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INVESTIGATING CELL DEATH PATHWAYS IN STEVENS-JOHNSON SYNDROME/TOXIC EPIDERMAL NECROLYSIS

by

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DEDICATION

I would like to dedicate this work to loving parents, Reza and Mehri Asemi, my amazing sister, Tina, and my grandfather, Karim. They are the most valuable thing in this world to me. They have mirrored ambition, persistence, patience, and compassion. Without them, I would not be who I am today. Thank you for always believing in me. To my parents, I think of the sacrifices you made immigrating to create a better life for Tina and I each day.

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Finally, I would like to dedicate this to the SJS/TEN victims and families. This work has given me so much purpose and I hope to give back through spreading awareness and my passion for evidence-based research in hopes of advancing the care and attention these patients and survivors deserve.

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INVESTIGATING CELL DEATH PATHWAYS IN STEVENS-JOHNSON SYNDROME/TOXIC EPIDERMAL NECROLYSIS

NATALIE ROSE ASEMI

ABSTRACT

BACKGROUND: Stevens-Johnson Syndrome/Toxic Epidermal Necrolysis (SJS/TEN) is the most severe form of cutaneous adverse drug reaction and is characterized by extensive epidermal destruction of the skin and mucosal surfaces. Controversy remains regarding the immunopathogenesis of disease. It has long been assumed that CD8 cytotoxic T cells mediate cell death by releasing cytotoxic granules and soluble granulysin that trigger keratinocyte apoptosis. However, this does not explain the massive cell death or inflammation that is observed clinically. We have preliminary evidence from transcriptional profiling of patient skin samples suggesting that the cell death pathways necroptosis and pyroptosis may mediate SJS/TEN. Herein we utilize retrospectively and prospectively collected patient samples to investigate these cell death pathways.

OBJECTIVE: The goals of this study are two-fold: (i) to investigate cell death pathways in retrospectively-collected (SJS/TEN) patient skin samples and (ii) to directly test the cell death mediators and pathways mediating SJS/TEN using a novel *in vitro* model.

METHODS: Clinically and histopathologically confirmed SJS/TEN skin specimens and control skin specimens from non-blistering T cell mediated drug reactions and healthy skin were obtained following retrospective analysis from a multi-centered patient database. Gene expression profiling is being performed using the NanoString nCounter®

System on these samples as a second patient cohort to confirm and expand on preliminary study findings. In parallel, we have optimized the use of a novel human skin platform for an *in vitro* model of SJS/TEN. We also collected human serum from a prospective study of SJS/TEN and control patients and have optimized and are actively collecting blister fluid from SJS/TEN and control patients in an ongoing prospective study for use in this model.

RESULTS: Through an extensive pathology database and medical record search of potential cases at Brigham and Women's Hospital, we identified a second patient cohort of SJS/TEN, non-blistering delayed-type drug hypersensitivity reactions and healthy controls. We identified and are collecting thorough demographic, clinical and laboratory data on 61 potential candidates for SJS/TEN, 4 for Drug Reaction with Eosinophilia Syndrome (DRESS), and 200 for Morbilliform Drug Eruptions (MDE). This second cohort is in the final step of analysis with review by an expert clinician to confirm cases. In parallel, we have designed an expansive gene panel to confirm cell death mediator and marker transcription in our bank of skin samples. This 815 gene panel uses the pre-designed panel from Nanostring, spiked with an additional 30 genes specific to apoptosis, pyroptosis, and necroptosis.

We reviewed multiple potential *in vitro skin* models and identified GenoSkin® as the most suitable human skin platform for our *in vitro* model. We collected serum from 6 SJS/TEN patients and 6 non-blistering drug reaction patients and 3 healthy controls, and are actively collecting blister fluid from SJS/TEN and thermal burn control patients for analysis in this model.

CONCLUSIONS: Our preliminary data suggest necroptosis and pyroptosis induced by soluble death mediators TNFα and TRAIL as the main cell death pathways responsible for SJS/TEN. We have successfully identified a large number of potential patient samples of both cases and controls to perform transcriptional profiling using a self-designed gene panel to confirm and expand upon our preliminary data. We have successfully collected prospectively patient serum and are actively collecting patient blister fluid for analysis in an optimized in vitro model using GenoSkin®. SJS/TEN is severely understudied and lacks a standard protocol for care. This stems from uncertainty surrounding disease pathobiology. It is critical that we use innovative approaches to interrogate the mechanism mediating disease to advance the field, and, most importantly, to improve the quality of care for these patients.

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LIST OF ABBREVIATIONS

AIM2.....Absent in Melanoma 2

ADRs.....Adverse Drug Reactions

APCs.....Antigen Presenting Cells

ASC.....Apoptosis-Associated Speck-Like Protein Containing a CARD

BCL-2.....B-cell lymphoma 2

BSA.....Body Surface Area

BMT.....Bone Marrow Transplant

CBZ.....Carbamazepine

CARD.....Caspase Activation and Recruitment Domain

cFLIP.....Cellular FLICE-Like Inhibitory Protein

CD95L.....Cognate Ligand 95 Ligand

CYLD.....Cylindromatosis

APAF-1.....Cytosolic Apoptotic Protease Inhibiting Factor 1

CTLs.....Cytotoxic CD8 T lymphocytes

DAMPS..... Damage-Associated Molecular Patterns

DED.....Death Effector Domains

DFNA5.....Deafness Autosomal Dominant 5

DHRs.....Drug Hypersensitivity Reactions

DISC.....Death Inducing Signaling Complex

dsDNA.....Double Stranded Deoxynucleotide Ribonucleic Acid

dtDHRs.....Delayed Type Drug Hypersensitivity Reactions

DRESS.....Drug Reaction with Eosinophilia Syndrome

EM.....Erythema Multiforme

NLRC4.....Family CARD Domain Containing 4

FADD.....Fas-Associated Protein with Death Domain

FasL.....Fas Ligand

FLICE.....FADD-ike Interleukin-1β-Converting Enzyme

FLIP.....Flice Inhibitory Protein

FOV.....Field of View

FDE.....Fixed Drug Eruptions

FFPE.....Formalin-Fixed Paraffin-Embedded

FPR1.....Formyl Peptide Receptor 1

GBFDE.....Generalized Bullous FDE

LL-37.....Human Cathelicidin 37

LPS.....Lipopolysaccharide

HIV.....Human Immunodeficiency Virus

HLA.....Human Leukocyte Antigen

HAT.....Hydrocortisone, Ascorbic Acid, and Thiamine

IRB.....Institutional Review Board

IFN-γ.....Interferon Gamma

IL-1β.....Interleukin 1-Beta

IL-15.....Interleukin-15

IL-18.....Interleukin-18

kDa.....Kilodalton

MDE.....Morbilliform Drug Eruptions

MLKL.....Mixed Lineage Kinase-Like

MOMP.....Mitochondrial Outer Membrane Permeabilization

MPE.....Maculopapular Exanthema

NETosis.....Neutrophil Extracellular Traps

NFκB.....Nuclear Factor Kappa B

NLRP.....Nucleotide-Binding Oligomerization Domain, Leucine Rich Repeat and Pyrin Domain Containing Protein

NOD.....Nucleotide-Binding and Oligomerization Domain

NK.....Natural Killer

NSAID.....Non-Steroidal Anti-Inflammatory Drugs

ODSR.....Ordinary Drug Skin Reactions

PAMPS.....Pathogen-Associated Molecular Patterns

PBMC.....Peripheral Blood Mononuclear Cells

PRR.....Pattern Recognition Receptor

p-i.....Pharmacological Interaction

PYD.....Pyrin Domain

RIPK1/RIPK3.....Receptor-Interacting Protein Kinase 1 and 3

RIP3.....Receptor-Interacting Protein Kinase-3

RegiSCAR.....Registry of Severe Cutaneous Adverse Reactions To Drugs and Collection of Biological Samples

SCAR.....Severe Cutaneous Adverse Drug Reactions

SCORTEN.....Severity of Illness Score for Toxic Epidermal Necrosis

SJS.....Stevens-Johnson Syndrome

SJS/TEN.....Stevens-Johnsons Syndrome/Toxic Epidermal Necrolysis

Th1.....T helper type 1

TCRs.....T-cell receptors

TNFR.....Tumor Necrosis Factor Receptor

TNFR1..... Tumor Necrosis Factor Receptor 1

TWEAK.....TNF-Like Weak Inducer of Apoptosis

TUNEL.....Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling

TBSA.....Total Body Surface Area

TEN.....Toxic Epidermal Necrolysis

TNF-α.....Tumor Necrosis Factor-Alpha

TRADD.....Tumor Necrosis Factor Receptor Type 1 Associated DEATH Domain

TRAIL.....Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand

Introduction

I. Overview of Stevens-Johnsons syndrome/Toxic epidermal necrolysis

Adverse drug reactions (ADRs) are defined as any noxious and unintended response to a drug and affect approximately 15% of hospitalized patients (Dykewicz 2019, Wilkerson 2022). ADRs may be predictable based on known pharmacologic properties of a drug, or unpredictable, related to immunologic response or genetic differences in patients. A subset of ADRs is drug hypersensitivity reactions (DHRs) (Dykewicz 2019). DHRs are immune-mediated reactions. There are four classes of drug hypersensitivity reactions; the focus of our research is the delayed-type, which begin days to weeks after exposure to drug. The organ most prominently affected in delayed-type DHRs is the skin (Warrington 2011). Clinical diagnoses can vary from a mild red rash to life threatening reactions such as Stevens-Johnsons syndrome/toxic epidermal necrolysis (SJS/TEN) (Redwood 2018). SJS/TEN is characterized by massive blistering and sloughing of skin and mucosal surfaces (eyes, mouth, esophagus, genitals) (Chung 2008) as seen in Figure 1 (Middendorf 2019). SJS/TEN is considered a disease continuum and is classified by the severity of the total body surface area (TBSA) of epidermal detachment (Kinoshita 2021, Grunwald 2020). Skin detachment affecting more than 30% of the TBSA is a characteristic feature of TEN. Involvement between 10% and 30% of TBSA is defined as SJS/TEN overlap. Skin detachment affecting less than 10% of BSA is classified as SJS (Grunwald 2020). Hereafter, we use “SJS/TEN” as the term including all these three conditions or each specific term to indicate the specific condition (Kinoshita 2021).

SJS/TEN causes significant morbidity and mortality, costing the US healthcare system 129 million dollars per year (Kinoshita 2021,Hsu 2016, Sekula 2013, Chang 2011). The associated mortality rates are as high as 5% for SJS and between 30-40% for TEN. The risk for death can be estimated using the SCORTEN scale, which takes a number of prognostic indicators into account (Table 1). Currently, no standardized treatment exists (Chang 2011, Mockenhaupt 2011).

Survivors suffer from long-term sequelae. These include severe ocular complications, strictures of mucous membranes, respiratory tract obstruction, and urogenital or gynecological involvement (Lee 2017, Mockenhaupt 2011). Psychological sequelae are also frequent with patients experiencing post-traumatic stress disorder in SJS/TEN (Hafez 2019). In addition to its physical long-term complications, Hsu et al completed a retrospective analysis and found that SJS/TEN is also a significant health care burden (Hsu 2016). The mean cost of hospitalization is greater than $20,000 for patients with SJS and $50,000 for SJS/TEN overlap and TEN; and up to 4-fold longer duration and 5-fold higher mean cost of hospitalization compared with that of an average hospital admission (Hsu 2016).

Insufficient understanding of SJS/TEN pathophysiology has contributed to limited treatment modalities and posed challenges in the management of care (Kinoshita 2021). Currently, it is assumed that CD8 cytotoxic T cells release cytotoxic granules and soluble granulysin which induce apoptosis in keratinocytes in SJS/TEN. However, apoptosis is non-inflammatory and moreover the targeting of keratinocytes by CD8 T cell granule exocytosis in a one-to-one fashion does not adequately explain the massive epidermal cell

death observed in disease. Recent literature has started to question the current dogma, proposing necroptosis as being responsible rather than the non-inflammatory apoptotic pathway. In 2014, Saito et al suggested that the interaction of soluble annexin 1 with its receptor, FPR1, contributed to SJS/TEN via necroptosis. Annexin A1 was abundant in SJS/TEN PBMC supernatant compared to supernatant from ordinary drug skin reactions (ODSR). Investigating its involvement, they depleted annexin A1 from SJS/TEN PBMC supernatant using an antibody. As a result, this significantly blocked SJS/TEN supernatant-induced keratinocyte death. Furthermore, they identified the downstream mediators of necroptosis. In SJS/TEN skin lesions, there was abundant RIP3 expression in keratinocytes in contrast to ODSR patients (Saito 2014). Moreover, our labs preliminary data support involvement of necroptosis as well as pyroptosis in SJS/TEN. Therefore, investigating the molecular mechanisms is imperative, and a prerequisite for establishing effective treatment options which are lacking to date (Panayotova-Dimitrova 2015). Notably, both FDA approved inhibitors of necroptosis and pyroptosis exist for other indications. Hence, gaining mechanistic insights by investigating the cell death pathways in SJS/TEN has significant potential to advance the field and lead to the testing of new treatments in clinical trials.

