determined for TrPEs. These results reveal a clinical context dependent effect of the IL-33/sST2 axis in PEs.

KEY WORDS: cell adhesion; cell migration; interleukin 33; mesothelial cells; pleural effusion; sST2.

INTRODUCTION

Interleukin 33 (IL-33) is an alarmin with multiple roles in immunity and cell homeostasis, by regulating transcription and acting as a pro-inflammatory cytokine in a paracrine fashion when released or secreted [1–3]. IL-33 is constitutively highly expressed in airway and alveolar epithelial cells, smooth muscle cells, fibroblasts, endothelial cells, and several leucocytes at barrier sites

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exposed to damaging stimuli or pathogens [2, 3]. IL-33 levels are elevated in the serum of patients with asthma, acute lung injury, COPD, infection, lung cancer and in the serum and bronchoalveolar lavage in experimental models of the above-mentioned diseases [1].

IL-33 binds to its receptor, suppression of tumorigenicity 2 (ST2), both in its transmembrane (ST2L) or soluble (sST2) form, expressed by a variety of cells [4]. The binding of IL-33 to ST2L leads to cytokine secretion, cell activation, and differentiation, while sST2 acts as a decoy receptor that binds IL-33 and blocks intracellular signaling and induced pro-inflammatory responses [4]. Increased sST2 levels in serum and bronchoalveolar lavage have been described in lung cancer, idiopathic pulmonary fibrosis, asthma, COPD, and acute lung injury [5–9]. Increased concentrations of serum sST2 serve to attenuate the systemic effects of IL-33, constituting an indirect measure of IL-33 activity [9].

The IL-33/sST2 axis has been scarcely investigated in the pathophysiology of pleural diseases. IL-33 has been reported to be significantly elevated in the pleural fluid from patients with tuberculous pleural effusion (TPE) as compared to pleural effusions of transudative (TrPE), malignant (MPE), and parapneumonic (PPE) origin [10–12]. On the contrary, in eosinophilic pleural effusions due to primary spontaneous pneumothorax (an acute inflammatory condition of the pleural space), the pleural fluid levels of IL-33 were significantly elevated as compared to subjects that served as controls (having turberculosis, cancer, pneumonia, and hyperhidrosis) [13]. On the other hand, regarding sST2, one study reported significantly higher levels in MPEs as compared to TPEs and no difference compared to PPEs [10], while another study reported significantly higher levels of MPEs as compared to TPEs and TrPEs [14]. There is only one study from the ones cited above that have concomitantly assessed IL-33 and sST2 levels in the pleural fluids of the same underlying causes [10]. In this study, TPE had higher levels of IL-33 than TrPEs, MPEs, and PPEs (that had comparable levels of IL-33), while sST2 levels were significantly higher only in the pleural fluids of MPE patients as compared to TPE ones and no different in the PPE patients. However, the levels of sST2 in TrPE patients were not assessed in this study. Moreover, no correlation was reported between IL-33 and sST2 levels in the pleural fluids of the patients included neither in the study as a whole nor in pathology-based subgroup analysis. From the above evidence, the role of the IL-33/sST2 axis in pleural effusions is not clear. In cases of TPE, the IL-33 levels are significantly increased as compared to TrPE, MPE, and PPE, while in cases of acute inflammatory non-infectious pleural effusions (as in the case of spontaneous hydropneumothorax) the IL-33 pleural fluid levels are higher than in all other underlying pathologies.

Tissue repair in the pleura involves focal remesothelialization by adhesion of floating mesothelial cells to the site of injury and subsequent cell migration for wound closure [15]. Pleural mesothelial cells therefore have a critical role in the resolution of pleural injury through wound healing mechanisms. When these mechanisms fail, pleural fibrosis, a debilitating disorder for the patients, occurs [16–18]. The effects of the levels of the IL-33/sST2 axis in pleural mesothelial cells in phenotypes pertinent to wound healing processes that follow the resolution of the local inflammation are unknown. IL-33 has a significant role in mucosal wound healing, while sST2 has also been reported to affect cell motility in metastatic cancer cells in the pleura and the peritoneum [19, 20]. We opted to investigate the effects of the IL-33/sST2 axis in cell adhesion and cell migration of pleural mesothelial cells in a pleural pathology-dependent context. For this we measured the levels of both IL-33 and sST2 in pleural effusions that based on the literature would not differ significantly, that is in TrPEs, MPEs, and PPEs, excluding TPEs and spontaneous hydropneumothoraces. After assessing the levels of IL-33/sST2 in the respective pleural effusions, we compared the effects of the pleural fluids from each group that had concomitantly high and low IL-33/sST2 levels in terms of cell adhesion and cell migration in human MeT-5A cells. Our results indicate a differential effect of the IL-33/sST2 axis depending on the pleural effusion underlying pathology.

MATERIALS AND METHODS

Subjects and Collection of Pleural Fluids. The study included 40 patients (14 with TrPE, 13 with MPE, and 13 with PPE) hospitalized at the University Hospital of Larissa from January 2016 to March 2016. All transudates (TrPEs) and exudates (MPEs and PPEs) were defined according to Light's criteria [21]. TrPEs were diagnosed based on the presence of congestive heart failure. MPEs were diagnosed when cancer cells were present in pleural fluid or pleural biopsies. PPEs were diagnosed based on the presence of pulmonary infection associated with acute febrile illness, pulmonary infiltrates, purulent sputum, and response to antibiotic treatment or identification of the organism in the pleural fluid. The pleural fluid (PF) samples were collected in sterile tubes by thoracentesis under ultrasound guidance. For each PF sample, half was sent to

the Biochemical Department of the University Hospital of Larissa for routine biochemical tests [glucose, total protein, albumin, lactate dehydrogenase (LDH), adenosine deaminase (ADA), pH, total white blood cell counts, red blood cell (RBC) counts, neutrophil and lymphocyte counts) as part of the clinical diagnosis algorithm, and the rest was centrifuged (1200×*g* for 10 min) within 60 min of collection and stored at − 80 °C until further use. The University Hospital of Larissa Ethical Committee approved all procedures and written informed consent was obtained from all individual patients participating in the study. The demographics and the pleural fluid characteristics of the study participants according to the pleural effusion type are presented in Table 1.

PF Measurements of IL-33 and sST2. The measurement of IL-33 and sST2 levels was performed by ELISA (#ab119547 and #ab100563, respectively; Abcam, UK) (detection range for IL-33: 7.8–500 pg/mL, and sST2: 1.65–1200 pg/mL), as per the manufacturer's instructions. The limit of detection (LOD) was 1.05 pg/mL for IL-33 and 148.24 pg/mL for sST2. Each sample was analyzed in duplicate. Appropriate dilutions were done in samples when measuring sST2. In samples that IL-33 or sST2 had values below the LOD, they were assigned a value equal to the LOD for comparison purposes among groups. The levels of IL-33 and sST2 in the PF were tested for potential differences in the different pleural effusions and their levels were correlated. The effect of age (65 years of age as a cutoff) and gender (males/females) was also assessed for potential differences in IL-33 and sST2 levels [23, 24]. Additionally, the levels of IL-33 and sST2 were tested for correlation with parameters of the standard PF biochemistry used in Light's criteria (total protein and LDH levels), and total and differential PF white blood cell counts as well as RBC counts in order to speculate for potential relationships among them [22].

