

The Role of IL-13, IL-15 and Granulysin in the Pathogenesis of Stevens-Johnson Syndrome/Toxic Epidermal Necrolysis

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Abstract

Stevens-Johnson Syndrome (SJS) and toxic epidermal necrolysis (TEN) are Severe Cutaneous Adverse Reactions (SCARS) characterized by fever and mucocutaneous lesions leading to necrosis and sloughing of the epidermis. Conjunctival lesions are reported in 85% of patients. The pathogenesis of SJS/TEN/SCARS is not completely understood. It is hypothesized that IL-13, IL-15 and Granulysin expressed in plasma and skin may play a role. We measured the circulating levels of these cytokines in the plasma using ELISA and their expression in the skin using immunofluorescence microscopy. A total of 12 SJS/TEN skin biopsy samples (8 SJS, 2 SJS/TEN overlap and 2 TEN) were analyzed. Biopsy samples from patients with Lichen Planus (an inflammatory condition of the skin and mucous membranes) served as controls. Studies were also performed in human corneal epithelial cells where expression of these cytokines were measured following a challenge with TNF- α (0, 1, 10 and 100 ng/ml). The intensity of immunofluorescence was measured Using Imaris[®] software. The results showed significantly increased expression of these cytokines in the skin biopsy samples as measured by the average intensities of IL-13 ($6.1 \times 133.0 \pm 4.231 \times 10^8$), and Granulysin ($4.2 \times 123.0 \pm 4.231 \times 10^8$) compared to Lichen planus control ($3.0 \times 123.0 \pm 1.62 \times 10^5$). Increased expression of IL-13 and IL-15 were noted in cell culture studies and in the plasma samples when compared to Normal Human Plasma as controls. It is concluded that IL-13, IL-15 and Granulysin play a role in the pathogenesis of SJS/TEN.

Keywords

Stevens-Johnson syndrome, toxic epidermal necrolysis, granulysin, IL13, IL-15, immunofluorescence microscopy

Introduction

Stevens-Johnson Syndrome (SJS) and toxic epidermal necrolysis (TEN) are rare life-threatening Severe Cutaneous Adverse Reactions (SCARS) with an estimated incidence ranging from 2 to 7 cases per million people per year.¹ Other examples of SCARS include Drug Reaction with Eosinophilia and Systemic Symptoms (DRESS syndrome) and Acute Generalized Exanthematous Pustulosis (AGEP). The SJS was first described in 1922, when the American pediatricians Albert Mason Stevens and Frank Chambliss Johnson reported the cases of 2 boys aged 7 and 8 year-old with “an extraordinary, generalized eruption with continued fever, inflamed buccal mucosa, and severe purulent conjunctivitis.”² SJS and TEN are most often drug-induced,