II. Stevens-Johnsons Syndrome/Toxic Epidermal Necrolysis (SJS/TEN)

- ***Clinical Features of SJS/TEN***

Stevens-Johnson Syndrome/Toxic Epidermal Necrolysis (SJS/TEN) is the most severe adverse drug reaction involving extensive keratinocyte death in the epidermis (Kim 2015, Kim 2014). In over 80% of cases, drugs are the main cause of SJS/TEN

(Oakey 2021, Roujeau 1995). The most commonly reported culprit drugs include allopurinol, trimethoprim, phenytoin, phenobarbital and non-steroidal anti-inflammatory drugs (NSAIDs) of the oxicam-type. Infection is causal in a smaller subset of cases, particularly in adults, and a smaller subset still are idiopathic (Harr 2010).

Symptoms usually begin 4-28 days after the onset of drug intake (Dodiuk-Gad 2015). SJS/TEN often manifests with a prodromal phase of malaise and fever, followed by the onset of a rash and mucosal involvement that evolves into painful blistering and sloughing. Mucosal involvement is prominent and severe affecting several organ systems including pulmonary, gastrointestinal, hepatic, oral, otorhinolaryngologic, gynecologic, genitourinary, and renal systems (Shanbhag 2020, Lerch 2017, Dodiuk-Gad 2015). SJS/TEN is of major concern because of the severe morbidity and high mortality rates (Sekula 2013). The mortality risk of SJS/TEN continues in the post-acute period: a significant number of deaths occurring after hospital discharge with 23% at 6 weeks, 28% at 3 months, and 34% at 1 year (Lee 2017). Systemic treatment for SJS/TEN varies widely with no clear consensus on acute or chronic systemic management (Shanbhag 2020).

Chronic complications in affected organ systems are prevalent in survivors, substantially lowering their health-related quality of life (Shanbhag 2020, Lee 2017). Often, they are left with debilitating multi-organ complications with mucous membrane involvement observed in approximately 90% of cases and 85% of cases involving conjunctival lesions with potential to result in corneal blindness (Shanbhag 2020, Abe 2016). Chronic ocular sequelae are the most disabling long-term complication of

SJS/TEN, occurring in 20-75% of survivors. It is estimated that 87% of patients with late ocular complications have difficulties reading, driving at night, and using a computer (Saka 2019, Lee 2017). Respiratory involvement occurs in about 40% of patients with SJS/TEN during the acute phase (Lee 2017). For survivors, late pulmonary sequelae include interstitial lung disease, respiratory tract obstruction, bronchiectasis, and bronchiolitis obliterans (Lee 2017). GI tract involvement is generally rare. Oesophageal strictures are the most common chronic GI complication and develop between 2 months to 2 years after the acute episode (Lee 2017). Furthermore, serious psychological consequences may arise in patients who have experienced SJS/TEN. Hefez et al. (2018) conducted one of the first prospective studies to assess the psychiatric consequences of SJS/TEN in adult populations. At the 6-month follow-up after the acute phase, their study confirmed the risk of PTSD with a prevalence of 23%. The devastating impact SJS/TEN places on survivors is evident.

Diagnosis of SJS/TEN relies on clinical features together and is often supported by histopathological analysis (Harr 2010, Charlton 2019). Histologically, SJS/TEN is characterized by full thickness epidermal necrosis and pauci-inflammatory mononuclear cell infiltrate, potentially with eosinophils (Lerch 2017, Orime 2017, Wetter 2010). Histopathology helps rule out alternative diagnoses such as autoimmune blistering diseases, bullous fixed drug eruption, and staphylococcal scalded skin syndrome, which can clinically mimic SJS/TEN (Hoetzenecker 2016, Harr 2010).

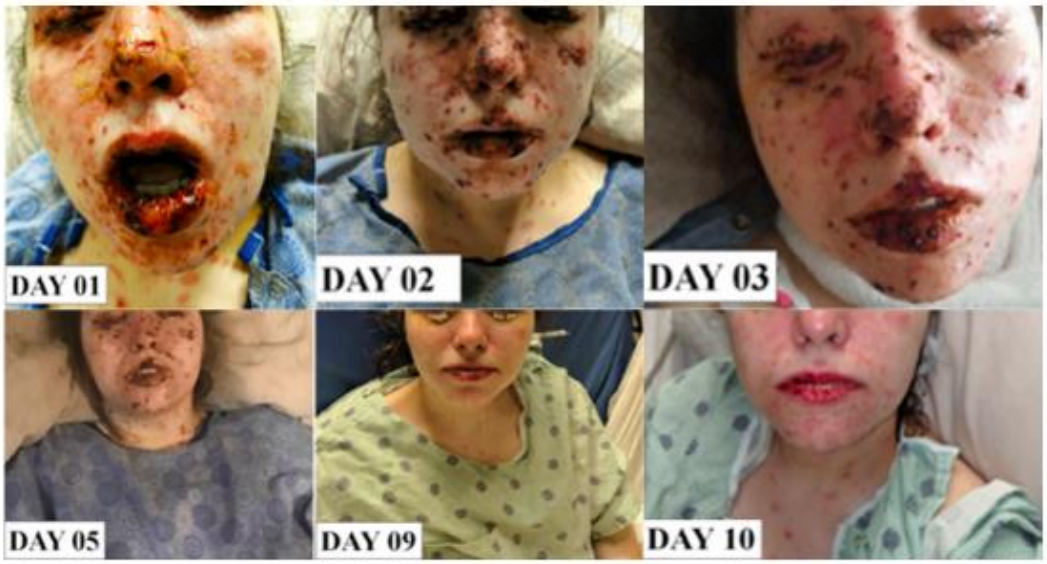

Figure 1. Progressive hospital course of a SJS/TEN patient; adapted from (Middendorf 2019). In the following case study from Middendorf et al, this patient was diagnosed with SJS/TEN overlap syndrome likely secondary to ibuprofen. A dramatic improvement was noted within 48 hours of initiating 'low-dose' intravenous hydrocortisone, ascorbic acid, and thiamine (HAT) therapy with daily improvement of her cutaneous and mucosal lesions.

- ***Epidemiology of SJS/TEN***

The incidence of toxic epidermal necrolysis is estimated at 0.4 to 1.2 cases per million person-years and Stevens-Johnson syndrome at 1 to 6 cases per million person-years. A newer USA-based study analyzing nationwide inpatient records from 2009 to 2012 calculated an incidence per million inhabitants of 8.61 to 9.69 for SJS, 1.46 to 1.84 for SJS/TEN, and 1.58 to 2.26 for TEN (Lerch 2017, Hsu 2016). In pediatric populations (20% of hospitalizations in the USA), the incidences of SJS, SJS/TEN, and TEN were a mean 5.3, 0.8, and 0.4 cases per million children per year, respectively (Lerch 2017, Hsu 2017). The prevalence of SJS/TEN appears to be lower in US children than in adults per year (Hsu 2017). Furthermore, SJS/TEN is more common in women than men (Charlton

2019, Sekula 2013, Gerull 2011). Other factors such as increased age and pre-existing comorbidities such as malignancy, particularly hematologic cancer, and human immunodeficiency virus (HIV) increase the risk of SJS/TEN (Charlton 2019). Hsu et al. (2016) studied the rates of SJS/TEN in different racial groups and found that Asians and Blacks had the highest rates of SJS/TEN, although Hispanics, Native Americans, and those who were multiracial/other had smaller but significant increases compared with Whites (Hsu 2016).

- ***SJS/TEN treatment***

With limited understanding behind the pathobiology of SJS/TEN, the optimal therapeutic strategy is still controversial and evidence for systemic treatment remains insufficient (Hasegawa 2020, Shahnbhag 2020, Zimmerman 2017, Rojeau 1995). Current care of SJS/TEN patients is multidisciplinary and similar to that of severe burn patients. Preventing the progression of the disease involves the identification and withdrawal of the culprit drug, transfer to a burn unit and supportive care (Charlton 2019, Dodiuk-Gad 2015). Prompt withdrawal of the suspected medication decreases mortality (Garcia-Doval 2000). Administration and use of systemic corticosteroids, intravenous immunoglobulins, and tumor necrosis factor (TNF)-alpha inhibitors have all been described in the acute phase, with mortality results varying from improved mortality, no benefit, to increased mortality (Shanbhag 2020, Rojeau 1995).

Zhang et al investigated the effect of biologic TNF-alpha inhibitors in the treatment of SJS/TEN and found that among 91 patients on this therapy, 79 patients, (86.8%) responded well and were discharged with few side effects and complications.

Although this opens up new possibilities, further investigations need to be pursued with larger cohorts to provide more evidence for clinical application (Zhang 2019).

Prognostic factors	Points
Age > 40 years	1
Tachycardia > 120 bpm	1
Neoplasia	1
Initial detachment > 10%	1
Serum urea > 10 mmol/L	1
Serum bicarbonate < 20 mmol/L	1
Blood glucose > 14 mmol/L	1

SCORTEN	Mortality (%)
0-1	3
2	12
3	35
4	58
≥ 5	90

Table 1. Score of Severity of TEN (SCORTEN); adapted from (Fracaroli 2013). SCORTEN was developed to predict the mortality risk in patients with SJS/TEN. It is a useful assessment to gauge the prognosis of patients and is done within the first 48 hours from the onset of the disease

- ***Genes in SJS/TEN***

In recent studies, drug-specific genetic susceptibility has been associated with human leukocyte antigen (HLA) alleles. HLA-B*15:02 is strongly associated with carbamazepine (CBZ)-induced SJS/TEN and HLA-B*58:01 with allopurinol-induced SJS/TEN in Han-Chinese (Chung 2010). These findings have led to the implementation of screening of patients for HLA-B*15:02 allele before the initiation of CBZ treatment to reduce incidence among Southeast Asian populations (Chen 2011). The relationship between HLA-B*58:01 and allopurinol-induced SJS/TEN has been reported in many ethnicities, including in Taiwanese, Japanese, Korean, Thai, and European individuals.

Therefore, this data suggested that HLA-B*58:01 genotyping may be useful to prevent allopurinol-induced SJS/TEN. A United States study found that testing for HLA-B*58:01 prior to allopurinol initiation was cost effective for Asians and African Americans but not for Caucasians or Hispanics (Hasegawa 2020). Evidently, HLA typing can help identify individuals at higher risk for SJS/TEN and reduce the burden for patients who lack susceptibility alleles. Furthermore, this reveals that HLA-restricted presentation of antigens (drugs or their metabolites) to T lymphocytes initiates the immune reaction of SJS/TEN (Chung 2010).

III. Non-blistering delayed-type DHRs

- ***Drug reaction with Eosinophilia and Systemic Symptoms (DRESS) Syndrome***

Drug reaction with eosinophilia and systemic symptoms (DRESS) syndrome is another life-threatening delayed-type DHR that does not blister (Kardaun 2013). DRESS develops typically from 2 weeks to as late as 2-3 months after initial exposure to the causative agent (Hoetzenecker 2015). The rash presents as a diffuse morbilliform eruption with prominent facial and ear involvement. The rash can become edematous and include purpuric lesions and pustules (Cabanas 2020). Systemically, fever is seen in 90% of patients along with a high internal organ involvement in 85-96% of patients (Cabanas 2020). The liver (70 %), kidneys (11 %), and lungs are the organs most frequently involved. The most common drug culprits include carbamazepine, dapsone, phenytoin, salazosulfapyridine, phenobarbital, allopurinol, and zonisamide (Hoetzenecker 2015). Data on the incidence of DRESS are scarce with the reported number of cases at 10 per million. In most cases, patients with DRESS are treated with systemic corticosteroids,

though the disease is not always responsive and the mortality rate is estimated at about 10% (Bellon 2019, Hoetzenecker 2015).