In Vitro Assays Using Pleural Fluids with High and Low IL-33/sST2 Levels. After the determination of IL-33 and sST2 levels in the PFs, they were stratified based on the median value in each pleural effusion category into High and Low IL-33/sST2 samples. IL-33 median values for TrPE, MPE, and PPE were 2.19, 3.14, and 4.08 pg/mL, respectively, while for sST2 were 1583, 1317, and 1109 pg/mL, respectively. Representative samples from each group (TrPE, MPE, and PPE) were used for *in vitro* assays of cell adhesion and cell migration (wound healing assay). The High TrPE sample had values of 9.48 and 6544.11 pg/mL for IL-33 and sST2, respectively, while the Low TrPE sample had values of 1.05 and 148.24 pg/mL for IL-33 and sST2, respectively. The High MPE sample had values of 7.25 and 9282.19 pg/mL for IL-33 and sST2, respectively, while the Low MPE sample had values of 1.21 and 518.29 pg/mL for IL-33 and sST2, respectively. The High PPE sample had values of 6.39 and 1589.14 pg/mL for IL-33 and sST2, respectively, while the Low PPE sample had values of 1.05 and 296.99 pg/mL for IL-33 and sST2, respectively. Therefore, these samples referring to the *in vitro* assays will be called High IL-33/sST2 and Low IL-33/sST2.

Cell Adhesion Assay. Cell adhesion assay was performed in 48-well plates pre-coated with plasma-fibronectin (FN, 50 μg/mL; #341631, Calbiochem, USA). MeT-5A cells (human benign immortalized pleural mesothelial cells) were grown in 10% FBS-RPMI cell medium supplemented with 1% Penicillin/Streptomycin, 0.5% w/v Plasmocin, and 1% L-glutamine at 37 °C in a humidified CO_2 incubator. Prior to experiments, cells were synchronized by 24-h serum starvation. Synchronized cells were suspended in filter sterilized high- and low-concentration IL-33/sST2 pleural fluids (0.25×10^5 cells in 100 μL of pleural fluid). Cells were allowed to attach for 90 min at 37 °C in a humidified CO_2 incubator and subsequently unattached cells were aspirated by 3× warm PBS washes. The attached cells were fixed with 4% PFA for 10 min followed by staining with 0.5% crystal violet for 10 min. Excess stain was removed by washing the plate in running tap water. Plates were air-dried overnight, followed by de-staining with 10% acetic acid. The de-stain solution was subject to OD measurements in a plate reader at 570 nm [25, 26]. Each experiment had a replicate of three.

Wound Healing (Migration) Assay. Assays were carried out in 48-well plates pre-coated with FN. The backside of the plates were pre-marked with a straight black line as a ruler in order ensure that microphotographs at each time point would be taken from the identical points of each well. In total 1.35×10^5 MeT-5A cells were seeded in each well and allowed to form a monolayer within 24 h. Upon 100% confluence, the cells were serum starved for 24 h followed by warm sterile PBS wash. The monolayer was scratched in a straight line with a 20-μL sterile pipette tip (vertical to the black ruler line) followed by washing with warm PBS and treatment with the appropriate pleural fluids. Monolayers wound borders were imaged at ×100 magnification at time $t = 0$ h (immediately after the scratch). The plates were then incubated for 8 h. Experiments were terminated by pleural fluid aspiration, fixation with 4% PFA for 10 min, and followed by image capture at $t = 8$ h. The wound edges at both time points were annotated with dotted white lines in the microphotographs. The area of the wound was measured in ImageJ using the polygon tool.

Table 1. Demographics, and Pleural Fluid Characteristics of Study Participants as Whole and According to Type of Pleural Effusion

	All patients $n = 40$	TPE $n = 14$	MPE $n = 13$	PPE $n = 13$	p value
Gender					
Males	28 (70)	9 (64.3)	8 (61.5)	11 (84.6)	0.371
Females	12 (30)	5 (35.7)	5 (38.5)	2 (15.4)	0.371
Age	67.7 ± 15.0	72.8 ± 13.1	69.2 ± 10.4	60.8 ± 18.7	0.063
Pleural fluid					
Glu (g/dL)	92.6 ± 48.3	121.6 ± 28.1	73.4 ± 49.3	73.5 ± 53.0*	0.016
Total protein (g/dL)	3.8 ± 1.3	2.4 ± 0.7	4.7 ± 0.6*	4.5 ± 1.0*	< 0.001
Albumin (g/dL)	2.1 ± 0.7	1.5 ± 0.6*	2.8 ± 0.4*	2.1 ± 0.5*	< 0.001
LDH (IU/L)	822 ± 1187	118 ± 39	952 ± 1069	1450 ± 1551*	< 0.001**
ADA (U/L)	11.0 ± 8.7	4.1 ± 1.8	12.2 ± 6*	17.4 ± 10.4*	< 0.001
pH	7.4 ± 0.1	7.4 ± 0.0	7.4 ± 0.1	7.3 ± 0.1	0.162†
N cells/mm^3	2823 ± 5277	1152 ± 1210	4338 ± 8543	3107 ± 3176	0.065†
N RBCs/mm^3	73,624 ± 183,329	15,574 ± 30,497	135,720 ± 273635*	74,148 ± 158,824	0.014†
N neutro/mm^3	1188 ± 1779	339 ± 475	1090 ± 1431	2200 ± 2455*	0.005†
N lymph/mm^3	1429 ± 4275	712 ± 696	2901 ± 7414	728 ± 649	0.303
Serum					
Total protein (g/dL)	6.7 ± 0.7	6.4 ± 0.5	7.1 ± 0.4*	6.7 ± 0.9	0.027
Albumin (g/dL)	2.1 ± 0.7	3.2 ± 0.7	3.6 ± 0.5	2.7 ± 0.7*	0.006
LDH (IU/L)	273 ± 154	278 ± 128	279 ± 81	284 ± 226	0.951
Type of MPEs					
Metastatic Ca	2 (15.4)				
Lung cancer	9 (69.2)				
Mesothelioma	1 (7.7)				
Lymphoma	1 (7.7)				

Data are expressed as mean ± SD or as frequencies (percentages). Percentage represents % within the type of pleural effusion
Abbreviations: *ADA* adenosine deaminase, *Glu* glucose, *LDH* lactate dehydrogenase, *lymph* lymphocytes, *N* number, *neutro* neutrophils, *RBCs* red blood cells
*Bonferroni; † Kruskal-Wallis H test

For comparisons, the migration index (MI) formula MI = ($A_0 - A_8)/A_0$ was used; A_0 represents the wound area measured at time 0 and A_8 represents wound area at the time of termination of the experiment [26]. Experiments with clinical samples had a replicate of three. In all experiments imaging of the wounded areas was performed at ×100 magnification. Microscopy was done with a Nikon Eclipse TS100 microscope that was interfaced with a Leica CCD camera and imaging was controlled with Leica application suite LAS version 3.6.

