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The Roles of IL-33 and TGF-B1 in the Pathogenesis of Stevens-Johnson Syndrome/ Toxic Epidermal Necrolysis: Potential Biomarkers for Disease Severity

Till SD*[1], Iqbal O[2,4], Dharan A[3], Campbell E[3], Bu P[4], Mudaliar K[2], Speiser J[2] and Bouchard C[4]

[1]Stritch School of Medicine, Loyola University Medical Center, USA
[2]Department of Pathology, Loyola University Medical Center, USA
[3]Department of Microbiology and Immunology, Loyola University Medical Center, USA
[4]Department of Ophthalmology, Loyola University Medical Center, USA

***Corresponding author:** Till SD, Stritch School of Medicine, Department of Pathology, Loyola University Medical Center, USA, Tel: 516-729-1346, E-mail: still@luc.edu

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Abstract

Stevens-Johnson syndrome/toxic epidermal necrolysis (SJS/TEN) is a disease continuum of potentially life threatening, severe allergic drug reactions which result in cellular apoptosis in the skin, mucous membranes, and ocular surface. The exact pathophysiologic mechanisms leading to this apoptosis is unclear but genetic predisposition and abnormal immune regulations play a role. Interleukin-33 (IL-33) and transforming growth factor beta 1 (TGF-β1) are known to be involved in many inflammatory and apoptotic diseases of the skin, but their roles in SJS/TEN remain unexplored. In the current study we use immunofluorescent microscopy and ELISA testing to investigate the presence of IL-33 and TGF-β1 in skin biopsy and plasma specimens of patients with SJS/TEN compared to lichen planus (LP) controls. The results of our study revealed statistically significant increased expression of IL-33 and TGF-β1 in the skin of SJS/TEN patients compared to LP controls ($p<0.0001$). Interestingly, IL-33 and TGF-β1 concentrations were mildly elevated in the plasma of SJS/TEN patients compared to LP controls, but these levels were not statistically significant ($p=0.4038$ and $p=0.3346$ respectively). We conclude that both IL-33 and TGF-β1 are key regulators in the pathogenesis of SJS/TEN in the skin, but their roles as systemic mediators warrant further investigation.

Keywords: Stevens-Johnson Syndrome; Pathogenesis; IL-33; TGF-Beta1; Immunofluorescence; Microscopy

Introduction

Stevens-Johnson syndrome/ toxic epidermal necrolysis (SJS/TEN) is a spectrum of drug-induced epidermal disorders. Over 200 different drugs have been implicated in the development of SJS/TEN, with the most common classes including antiepileptics, antibiotics, and NSAIDs [1-3]. SJS/TEN is characterized by blistering and erosion of the mucous membranes and skin, resulting from keratinocyte apoptosis [1,4]. This disease spectrum is classified by the percentage of body surface area (BSA) that undergoes epidermal detachment, with SJS defined to involve <10% BSA, SJS/TEN overlap involving 10-30% BSA, and TEN involving >30% BSA. SJS/TEN is rare with incidences ranging from 1 to 9.2 cases per million individuals [5,6]. It commonly affects female (increased F:M ratio), African American, Asian, Hispanic and Native American patients [6]. The prevalence of SJS/TEN in Asian patients is well established due to its link with HLA-A*0206, HAL-B*44:03, HLA-B*1502 and HLA-B*5801 genotypes [7]. The treatment of SJS/TEN has also placed a burden on US healthcare expenditures, with average length of stay ranging from 9.8 days for SJS patients to 16.2 days for TEN patients [6]. Various studies have noted average hospitalization costs between $21,000-$100,000 per stay for these patients [6,8].

Keratinocyte apoptosis associated with SJS/TEN also occurs in the ocular surface epithelium. This process is regulated by activated CD8+ T cells and Fas:Fas-L pathway dysregulation [7]. The incidence of ocular involvement in SJS/TEN is common with 50-88% of acute SJS cases involving the eyes [9]. The conjunctiva is most commonly affected, followed by the cornea, iris and ciliary body [6,10]. Ocular involvement in SJS/TEN is especially concerning because if left untreated (within the first week in the more severe cases) it can lead to severe sequelae including dry eye syndrome, chronic conjunctivitis, chronic ocular scarring, corneal erosions,

permanent vision loss, and blindness [10-13]. Yang *et al.* report that long-term cutaneous and ocular sequelae are common in SJS/TEN patients with reported incidence of 81% in SJS/TEN overlap and TEN patients versus 63% in SJS patients [12].