- ***Morbilliform Drug Eruption (MDE)***

Morbilliform Drug Eruptions (MDE) are the most common type of delayed-type DHR. The reaction develops typically days to three weeks after the offending drug is first given, although the timing can differ if previously sensitized (Alboud 2021, Fitzpatrick 2018). The rash consists of pink to red maculopapular lesions with no mucosal involvement and no blistering (Alboud 2021, Fitzpatrick 2018). Lesions usually appear first on the trunk or in areas of pressure or trauma (Alboud 2021, Fitzpatrick 2018). They spread to involve the extremities, usually in a symmetrical distribution (Alboud 2021, Fitzpatrick 2018). The most frequent drug offenders include antibiotics (beta-lactams, sulfonamides), NSAIDS, antiepileptics (carbamazepine, hydantoins), and allopurinol (Alboud 2021, Fitzpatrick 2018).

IV. Proposed Modes of Pathogenesis in SJS/TEN

- ***Models of T Cell Activation and its Effects***

SJS/TEN is traditionally thought to be a T-cell-mediated disorder. T cells are activated by the binding of drugs to T-cell receptors (TCRs) from antigen-presenting cells (APCs). There are currently three hypotheses on T-cell activation in SJS/TEN: (1) the hapten/pro-hapten model, (2) the pharmacological interaction (p-i) concept, and (3) the altered peptide model (Hasegawa 2020). Yet, among these hypotheses, the initiating phase of cell death is attributed to interactions between HLA determinants, drug epitopes, and TCRs of yet poorly characterized T-cell subsets (Panayotova-Dimitrova 2015). This

leads to unwanted immune cell activation (Panayotova-Dimitrova 2015). Recent advances in pharmacogenomic studies have provided strong evidence for the association of HLAs with specific drug-induced SJS/TEN (Abe 2016). An interaction with the drug or drug metabolite alters the repertoire of self-peptides bound to the HLA on the cell surface, activating a wide range of CD8 T cells (Abe 2016). The predominance of the CD8 phenotype in blister fluid and skin biopsies has validated this (Iwai 2012, Harr 2010, Correia 1993). Further, evidence suggests a pathogenic role for a disease-specific clonotype. For example, CD8 T cells isolated from SJS/TEN patients were expanded upon drug re-stimulation *in vitro* (Ko 2011). Ko et al. (2011) found that when re-challenging isolated PBMCs from subjects with CBZ-SJS/TEN, their CD8 T cells expanded and released granulysin. Furthermore, SJS/TEN blister fluid and skin biopsies have been found to contain a predominant fraction of CTLs, and to a lesser extent, natural killer (NK) cells, and macrophages (Kujper 2020).

Cytotoxic CD8 T lymphocytes (CTLs) are best known for the secretion of cytolytic granules containing perforin, granzymes and granulysin. Perforin plays an important role in the cytotoxic activity of CD8 T cells and NK cells by inducing pore formation in the cell membrane of target cells (Voskoboinik 2015, Osinska 2014). This allows for the entry of granzyme B and other deadly proteins such as granulysin. As a result, target cell apoptosis is triggered by an enzymatic cascade (Voskoboinik 2015, Tewary 2010, Janeway 2001).

- ***Other Potential Mediators of Keratinocyte Death in SJS/TEN***

Granulysin, in particular, has received attention for its role in cell-mediated immunity. High levels of the uncleaved 15-kDa form have been reported as a serum marker for SJS/TEN (Abe 2016). For example, Chung et al. (2008) reported that granulysin is strongly expressed in SJS/TEN skin lesions and plays a crucial role in keratinocyte death. The cytotoxic effect of SJS/TEN blister fluids on keratinocytes was reduced by depletion of granulysin (Abe 2016, Chung 2008). In addition, an intra-dermal injection of recombinant uncleaved 15 kDa granulysin in mice induced TEN in a matter of hours, apparent both grossly and histologically (Chung 2008). Moreover, another study found that serum levels of granulysin were found to increase during the early stage of SJS/TEN, but not in patients with MDE (Carr 2019, Abe 2016).

Other suspected mediators of keratinocyte death include interferon-γ, TNF-α and TRAIL proteins. For example, Araujo et al. (2011) found high concentrations of these proteins in SJS/TEN blister fluids compared to normal sera, SJS/TEN sera, and eosinophilic pustular folliculitis blister fluids. They suggest these proteins act in synergy to induce cell death in vivo (Araujo 2011). Gupta et al. (2016) showed support for these factors along with TNF-like weak inducer of apoptosis (TWEAK) in the involvement of SJS/TEN pathogenesis (Gupta 2016). Moreover, CTLs and NK cells can express Fas ligand (FasL) which binds the Fas receptor on target cells resulting in apoptosis. Viard et al. (1998) found that Fas is present on the surface of keratinocytes of patients with TEN which differentiated TEN patients to those with other dermatological conditions (Viard 1998). Additional cell death molecules have been implicated in SJS/TEN, including

annexin A1, and micro-RNA 18a-5p regulation (Panayotova-Dimitrova 2021, Ichihara 2014, Saito 2014, Chung 2008, Viard 1998).

- ***Other Potential Cell Types involved in Keratinocyte Death in SJS/TEN***

CTLs have been implicated as the associated effector cell molecule known to drive SJS/TEN but recent studies are beginning to challenge this paradigm. Along with T cells, NK cells and monocytes are consistently observed in patient samples and may be contributory to cell death (Bellon 2019). As noted above, NK cells and monocytes/macrophages are capable of producing some of the same cell death molecules as CTLs. Notably, evidence for neutrophil involvement is now bringing a shift in direction of our understanding of the etiology of SJS/TEN. Recently, Kinoshita et al described a new mechanism by which neutrophils trigger inflammation during early phases of SJS/TEN. They found that the initiation and progression of keratinocyte death is potentially mediated by innate and adaptive immune responses. Here, they discovered CD8 T cells produced lipocalin-2 in a drug specific manner which triggered neutrophil extraceullar traps (NETs) in early lesional skin. As a result, neutrophils undergoing NETosis released LL-37 to induce formyl peptide receptor 1 (FPR1) expression. Furthermore, this led keratinocytes to be vulnerable to cell death by necroptosis (Kinoshita 2021).

Nevertheless, the pathobiology of this disease remains obscure, and contradictory evidence from various studies signifies the need for rigorous analysis to overcome these barriers in our understanding of SJS/TEN and its pathogenesis.

V. Overview of Cell Death Pathways

It is well known that apoptosis is an active and highly regulated cell death process that is non-inflammatory. In contrast, necrosis, a morphologically distinct form of cell death, has traditionally been regarded as passive, unregulated, and pro-inflammatory (Kung 2011). Recent evidence over the last few decades has revealed other mechanisms of cell death including two other programmed forms of cell death, pyroptosis and necroptosis.

- ***Apoptosis***

Apoptosis was the first programmed cell death pathway to be described and was morphologically characterized in 1972. It is highly coordinated and immunologically inert. It can be triggered through two major pathways referred to as the intrinsic and extrinsic pathways (Bertheloot 2020).

The intrinsic pathway is triggered by dysregulation or imbalance in intracellular homeostasis caused by toxic agents or DNA damage. It is characterized by mitochondrial outer membrane permeabilization (MOMP), resulting in the release of cytochrome C into the cytosol, which triggers the formation of the apoptosome. The apoptosome consists of Apaf-1 and pro-caspase 9. Autocleavage of pro-caspase 9 leads to caspase 3 activation and the point of no return in apoptotic cell death (Bertheloot 2020, Galluzi 2018).

The extrinsic pathway is activated by the engagement of membrane receptors such as the TNF receptor 1 (TNFR1), death receptors, or toll like receptors. These proteins induce the formation of signaling complexes involving TNFR1-associated death domain protein (TRADD) and Fas-associated death domain protein (FADD), receptor-

interacting serine/threonine protein kinase 1 (RIPK1) and pro-caspase 8 (Bertheloot 2020). Ubiquitylation of RIPK1 by cellular inhibitors (cIAPs) stabilizes the complex and induces the activation of the transcription factor NFκB (Bertheloot 2020). Flice inhibitory protein (FLIP), also present in the death inducing signaling complex (DISC), limits caspase-8 activity while promoting cell survival, cell proliferation, and the production of proinflammatory cytokines (Bertheloot 2020). Imbalances in this pathway such as those induced by cellular stress, activates caspase-8 and caspase-10 which in turn triggers the caspase activation cascade, cleaving pro-caspase 3 where both pathways converge (Wang 2021, Bertheloot 2020).

The activation of caspase 3 in both pathways induces chromatin condensation and nuclear fragmentation (pyknosis), plasma membrane blebbing, and cell shrinkage. Eventually, the cell breaks into small membrane-surrounded fragments (apoptotic bodies), which are cleared by phagocytosis without inciting an inflammatory response (Reed 2000). Notably though, when apoptotic cells are not cleared in an efficient and timely manner, it progresses to secondary necrosis, an inflammatory process (Sachet 2017). Conversion to secondary necrosis is mediated by deafness autosomal dominant 5 (DFNA5) which is cleaved by caspase-3 to generate DFNA5 N-terminal fragment. This leads to the formation of non-selective pores and finally secondary necrosis (Zhang 2018).

- ***Necroptosis***

Necroptosis serves as a critical cell-killing mechanism in response to severe stress (Figure 2). Unlike apoptosis, necroptosis elicits a more robust immune response. It can be

induced by inflammatory cytokines or chemotherapeutic drugs, or by blocked apoptosis, and as such, both apoptotic and necroptosis have overlapping molecular machineries (Panayotova-Dimitrova 2015). Most notably, TNF-α, TRAIL, and FasL can induce necroptosis if apoptosis is blocked. Interestingly, caspase 8 drives apoptosis; however, in its absence, cell death will shift to necroptosis (Dhuriya 2018). This requires assembly of the receptor-interacting protein kinase 1 and 3 (RIPK1/RIPK3) complex, called the Necrosome. Auto- and trans-phosphorylation of RIPK1 and RIPK3 enables recruitment and activation of the pseudokinase, mixed lineage kinase-like (MLKL), followed by oligomerization, binding to plasma membrane-associated phosphatidylinositol phosphates, and the disruption of plasma membrane integrity (Wang 2021, Kim 2018). This leads to the loss of membrane integrity, swelling of the mitochondria, and passive leakage of intracellular contents ultimately leading to inflammation (Bertheloot 2021, Chen 2016).

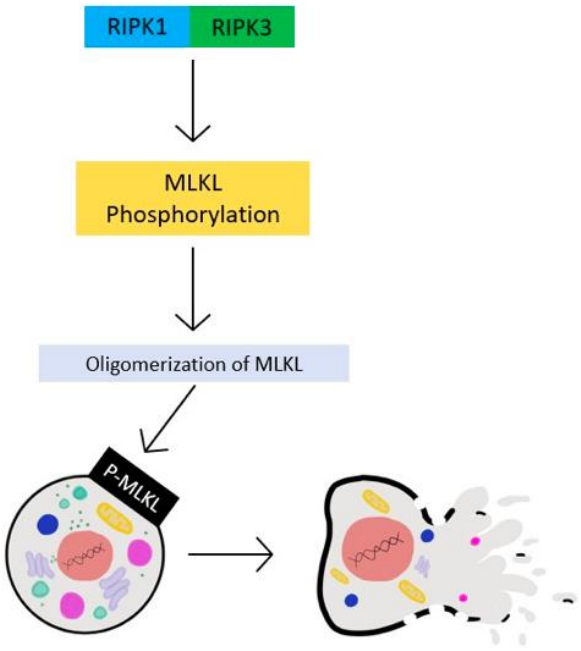

Figure 2. General overview of key mediators in necroptosis pathway. A simplified molecular mechanism of necroptosis. Inside the cell, RIPK1 and RIPK3 interact to create a complex that will promote the oligomerization of MLKL by phosphorylating it. Following MLKL phosphorylation, the protein will then insert itself in the plasma membrane creating a pore. As a result, the cell ruptures and sparks an inflammatory response.