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characterized by fever and mucocutaneous lesions resulting in necrosis and sloughing of the epidermis. SJS and TEN are distinguished based on the severity and involvement of the body surface area. SJS is less severe and the skin sloughing is less than 10% of the body surface (BSA). TEN is more severe and the sloughing is more than 30% of BSA. When the involvement is greater than 10% but less than 30% of the body surface, it is referred to as SJS/TEN overlap syndrome.¹ SJS and TEN are immune-mediated reactions, considered to be T-cell mediated, characterized by a painful blistering rash often associated with multiorgan involvement and commonly fever, hematologic abnormalities, ophthalmologic and genitourinary involvement.³ Drugs are the leading cause of SJS and TEN in children. However, in addition to the drugs, infections particularly *Mycoplasma pneumoniae* and herpes viruses are associated with greater proportions of pediatric population.^{4,5} A wide variety of over 200 different drugs are implicated in its pathogenesis. Some of the most common culprit drugs include anti-gout agents, antibiotics, anti-psychotic and antiepileptics, and non-steroidal anti-inflammatory drugs (NSAIDs).⁶⁻⁹ The drug exposure generally precedes the onset of symptoms by 1 to 3 weeks. Signs and symptoms such as confluent erythema, facial edema, skin rash, palpable purpura, skin necrosis, blister with or without epidermal detachment, mucous membrane erosions and crusting and swelling of tongue should usually alert the physician.¹⁰ Several strong associations of prevalence of SJS/TEN in Southeast Asia have been documented with associations of HLA-A*0206, HLA-B*44:03, HLA-B*1502 (carbamazepine) and HLA-B*5801 (allopurinol) genotypes.¹¹⁻¹⁴ In the United States, 10% of the total 12 million people, self-identified as Asian in the 2000 census are positive for HLA-B*1502 and its association with carbamazepine-induced SJS/TEN. Owing to the data implicating the HLA-B*1502 as a marker for carbamazepine-induced-SJS/TEN in Han Chinese but not in Caucasian patients, the FDA recommends genotyping all Asians for the allele before initiation of carbamazepine therapy.¹⁵ Despite this progress, there are significant clinical research gaps. Given that the pathogenesis of SJS/TEN is not completely understood, there is a need to explore these gaps. There are believed to be immune-mediated specific drug hypersensitivity reactions initiated by cytotoxic T-lymphocytes.^{16,17} Cytotoxic T cells and NK cells were found to infiltrate the skin lesions of subjects with SJS/TEN.¹⁸ Recognizing the fact that the number of infiltrating cells in the skin lesions is far too few to explain the extensive keratinocyte apoptosis, it was thought that soluble mediators may contribute to keratinocyte apoptosis.¹⁹ Granule-mediated exocytosis mainly of perforin and granzyme B and Fas and Fas Ligand (Fas L) interactions were reported to be 2 pathways to explain keratinocyte apoptosis.^{18,20-22} Other reports refute the crucial roles Fas-FasL or perforin and granzyme B in SJS/TEN as they occur in drug-induced eruptions such as maculopapular erythema, where massive apoptosis in skin is not observed.^{23,24} Increased expression of the death cell receptor TNF- α or TNF receptor 1 has been reported.²⁵ Granulysin (15-kDa) form secreted by the NK cells and cytotoxic T cells was reported to be present in the blister fluid, which was shown to be responsible

for the widespread keratinocyte apoptosis.²⁶ The results of a pilot study on patients with SJS/TEN develop coagulopathies like those seen in burn patients was reported earlier.²⁷ Ueta et al reported on the associations of combined IL-13/IL-4 R signaling pathway gene polymorphism with Stevens-Johnson Syndrome, accompanied by ocular surface complications.²⁸ Our laboratories earlier reported on the roles of IL-33 and TGF-beta1 in the pathogenesis of SJS/TEN as potential biomarkers for disease severity.²⁹ Interleukin-15 was found to be associated with severity and mortality in SJS/TEN.³⁰ This study is designed to better understand the roles of IL-13, IL-15 and Granulysin in the pathogenesis of SJS/TEN. It is hypothesized that IL-13, IL-15 and Granulysin have crucial roles in the pathogenesis of SJS/TEN.

Materials & Methods

The study is designed to study a) The corneal sample study- the histopathological staining of archived skin biopsy slides of patients with SJS/TEN, b) plasma samples study of patients with SJS/TEN and c) cell culture study using human corneal epithelial cells. The plasma samples of patients with SJS/TEN were obtained from a recent prospective study, however, the corneal biopsy slides are from patient samples which are archived in the pathology department of Loyola University Medical Center, after the patients had undergone corneal transplantation. Hence, the plasma samples and archived corneal biopsy samples may be from different patients with SJS/TEN. In this study we highlight the immunofluorescent staining of the corneal biopsy slides.