SJS/TEN is currently classified as a T cell mediated type IV hypersensitivity disorder, although recent studies suggest that natural killer (NK) cells, monocytes, macrophages, and dendritic cells also play a key role in disease pathogenesis [7,11,14]. Interleukin-15 (IL-15) may also play a key role in the pathogenesis of SJS/TEN because it activates both T cells and NK cells via the JAK-STAT and mTOR pathways [15,16]. Recent work by Su et al. has even reported a direct correlation between IL-15 levels and disease severity of SJS/TEN [17]. Even with these discoveries, the exact mechanism of T and NK cell involvement in the pathogenesis of SJS/TEN remains unknown.

The need for unique biomarkers to understand the disease mechanisms of SJS/TEN is paramount. Interleukin-33 (IL-33) is a recently discovered cytokine protein with an increased presence in skin, bronchial and intestinal epithelial and endothelial cells [18]. Specifically, IL-33 expression was localized to the nucleus and cytoplasm of intestinal epithelial cells [19]. During instances of cell damage or necrosis, its expression is upregulated to promote immune and pro-inflammatory responses through a ST2L receptor pathway by activating NF-κB and AP-1 [18,20]. IL-33 also plays a key role in the pathogenesis of many inflammatory diseases including atopic dermatitis, rheumatoid arthritis, inflammatory bowel disease (IBD), COPD, and asthma [2,18-21]. Although the exact role of IL-33 in inflammatory disease remains uncertain, it appears to have a role in promoting both inflammation and cutaneous wound healing [18,20,22].

Transforming growth factor beta 1(TGF-β1) is a ubiquitous cytokine that participates in a variety of pathophysiological responses, including the promotion of apoptosis. TGF-β1 is also pleiotropic in nature, promoting either pro-inflammatory or anti-inflammatory conditions depending on activating agents and its pathogenesis in each disease [23,24]. During inflammatory states, TGF-β1 is released from damaged epithelial cells leading to the recruitment of neutrophils, macrophages, and fibroblasts to promote tissue repair [23,24]. In addition, over or under expression of TGF-β1 can lead to inflammation. Increased TGF-β1 expression has been observed in a variety of inflammatory diseases including IBD and asthma [25, 26]. On the other hand, McCormick et al. created a successful therapeutic graft-versus-host disease mouse model demonstrating that the inhibition of TGF-β1 expression leads to scleroderma [27]. Recent work by Gurumurthy et al. revealed elevated expression of TGF-β1 in both the tears and conjunctival cells of SJS patients with ocular involvement [28]. Yet its exact role in the pathogenesis of SJS/TEN, either pro- or anti-inflammatory, remains undefined. Based on this information, we hypothesize that IL-33 and TGF-β1 play a role in the pathogenesis of SJS/TEN.

Materials and Methods

Immunofluorescence Microscopy

Under a current, Loyola institutional review board (IRB) protocol, 20 paraffin embedded skin tissue samples from one biopsy confirmed SJS, two biopsy confirmed SJS/TEN overlap and two biopsy confirmed TEN patients (4 slides per patient) were selected for this study. These paraffin embedded skin samples were obtained from the Loyola Univeristy, Medical Center core pathology lab archives. All tissue samples were de-identified. The pathologists participating in this study maintained a list of pertinent personal health information (PHI) tied to these samples. All samples were sectioned using an American Optical model 820 microtome at four microns thick and placed on positively charged glass slides.

All slides were stored and processed in the same manner. Slides were first deparaffinized by washing three times with xylene for 5 minutes each. The slides were then rehydrated using a progressive ethanol gradient. They were washed twice in 100% ethanol (EtOH) for 2 minutes, once in 95% EtOH for 5 minutes, and once in 70% ethanol for 5 minutes. Slides were then rinsed with distilled water for 1 minute and washed for 5 minutes in a phosphate buffer solution (PBS). All slides were blocked using 10% normal donkey serum (NDS) with 0.01% sodium azide for 1 hour. Slides were then treated with either IL-33 primary antibody (1:100; R&D systems, Minneapolis, MN) or TGF-β1 primary antibody (1:200; PeproTech, Rocky Hill, NJ) and incubated overnight in a humidified dark box at 4oC. Following incubation, the biopsies were washed 3 times with PBS and incubated with secondary donkey anti goat IgG (Jackson ImmunoResearch, West Grove, PA) fluorescein isotiocyanate (FITC), and diamino-2 phenylindole (DAPI) antibodies for 30 minutes. After washing with PBS, slide covers were mounted with fluorogel. For each slide stained with primary antibody, an additional control slide was prepared. These control slides were incubated with 10% NDS instead of IL-33 or TGF-β1 primary antibody and stained with secondary donkey anti goat IgG, FITC, and DAPI antibodies. These control slides were utilized to determine the level of background auto-fluorescence in all tissue samples.