Statistical Analyses. Statistical analyses were performed with GraphPad Prism version 6 for Mac. Quantitative variables were presented as frequencies or mean ± standard deviation (SD). Outliers were detected by the Grubbs test. Comparisons of frequencies were performed with χ^2 test. Normality of the data was assessed with the D'Agostino-Pearson Omnibus normality test where appropriate. For data with small size number ($n = 3$), the similarity of the mean and median values was compared. Parametric data comparing two groups were analyzed with unpaired t test while non-parametric data were analyzed with the Mann-Whitney U test. Parametric data comparing three or more groups were analyzed with one-way ANOVA and Tukey's multiple comparisons test, while non-parametric were analyzed with Kruskal-Wallis test and Dunn's multiple comparison test. Spearman's correlation was used for correlation analysis. A value of $p < 0.05$ was considered significant.

RESULTS

Levels of IL-33 and Its Soluble Receptor sST2 Do Not Differ Significantly in TrPEs, MPEs, and PPEs. There were no differences in the fraction of samples in each pleural effusion category that IL-33 was found below the LOD (TrPE, 3/14; MPE, 1/13; PPE, 4/13) ($\chi^2 = 2.19$; $p = 0.33$). One value from the TrPE group was excluded as an outlier

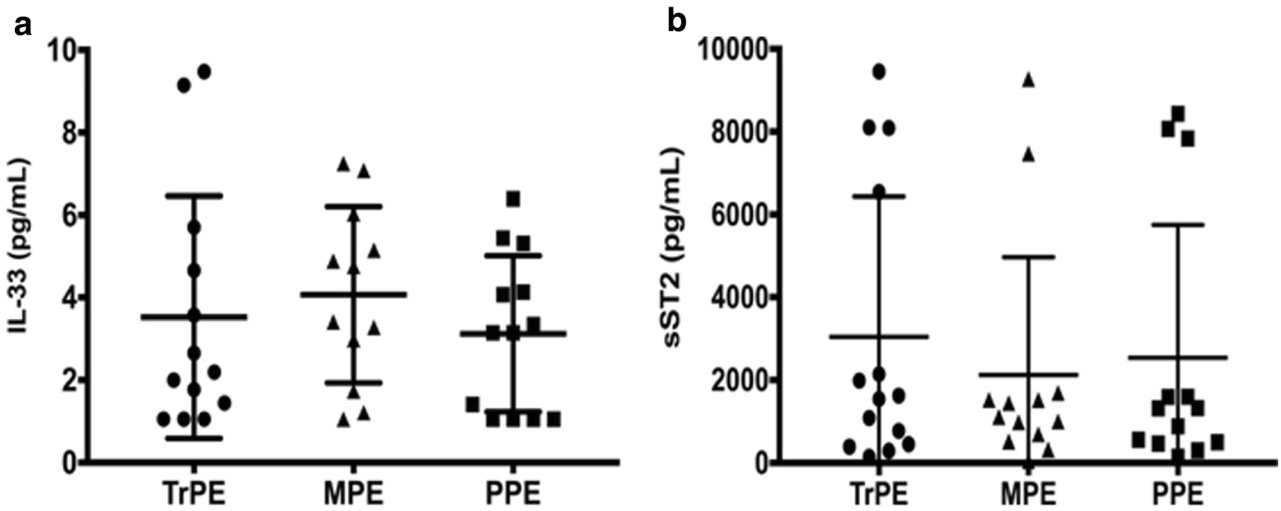

Fig. 1. **a** Pleural effusion levels of IL-33 and **b** sST2, as measured by ELISA in transudative (TrPE; $n = 14$), malignant (MPE; $n = 13$), and parapneumonic (PPE; $n = 13$) pleural effusions. No differences were found among groups in both cases. Values are presented as mean ± SD.

(113.42 pg/mL) and one value was excluded by the MPE group as an outlier (12.76 pg/mL). There were also no differences in the fraction of samples in each pleural effusion category that sST2 was found below the LOD (TrPE, 1/14; MPE, 1/13; PPE, 1/13) ($\chi^2 = 0.004$; $p = 0.99$). Comparisons of IL-33 concentrations showed no significant differences among TrPEs (3.52 ± 2.94 pg/mL), MPEs (4.07 ± 2.14 pg/mL), and PPEs (3.12 ± 1.89 pg/mL) (Fig. 1a). Similarly, there were no significant differences in sST2 levels among TrPEs (3044 ± 3390 pg/mL), MPEs (1536 ± 1936 pg/mL), and PPEs (2538 ± 3215 pg/mL) (Fig. 1b). No significant differences were found between the levels of IL-33 or sST2 when patients were stratified for age (above and below 65 years of age) and gender.

Correlations Between IL-33 and sST2 in Each Pleural Effusion Group and of IL-33 and sST2 with Standard Pleural Fluid Biochemistry and Cytology. The levels of IL-33 and sST2 were correlated in each pleural effusion patient group, while in order to investigate potential cellular sources of IL-33 and sST2 their levels were correlated with LDH (an intracellular marker that implies cellular necrosis), with white blood cells and RBCs. In TrPEs, IL-33 and sST2 did not correlate ($p = 0.98$); however, the concentration of IL-33 positively correlated with pleural fluid LDH ($r = 0.71$; $p = 0.008$) (Fig. 2a), while the levels of sST2 positively correlated with pleural fluid lymphocyte count ($r = 0.64$; $p = 0.017$) (Fig. 2b).

As shown in Fig. 3 a in MPEs, the levels of IL-33 significantly correlated with the levels of sST2 with a correlation coefficient $r = 0.71$ ($p = 0.012$). Furthermore, IL-33 levels positively correlated with the pleural fluid RBC count ($r = 0.68$; $p = 0.024$) (Fig. 3b). No correlations were detected in PPEs neither between IL-33 and sST2 ($p = 0.84$) nor among them and routine pleural fluid biochemical and cytological indices.

In Vitro Effects of Low and High IL-33/sST2 PFs Within and Among the Three Patient Groups on MeT-5A Cell Adhesion. PF samples from two patients per group with IL-33/sST2 levels below and over the median value in each group as previously defined by ELISA (concentrations described in Materials and Methods section) were used for the MeT-5A cell adhesion experiments. MeT-5A cells in complete 10% FBS-RPMI served as controls. As shown in Fig. 4 a, PFs from TrPE patients with either Low or High IL-33/sST2 concentrations resulted in significantly decreased cell adhesion of MeT-5A cells as compared to Control (0.11 ± 0.01 and 0.12 ± 0.02 *vs.* 0.18 ± 0.01, respectively; $p < 0.01$ in both cases). No differences in cell adhesion were observed between Low and High IL-33/sST2 samples ($p > 0.05$). In MPE samples, the cell adhesion of MeT-5A cells was significantly decreased in the Low IL-33/sST2 (0.03 ± 0.01) as compared to Control (0.18 ± 0.01; $p < 0.001$) and to High IL-33/sST2 (0.16 ± 0.02; $p < 0.001$). No differences occurred between the High IL-33/sST2 and Control ($p > 0.05$; Fig. 4b). Finally, in PPEs the cell adhesion of MeT-5A cells was significantly decreased both in Low and High IL-33/sST2 as compared to Control (0.08 ± 0.01 and 0.04 ± 0.01 *vs.* 0.18 ± 0.01,