Immunofluorescence Microscopy of Skin Biopsy Samples

Under a current Loyola institutional review board (IRB) protocol, a total of 12 SJS/TEN skin biopsy samples (8 SJS, 2 SJS/TEN overlap and 2 TEN) were analyzed. Due to the limitations of our IRB protocol, we were unable to obtain skin biopsy and plasma samples from the same patient. Biopsy samples from patients with Lichen Planus (an inflammatory condition of the skin and mucous membranes) served as controls. These paraffin embedded skin samples were obtained from the Loyola University 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 4 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 3 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

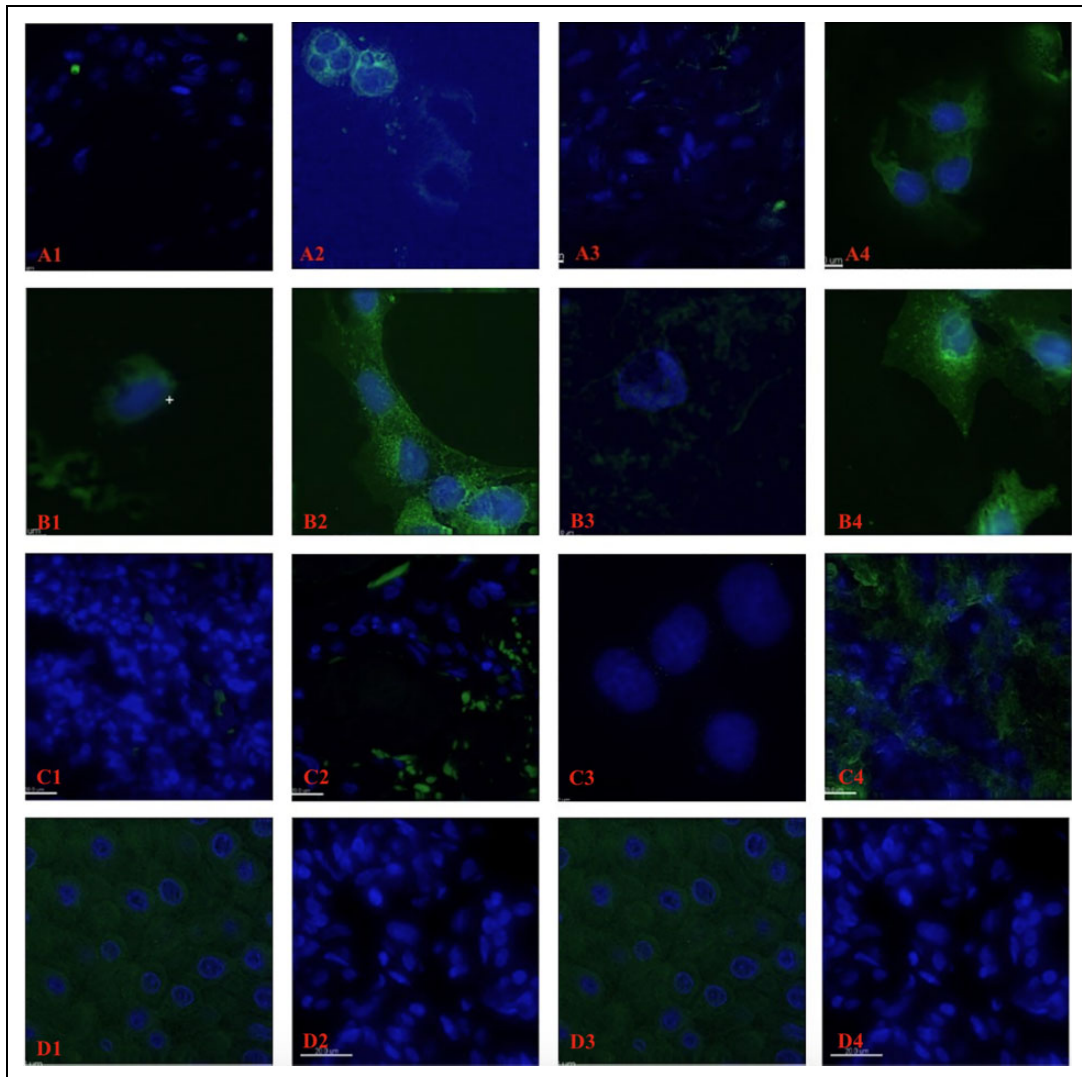


Figure 1. A1-A4 shows expression of IL-13 in SJS Patients. B1-B4 shows expression of Granulysin in SJS patients. C1-C4 shows expression of IL-13 in TEN patients. D1-D4 shows expression of IL-13 and Granulysin in LP patients.