Deconvolution immunofluorescence (IF) was performed on all slides using a DeltaVision microscope equipped with a digital camera. Exposure times and settings were kept constant for all samples. After imaging, fluorescent intensity sum per punctum was determined using Imaris® software. Following the subtraction of background auto-fluorescence fluorescence from all samples, the degree of IF above baseline was quantified using the surface function in the 488-nm channel (FITC stained IL-33 or TGF-β1). Statistical analysis was performed using Graph Pad Prism software. Seen below sample images depicting how Imaris® was used to quantify data (Figure 1).

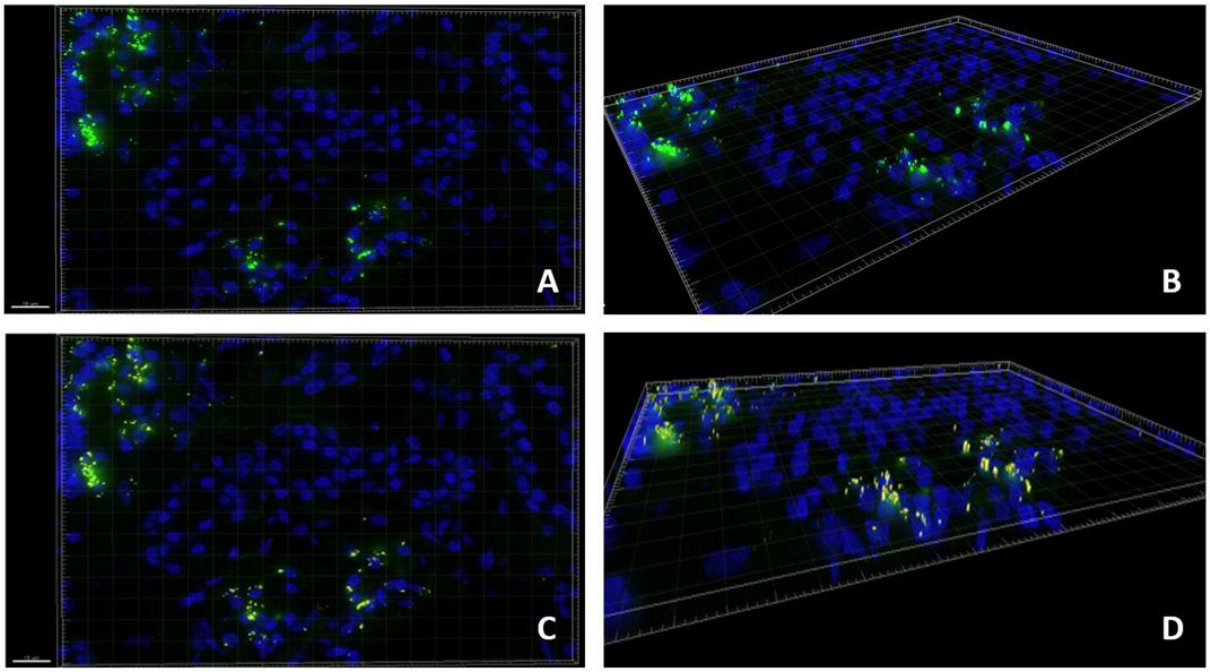

Figure 1: Example of Imaris® Methodology. Deconvolved images obtained from a DeltaVision microscope equipped with a digital camera were analyzed with Imaris® software. The software was used to remove background tissue immunofluorescence (IF) revealing IF due to either IL-33 or TGF-β1 (A and B). To determine the intensity of IL-33 or TGF-β1 in each punctum, surfaces were created around Imaris® in the 488-nm channel and the sum intensity was quantified within these volumes. These surfaces are highlighted in yellow above (C and D).

Twenty paraffin embedded unstained slides of skin from biopsy confirmed Lichen planus (LP) patients (4 slides per patient) were used as a positive control group. These slides were prepared and analyzed using the methodology described above. For each LP skin sample, a second slide was also prepared without TGF-β1 or IL-33 antibody to determine the level of auto-fluorescence in each sample. Of note, these skin biopsy and plasma samples are not always obtained from the same patient. The plasma samples used in this study came from the blood samples of patients determined to have SJS/TEN that were being discarded by the Loyola University core lab forty-eight hours after all clinically relevant blood tests were completed. As previously mentioned, the skin biopsy samples came from the core pathology lab archives. Due to the limitations of our IRB protocol, we were unable to obtain skin biopsy and plasma samples from the same patient.