- ***Pyroptosis***

Pyroptosis is becoming a widely studied inflammatory disease model (Wang 2019, Figure 3). In the canonical caspase-1-mediated pathway, intracellular sources known as inflammasome sensors become activated by pathogen-associated molecular patterns (PAMPS), damage-associated molecular patterns (DAMPS), or dysregulated cellular pathways (Bertheloot 2020, Wang 2019). Inflammasome sensor composition can differ depending on the stimuli but are broadly composed of the Nod-like receptor (NLR) family, the DNA receptor Absent in Melanoma 2 (AIM2), and the pyrin receptor. In particular, the nod-like receptor family pyrin domain-containing 3 (NLRP3)

inflammasome is the sensor most strongly associated with the development of uncontrolled inflammation (Bertheloot 2020). Upon stimulation, the inflammasome sensor oligomerizes and recruits the adaptor protein apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC) (Bertheloot 2020). As a result, this forms the polymeric inflammasome structure that activates caspase-1. Active caspase-1 cleaves the pro-forms of the interleukin family cytokines interleukin 1-beta (IL-1β) and interleukin-18 (IL-18) as well as gasdermin D. Cleavage of gasdermin D activates and unmasks the N-terminal domain which inserts itself in the plasma membrane forming pores. As a result, this enables the release of mature IL-1β and IL-18. These inflammatory cytokines further stimulate interferon gamma (IFN-γ) release from T helper type 1 (Th1) cells, amplifying systemic inflammatory effects (Wang 2019, Wang 2021, Galluzi 2018, Kim 2018).

In contrast to canonical pyroptosis, non-canonical pyroptosis is mediated by activation of caspases-4/5 through binding of cytosolic lipopolysaccharide (LPS) to the CARD domain, which results in cleavage of gasdermin D to induce pyroptosis. Additionally, activated caspase 3 can cleave gasdermin E, releasing its active N-terminal fragment to induce pyroptosis (Wang 2019).

The downstream effects of pyroptosis lead to cytoplasmic swelling, membrane rupture, and the release of the cytosolic contents of the cell (Yan-Yang Wang 2019, Kim 2018).

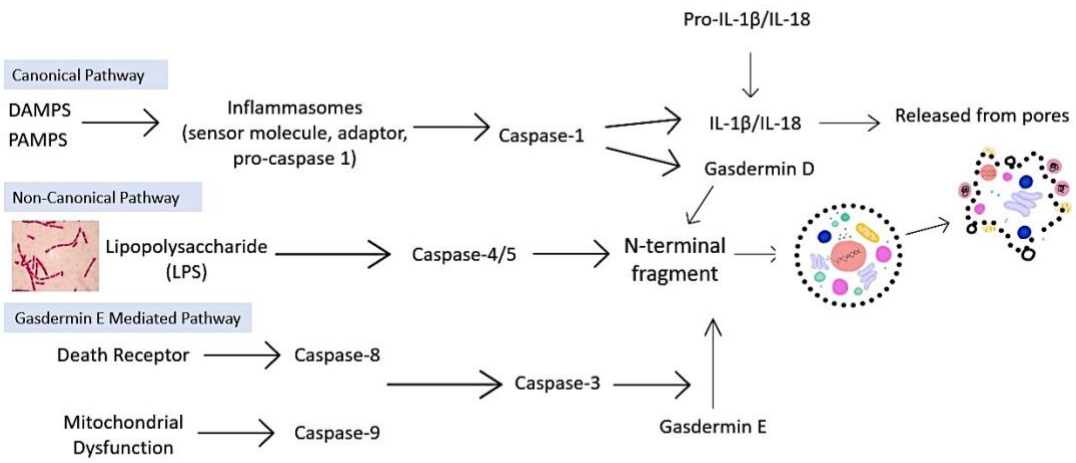

Figure 3. General overview of 3 known pathway arches in pyroptosis. A simplified schematic diagram of the three known pyroptotic pathways.

VI. What is Known about the Cell Death Pathways in SJS/TEN

It is assumed that the cell death pathway apoptosis is responsible for keratinocyte cell death in SJS/TEN (Bellon 2019, Dodiuk-Gad 2015). However, to associate a classically non-inflammatory pathway such as apoptosis to a highly inflammatory disease like SJS/TEN is contradictory (Romar 2020, Ueta 2018).

In 1996, Paul et al. reported that keratinocyte cell death seen in SJS/TEN was due to apoptosis. In his investigation, terminal deoxynucleotide transferase (dUTP) nick end labeling (TUNEL) staining was applied to SJS/TEN skin samples and demonstrated DNA fragmentation (Paul 1996). However, we now know that TUNEL staining/DNA fragmentation is not limited to apoptosis (Mirzayans 2020). Other cell death pathways such as necroptosis and pyroptosis also result in TUNEL-positive staining (Jia 2019, Man 2017, Wen 2017). In 2014, Saito et al challenged the dogma regarding keratinocyte apoptosis in SJS/TEN with evidence supporting necroptosis instead. Saito et al suggested

that causative drug exposure induces annexin A1 secretion from monocytes in SJS/TEN which binds to FPR1, which in turn induces keratinocyte death via necroptosis. The importance of FPR1 to keratinocyte death in SJS/TEN gained additional support from Kinoshita et al. Their study demonstrated that neutrophils forming neutrophil extracellular traps (NETosis) released human cathelicidin 37 (LL-37), an anti-microbial peptide, which induced FPR1 expression by keratinocytes and activated downstream necroptosis (Kinoshita 2021).

In apoptosis, proteins such as cellular Fas-associated death domain-like interleukin-1β-converting enzyme (FLICE)-like inhibitory protein (c-FLIP) control the extrinsic apoptosis pathway through caspase-8 to prevent uncontrolled cell death. Epidermal loss of c-FLIP was suggested to be associated with epidermal cell death in SJS/TEN. c-FLIP is responsible for keeping caspase-8 active. Interestingly, loss of c-FLIP results in spontaneous skin inflammation which supports a role for cFLIP in maintaining the integrity of the epidermis (Panayotova-Dimitrova 2013). Furthermore, c-FLIP functions to regulate caspase 8 activity and together they suppress the lethal effects of RIPK3 in the necroptotic pathway (Weinlich 2014). Kim et al. (2015) found that RIPK3 expression was highly upregulated along with elevated MLKL phosphorylation in skin sections from TEN patients (Kim 2015), suggesting a potential role for necroptosis in TEN (Panayotova-Dimitrova 2015). Moreover, Saito et al. (2014) suggested that the necroptosis pathway was mediated by annexin 1 acting through FPR1 (abundant on keratinocytes), thereby contributing to SJS/TEN keratinocyte death. Indeed, blocking annexin 1 with an antibody led to attenuation of the disease. Further studies regarding

specific receptors or receptor polymorphisms related to SJS/TEN have yet to be carried out (Cheng 2021, Saito 2014). To our knowledge, no studies have pursued the role of pro-inflammatory pyroptosis in SJS/TEN, though its potential role in disease pathogenesis is worth unraveling.

VII. Challenges in researching SJS/TEN

There are multiple obstacles limiting investigation of SJS/TEN. First, SJS/TEN is a rare disease, limiting available sample sizes for both retrospective and prospective studies. Second, is the difficulty of obtaining usable skin samples. For retrospective studies, these tissues are most often formalin-fixed paraffin-embedded (FFPE) specimens. Although this method supports pathological diagnosis by preserving tissue architecture and cell morphology, it impedes most research applications. Furthermore, there are currently no model systems available for mechanistic studies. Our group hopes to overcome these barriers by utilizing a large patient database and a bank of FFPE skin specimens from multiple Harvard teaching hospitals and outside institutional partnerships, collection of fresh samples across multiple Harvard hospitals for prospective studies, and development of a novel *in vitro* model of disease. Microscopically, characteristics of cell death (Table 2) coupled with experimental indicators of cell death per pathway (Table 3) can allow for effective discrimination. In addition, the activation and cleavage of certain proteins and molecules gathered from our preliminary findings and published studies will allow to us further distinguish among these three cell death pathways in tissue samples (Table 4).

Characteristics of Cell Death									
	Cell Lysis	Cell Swelling	Pore Formation	Membrane Rupture	Membrane Blebbing	DNA Fragmentation	Nucleus Intract	Inflammation	Programmed Cell Death
Apoptosis	×	×	×	×	✓	✓	×	×	✓
Pyroptosis	✓	✓	✓	✓	✓	✓	✓	✓	✓
Necroptosis	✓	✓	✓	✓	×	✓	×	✓	✓

Table 2. Characteristics of cell death among different cell death pathways. Check marks indicate which characteristics of cell death are viewed microscopically for each distinct pathway. The "x" mark means this characteristic is absent. This chart will guide us in differentiating these pathways through tissue staining.

Experimental Indicators of Cell Death								
	TUNEL	Annexin V	Propidium Iodide	7 AAD	HMGB1	LDH	Cleaved IL-1α	Other Markers
Apoptosis	+	+	-	-	-	-	-	Cytochrome C, Cleaved ICAD, Mitochondrial Membrane Assay, WB of Cleaved Gelsolin or ROCK1
Pyroptosis	+	+	+	+	+	+	+	Cleaved IL-1β, TNFα, IL-6, ASC specks, β-Actin, Cleaved N-GSDMD or N-GSDME, or Lysosomal Cathepsin B
Necroptosis	+	+	+	-	+	-	+	Cleaved IL-33, ATP, P-MLKL, High RIPK1 ratio to Pro-Caspase-8

Table 3. Experimental indicators of cell death by pathway. The "+" mark indicates that a pathway is positive for release one of the following mediators shown in the column or can be detected by an assay. The "-" shows no presence. ICAD, otherwise known as DFFA is a substrate for caspase-3 and triggers DNA fragmentation during apoptosis. WB = Western Blot. ROCK1 = Rho-Associated Kinase 1, IL-6 = Interleukin 6, N-GSDMD = N-terminal fragment of gasdermin D, N-GSDME = N-terminal fragment of gasdermin E, IL-33 = interleukin 33, ATP= adenosine triphosphate, and p-MLKL = phosphorylated MLKL.

Intrinsic Apoptosis	Extrinsic Apoptosis	Secondary Necrosis	Necroptosis	Pyroptosis
BCL-2	TNFR1	DFNA5	cFLIP	AIM2
Bak	TLR		Annexin A1	CARD
BAX	Fas		FRP1	Caspase 1
MOMP	FADD		RIP1	Caspase 3
Cytochrome C	Caspase 8		RIP3	Caspase 4/5
Smac	Caspase 10		p-MLKL	Caspase 8
HTRA2	cFLIP		Caspase 10	NLRC4
Apaf-1	Caspase 3			NLRP1
Caspase 9	Caspase 6			NLRP3
Caspase 3	Caspase 7			PYCARD
ROCK1	Lamin B1			ASC
Caspase 7	Caspase 10			LPS
				Gasdermin D
				Gasdermin E
				IL-1β
				IL-18

Table 4. Key proteins and soluble mediators in different cell death pathways. Results from preliminary data (unpublished) in conjunction with literature analysis yielded key downstream mediators in pathways. This will be utilized for tissue staining studies.

SPECIFIC AIMS

The purpose of this study is to investigate immune-mediated cell death pathway(s) in SJS/TEN. We hypothesize that soluble death molecules TNFα and TRAIL from multiple immune cell types (CTLs, NK cells and monocytes/macrophages) induce necroptosis and pyroptosis in keratinocytes in SJS/TEN.

Aim 1. To investigate immune-mediated cell death pathway(s) in SJS/TEN retrospectively using human skin samples.

- ***Subaim 1.1*** Conduct gene expression profiling with the NanoString nCounter® System on our newly established secondary cohort using an expansive gene panel to build on preliminary data from a previous cohort of patients

Aim 2. To interrogate immune-mediated cell death pathway(s) in SJS/TEN using a novel *in vitro* model of disease.