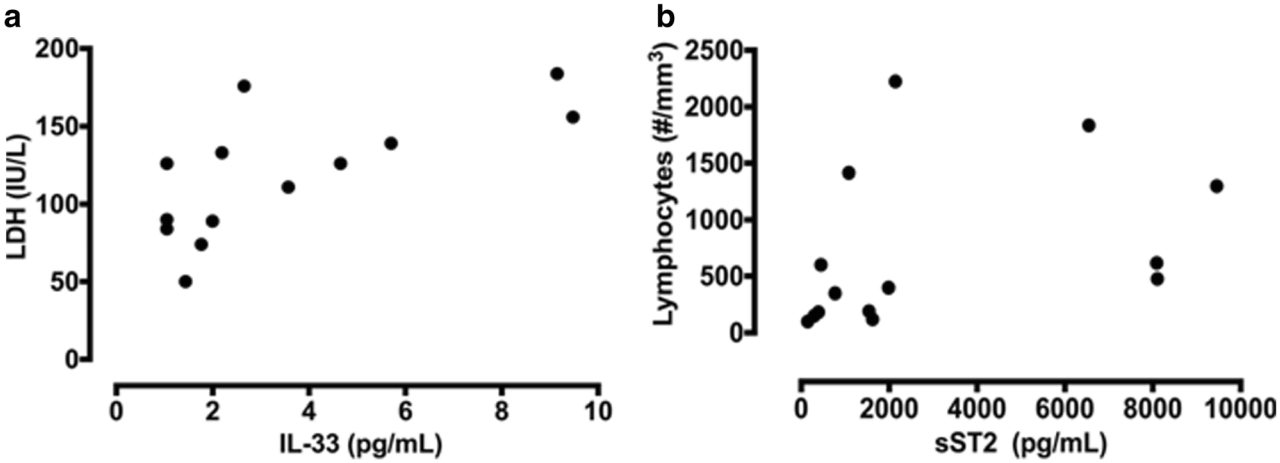

Fig. 2. **a** Correlation of pleural fluid IL-33 levels and LDH levels in TrPEs ($r = 0.71$; $p = 0.008$). **b** Correlation of pleural fluid lymphocytes counts and sST2 levels in TrPEs ($r = 0.64$; $p = 0.017$).

respectively; $p < 0.001$), while in High IL-33/sST2 cell adhesion was significantly decreased compared to Low IL-33/sST2 ($p < 0.001$; Fig. 4c).

An additional analysis was performed where MeT-5A cell adhesion in Low and High IL-33/sST2 was compared among the three groups of pleural effusions. As shown in Fig. 4 d, samples with Low IL-33/sST2 pleural effusions resulted in significantly reduced cell adhesion in MPE and PPE samples as compared to TrPE ones (0.03 ± 0.01 and 0.08 ± 0.01 *vs.* 0.11 ± 0.01, respectively; $p < 0.001$ and $p < 0.05$), while adhesion in MPEs was significantly decreased as compared to PPEs ($p < 0.001$). On the other hand, as shown in Fig. 4 e, TrPE samples with High IL-33/sST2 pleural effusions (0.12 ± 0.02) resulted in significantly less adhesion compared to MPE (0.16 ± 0.02; $p < 0.05$) and significantly increased adhesion compared to PPE (0.04 ± 0.01; $p < 0.01$), while PPE samples had significantly decreased adhesion compared to MPEs ($p < 0.001$).

In Vitro Effects of Low and High IL-33/sST2 PFs Within and Among the Three Patient Groups on MeT-5A Cell Migration. The same PF samples from two patients per group with IL-33/sST2 levels below and over the median value in each group that were used for cell adhesion experiments were also used for the MeT-5A cell migration experiments by the wound healing assay. MeT-5A cells in

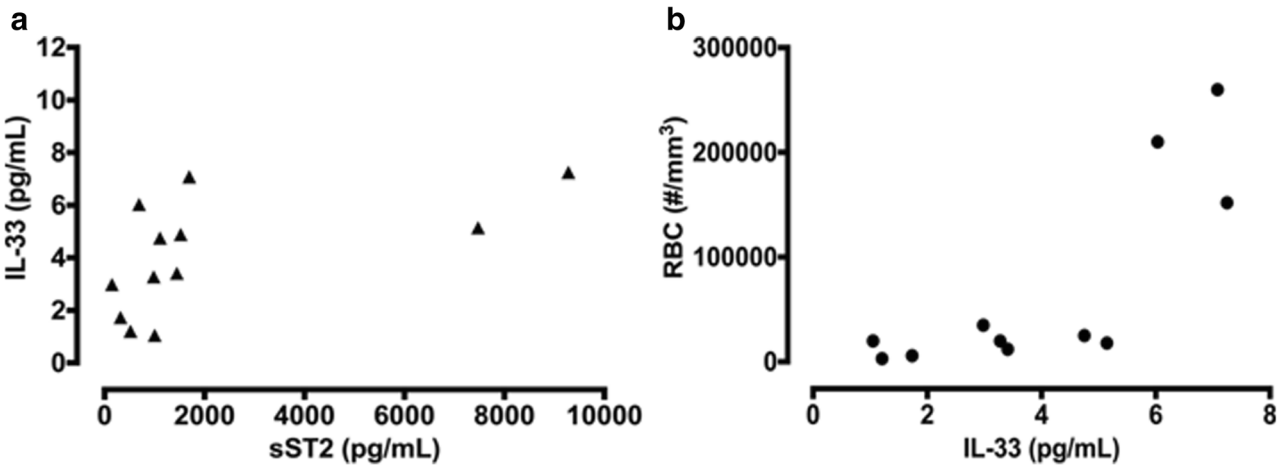

Fig. 3. **a** Correlation of pleural fluid IL-33 levels and sST2 levels in MPEs ($r = 0.71$; $p = 0.012$). **b** Correlation of pleural IL-33 levels and red blood cell counts in MPEs ($r = 0.68$; $p = 0.024$).