ELISA Analysis

Under a current, Loyola IRB protocol, a total of 76 deidentified platelet-poor plasma samples from the Loyola core pathology lab (12 patients) with a diagnosis of SJS, SJS/TEN overlap or TEN were analyzed using ELISA. One patient (2 plasma samples) was excluded from analysis due to confirmed final surgical pathology diagnosis that was negative for SJS, SJS/TEN overlap or TEN. All plasma samples were stored at ≤ -70oC and contained either EDTA or citrate as an anticoagulant. Normal human plasma and pathologic pooled plasma were used as negative controls.

ELISA analysis of plasma samples was performed using a Quantitative Human IL-33 kit (Abcam, Cambridge, UK) and a Quantitative Human TGF-β1 kit (Abcam, Cambridge, UK). All reagent preparation and ELISA experimentation was preformed according to the manufacturer's instructions. Both IL-33 and TGF-β1 plates were analyzed on a SpectraMax plus 384 microplate reader (Molecular Devices, Sunnyvale, CA) set at 450 nm. Statistical analysis was performed using Graph Pad Prism software.

Results

TGF-β1 and IL-33 are two well-studied cytokines that play important roles in a variety of inflammatory diseases. To investigate the expression of TGF-β1 and IL-33 in patients with SJS/TEN and LP, we performed the experimentation outlined in the previous section. The results of those experiments are discussed below.

TGF-β1 Intensities in SJS/TEN and Lichen Planus Tissue Samples

A total of 1 SJS, 2 SJS/TEN overlap, 2 TEN and 5 LP skin biopsy samples were analyzed. All puncta for SJS/TEN patients above background auto-fluorescent intensity were pooled (N=5, 1572 sample points) and compared against pooled LP puncta (N=5, 1250 sample points) using a Mann-Whitney test. There was a significantly increased expression of TGF-β1 in the epithelium of pooled SJS, SJS/TEN overlap or TEN patients (average IF intensity of 3.677x107 ± $8.248x10^8$) compared to the epithelium of pooled LP patients (average IF intensity of 2.647x104 ± 1.439x105) ($p<0.0001$) (Figure 2A, 2B).