- ***Subaim 2.1*** Identify the best source of skin for creating an in vitro model of disease
- ***Subaim 2.2*** Test the selected source of skin in an optimization assay
- ***Subaim 2.3*** Use the selected source of skin to investigate cell death mediators and downstream cell death pathways in disease

METHODS

Retrospective Study Patients for Gene Expression Profiling and Tissue Staining

A retrospective study was conducted on FFPE skin samples obtained from adult and pediatric patients from January 1, 2014 to present with clinically-diagnosed and dermatopathology-confirmed cases of SJS/TEN, DRESS, and MDE. Pathology department databases at Massachusetts General Hospital, Brigham and Women's Hospital, and Boston Children's Hospital were first searched using the following terms: "toxic epidermal necrolysis," "toxic," "epidermal," "necrolysis," "Stevens-Johnson syndrome," "Stevens- Johnson," "full-thickness necrosis," "drug reaction with eosinophilia and systemic symptoms," "drug rash with eosinophilia and systemic symptoms," "DRESS," "drug hypersensitivity reaction," "hypersensitivity," and "drug." This search was carried out in two phases. The first phase was to identify patient cases from the database that exclusively had skin biopsies and received a dermatology consult. Pathology records and clinical data included in the biopsy results were read. Biopsies were taken either before or after systemic treatment was administered following disease onset. Patients without skin biopsies, no records found, missing medical record numbers, duplicates in our lab database, or restricted cases to access were excluded. Extracted information from phase one of the database search were reviewed by an experienced dermatopathologist to identify likely candidate cases to move on to the second phase.

In the second phase, selected cases with confirmed skin biopsies and dermatology consults were cross referenced and screened using the electronic medical record at its respective institution to collect extensive clinical data. Patient medical record data were

collected including age, race/ethnicity, survival or death from causes related to SJS/TEN, loss to follow-up, pre-existing comorbidities including HIV, cancer, bone marrow transplant (BMT), autoimmune disease, history of drug allergies, the timing of the emergence of the rash and treatment, infections (if any), consults, TBSA affected, organ system involvement, causative drugs, treatments, and suspected drugs or causes (Figure 4, Figure 5, Figure 6). Alternative pathology or clinical diagnoses, cases lacking sufficient clinical data to confirm diagnosis, and cases of pure SJS (<10% body surface area involvement) or erythema multiforme (EM) were excluded. Medical cases from phase two collection for SJS/TEN, DRESS, and MDE are currently being reviewed by an expert dermatopathologist for final confirmation in order to be included in our study. These cases will expand the current bank of 45 FFPE dtDHR specimens collected previously from preliminary work.

Prospectively collected cases were included in this database following the diagnosis made by a board-certified dermatologist during the patient's on-site care. After receiving patient consent, tissue specimens were collected.

The processing of tissues was done under sterile conditions. The biopsy specimen was sectioned into three parts, one for formalin fixation, and the other two sections were frozen separately in 1.5 ml cryotubes (ThermoFisher Scientific, Waltham, MA, USA) filled with 1200 ul of CryoStor® cell cryopreservation media (Sigma-Aldrich, St. Louis, MO, USA) and placed in the -80°C freezer. After 24 hours, the two cryotubes with skin were placed in a liquid nitrogen tank for preservation for future use.

For formalin fixation, the remaining one-fifth of the tissue was placed in 10 ml of 10% neutral buffered formalin overnight. The following day the skin was washed with distilled water for 1 hour and then placed in 70% ethanol solution. This was then embedded into paraffin and sectioned onto slides by the Harvard Pathology Core.

Healthy human skin discarded during plastic surgeries and healthy human blood serum served as controls. The excess human skin was processed in the same manner as prospective tissue specimens. Blood collars from Boston Children's Hospital were used to extract healthy serum. To a 50 ml falcon tube, 12.5 ml of Ficoll® (Sigma-Aldrich, St. Louis, MO, USA) was transferred. The blood from the collar was gently overlaid on the Ficoll® to prevent mixing and then centrifuged at 25°C for 20 minutes at 2000 rpm with a speed acceleration of 9 and speed deceleration of 1. Four distinct layers appeared and with a sterile pipette, 1 ml of serum, from the first layer was transferred into a sterile 2 ml Eppendorf Tube® (Sigma-Aldrich, St. Louis, MO, USA) and then stored in the -80°C freezer. This study was approved by the Institutional Review Board (IRB) of Partners Human Research Committee.

Designing a NanoString nCounter® Gene Panel to Study Cell Death Pathways in SJS/TEN

An extensive literature search was conducted to identify genes involved in cell death pathways, in particular genes critical to distinguishing between cell death pathways. These are referred to as genes of interest, immune cells of interest, necroptosis, pyroptosis, and apoptosis, mediators, and markers (Table 5). NanoString® Technologies (Seattle, WA, USA) nCounter® Panel is a robust platform allowing for gene expression

profiling from FFPE skin samples. Three pre-designed Nanostring panels, the Human Immunology V2, Human Inflammation V2, and Human Host Response panels were analyzed for the number of genes of interest each panel included. This required identifying the associated protein encoded by each gene. Panels were then compared to identify the panel with the highest number of genes for of interest. The Human Host Response panel was identified as containing the highest number of desired genes of interest. Genes of interest not included in the pre-designed panel, were then added to the panel. The final custom designed panel is listed in Table 6.

In Vitro Model Development

The *in vitro* model uses the human tissue product NativeSkin Access® (Genoskin, Salem, MA, USA), a commercially available 3D full thickness human skin with human appendages preserved with patented technology. The ultimate goal is to apply patient blister fluid or serum (both devoid of cells), or recombinant proteins including granulysin, TNF-α, or TRAIL to the skin to recapitulate keratinocyte cell death and epidermal necrosis.

Prospective samples are collected in two capacities. Currently, we are engaged in a cross-disciplinary effort with the burn teams at Mass General Hospital and Shriners Hospital. This is part of our prospective study to obtain blister fluid from patients with SJS/TEN that have over 10% of TBSA affected, and compare with specimens from thermal burn patients as controls. Patients of all ages, races and ethnicities, and sex are included.

Blister fluid collected from patients was processed under sterile conditions. The fluid was transferred into a sterilized 2 ml LoBind Eppendorf Tube® (Eppendorf North America, Framingham, MA, USA) and then spun down at 1400 rpm at 4°C for 5 minutes. The supernatant was then transferred into another sterilized 2 ml LoBind Eppendorf Tube® and frozen at -80°C for later analysis and future use as a control that will be applied to the NativeSkin Access® *in* vitro model.

To optimize our assay, a trial run was conducted with 8 tissues. Tissues were acclimated at 37°C, 5% CO2, and 95% relative humidity in the incubator for one hour with 1 ml of GenoSkin media prior to experimentation per manufacturer's instructions. At time point zero, the media added for the pre-incubation period was removed. 1 ml of healthy human serum (Biochemed25, Winchester, VA, USA) was added to the well of 3 NativeSkin Access® tissues with 20 µl added to the epidermal chamber. This was repeated for 3 other tissue wells using the provided GenoSkin media instead. The seventh tissue specimen had 800 µl (80%) of serum and 200 µl (20%) of GenoSkin media added to the well with 20 µl of media to the epidermal chamber. Finally, the eighth tissue was processed at time point zero to be utilized for comparison between other time points.

Tissues and well plate solution were observed grossly for changes at multiple time points following time point zero (1 hour, 3 hours, 6 hours, and 24 hours). At 3, 6 and 24 hours, tissue was harvested using an 8mm punch biopsy tool and cut into 4 pieces, one each for formalin fixation, OCT embedding, RNA isolation, and protein isolation (Figure 4).

For formalin fixation, each tissue section was added to 5 ml of 10% neutral buffered formalin overnight and washed the following day with distilled water for 1 hour, and then stored in 70% ethanol solution then embedded into paraffin and sectioned onto slides by the Harvard Pathology Core. Hematoxylin and eosin stains (H&E) were carried out on the FFPE tissue sections (4 µm) by standard immunohistochemical techniques for slides at time point 0 with media and time point 24 with serum only (Figure 8).

For RNA isolation, a piece of tissue was added in 500 µl of RNAlater (ThermoFisher Scientific, Waltham, MA, USA) in a 1.5 ml cryotubes (ThermoFisher Scientific, Waltham, MA, USA) and stored in a -80°C freezer for future use. A piece of tissue for protein isolation were added to empty cryotubes then frozen in the -80°C freezer. Finally, tissues sections for OCT (Sakura Fintek USA, Torrance, CA, USA) were added to a 15 mm x 15 mm x 5 mm disposable vinyl specimen mold (Sakura Fintek USA, Torrance, CA, USA) filled with OCT embedding matrix, frozen on dry ice, and stored in a -80°C freezer.

Following our optimization, we plan to perform repeat experimentation using the collected SJS/TEN and control patient serum and blister fluid with this model. Treated epidermis will be assayed by histology, immunofluorescence staining and microscopy, bulk RNA sequencing and protein assay by multiplex immunoassay for 50 relevant analytes, colorimetric assay for activated/cleaved caspases 1, 3, 4, 5, 6, 8, 9, and 10 and western blot for activated RIPK3, MLKL, DFNA5, gasdermin D, ROCK1, Lamin B, along with loading controls. Other plans include injecting recombinant proteins such as

granulysin, TNF-α, and TRAIL to see if it induces epidermal necrosis in this *in vitro* model.

Thus far, an extensive literature search was conducted to identify the physiological amounts of granulysin, TNF-α, and TRAIL in SJS/TEN patient blister fluid and blood serum. In parallel, we compared different recombinant protein options based on their use for functional studies, endotoxin levels, molecular structure, use for bioactivity assays, evaluating published reports on their use, and discussion with industry. This led to the identification of recombinant proteins for future use and assessment of amounts that can be injected for experimental planning.

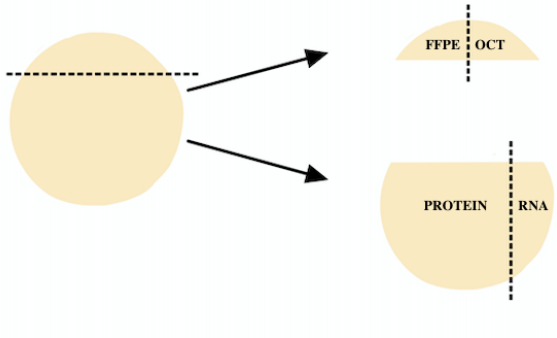

Figure 4. Sectioning of NativeSkinAccess® Tissues. This figure shows the how skin biopsy specimens were sectioned for FFPE, OCT, protein, and RNA analysis.

RESULTS

Aim 1 Rationale

The gene panel selected for was the Human Host Response panel which contains 785 genes associated with over 50 pathways. Following comparison with other panels, we found that this specific panel will allow us to best interrogate the complexities of the immune response. Additionally, this panel contains more genes of interest related to cell death pathways than other available panels (Immunology V2 and Inflammation V2). To provide the most comprehensive analysis we spiked in an additional 30 genes (Table 6) shown in red.

In order to investigate immune-mediated cell death pathways in SJS/TEN, access to samples of diseased tissues is required. The rarity of SJS/TEN and other severe dtDHRs such as DRESS and MDE limits the availability of clinical specimens for investigation. To overcome this challenge, we retrospectively analyzed a large patient database to accumulate enough formalin fixed paraffin embedded (FFPE) clinical specimens. To further leverage this approach, our multi-institutional design has allowed for greater access to dtDHR specimens. By having a larger pool of samples, we can obtain reliable results with increased precision and power. Currently, these cases are being evaluated by an expert dermatopathologist to identify candidates for our study.

Pathology department databases at Brigham and Women's Hospital (BWH), Massachusetts General Hospital (MGH), and Boston Children's Hospital (BCH) were first searched for pathology reads possibly consistent with SJS/TEN, DRESS, and MDE from January 1, 2014 to the present with a maximum of 10,000 hits. Search terms used

were as listed in the Methods section. For SJS/TEN this yielded an initial caseload of 194 reads (Figure 5). Data extraction and application of exclusion criteria yielded a total of 61 cases. For DRESS, the search terms yielded 246 cases (Figure 6). Since the search terms "drug" and "eosinophilia" were used, this brought in a broad range of cases unrelated to DRESS based on the pathology report and clinical data. Among the 6 dermatopathology reports, 4 cases were further evaluated. For MDE, the terms "hypersensitivity" and "drug" were searched with a set maximum of 10,000 records. This yielded an initial caseload of 2650 reads and 364 from 2021-2020 alone. Information was gathered from 200 out of these 364 cases (Figure 7).