IL-13 primary antibody (1:100; R&D systems, Minneapolis, MN) or Granulysin primary antibody (1:200; PeproTech, Rocky Hill, NJ) and incubated overnight in a humidified dark box at 4 degrees Celsius. Following incubation, the biopsies were washed 3 times with PBS and incubated with secondary donkey anti goat IgG (Jackson Immuno Research, West Grove, PA) fluorescein isothiocyanate (FITC), and 4',6'-diamidino-2 phenylindole (DAPI) stain 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-13 or Granulysin 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 from all samples, the degree of IF above baseline was quantified using the surface function in the 488-nm channel (FITC stained IL-13 or Granulysin). Statistical analysis was performed using Graph Pad Prism software. See below sample images depicting how Imaris[®] was used to quantify data (Figure 1).

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 granulysin or IL-13 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.

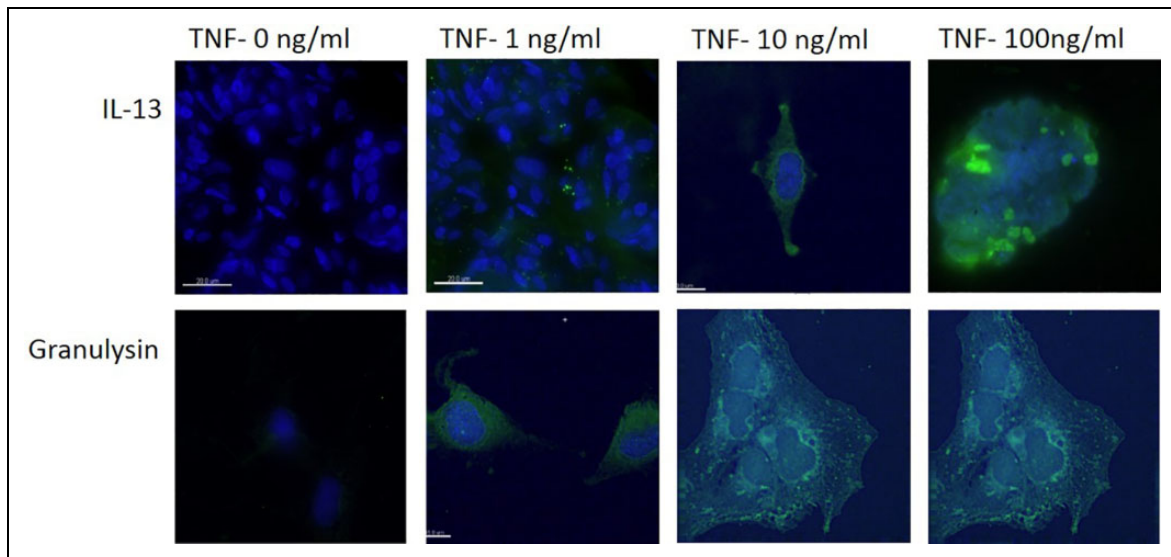


Figure 2. Increase in expression of IL-13 and granulysin with increasing concentration of TNF α .

Cell Culture Study

Human Corneal Epithelial Cell Cultures were maintained. The human corneal epithelial cells were grown to confluency and were challenged with different concentrations of TNF- α ranging from 0,1,10,100 ng/ml. Then the cells were plated and challenged with plasma from patients with Stevens Johnson Syndrome and toxic epidermal necrolysis. Following incubation, the supernatants were collected, and the cells stained with IL-13 and Granulysin antibodies and immunofluorescence was determined. The collected supernatants were aliquoted and stored at -70 °C freezer. The samples were later thawed together with SJS/TEN patient plasma samples and the quantification of IL-13, Granulysin and IL-15 analyzed by ELISA.