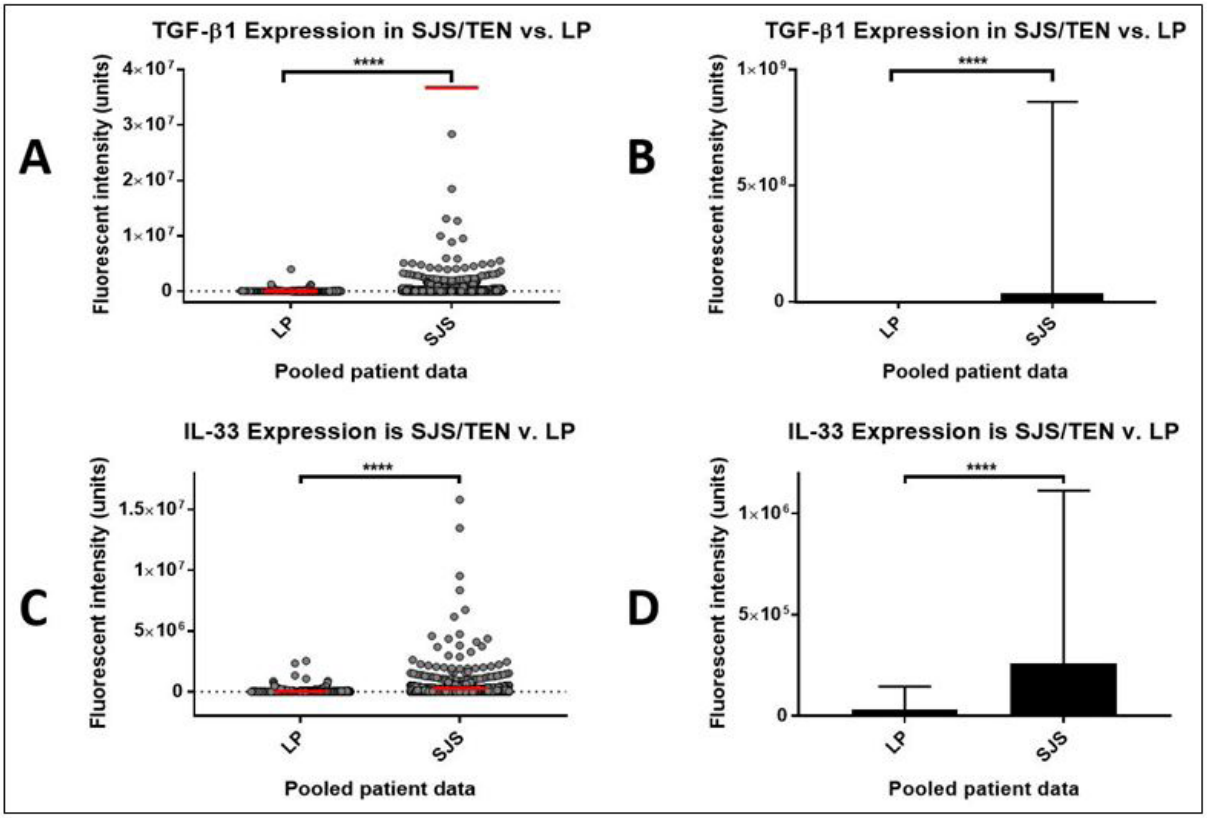

Figure 2: IL-33 and TGF-β1 expression in SJS/TEN and LP patient skin samples. Immunofluorescent puncta above baseline fluorescence for 1 SJS, 2 SJS/TEN overlap, and 2 TEN patients were pooled and compared against pooled data from 5 LP patients. The results are depicted above in both dot plot and bar graph formats. Experimentation revealed increased expression of both IL-33 and TGF-β1 in the skin of SJS/TEN patients compared to LP controls.

IL-33 Intensities in SJS/TEN and Lichen Planus Tissue Samples

A total of 1 SJS, 2 SJS/TEN overlap, 2 TEN and 5 LP skin biopsy slides were analyzed. All puncta above baseline auto-fluorescence were pooled for both SJS/TEN (N=5, 1292 sample points) and LP (N=5, 1772 sample points) samples. Pooled SJS/TEN data were compared against pooled LP data using a Mann-Whitney test. Results revealed a significantly increased expression of IL-33 in the epithelium of pooled SJS, SJS/TEN overlap or TEN patients (average IF intensity of $2.612x10^5 \pm 8.524x10^5$) compared to the epithelium of pooled LP patients (average IF intensity of $3.181x10^4 \pm 1.123x10^5$) ($p<0.0001$) (Figure 2C, 2D).

TGF-β1 Levels in Plasma Samples

All patient samples showed elevated average [TGF-β1] compared to NHP control (0.1937 ng/ml) except for SJS sample 20 (0.1934 ng/ml). However, both ordinary one-way ANOVA ($p=0.3346$) and Dunnett's multiple comparisons tests revealed no statistical significance in TGF-β1 expression in all SJS plasma samples when compared to the NHP control (Figure 3A).

IL-33 Levels in Plasma Samples

Only SJS patient sample 38 showed elevated average [IL-33] compared to NHP control (0.1313 pg/ml and 0.1312 ng/ml respectively). Both ordinary one-way ANOVA ($p=0.4038$) and Dunnett's multiple comparisons tests revealed no statistical significance in IL-33 expression in all SJS plasma samples when compared to the NHP control. Interestingly, this contrasts with the markedly increased expression of IL-33 in SJS/TEN patient skin biopsy samples (Figure 3B).

Discussion

SJS/TEN is a devastating autoimmune disease with severe clinical consequences. However, little is still known about the pathogenesis of this disease spectrum. Recent studies have emphasized the importance of identifying cytokines and other molecular mediators and their roles in SJS/TEN. The results of our study suggest that both IL-33 and TGF-β1 play a key role in the pathogenesis of SJS/TEN, as they show statistically significant increased levels of expression in the skin of SJS/TEN patients. To our knowledge (aside from recent work by Su et al.) reports of IL-33 and TGF-β1 acting as disease mediators in SJS/TEN have not been previously reported [17].

IL-33 has been proven to promote inflammation in a variety of dermatologic, GI, pulmonary and autoimmune disease processes [18-21]. Various studies have proposed that IL-33 may act as an alarmin, meaning high concentrations of it are released from