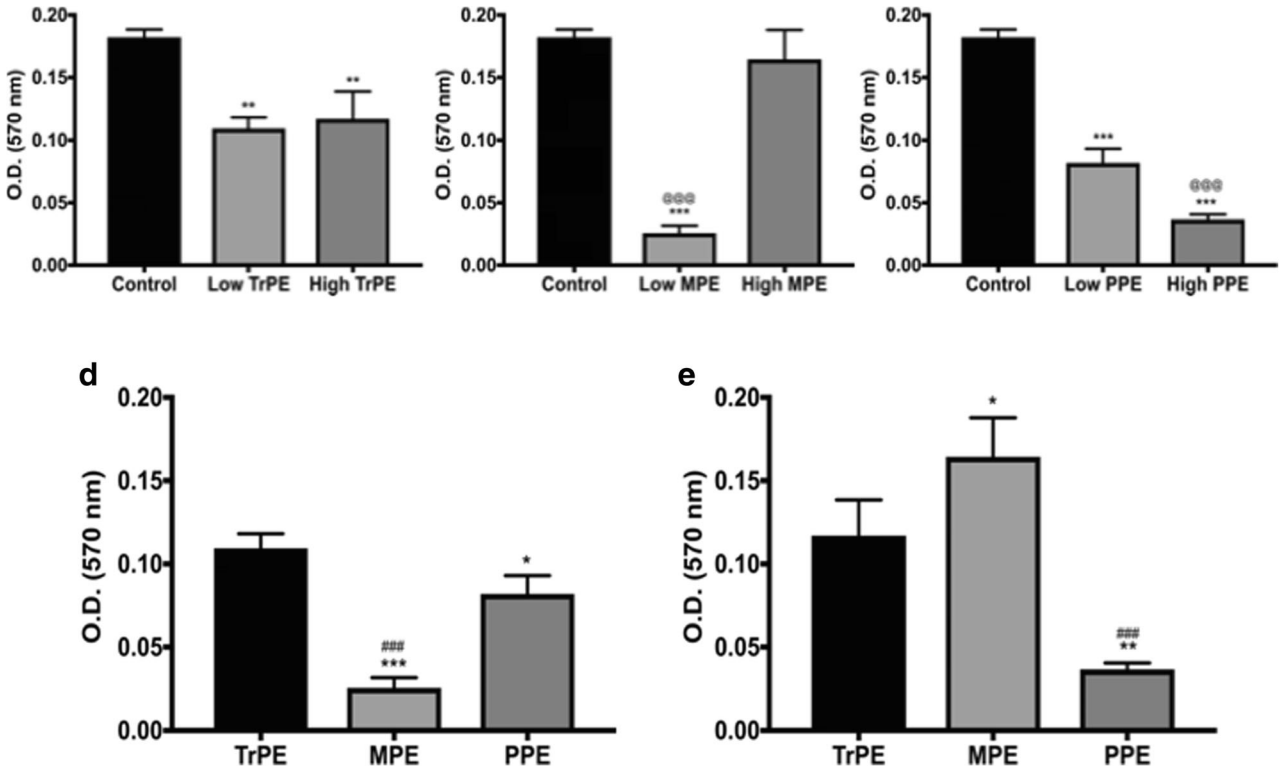

Fig. 4. **a** Differences in cell adhesion expressed in O.D. values in TrPEs, **b** in MPEs and **c** PPEs. $^{**}p < 0.01$ and $^{***}p < 0.001$ *versus* Control. $^{@@@}p < 0.001$ *versus* High MPE (in **b**) and Low PPE (in **c**). **d** Differences in cell adhesion expressed in O.D. values among Low IL-33/sST2 levels in TrPEs, MPEs, and PPEs. $^{*}p < 0.05$ and $^{***}p < 0.001$ *versus* TrPE, $^{###}p < 0.001$ *versus* PPE. **e** Differences in cell adhesion expressed in O.D. values among High IL-33/sST2 levels in TrPEs, MPEs, and PPEs. $^{*}p < 0.05$ and $^{**}p < 0.01$ *versus* TrPE, $^{###}p < 0.001$ *versus* MPE.

complete 10% FBS-RPMI served as Controls. As shown in Fig. 5 a, PFs from TrPE patients with High IL-33/sST2 concentrations resulted in significantly decreased cell migration of MeT-5A cells as compared to Control (0.14 ± 0.07 *vs.* 0.24 ± 0.03; $p < 0.05$). No differences in cell migration occurred between Low (0.17 ± 0.04) and High IL-33/sST2 samples as well as between Control and Low IL-33/sST2 ($p > 0.05$ in both cases). In Fig. 5 b, representative microphotographs of the TrPE group wound healing assay are shown. In MPE samples, the cell migration of MeT-5A cells was significantly decreased in the Low IL-33/sST2 (0.02 ± 0.02) as compared to Control (0.24 ± 0.03; $p < 0.001$) and to High IL-33/sST2 (0.21 ± 0.07; $p < 0.001$). No differences occurred between the High IL-33/sST2 and Control ($p > 0.05$; Fig. 5c). In Fig. 5 d, representative microphotographs of the MPE group wound healing assay are shown. Finally, in PPEs the cell migration of MeT-5A cells was significantly decreased in High IL-33/sST2 as compared to Control and Low IL-33/sST2 (0.01 ± 0.02 *vs.* 0.24 ± 0.03 and 0.22 ± 0.01, respectively; $p < 0.001$ in both comparisons). No differences occurred between the Control and the Low IL-33/sST2 cell migration (Fig. 5e). In Fig. 5 f, representative microphotographs of the PPE group wound healing assay are shown.

Furthermore, another analysis was done where MeT-5A cell migration in Low and High IL-33/sST2 was compared among the three groups of pleural effusions. As shown in Fig. 6 a, samples with Low IL-33/sST2 pleural effusions resulted in significantly reduced cell adhesion in MPE samples as compared to TrPE and PPE ones (0.02 ± 0.02 *vs.* 0.17 ± 0.04 and 0.22 ± 0.01, respectively; $p < 0.01$ and $p < 0.001$). In Fig. 6 b, representative microphotographs of the Low IL-33/sST2 wound healing assay are shown. On the other hand, as shown in Fig. 6 c, PPE samples with High IL-33/sST2 pleural effusions (0.01 ± 0.02) resulted in significantly decreased migration compared to MPE (0.21 ± 0.07; $p < 0.05$). In