Plasma Samples

The de-identified and discarded plasma samples from patients with SJS/TEN were obtained from the Loyola University core labs. Under a current, Loyola IRB protocol, a total of 76 de-identified 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 -70 °C 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-13 kit (Abcam, Cambridge, UK) and a Quantitative Human Granulysin kit (Abcam, Cambridge, UK). All reagent preparation and ELISA experimentation was performed according to the manufacturer's instructions. The Quantikine Human IL-13 Immunoassay is a 4.5- hour solid phase ELISA designed to measure IL-13 levels in cell culture supernates, serum, and plasma. It contains

E. coli-expressed recombinant human IL-13 and antibodies raised against the recombinant factor. This immunoassay has been shown to quantitate recombinant human IL-13 accurately. Results obtained using natural human IL-13 showed dose response curves that were parallel to the standard curves obtained using the recombinant kit standards. These results indicate that this kit can be used to determine relative mass values for natural human IL-13. Both IL-13 and IL-15 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

The results presented reflect the 3 aspects of the study a) Immunofluorescence study, b) Cell culture study and c) Plasma cell study.

a): Immunofluorescence study:

IL-13 Intensities in SJS/TEN and Lichen Planus Tissue Samples

A total 12 SJS/TEN (8 SJS, 2 SJS/TEN overlap and 2 TEN) and 5 LP biopsy samples were analyzed. All puncta for SJS/TEN patients above background auto-fluorescent intensity were pooled (N = 12, 1600 sample points) and compared against pooled LP puncta (N = 8, 1300 sample points) using a Mann-Whitney test. There was a significantly increased expression of IL-13 ($p = 0.00001$) in the epithelium of pooled SJS, SJS/TEN overlap or TEN patients (average IF intensity of $6.1 \times 133.0 \pm 4.231 \times 10^8$ compared to the epithelium of pooled LP patients (average IF intensity of $3.0 \times 111.0 \pm 1.73 \times 110.0$ ($p < 0.00001$) (Figure 1).

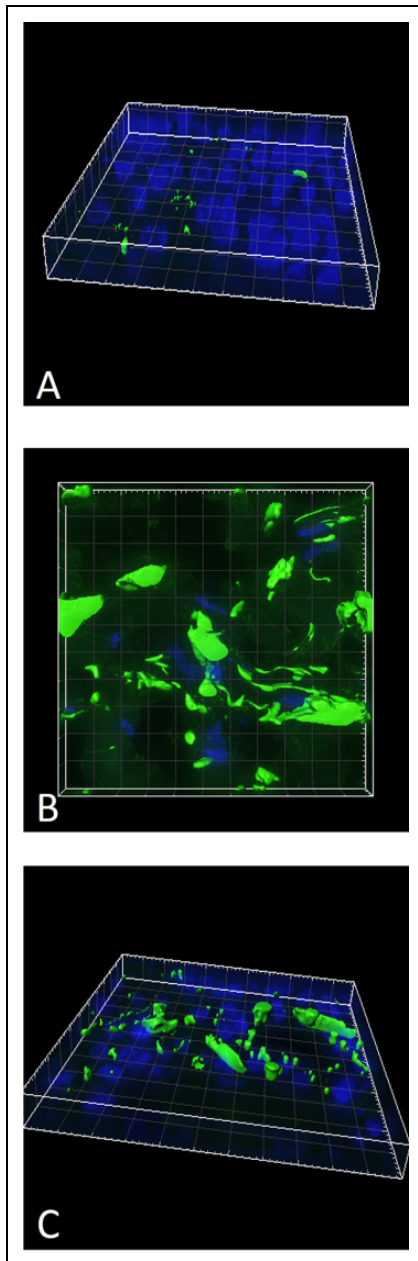


Figure 3. Example of Imaris[®] Methodology. Deconvolved images obtained from a Delta Vision microscope equipped with a digital camera were analyzed with Imaris software. The software was used to remove background tissue immunofluorescence revealing IF due to either IL-13 or Granulysin (B and C). To determine the intensity of IL-13 or Granulysin in each punctum, surfaces were created around Imaris[®] in the 488-nm channel and the sum intensity was quantified within these volumes.