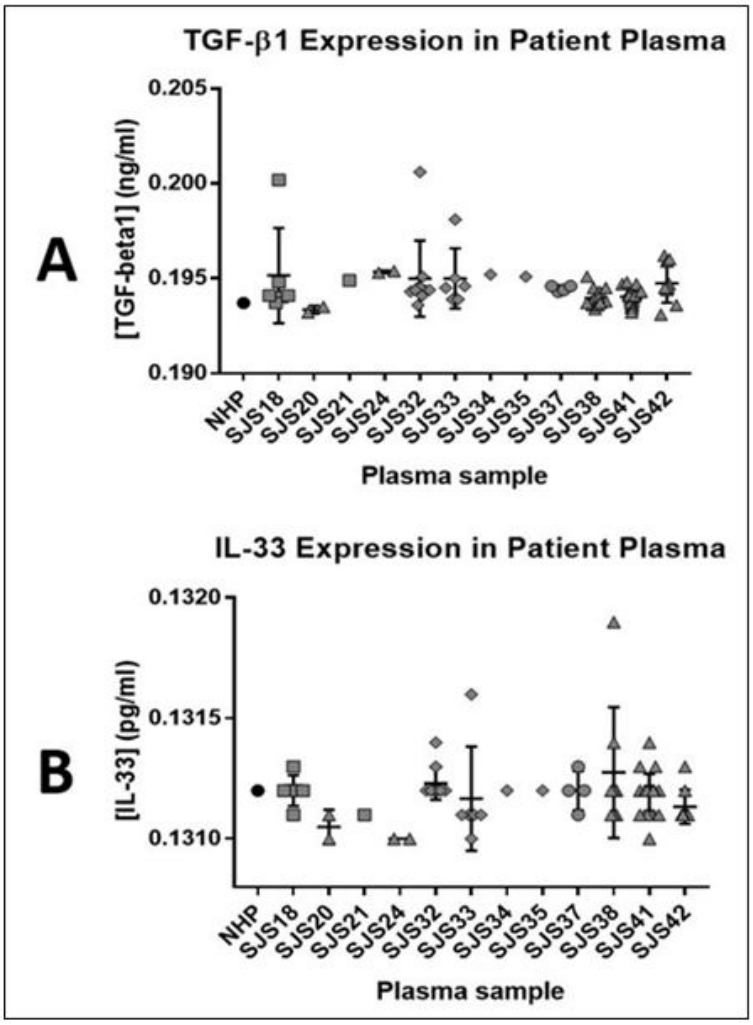

Figure 3: ELISA analysis of STS/TEN citrated plasma samples. A total of 76 plasma samples from 12 SJS/TEN patients were analyzed using Abcam Quantitative IL-33 and Quantitative TGF-β1 ELISA kits. Normal human plasma (NHP) was used as a healthy control. Results reveal mildly elevated levels of TGF-β1 and IL-33 in the plasma of SJS/TEN patients compared to NHP. However, ANOVA and Dunnett's multiple comparisons tests revealed no statistical significance in IL-33 or TGF-β1 expression in all SJS plasma samples when compared to NHP control.

damaged cells promoting the recruitment, activation and proliferation of immune cells [21,29]. Given the elevated expression of IL-33 seen in our SJS/TEN skin samples, we propose that IL-33 plays a similar role in the pathogenesis of SJS/TEN. Schmirtz et al. have also reported increased expression of IL-33 in epithelial cells that have direct contact with the external environment [30]. The elevated expression of IL-33 in the skin of SJS/TEN patients corroborates this finding. Further, we hypothesize that similar expressions of IL-33 may be found in the ocular epithelium given its exposure to the outside world. Impression cytology specimens might be used to determine the levels in the acute stage.

TGF-β1 has been shown to have pro- or anti-inflammatory effects depending on the tissue or disease in which it is involved. Loboda et al. note that elevated TGF-β1 expression in the kidney plays a key role in renal inflammation and the eventual development of fibrosis whereas a TGF-β1 knockout mouse model leads to inflammation in the heart and lungs [24,31,32]. In the skin, TGF-β1overexpression leads to severe inflammation in the basal layer of the epidermis and delays wound healing [33,34]. Elevated levels of TGF-β1 in the epidermis of SJS/TEN patients suggests that TGF-β1 plays an inflammatory role in the pathogenesis. With paraffin embedded slides, auto-fluorescence is a known issue [35]. However, the measured relative fluorescence in tissue samples cannot be attributable to auto-fluorescence because there was a lack of significant auto-fluorescence in control samples that were not incubated with a primary antibody (Figure 4).

Recognizing elevated expression of IL-33 and TGF-β1 in the skin of SJS/TEN patients can be a useful diagnostic tool. Although the early recognition and acute treatment of SJS/TEN is time sensitive, we believe that IL-33 and TGF-β1 might serve as reliable disease biomarkers. While these cytokines are not exclusive to this disease process, they are markedly expressed compared to other inflammatory skin disorders like LP. Further work is needed to confirm that both elevated IL-33 and TGF-β1 expression are unique to SJS/TEN, but their clinical implications in skin biopsy samples show great promise. The increased expression of TGF-β1 and IL-33 in SJS/TEN also warrants investigation into the use of IL-33/ST2 and TGF-β/SMAD pathway inhibitors as potential

treatments for this disease spectrum. Previous studies have shown that inhibition of the IL-33/ST2 pathway in mouse models led to decreased inflammation in both RA and asthma [35-37]. Therefore, the use of IL-33/ST2 pathway inhibitors, such as Infliximab/ Remicade, could prove useful in the treatment of SJS/TEN. There is a prospective multicenter study (which will start in 2018) that will compare the outcomes of supportive care, systemic cyclosporine and systemic etanercept (Enbrel) in patients with SJS/ TEN sponsored by the Canadian Dermatology Foundation and NIH. Similarly, casticin, fucoidan and ursodeoxycolic acid have been shown to inhibit the TGF-β1/Smad pathway in mice with liver fibrosis [38-40]. Endogenous sulfur dioxide and tacrolimus also act as TGF-β1/Smad inhibitors with proven efficacy in vascular smooth muscle cells, keloid scar formation and scleroderma respectively [41,42]. These compounds, used either as monotherapy or in combination with IL-33/ST2 inhibitors, could also prove useful in the treatment of SJS/TEN.