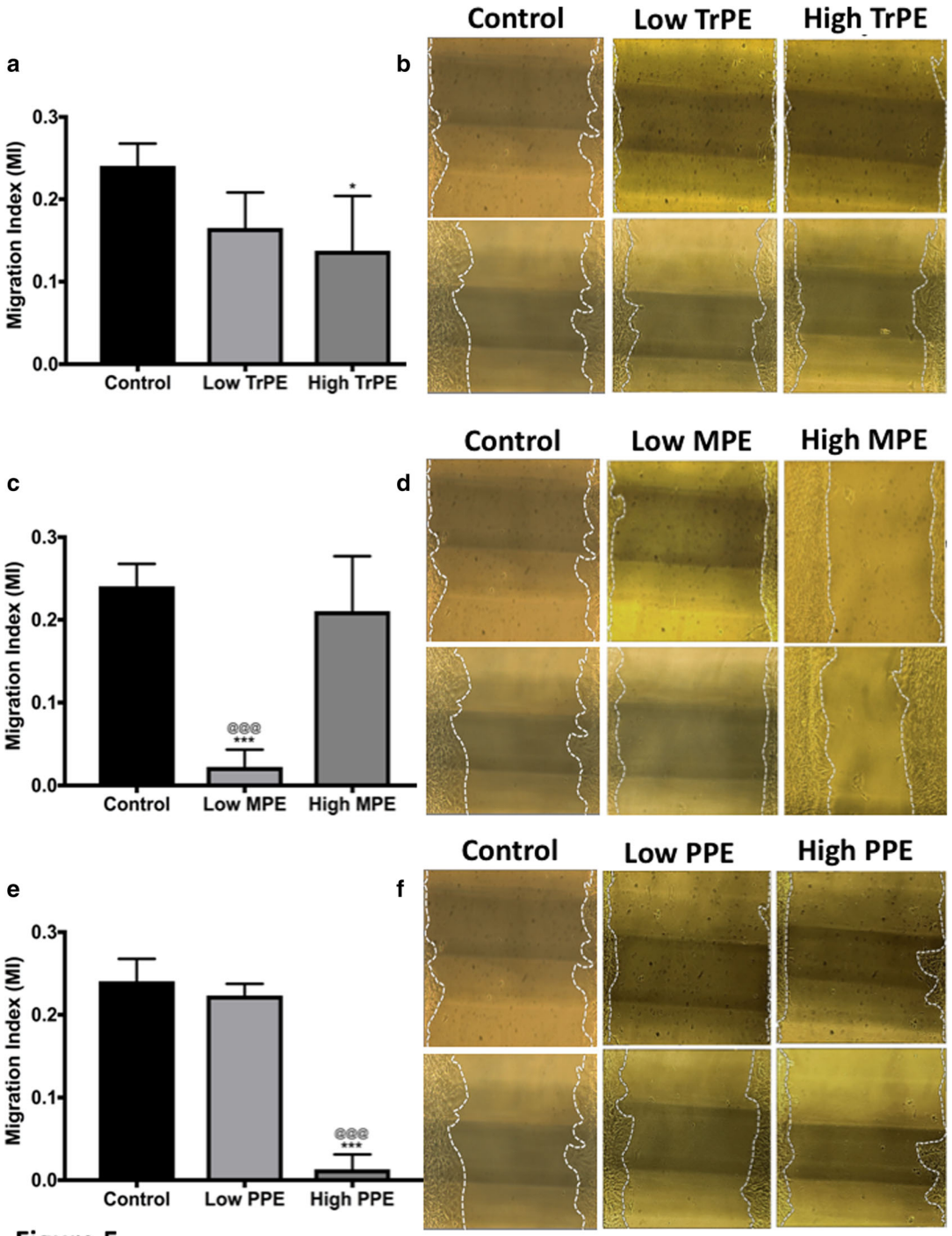

Figure 5

Fig. 5. **a** Differences in cell migration expressed as M.I. in Controls, Low IL-33/sST2, and High IL-33/sST2 TrPEs and **b** representative microphotographs of wound healing assay in Controls, Low IL-33/sST2, and High IL-33/sST2 TrPEs. $*p < 0.05$ *versus* Control. **c** Differences in cell migration expressed as M.I. in Controls, Low IL-33/sST2, and High IL-33/sST2 MPEs and **d** representative microphotographs of wound healing assay in Controls, Low IL-33/sST2, and High IL-33/sST2 MPEs. $***p < 0.001$ *versus* Control, $^{@@@}p < 0.001$ *versus* High MPE. **e** Differences in cell migration expressed as M.I. in Controls, Low IL-33/sST2, and High IL-33/sST2 PPEs and **f** representative microphotographs of wound healing assay in Controls, Low IL-33/sST2, and High IL-33/sST2 PPEs. $***p < 0.001$ *versus* Control, $^{@@@}p < 0.001$ *versus* Low PPE.

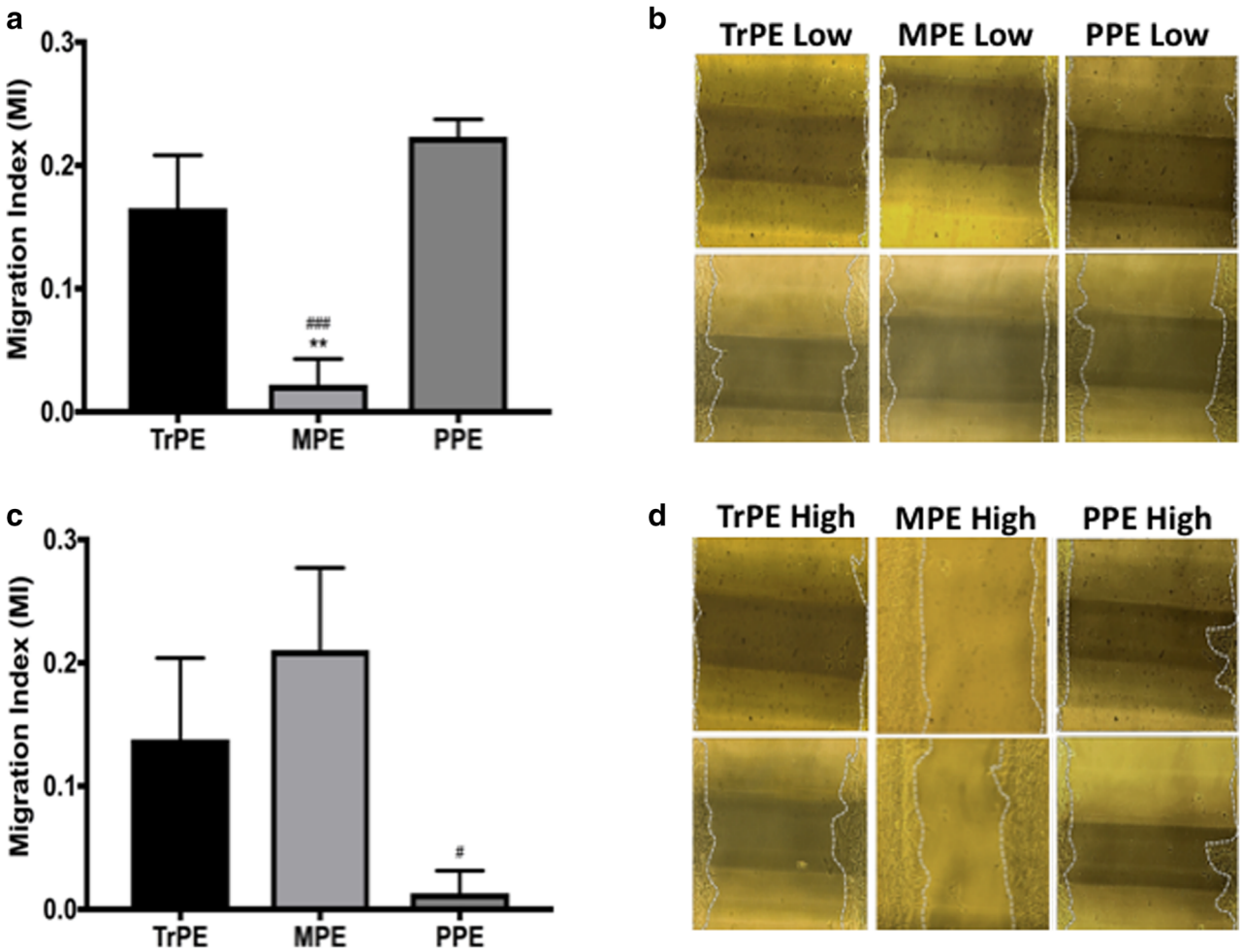

Fig. 6. **a** Differences in cell migration expressed as M.I. among Low IL-33/sST2 levels in TrPEs, MPEs, and PPEs and **b** representative microphotographs of wound healing assay of the corresponding groups. $^{**}p < 0.01$ *versus* TrPEs and $^{###}p < 0.001$ *versus* Low IL-33/sST2 PPE. **c** Differences in cell migration expressed as M.I. among High IL-33/sST2 levels in TrPEs, MPEs, and PPEs and **d** representative microphotographs of wound healing assay of the corresponding groups. $^{#}p < 0.05$ *versus* High IL-33/sST2 MPE.