The data collected for each likely candidate included documentation of sex, gender, race or ethnicity, age at presentation, survival of incident, current living status or loss to follow up, presence of malignancy, history of drug allergy, HIV status, history of bone marrow transplant and if allogeneic or autologous, history of autoimmune disorder, whether on immunosuppressive medications, other co-morbidities, recent history of infection prior to start of rash, date rash began, date disease began if different than rash, administration of immunosuppressive medication or treatments prior to biopsy, date of biopsy, biopsy results, fever >38degC , eosinophilia, positive testing for HHV6/EBV/HSV/*Mycoplasma pneumoniae*, whether allergy service was consulted, whether dermatology service was consulted, diagnosis of dermatology consulting service, maximum total body surface area involved, obvious or suspected culprit drug(s), other medications taken, treatment received, whether patient was listed as allergic to obvious or suspected culprits, whether listing(s) persist today, and availability of photos. For

SJS/TEN cases, also documented were the SCORTEN (if calculated) and involvement of eyes, oropharynx, vagina, penis/scrotum, urethra, respiratory tract, and gastrointestinal tract. For DRESS cases, also documented were presence of facial or ear erythema and edema, lymphadenopathy, eosinophilia, atypical lymphocytes, and involvement of kidneys and other organs. For MDE, the date of the biopsy, biopsy type, results, and dermatology consultation were included. Pathology reports for MDE and clinical data were read.

Overall, from this retrospective analysis, there are 61 cases being analyzed for SJS/TEN, 4 for DRESS, and 200 for MDE. A board-certified dermatologist with expertise in dtDHRs is evaluating each candidate case to confirm or exclude the diagnosis, to give our final yield of cases of dtDHRs that were clinically-diagnosed and biopsy-confirmed cases of disease. Patients will be excluded if there was <10% TBSA for SJS to eliminate or erythema multiforme (EM). This exclusion criteria would eliminate any chance of questionable diagnosis or an infection-induced disease.

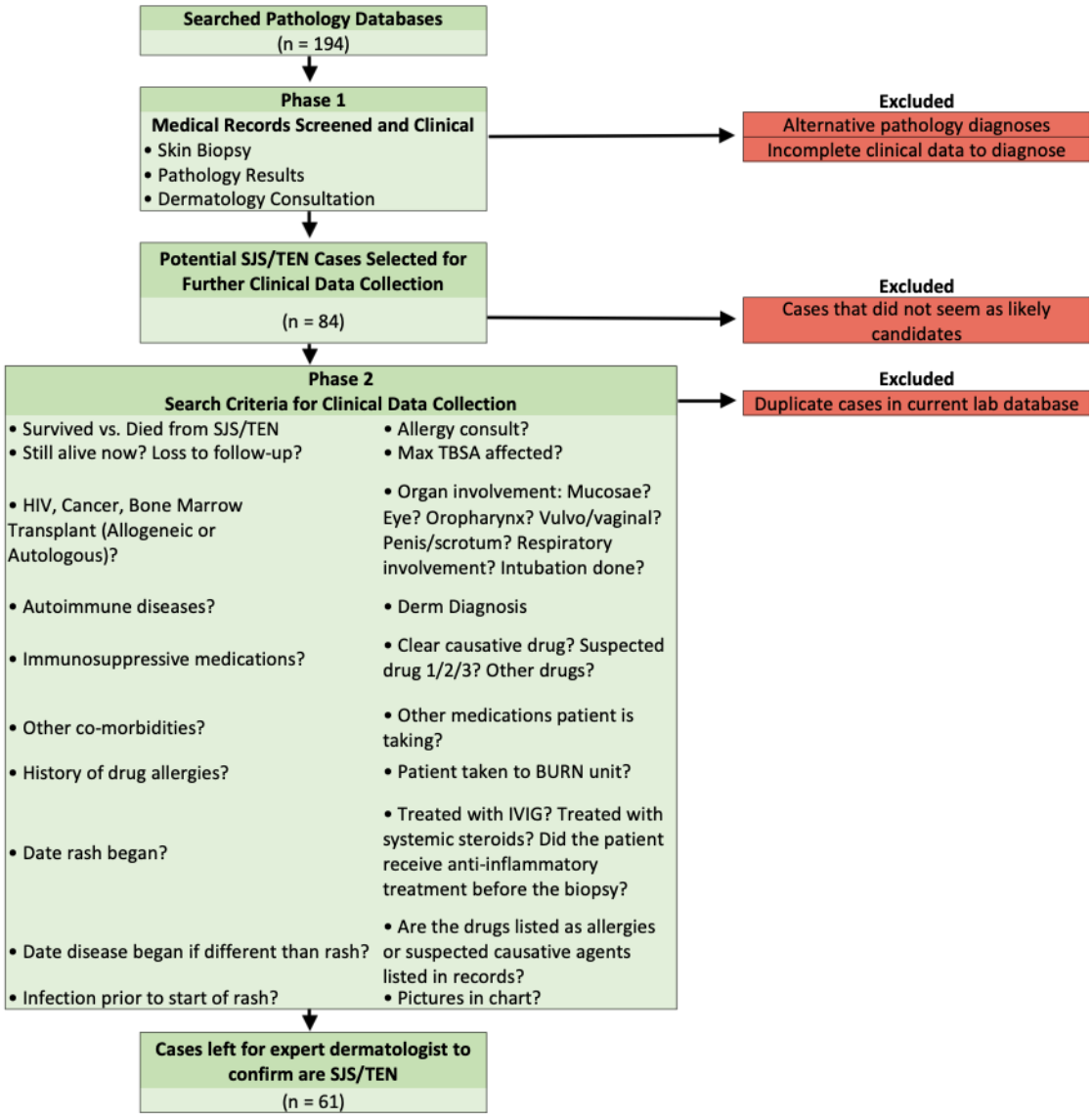

Figure 4. SJS/TEN database creation schematic. This illustration shows the stepwise process to generate the database of cases that are currently being validated by an expert dermatologist.

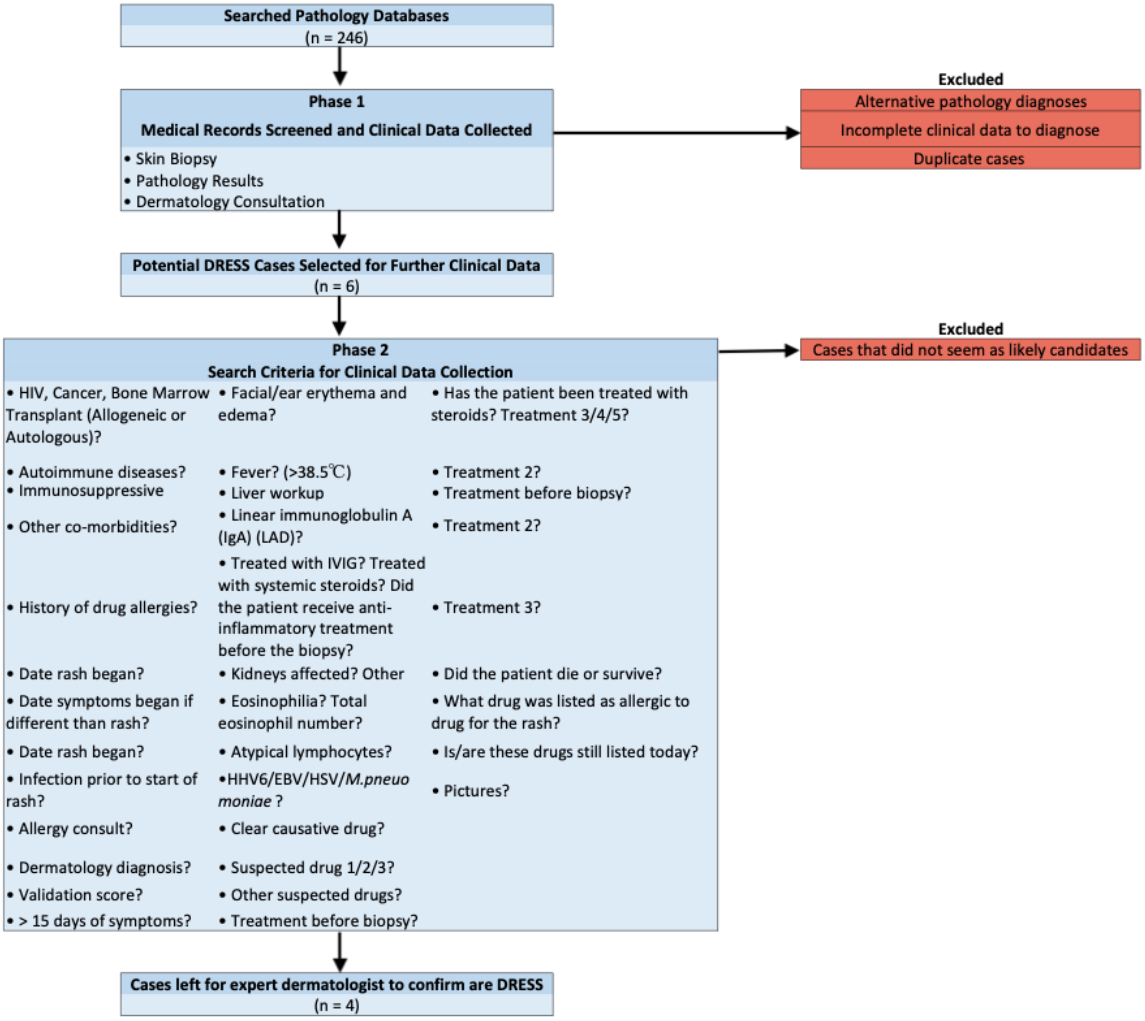

Figure 5. DRESS database creation schematic. This illustration shows the stepwise process to generate the database of DRESS cases that are currently being validated by an expert dermatologist.

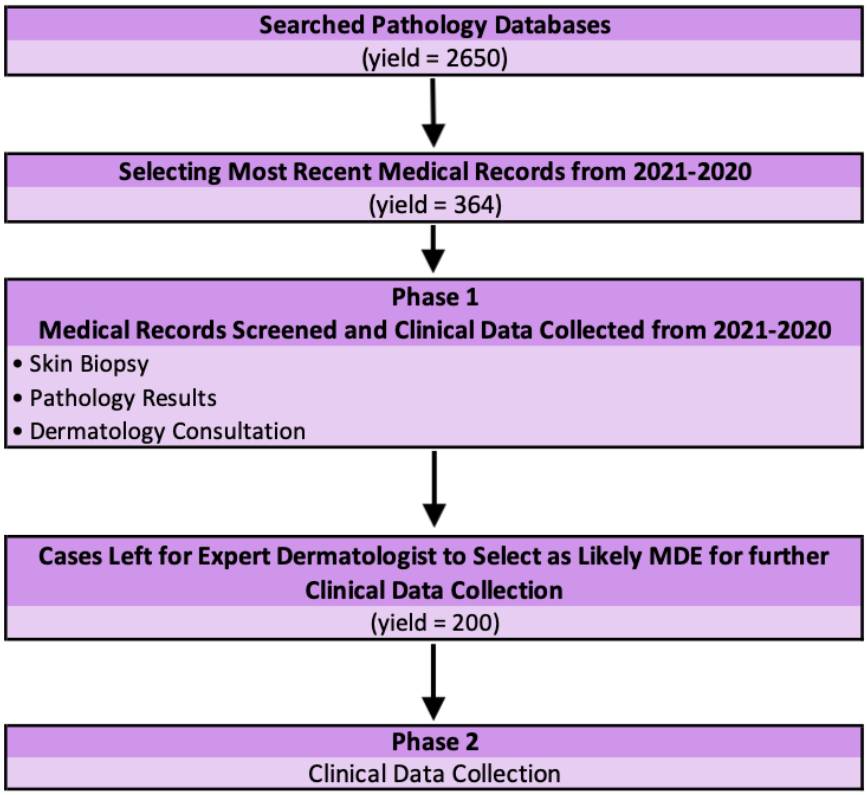

Figure 6. MDE database creation schematic. This illustration shows the stepwise process to generate the database of MDE cases that are currently being evaluated by an expert dermatologist for the second phase to collect additional clinical information.

Subaim 1.1 To Interrogate Cell Death Pathways at the Transcriptional Level in SJS/TEN

Preliminary data from previous gene expressional analysis with the NanoString nCounter® system in FFPE skin samples taken from 14 SJS/TEN patients suggests that death receptor and ligand-mediated cell death, potentially via necroptosis or pyroptosis, may be a key cell death pathway in SJS/TEN. These 14 SJS/TEN patients were compared to 10 healthy controls, and to DRESS, MDE, and fixed drug eruption (FDE) patients (unpublished). These results have shown key mediators that were highly upregulated compared to DRESS, MDE, and FDE (indicated by the color blue in Table 6). In contrast, apoptosis-associated caspases 2, 3, and 8 were not significantly upregulated (unpublished). We aim to validate these findings in a second patient cohort with an expanded gene panel.