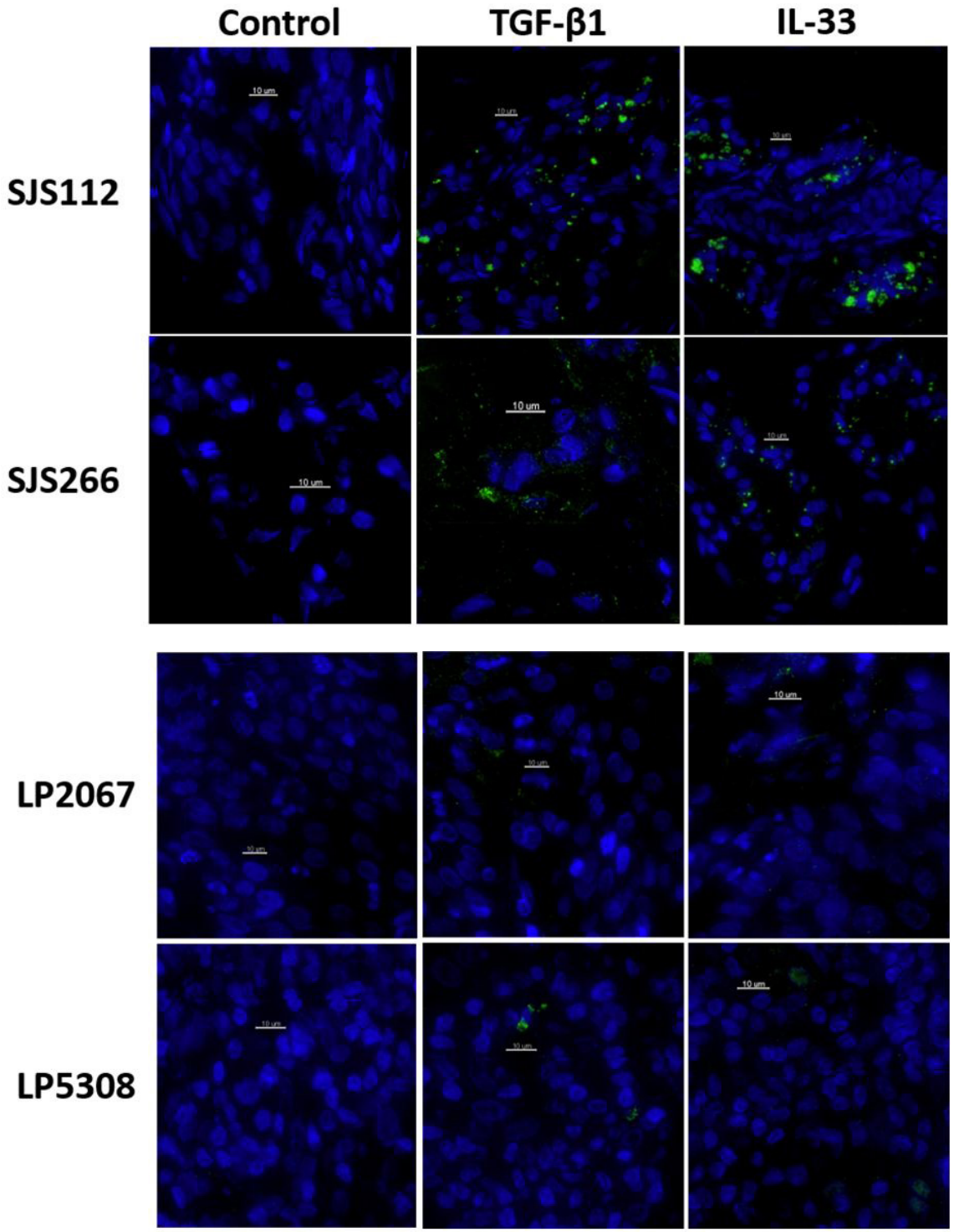

Figure 4: Representative images from Immunofluorescent (IF) microscopy. A total of 5 SJS/TEN and 5 LP skin biopsy samples were analyzed using IF microscopy. Two SJS/TEN and LP samples are shown above. Control slides lacked anti-TGF-β1 or anti-IL-33 primary antibody. All scale bars are set to 10 μm.