Fig. 6 d, representative microphotographs of the Low IL-33/sST2 wound healing assay are shown.

DISCUSSION

In this report we investigated the levels of IL-33/sST2 in pleural fluids of patients with TrPEs, MPEs, and PPEs and furthermore assessed the effects that pleural fluid with high and low IL-33/sST2 levels from each group had in cell adhesion and cell migration of the human pleural mesothelial cell line MeT-5A. We found that both the pleural fluid levels of IL-33 and sST2 did not differ significantly among the three groups that we assessed. Only one previous study had measured both molecules in pleural fluids of patients finding that there were no significant differences among IL-33 levels of pleural fluids from TrPE, MPE, and PPE patients that is in accordance with our findings [10]. In the same study, the pleural fluid levels of sST2 were measured only in MPE and PPE patients (but not in TrPE ones as in ours) where no difference was found in accordance with our findings. In another study investigating differences in the levels of IL-33 between TPE (tuberculous pleural effusion) and non-TPE patients, the three groups that are presented in our study were grouped as one (the non-TPE) so no comparison with our results

can be made [11]. One more study had reported comparison of the levels of IL-33 between TPE and MPE patients alone finding significantly higher levels in the first group, however in our study no TPE patients were included [12]. As far as sST2 measurements in pleural fluids are concerned, there is one study that compared patients with pleural effusion due to spontaneous pneumothorax and a control group that included patients with pleural effusions due to tuberculosis, cancer, pneumonia, and hyperhydrosis. The first group had significantly higher pleural fluids levels of sST2 than the second; however, no comparisons can be done with our results because no individual values were provided for the control group that included patients comparable with our groups [13]. Finally, in another study the levels of pleural sST2 were significantly higher in MPE compared to TPE patients that was not the case in our study where we found no differences [14]. In the aforementioned study however, the TrPE group had significantly less patients than our study ($n = 5$ *versus* $n = 14$) and potentially this could explain the difference in our findings. One interesting point in our study that deserves further investigation was the fact that in all groups a fraction of patients had substantially high levels of sST2, although it could not be explained by any correlation with our biochemical and cytological data.

However, we identified some interesting correlations of both IL-33 and sST2 with biochemical and cytological indices in the pleural fluid of the patients of TrPE and MPE groups. In TrPE patients, the levels of pleural fluid IL-33 positively correlated with pleural fluid LDH, while sST2 levels positively correlated with the number of lymphocytes in the pleural fluid. LDH is measured as an index of local cell necrosis and thus tissue injury within the pleural cavity, and since we did not find any correlation of IL-33 with total and differential white blood cell counts in the pleural fluid, the most likely source would be from pleural mesothelial cells undergoing necrosis leading to release of IL-33 in the fluid [21]. In TrPEs, we also found a positive correlation of sST2 levels with the pleural fluid lymphocyte count, suggesting that the source of sST2 in this group of patients is mainly lymphocytes. Although we did not perform a lymphocyte type subset analysis, it is likely that the source of sST2 in our study were CD4 T cells since this finding was also reported in a previous study [14].

In pleural fluids of MPE patients, we found a positive correlation between IL-33 and sST2 levels. As previously mentioned, there is only one study that has assessed IL-33 and sST2 in patients with MPEs; however, the authors do not provide any information on testing a potential correlation of the two molecules in their study [10]. Interestingly, there is one study that has reported a significant positive correlation between IL-33 and sST2 levels in the peritoneal fluid of women undergoing surgery due to endometriosis [27]. Both malignancy and endometriosis are conditions characterized by chronic inflammation, therefore potentially in such chronic conditions there are balanced signaling events in the IL-33/sST2 axis mediated both by the mesothelial cells and inflammatory cells that are present. In connection to the above, it has been shown in a mouse model that mast cells mediate the formation of MPEs [28]. Furthermore, the authors showed that in MPEs from patient samples the count of mast cells was significantly elevated compared to benign effusions [28]. Mast cells are a known source of IL-33 and also IL-33 can activate mast cells, while IL-33 primed mast cells produce significant amounts of sST2 [4]. Thus, IL-33/sST2 signaling promotes the activation and maturation of mast cells that in turn can propagate the formation of the MPE. Nonetheless, this is an observation that requires further detailed investigation where mast cells will be also evaluated in MPEs along with IL-33/sST2 levels. Furthermore, a significant positive correlation of pleural fluid IL-33 levels and RBC count was found in MPE patients, indicating that RBCs may be a possible cellular source of IL-33 in MPEs. In support of this, recent studies have shown that IL-33 is released after RBCs hemolysis [29]. An elevated RBC count is a frequent finding in MPEs as compared to benign pleural effusions (like TrPEs and PPEs), and has been demonstrated as an independent prognostic factor of malignancy when undiagnosed large or massive pleural effusions are investigated clinically [30]. Previous studies demonstrated that IL-33 augments vascular endothelial growth factor (VEGF) expression that is markedly increased in MPEs [31–33]. Both VEGF and IL-33 have been shown to impair the endothelial barrier of the pleural capillaries and this increased vascular permeability could account for the increased RBC counts found in MPEs [17]. Therefore, it is possible that IL-33 due to increased RBCs functions as a loop of sustained vascular permeability by itself and indirectly by inducing the expression of vasoactive factors like VEGF that further increase the supply of RBCs in the pleural cavity [17, 34]. One limitation of our study with respect to MPEs effects on MeT-5A cells is that potentially MPEs that are metastatic (due to lung cancer, breast cancer, *etc.*) as compared to MPEs due to primary tumor of the pleura (mesothelioma) could have differential effects on cell phenotypes.

In sites of pleural injury, mesothelial cells are involved in remesothelialization by focal adhesion of free-floating mesothelial cells and subsequent 2D migration that