Granulysin Intensities in SJS/TEN and Lichen Planus Tissue Samples

A total 12 SJS/TEN (8 SJS, 2 SJS/TEN overlap and 2 TEN) and 5 LP biopsy samples were analyzed. All puncta for SJS/TEN patients above background auto-fluorescent intensity were pooled (N = 12, 1600 sample points) and compared against

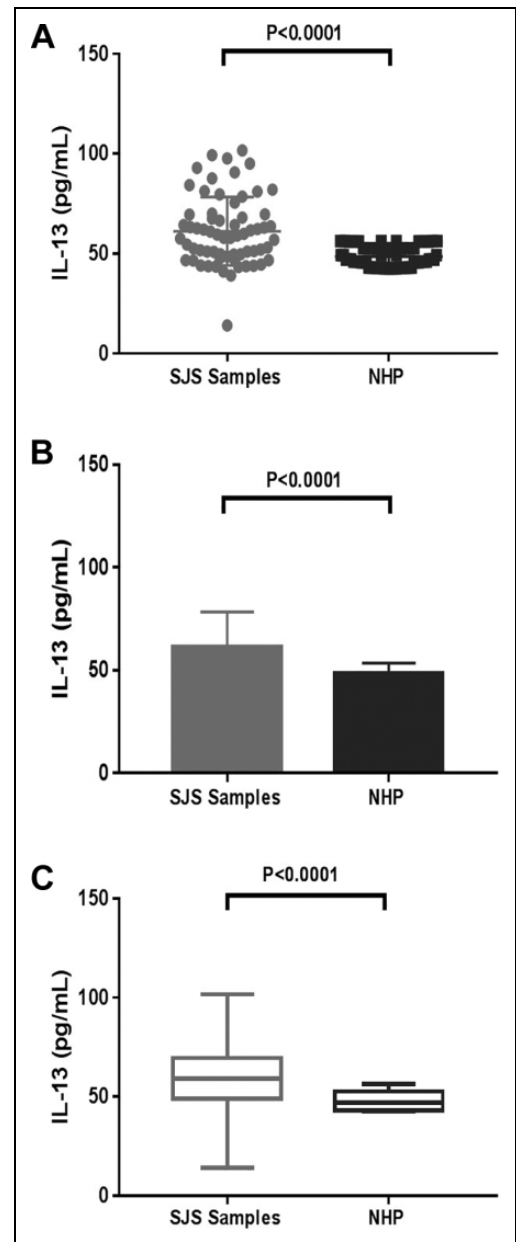


Figure 4. IL-13 expression in SJS/TEN patients. A total of 76 plasma samples from 12 SJS/TEN patients were analyzed using Abcam Quantitative IL-13 and Quantitative IL-15 ELISA kits. Normal human plasma (George King Inc.) was used as a healthy control. The results are depicted in dot plot, bar graph and box plot. Experimentation revealed increased expression of IL-13 in the plasma of SJS/TEN patients compared to NHP controls.

pooled LP puncta (N = 8, 1600 sample points) using a Mann-Whitney test. There was a significantly increased expression of Granulysin ($p = 0.00003$) in the epithelium of pooled SJS, SJS/TEN overlap or TEN patients (average IF intensity of $4.2 \times 123.0 \pm 8.426 \times 10^8$ compared to the epithelium of pooled LP patients (average IF intensity of $3.0 \times 111.0 \pm 1.62 \times 10^8$ ($p < 0.00001$)) (Figure 1).

b): Cell Culture study:

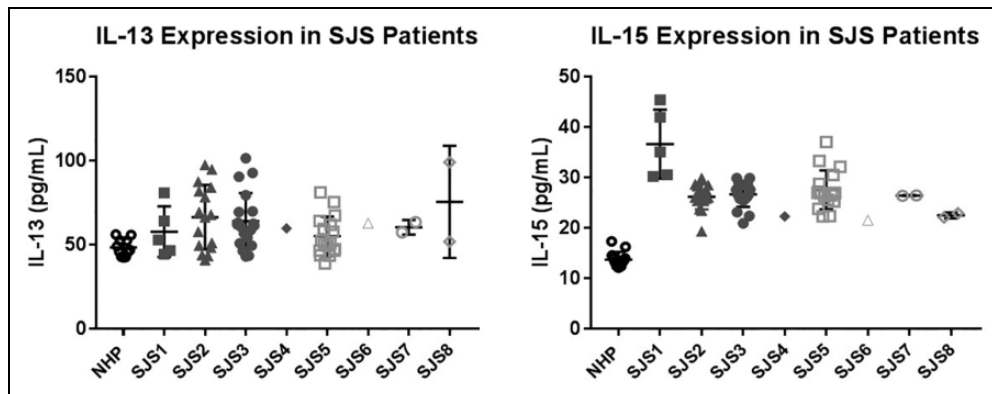


Figure 5. IL-13 and IL-15 ELISA analysis of SJS/TEN citrated plasma samples. A total of 76 plasma samples from 12 SJS/TEN patients were analyzed using Abcam Quantitative IL-13 and Quantitative IL-15 ELISA kits. Normal human plasma (George King Inc.) was used as a healthy control. Results reveal mildly elevated levels of IL-13 and IL-15 in the plasma of SJS/TEN patients compared to NHP. ANOVA and Dunnett's multiple comparisons tests revealed statistical significance in IL-13 and IL-15 expression in all SJS plasma samples when compared to NHP control (George King Inc.).

Expression of IL-13 and Granulysin Following a TNF- α Challenge in Human Corneal Epithelial Cell Cultures

The cells were studied for the expression of IL-13 and Granulysin using immunofluorescence microscopy. The intensities of immunofluorescence of IL-13 and Granulysin were calculated using Imaris[®] methodology (Figures 2 and 3).

c): Plasma Analysis:

IL-13 and IL-15 Expressions in plasma samples: All patients showed elevated average intensities (IL-13) compared to the normal human plasma (NHP) control $P < 0.0001$ (Figure 4 and 5). All patients showed elevated average intensities of IL-15 as compared to the normal human plasma (NHP) control $p < 0.0001$ (Figure 4, 5, and 6).

Discussion

In this study we investigated the roles of IL-13, IL-15 and Granulysin in the pathogenesis of SJS/TEN. The association of combined IL-13/IL-4 R signaling pathway gene polymorphism with Stevens Johnson Syndrome accompanied by ocular surface complications has already been reported earlier.²¹ The role of Granulysin in the pathogenesis of SJS/TEN was earlier reported.^{6,19} We studied the role of IL-13 and Granulysin in the pathogenesis of SJS/TEN in terms of their expression as studied by immunofluorescence microscopy and their quantitative determination using ELISA technique.

The immunofluorescence microscopy study: There was a significantly increased expression of Granulysin ($p = 0.00003$) in the epithelium of pooled SJS, SJS/TEN overlap or TEN patients (average IF intensity of $4.2 \times 123.0 \pm 8.426.0 \times 10^8$, compared to the epithelium of pooled LP patients (average IF intensity of $3.0 \times 111.0 \pm 1.62 \times 10^8$ ($p < 0.00001$). The increased expression of IL-13 and Granulysin suggests that these cytokines have a role in the pathogenesis of SJS/TEN.

Cell Culture Study: Given that TNF- α levels are increased in patients with SJS/TEN,²⁴ we stimulated the cultured human corneal epithelial cells with TNF- α at different concentrations ranging from 0, 1, 10 and 100 ng/ml and studied the expression of IL-13 and Granulysin using anti-IL-13 antibody and anti-Granulysin antibody. As seen in Figure 2 there was a concentration-dependent response with TNF- α -induced expression of IL-13 and Granulysin. Our findings related to TNF- α -induced expression of IL-13 and Granulysin by cultured human corneal epithelial cells is a first reported finding. TNF- α -induced expression of IL-13 and Granulysin in human corneal epithelial cell culture serves as a model to study the expression of other cytokines involved in the pathogenesis of SJS/TEN. Further studies are ongoing in our labs to study the TNF- α -induced expression of other cytokines using human corneal epithelial cells cultures.