Although TNF-α is a well-recognized marker of inflammation in SJS/TEN, its levels are also increased in other inflammatory disorders such as rheumatoid arthritis, multiple sclerosis, and inflammatory bowel disease. Previous studies have demonstrated that TNF-α plays a central role in SJS/TEN pathology, but these studies have not shown adequate results. In TNF-mediated pathologies, it is crucial to identify other cytokines that may have been triggered by TNF-α. In this study we have identified that both IL-33 and TGF- β1 play a role in SJS/TEN pathogenesis. We propose that increased TNF-α expression in SJS/TEN patients leads to triggering the expression of other cytokines including IL-33 and TGF- β1. Further research in this field may unravel the exact roles these cytokines are playing in the pathogenesis of SJS/TEN.

While our results show increased expression of both TGF-β1 and IL-33 in the skin of SJS/TEN patients, the concentrations of these cytokines are mildly increased in the blood. Recent work by De Salvo *et al,* Saadah *et al,* and Menegat *et al.* reports increased concentrations of IL-33 in both the peripheral blood and intestinal mucosa of patients with IBD [43-45]. Similarly, pediatric IBD patients showed increased expression of IL-12p40 and TGF-β1 in the peripheral blood [46]. While this study was conducted in patient populations with a different inflammatory disease, it suggests that IL-33 and TGF-β1 concentrations should be markedly elevated in the plasma of SJS/TEN patients. We propose the discrepancy between blood and skin expression of these cytokines is due to their roles in the TNF-α inflammatory cascade. It is known that TNF-α is a key regulator is SJS/TEN pathogenesis with high concentrations in the peripheral blood and blister fluid [47,48]. It is possible that both IL-33 and TGF-β1 are downstream targets of TNF-α that are located specifically in the mucosa and epithelium. These results can also be attributed to specificities of plasma sample collection. In our IRB approved protocol, deidentified plasma samples are obtained from the Loyola core pathology labs 24-48hrs after they were originally drawn. After obtaining these samples they are transferred and stored in a -70°C freezer until needed. It is possible that prolonged storage in the core pathology lab refrigerators and repeated freeze-thaw cycles could denature these cytokines, thus falsely decreasing their measured expression via ELISA. This is in direct contrast to skin biopsy samples which are immediately formalin fixed and embedded in paraffin, thus preventing denaturation. In the future, efforts should be made to study the expression of IL-33 and TGF-β1 in fresh plasma samples of SJS/TEN patients. To date we recommend the use of skin biopsy samples to determine pathologic SJS/TEN disease severity.

Originally developed in 2000, severity-of-illness score for toxic epidermal necrolysis (SCORTEN) has become the gold standard in predicting mortality for SJS/TEN patients [49]. Despite its clinical utility, SCORTEN does not include pathologic criteria. A recent study by Morales et al. even determined that SCORTEN does not correlate with the severity of SJS ocular involvement in the acute setting [50]. While SCORTEN remains an excellent predictor of mortality, increased scores do not necessarily correlate with disease progression. Therefore, we propose the addition of pathologic criteria to the SCORTEN system. Recent studies have shown that increased levels of IL-15 in SJS/TEN correlate closely with SCOTEN scores ≥3, indicating increased mortality [49,51]. Results of our experimentation, however, failed to demonstrate a correlation between TGF-β1 or IL-33 concentrations and SCORTEN scores for the 5 SJS/TEN patients included in this study (Table 1). We believe this lack of correlation is due to a limited number of patient samples (given the rarity of SJS/TEN as a disease), inconsistencies in electronic medical record documentation, and the time at which skin biopsy samples were obtained during the disease course. Despite these results, we encourage further investigation into the correlation between IL-33/TGF-β1 and mortality in SJS/TEN, as well as the incorporation of cytokine expression into the SCORTEN scoring system.

Conclusion

Our results show a statistically significant increase in IL-33 and TGF-β1 expression in the skin of SJS/TEN patients compared to LP controls. However, plasma samples obtained from SJS/TEN patients showed mild elevation of both IL-33 and TGF-β1 compared to normal human plasma controls. Therefore, we conclude that both IL-33 and TGF-β1 are key regulators in the pathogenesis of SJS/TEN in the skin, but their roles as systemic mediators remain undefined. Additional studies should be performed to validate these results with the eventual goal of discovering an appropriate TGF-β1/Smad or IL-33/ST2 inhibitor that can slow the progression of SJS/TEN.

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