leads to tissue repair [15, 35]. Given that in all pleural effusion there are various degrees of tissue injury and subsequent wound healing, we aimed at elucidating whether there would be differences in the wound repair mechanisms mentioned above in MeT-5A cells, a human pleural mesothelial cell line, incubated with pleural fluids from TrPE, MPE, and PPE patients that had high or low levels of IL-33/sST2. There are no studies in the literature shedding light in these processes using actual pleural fluids from patients. There is only one study that has tested the biological effects of pleural fluids from patients with malignant pleural mesothelioma (MPM) on cell proliferation and cell migration of human MPM cell lines [36]. In pilot experiments of this study, pleural fluids from MPM patients (not diluted with cell media) induced an increase in the cell proliferation after 72 h of incubation that was similar to that induced by cell culture medium with 10% FCS and significantly higher than serum free cell medium. In the subsequent experiments [with 30% (v/v) diluted MPM fluids], the pleural fluid from MPM patients induced significant increases in 5 different human MPM cell lines in terms of cell proliferation and cell migration as seen by the wound healing assay. In our study we used MeT-5A cells, a human benign pleural mesothelial cell line, and incubated them with pleural fluids from TrPE, MPE, and PPE patients with known amounts of IL-33/sST2 concentrations. In each group the pleural fluids used had high levels of IL-33/sST2 and low levels of IL-33/sST2 (that is above and below the median values measured in each group) so as to compare their effects in a cell adhesion assay and a cell migration assay (wound healing assay). In all *in vitro* studies, we used undiluted pleural fluids in order to maintain the fluid's native environment unaffected, while as controls we used complete cell culture medium with 10% FBS. For both phenotypes tested, we used time points (90 min for cell adhesion and 8 h for cell migration) that were established for MeT-5A in our laboratory as published previously [25, 26]. In MeT-5A cells incubated with TrPE fluids, there were no differences in the cell adhesion towards fibronectin substrate when comparing high and low in IL-33/sST2 fluid levels while both groups had significantly less adhesion when compared with controls. In MPEs, Controls did not differ significantly than High IL-33/sST2 fluids, while Low IL-33/sST2 fluids led to significantly less adhesion than both Controls and High IL-33/sST2 fluids. On the contrary in PPEs, Low IL-33/sST2 fluids led to significant decrease in cell adhesion as compared to Controls and High IL-33/sST2 fluids, and moreover Low IL-33/sST2 fluids led to significant decrease as compared to High IL-33/sST2 fluids. Furthermore, we conducted a comparison within the group of Low IL-33/sST2 fluids and found that both MPEs and PPEs lead to significantly less cell adhesion of MeT-5A cells and also that MPE fluids result in significantly lower cell adhesion than PPE fluids. Comparing the three different patient groups in fluids with High IL-33/sST2 fluids, we found that in MPE fluids the cell adhesion in significantly higher than TrPE and PPE fluids and that in PPE fluids the adhesion is significantly less than TrPE fluids. Importantly, we had the same results in the wound healing assay in MeT-5A cells when we tested Low and High IL-33/sST2 fluids within each pathological condition as well as when we compared the Low and High IL-33/sST2 fluids among the three groups, TrPEs, MPEs, and PPEs.

These data are suggestive of a potential role for IL-33/sST2 in pleural mesothelial cell adhesion and migration that dependents on the underlying disease and concentration levels. Therefore, IL-33/sST2 along with other disease specific cytokines and growth factors present in the fluid lead to either increased or decreased mesothelial cell adhesion and migration, differentiated by the etiology of the pleural effusion and the concentration of the axis components. There are no similar studies that we could compare our results with. However, there are some data in the literature in other cell types that can aid in the understanding of our findings. In human coronary artery and human umbilical vein endothelial cells (HUVEC), IL-33 has been reported to induce the expression of intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), endothelial selectin, and monocyte chemoattractant protein-1 (MCP-1) [2, 17, 37, 38]. Although we did not assess any cell adhesion molecules at the molecular level, the above studies show that IL-33 can modulate molecules critical for adhesion in endothelial cells and that could potentially be the case in mesothelial cells as well. However, our experimental setup was more complicated as we had known concentrations of IL-33 and sST2 in our samples that were used for cell adhesion and migration assays. It is important to point out that in our clinical samples the composition of the fluids apart from the measured parameters is not known (as this would require extensive proteomics studies) and it is very likely that the combination of IL-33/sST2 with other, disease specific components present in each pleural fluid, can differentially alter cell adhesion molecules turnover as they differentially altered the phenotypes of cell adhesion and migration in MPEs and PPEs. Chronic inflammatory conditions (encountered in MPEs) and acute inflammatory conditions (encountered in PPEs) are known to lead to different pleural composition of biological factors and this

is probably why we detect the differences between the two groups in our study [39]. A very important piece of information is that recently focal adhesion kinase (FAK), a critical component for cell adhesion and migration, has been shown to enhance the expression of inflammatory molecules among which both IL-33 and sST2 in murine squamous cell carcinoma [40].

Furthermore regarding the role of IL-33 in cell migration, there is more information available. IL-33 has been shown to favor tissue recovery through modulation of epithelial repair in intestinal and cutaneous wound healing in human and murine models [20, 41]. Additionally, it was observed that sST2 receptor neutralization maintains wound inflammation, delays healing, and increases fibrosis [42, 43]. IL-33 has been shown to promote cell migration and invasion in glioma, head and neck squamous cell carcinoma, and gastric cancer, outlining its important role in tumor progression and metastases [20, 44–47]. On the other hand, inhibition of IL-33 in colon cancer cells resulted in reduced tumor growth, migration and colony formation *in vitro*, and smaller tumors *in vivo* [48]. Although in these studies the combination of IL-33/sST2 levels in terms of cell migration was not assessed as in our MPE group, we agree with the above findings since High IL-33/sST2 levels in the context of MPE increased cell migration of MeT-5A cells. In contrast to our MPE results, MeT-5A cells exposed to High IL-33 PPE fluid migrated significantly less than counterparts exposed to Low IL-33 PPE fluids. In connection to this observation in an experimental sepsis animal model, animals that did not recover had significantly more serum sST2 bound to IL-33 than those who recovered [49]. A possible explanation for our finding is that in cases of acute inflammation, IL-33 signaling aims initially at accumulating anti-inflammatory factors to eliminate the *pathogenic* initiator, than in tissue recovering. Importantly, IL-33 is identified as a "dual cytokine" by exerting its protective and deleterious effect, either as a pro- or anti-inflammatory or pro-fibrotic factor; therefore, the context of the microenvironment is critical [50].

Our report is the first to take into consideration the combination of IL-33/sST2 in mesothelial cell adhesion and migration under the influence of the native microenvironment that mesothelial cells reside in. Still it has an inherent limitation that we already commented above and relates to the fact other factors present in the pleural fluids may well influence the effects that we observed. More detailed investigation employing proteomic analysis prior to the *in vitro* experiments is needed to further elucidate our results.

In conclusion, the present study showed IL-33 and sST2 pleural fluid levels do not differ significantly among patients with TrPE, MPE, and PPE. In TrPEs, necrotic mesothelial cells were probably the source of IL-33 while sST2 was derived from pleural fluid lymphocytes. In MPEs, IL-33 pleural fluid levels significantly correlated with that of sST2 while IL-33 levels correlated with pleural fluid RBCs suggesting that they are the cellular source of IL-33. Pleural fluids of TrPE patients that had high and low IL-33/sST2 concentrations did not result in different rates of cell adhesion and cell migration. On the other hand, we report significant differences in cell adhesion and migration being greater under the influence of MPEs with concentrations high in IL-33/sST2 as compared to the ones with low IL-33/sST2. Finally in PPEs we observed the opposite effects, namely higher cell adhesion and migration of MeT-5A cells under the influence of pleural fluids low in IL-33/sST2 as compared to the ones with high IL-33/sST2. Our results warrant further investigation of the role of IL-33/sST2 axis in mesothelial pathophysiology.

COMPLIANCE WITH ETHICAL STANDARDS

All procedures performed involving human participants were in accordance with the ethical standards of the University Hospital of Larissa Ethical Committee and written informed consent was obtained from all individual patients participating in the study.

Conflict of Interest. The authors have no conflict of interests to declare.

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