Plasma Study: All patients studied showed elevated average levels of IL-13 and IL-15 compared to the normal human plasma (NHP) control ($p < 0.0001$). The plasma levels of Granulysin by ELISA were not performed in this study since its increased levels have been reported earlier.^{6,19} IL-13 also plays a key role in the pathogenesis of allergy, cancer, and tissue fibrosis. Similarly, IL-15 is a proinflammatory cytokine. IL-15 binds to its receptor complex and activates an immune response through a signaling cascade in natural killer cells and subsets of T cells.²⁵ IL-15 is associated with severity and mortality in Stevens-Johnson Syndrome/Toxic Epidermal Necrolysis.²³ Understanding of the roles of IL-13, IL-15 and Granulysin in the pathogenesis of SJS/TEN is crucial in harnessing the adverse effects associated with this condition in order to formulate better treatment strategies. It is also important to identify other cytokines which might be playing a role in the pathogenesis of SJS/TEN such that the some of the adverse effects which cause long-term sequelae such as ocular sequelae may be prevented or better treated.

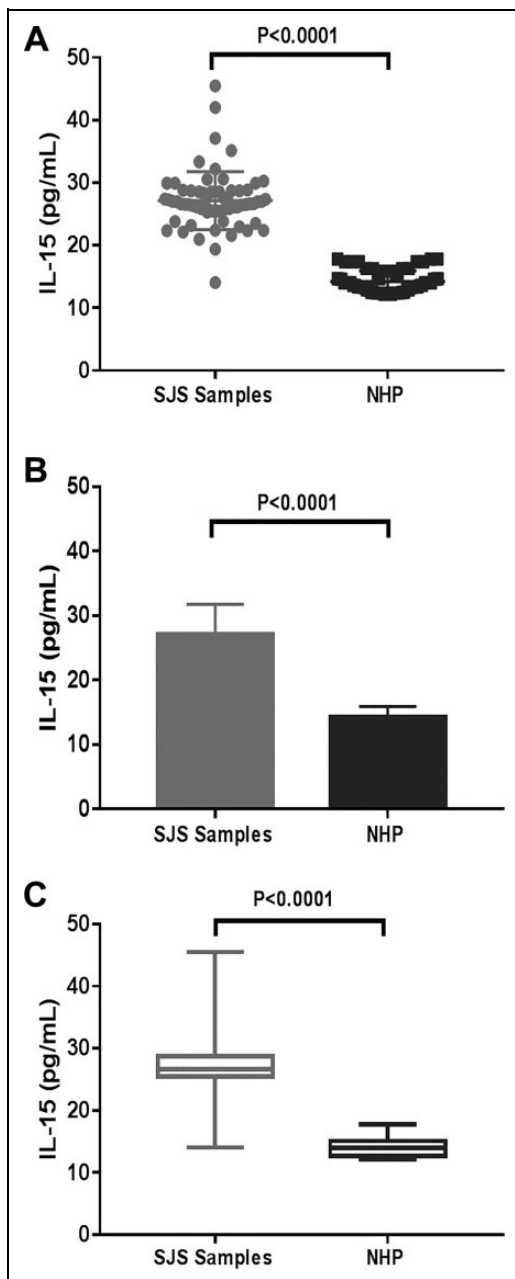


Figure 6. IL-15 expression in SJS/TEN patients. A total of 76 plasma samples from 12 SJS/TEN patients were analyzed using Abcam Quantitative IL-13 and Quantitative IL-15 ELISA kits. Normal human plasma (George King Inc.) was used as a healthy control. The results are depicted in dot plot, bar graph and box plot. Experimentation revealed increased expression of IL-15 in the plasma of SJS/TEN patients compared to NHP controls.

Conclusion

Skin biopsy samples from patients with SJS/TEN showed increased expression of IL-13 and granulysin compared to Lichen planus controls. Plasma samples obtained from patients with SJS/TEN also showed increased expression of IL-13 and IL-15 as determined by ELISA assays. IL-13, IL-15 and granulysin play a role in the pathogenesis of SJS/TEN. Increased

expression of IL-13 and granulysin were noted when cultured human corneal epithelial cells were challenged with TNF- α and studies using immunofluorescence microscopy.

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Declaration of Conflicting Interests

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