Panels	Cost	Reactions	Number of Genes	Ideal Use For
Human Immunology V2 Panel	$2,143	12	594	Allergy Autoimmune Disease Innate Immune Cell Activation Immune Response to Infectious Disease
Human Inflammation V2 Panel	$1,703	12	255	Asthma Allergy Arthritis Neurological-related Inflammation
Human Host Reponse Panel	$3,003	12	785	Host Susceptibility Interferon Response Innate Immune Cell Activation Adaptive Immune Response Homeostasis

Table 5. Comparison of NanoString's nCounter Panels & Assays.

ABCF1	ATP6V1B2	CCL26	CD79A	CXCL9	FOXP3	HMOX1	IL12A	IL27	JUN	MAP3K5	NFKB2	PLCG2	RNF135	TANK	TRAF6
ACE	BATF	CCL27	CD79B	CXCR1	FPR1	HPGD	IL12B	IL27RA	JUNB	MAP3K7	NGLY1	PLEK	RNF31	TAP1	TRAM1
ACKR2	BCL2	CCL28	CD80	CXCR2	FPR2	HPRT1	IL12RB1	IL2RA	KDM6B	MAP3K8	NKG7	PLEKHA1	RPS6KA1	TAP2	TRAT1
ACKR3	BCL2L1	CCL3/L1/L3	CD81	CXCR3	FURIN	HSD11B1	IL12RB2	IL2RB	KIR2DL1	MAPK1	NLRC4	PLG	RPS6KA3	TBK1	TRIM21
ACKR4	BCL3	CCL4/L1/L2	CD84	CXCR4	FYN	HSP90AA1	IL13	IL2RG	KIR2DL3	MAPK13	NLRC5	PLIN4	RPS6KB1	TBP	TRIM22
ACOX1	BCL6	CCL5	CD86	CXCR5	GAB2	HSP90AB1	IL13RA1	IL3	KIR3DL1/2	MAPK14	NLRP1	PNOC	RSAD2	TBX21	TRIM25
ACSL1	BCR	CCL7	CD8A	CXCR6	GADD45B	HSP90B1	IL13RA2	IL31	KLRB1	MAPK8	NLRP3	PPIA	RUNX3	TBXAS1	TRIM33
ACSL3	BDKRB1	CCL8	CD8B	CYP2E1	GATA3	ICAM3	IL15	IL31RA	KLRC1	MAPK9	NMT1	PRCP	S100A12	TCF7	TRIM5
ACSL4	BDKRB2	CCNC	CDH1	CYSTM1	GBA	ICOS	IL15RA	IL32	KLRD1	MAPKAPK2	NOD2	PRDM1	SAMHD1	TCIRG1	TRIM56
ACVR1	BECN1	CCR1	CDK4	DDAH2	GBP1	ICOSLG	IL16	IL33	KLRK1	MARCKS	NOS2	PRF1	SCARB2	TCL1A	TRIM6
ADAR	BLK	CCR10	CEACAM3	DDIT3	GBP2	IDO1	IL17A	IL34	KPNB1	MARCO	NOTCH1	PRKCA	SDHA	TCN2	TXK
ADGRE5	BNIP3	CCR2	CEBPB	DDOST	GBP4	IFI16	IL17B	IL36A	KRAS	MAVS	NOX1	PRKCD	SELE	TGFB1	TXN
ADGRG3	BPI	CCR3	CFLAR	DDX5	GBP5	IFI27	IL17C	IL36B	LAG3	MCL1	NPC2	PRKCQ	SELENOS	TGFB2	TXNIP
ADORA2A	BST2	CCR4	CGAS	DDX58	GCA	IFI35	IL17D	IL36G	LAMP1	MDFIC	NRAS	PRKCSH	SELL	TGFB3	TYK2
AGT	C1QBP	CCR5	CHUK	DEFA4	GK	IFI44	IL17F	IL36RN	LAMP2	MEFV	NRDE2	PSAP	SEM1	TGFBR2	TYROBP
AHR	C2	CCR6	CPA3	DEFB103A/B	GLA	IFI6	IL17RA	IL37	LAMP3	MGAM	NT5E	PSEN1	SERPINA1	THBS1	UBA52
AIF1	C3	CCR7	CR1	DERL1	GLB1	IFIH1	IL17RB	IL3RA	LANCL1	MIF	NTNG2	PSMB10	SH2D1A	THOP1	UBE2L6
AIM2	C3AR1	CCR8	CREBBP	DHX58	GNLY	IFIT1	IL17RC	IL4	LAT	MKNK1	OAS1	PSMB8	SIGIRR	TIFA	UBE2N
AKT1	C5	CCR9	CRK	DIABLO	GNS	IFIT2	IL17RD	IL4R	LAT2	MLKL	OAS2	PSMB9	SIGLEC5	TIGIT	ULK1
AKT2	C5AR1	CCRL2	CRP	DNAJA2	GPX7	IFIT3	IL17RE	IL5	LCK	MME	OAS3	PSTPIP1	SIRPA	TIMP2	ULK2
AKT3	CALM1	CD14	CSF1	DNAJC10	GSK3B	IFITM1	IL18	IL5RA	LCN2	MRC1	OASL	PTGER2	SLC11A1	TLN1	CASP2
ALAS1	CAP1	CD163	CSF1R	DTX3L	GSTM4	IFITM2	IL18BP	IL6	LCP1	MRPS7	OAZ1	PTGER4	SLC2A3	TLR1	CASP6
ALOX12	CARD11	CD19	CSF2	DYSF	GUCY1A1	IFITM3	IL18R1	IL6R	LCP2	MS4A1	OS9	PTGS2	SMAD3	TLR2	CASP7
ALOX15	CARD16	CD1E	CSF2RA	EBI3	GUCY1B1	IFNA1/13	IL18RAP	IL6ST	LDHB	MS4A2	OSM	PTK2B	SMAD4	TLR3	CASP9
ALOX5	CARD17	CD2	CSF2RB	EGLN1	GUSB	IFNA14/16	IL19	IL7	LEF1	MS4A4A	P2RX7	PTPN4	SMAD5	TLR4	BAK1
ALOX5AP	CASP1	CD209	CSF3	EIF2AK2	GZMA	IFNA2	IL1A	IL7R	LGALS3	MS4A7	PAK1	PTPN6	SOCS1	TLR5	BBC3
ALPK1	CASP10	CD22	CSF3R	EIF2AK3	GZMB	IFNA4/7/10/17/21	IL1B	IL9	LIF	MSRA	PANX1	PTPRC	SOCS3	TLR6	PMAIP1
ALPL	CASP3	CD244	CTLA4	EIF3F	GZMH	IFNA5	IL1F10	IL9R	LILRA3	MT2A	PARP1	PXN	SOD1	TLR7	TRADD
ANPEP	CASP4	CD247	CTSA	ELANE	HAMP	IFNA6	IL1R1	IRAK1	LILRA5	MTOR	PARP9	PYCARD	SOD2	TLR8	ANXA1
AP1G1	CASP5	CD27	CTSG	ENTPD1	HAVCR2	IFNA8	IL1R2	IRAK3	LILRA6	MVP	PDCD1	RAB31	SORT1	TLR9	GSDMD
AP1M1	CASP8	CD274	CTSL	EOMES	HCK	IFNAR1	IL1RAP	IRAK4	LILRB2	MX1	PDCD1LG2	RAB5C	SP1	TMEM140	GSDME
AP1S2	CBFB	CD276	CTSS	EPHX2	HCST	IFNAR2	IL1RAPL1	IRF1	LIMK2	MYC	PDHB	RAB7A	SP100	TMPRSS2	BID
APBB1IP	CBL	CD28	CTSW	ERN1	HDC	IFNB1	IL1RAPL2	IRF3	LITAF	MYD88	PECAM1	RAC2	SPI1	TNF	LMNB1
APEX1	CBLB	CD36	CTSZ	ETS1	HERC5	IFNG	IL1RL1	IRF4	LRG1	NAE1	PELI1	RACK1	SPIB	TNFRSF10B	ROCK1
APOBEC3G	CCL1	CD38	CUL1	EVL	HK3	IFNGR2	IL1RL2	IRF7	LRRK2	NAMPT	PELI2	RAF1	SSR1	TNFRSF17	BAX
APOL6	CCL11	CD3D	CX3CL1	F5	HLA-A	IFNK	IL1RN	IRF9	LTA4H	NCF1	PFKFB3	RASGRP1	STAT1	TNFRSF18	BCL10
APP	CCL13	CD3E	CX3CR1	FAM30A	HLA-B	IFNL1	IL2	ISG15	LTB	NCF2	PGK1	RASGRP4	STAT2	TNFRSF1A	BCL2L11

ARRB2	CCL14	CD3G	CXCL1	FAS	HLA-C	IFNL2/3	IL20	ITGAE	LTBR	NCF4	PIK3C3	RB1CC1	STAT3	TNFRSF25	CD207
ATF2	CCL15	CD4	CXCL10	FASLG	HLA-DMA	IFNL4	IL20RA	ITGAL	LTC4S	NCR1	PIK3CA	RBCK1	STAT4	TNFRSF4	FADD
ATF4	CCL16	CD40	CXCL11	FBXO6	HLA-DMB	IFNLR1	IL20RB	ITGAM	LTF	NCR3	PIK3CB	RBPJ	STAT5A	TNFRSF9	GZMK
ATF6	CCL17	CD40LG	CXCL12	FCAR	HLA-DOB	IFNW1	IL21	ITGAX	LYN	NDUFS8	PIK3CD	REL	STAT5B	TNFSF10	HMGB2
ATG10	CCL18	CD44	CXCL13	FCGR1A/B	HLA-DPA1	IGFBP7	IL21R	ITGB2	MAF	NEO1	PIK3CG	RELA	STAT6	TNFSF13B	NCAM1
ATG12	CCL19	CD45R0	CXCL14	FCGR2A	HLA-DPB1	IKBKB	IL22	ITGB7	MAFB	NEU1	PIK3R3	RELB	STING1	TNFSF18	TNFRSF1B
ATG13	CCL2	CD45RA	CXCL16	FCGR3A/B	HLA-DQA	IKBKE	IL22RA1	ITK	MAP1LC3A	NFAT5	PIK3R4	RGMA	STK11IP	TNFSF4	TNFSF11
ATG3	CCL20	CD45RB	CXCL17	FCGRT	HLA-DQB1	IKBKG	IL22RA2	ITLN1	MAP2K2	NFATC1	PIK3R5	RHOG	STRAP	TNFSF9	TNFSF12
ATG4A	CCL21	CD59	CXCL2	FCRL2	HLA-DRA	IL10	IL23A	ITPR3	MAP2K3	NFATC2	PIK3R6	RIPK1	STT3B	TOLLIP	KLRC2
ATG7	CCL22	CD6	CXCL3	FCRL4	HLA-DRB	IL10RA	IL23R	JAK1	MAP2K4	NFATC3	PLAT	RIPK2	SUGT1	TPP1	KLRC3
ATM	CCL23	CD68	CXCL5	FGR	HLA-E	IL10RB	IL24	JAK2	MAP2K7	NFATC4	PLAU	RIPK3	SYK	TPSAB1/B2	KIR_Activating_Subgroup_2
ATP6AP2	CCL24	CD69	CXCL6	FOS	HLX	IL11	IL25	JAK3	MAP3K1	NFE2L2	PLAUR	RNASEL	TAB1	TRAF2	KLRG1
ATP6V0D1	CCL25	CD70	CXCL8	FOXO1	HMGB1	IL11RA	IL26	JAML	MAP3K3	NFKB1	PLCG1	RNF114	TAB2	TRAF3	B3GAT1
VAMP3	VCAM1	VEGFA	VRK3	VSIR	VWF	WAS	WIPI1	XAF1	XBP1	XCL1/2	XCR1	YWHAQ	ZAP70	ZBP1	

Table 6. List of 815 genes in customized NanoString nCounter® Human Host Response Panel. Red = genes spiked in, blue = genes that were highly upregulated in preliminary studies, black = genes of over 50 different relevant immune response pathways.

Future Work for Aim 1

Subaim 1.1 Conduct Gene Expression Profiling with NanoString nCounter® System

Currently, we are in the process of finalizing the selection of confirmed dtDHR cases. Upon the completion of this task, we plan to utilize the Human Host Response panel to conduct gene expression profiling of these FFPE specimens. We seek to assess cell death mediator and marker transcription along with the validation of preliminary gene expression findings. We plan to run our customized NanoString nCounter® panel on the specimens we select for after the final confirmation of cases.

Aim 2 Rationale

Currently, the field lacks a reproducible model system for mechanistic studies of SJ/TEN. Due to the rarity of SJS/TEN cases, accessing fresh skin specimens are difficult to obtain. In this second aim, we plan to mitigate this by generating an *in vitro* model and using it to test cell death pathways and potential treatments in SJS/TEN. We will generate this *in vitro* model by separately applying the supernatant of SJS/TEN blister fluid and blood serum to NativeSkin Access®, a commercially available human skin model. Blister fluid and healthy blood serum from thermal burn patients will serve as our controls.

Subaim 2.1 To identify the best source of skin for a novel in vitro model of SJS/TEN

Prior to selecting NativeSkin Access®, we did a comparative analysis of two commercially available full thickness skin models. In contrast to NativeSkin Access® from GenoSkin, EpidermFTTM from MatteckTM is a validated 3D full thickness tissue engineered human skin model that does not contain immune cells (Table 7). In contrast, NativeSkin Access® maintains a normal skin barrier function, a mature stratum corneum, a functional basal layer, and all cell types and skin appendages of in vivo human skin. This provides us with the most realistic human skin model.

Company	Genoskin	Mattek
Product	NativeSkin, 11mm + silicone ring	EpidermFt-412
Details of Product	Full thickness (organization, skin barrier function, skin cells, appendages, metabolism, immune cells)	Full thickness (organization, skin barrier function, metabolism, appendages)
Working Surface Area	.5 cm2	1 cm2
Recommended Topical Application	10-20 uL	25-30 uL
Plate Format	12 wells	6 well plates
Media Contents	Free of serum, growth factors, hydrocortisone, and phenol red	Free of phenol red and hydrocortisone
Required Volume of Media Per Day	1 ml	2.5 ml
Tissue Viability	7 days	4 days
Storage	Tissues incubated at 37°C, 5% CO2, media in 4C	Tissues incubated at 37°C, 5% CO2, media in 4C

Table 7. Comparison of full thickness tissue models from different companies.

Subaim 2.2 To Test the Selected Source of skin in an Optimization Assay

Following our selection of the tissue, we optimized our protocol for the development of our *in vitro* model. We received 8 NativeSkin Access® tissues in a 12 well plate that were 11 mm in diameter and have a working surface of 0.5 cm^2 for topical application. We processed the first tissue at time zero to assess how healthy and viable the skin appears grossly and histologically. Next, 3 tissues were used as controls having GenoSkin media only. The effect of healthy female serum was evaluated grossly for the remaining 3 tissues with the 24 hour serum based tissue evaluated histologically. Multiple injections were practiced using distilled water with a 30 mm gauge needle and 10 ml syringe on the combined serum and media tissue. This was done to assess how smoothly injections could be successfully made using this tissue product. Evaluations were made at time points of 1 hour, 3 hours, 6 hours, and 24 hours in. No blistering or sign of contamination were seen grossly.

Histopathology of the skin sections at time point zero and 24 hours in (Figure 8) stained with hematoxylin and eosin showed no sign of inflammatory cell infiltration or dermal necrosis as expected.

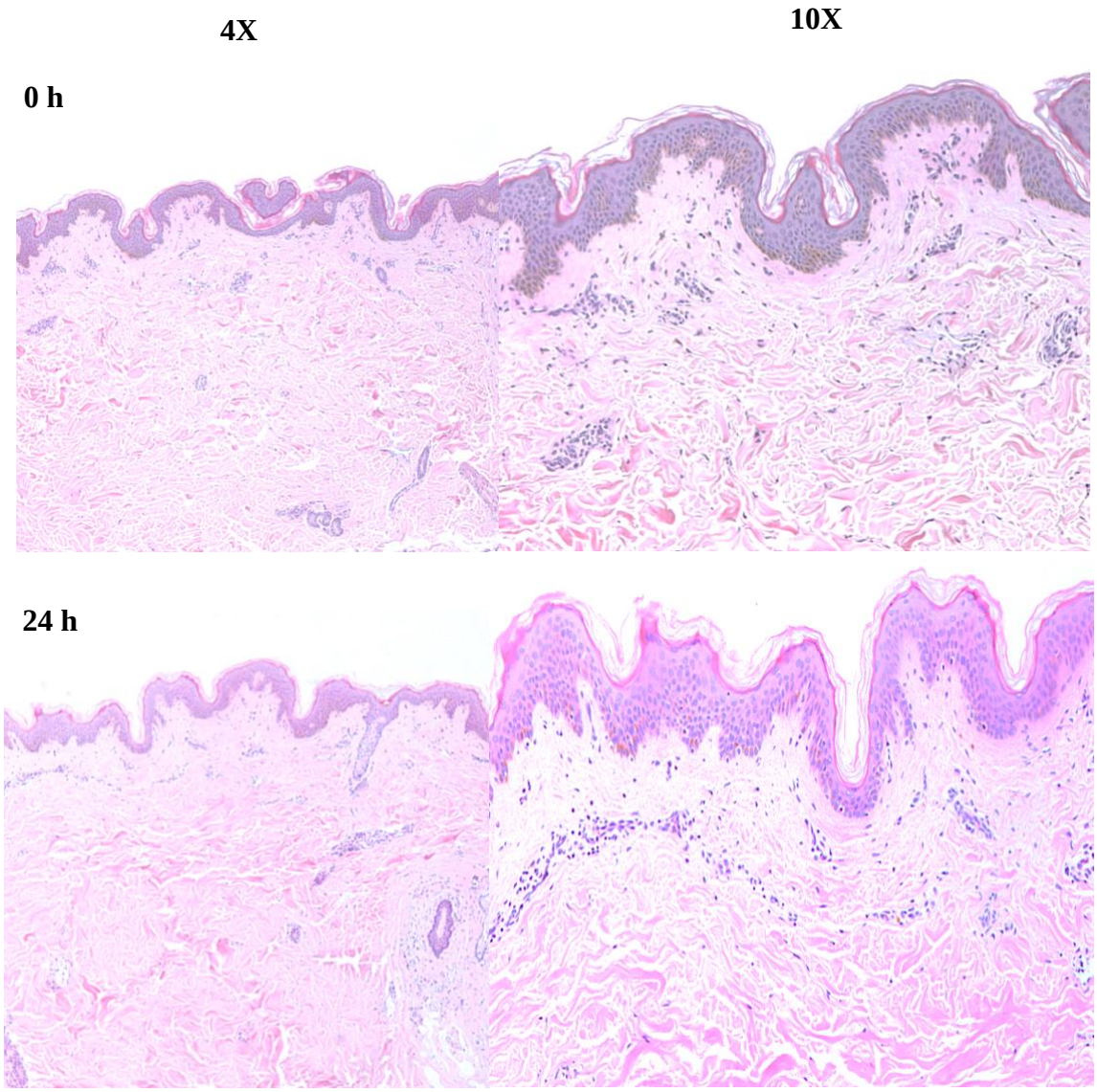

Figure 8. Optimization of *ex vivo* tissue model. Histopathology of the Genoskin NativeSkin Access® at time point zero for tissues that received GenoSkin media (1 ml to the well and 20 ul to the surface of the epidermis) and healthy human serum from Biochemmed® (1 ml to the well and 20 ul to the surface) at the 24 hour time point. Tissue slides were stained with hematoxylin and eosin (H&E). Views from 4X magnification (top left and bottom left) and 10X magnification exhibit a normal overall appearance.

Future Work for Aim 2

Subaim 2.3 To Use the Selected Source of Skin to Test Effects of Recombinant Proteins, Blockers, and Inhibitors

In order to gain insight into the mechanisms mediating disease in SJS/TEN, we will apply recombinant proteins along with various inhibitors, including FDA-approved medications to the NativeSkin Access® *in vitro* model. These commercially available agents and inhibitors were designed for other uses to target different cell death pathway mediators of interest (Table 8). We predict their application will inhibit epidermal destruction.

Furthermore, we hypothesize that soluble mediators, particularly TNF-, TRAIL, and FasL will induce necroptosis or pyroptosis in keratinocytes. Assessment will be made through intradermal injections containing concentrations of recombinant proteins that are resemble the physiological amount found in SJS/TEN blister fluid samples.

Ultimately, our goal is not only to advance our knowledge of SJS/TEN mechanistically, but also to explore potential novel treatments for this disease. This model can serve to transcend the challenges caused by the scarcity of fresh SJS/TEN samples. Importantly, it can help assess treatment options which may improve the clinical management and the quality of life of survivors.

Pathway	Drug	Inhibition
Necroptosis	Debrafenib	Inhibits RIPK3
	GSK'872	Inhibits RIPK3
	Necs1	Inhibits MLKL
Pyroptosis	Canakinumab	Anti IL-1β antibody
	Rilonacept	IL-1 trap
	Tranilast	Inhibits inflammasome formation
	Disulfiram	Inhibits gasdermin D
	Punicalagin	Inhibits inflammasome formation

Table 8. Commercially available agents and FDA-approved medications inhibiting necroptosis and pyroptosis. This will be utilized in future work. Red text indicates FDA-approved medications used to treat other conditions.

DISCUSSION

In SJS/TEN, most studies have assumed that the severe keratinocyte death and epidermal destruction is mediated by apoptosis. Emerging evidence is beginning to point to the involvement of other cell death pathways, yet insufficient number of samples and available model systems hamper thorough mechanistic analysis. We are utilizing innovative technologies and uniquely available patient specimens to overcome these barriers. Limitations include the difficulty of obtaining samples that were biopsied at one time point before treatment administration, the rarity of the disease, and the challenge of extracting RNA from difficult sample types. However, our multi-institutional database allows for sufficient n value. The application of the NanoString nCounter® technologies optimizes performance by overcoming the challenge of extracting RNA from FFPE samples. Ultimately, confirmation of our results at the protein level will be necessary

In our first aim, we began our retrospective analysis of SJS/TEN patients. Meanwhile, we modified a gene panel using NanoString's nCounter® Technology that will be extremely valuable in providing a technique to overcome the difficulty of extracting RNA from FFPE samples, especially those that are aged or not well preserved. We expanded the gene pool panel to analyze over 785 genes with 30 genes spiked in. We hypothesize that we will see the preferential expression and activation of genes and proteins that are related to the cell death pathways, necroptosis and pyroptosis over apoptosis.

In our second aim, we began the development and optimization of our *in vitro* model. Using a real human skin structure that includes normal skin barrier function, a

mature stratum corneum, a functional basal layer, and all cell types and skin appendages of in vivo skin, will provide us with the most realistic model. The future goal is to test cell death pathways and potential treatments in SJS/TEN using this novel *in vitro* model. We anticipated that this model would reveal that soluble mediators, specifically TNF-alpha, TRAIL, and FasL would induce keratinocyte death via necroptosis or pyroptosis. We hope to elucidate this in future work with multiplex immunoassays, colorimetric assays, and western blot.

We identified and optimized the maintenance and processing protocol our *in vitro* skin model. Next, we will test the effects of proteins hypothesized to induce SJS/TEN. Furthermore, we will apply patient serum and blister fluid in the presence or absence of specific cell death inhibitors to interrogate the role of each cell death mediator in disease.

CONCLUSION

We have made significant strides in this challenging project by identifying sufficient case numbers, creating an expansive yet targeted gene panel, and identifying and optimizing an in vitro skin platform. In addition, we have identified the best reagents for our studies, and begun collecting patient specimens In summary, we have successfully identified and obtained all the necessary tools to achieve our stated aims and are ready to complete the proposed experiments and analysis.

This work has significant potential to advance the field. It will identify new pathways potentially targetable with already clinically available treatments. Moreover, it develops a novel human skin model that can serve as a valuable resource to investigate pathogenesis of disease and also to directly test potential treatments. Application of these methods will help address fundamental questions surrounding the pathobiology of SJS/TEN, with the goal of improving clinical outcomes and quality of life of survivors.

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