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The Combined Utility of *Ex vivo* IFN-g Release Enzyme-Linked ImmunoSpot Assay and *In vivo* Skin Testing in Patients With Antibiotic-Associated Severe Cutaneous Adverse Reactions

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Abstract

BACKGROUND—For severe cutaneous adverse reactions (SCARs) associated with multiple antibiotics dosed concurrently, clinical causality is challenging and diagnostic approaches are limited, leading to constricted future antibiotic choices.

OBJECTIVE—To examine the combined utility of *in vivo* and *ex vivo* diagnostic approaches at assigning drug causality in a cohort of patients with antibiotic-associated (AA)-SCARs.

METHODS—Patients with AA-SCARs were prospectively recruited between April 2015 and February 2017. *In vivo* testing (patch testing or delayed intradermal testing) was performed to the implicated antibiotic(s) at the highest nonirritating concentration and read at 24 hours through 1 week. Ex vivo testing used patient peripheral blood mononuclear cells (PBMCs) stimulated with a

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range of pharmacologically relevant concentrations of implicated antibiotics to measure dose-dependent IFN-g release from CD4D and CD8D T cells via an enzyme-linked immunoSpot assay.

RESULTS—In 19 patients with AA-SCARs, combined *in vivo* and *ex vivo* testing assigned antibiotic causality in 15 (79%) patients. Ten patients (53%) with AA-SCARs were positive on IFN-g release enzyme-linked immunoSpot assay, with an overall reported sensitivity of 52% (95% CI, 29-76) and specificity of100% (95% CI, 79-100), with improved sensitivity noted in acute (within 1 day to 6 weeks after SCAR onset) testing (75%) and in patients with higher phenotypic scores (59%). There was increased use of narrow-spectrum beta-lactams and antibiotics from within the implicated class following testing in patients with a positive *ex vivo* or *in vivo* test result.

CONCLUSIONS—We demonstrate the potential utility of combined *in vivo* and *ex vivo* testing in patients with AA-SCARs to assign drug causality with high specificity.

Keywords

Antibiotic allergy; Delayed hypersensitivity; Stevens-Johnson syndrome; toxic epidermal necrolysis; drug reaction with eosinophilia and systemic symptoms

Severe cutaneous adverse reactions (SCARs), such as Stevens-Johnson syndrome (SJS), toxic epidermal necrolysis (TEN), and drug reaction with eosinophilia and systemic symptoms (DRESS), are associated with significant mortality and short-term and long-term morbidity[1,2] and may be caused by a range of medications including antibiotics.[1] SJS and TEN are considered the same condition representing different severities across a spectrum. The hallmarks of SJS/TEN are skin detachment (1%-10% for SJS, 10%-30% for SJS/TEN overlap, and >30% for TEN) and blistering of mucous membranes accompanied by other serious manifestations of systemic involvement.[3] Patients experiencing DRESS exhibit an exanthematous rash, fever, internal organ involvement, and possible eosinophilia.[3] Acute generalized exanthematous pustulosis (AGEP), another SCAR, is an acute widespread erythematous reaction that is followed by a pustular eruption together with fever.[3] To avoid the recurrence of SCARs, culprit drugs are traditionally avoided in the future.

Often in SCARs multiple antibiotics are prescribed concurrently, creating uncertainty in ascribing causality, which can lead to significant constriction of future therapeutic choices.[1,2] Current diagnostic options such as *in vivo* patch testing (PT) or delayed intradermal skin testing (IDT) have been limited by lack of experience, lack of validated concentrations and approaches, limited availability to providers, and poor sensitivity.[4–6] *Ex vivo* and *in vitro* testing using a range of research platforms including lymphocyte transformation testing and IFN-g release enzyme-linked immunoSpot (ELISpot) assay have been used in small cohorts of antibiotic-associated (AA) delayed hypersensitivities with varied success.[7–12] Furthermore, there is scarce published literature on the utility of combination *in vivo* and *ex vivo/in vitro* approaches such as PT and/or delayed IDT with IFN-g release ELISpot assay or lymphocyte transformation testing in AA-SCARs. The objectives of this pilot study were to examine the potential combined utility of IFN-g release ELISpot assay and *in vivo* skin testing in defining antibiotic causality assessments in patients with AA-SCARs.

METHODS

Patient recruitment and definitions

Study patients were prospectively recruited at Austin Health, Alfred Health and Peter MacCallum Cancer Centre from April 2015 until February 2017. Inclusion criteria were patients 18 years or older with a history of AA-SCARs. Patients with AA-SCARs with an antibiotic identified as the primary implicated drug(s) and corresponding Naranjo adverse drug reaction score of 5 or more (probable adverse drug reaction)[13] were recruited. For the SJS/TEN phenotypes, an ALDEN score of 4 or more was required (as per published definitions14), with an antibiotic having to carry the highest ALDEN (algorithm for assessment of drug causality for epidermal necrolysis) score. For phenotypes of DRESS and AGEP, a RegiSCAR score of 2 or more and an AGEP score of 2 or more, respectively, were required.[15,16]. All cases had the diagnosis and phenotype confirmed by a dermatologist and were reviewed in the respective hospital antibiotic allergy clinics (Austin Health and Peter MacCallum Cancer Centre). Patients with an alternative viral, bacterial, or autoimmune SCAR etiology were excluded, evidenced by any one of the following: (1) positive plasma PCR for herpesvirus (HSV1/2, cytomegalovirus, EBV) or Enterovirus, (2) positive Mycoplasma species PCR (respiratory specimen) or serology, or (3) detectable antinuclear antigen antibody titer of more than 1:64. Patients were also excluded if skin biopsy (histopathology or direct immunofluorescence) was not consistent with a drug reaction or clinical picture was consistent with an alternative diagnosis. There were 2 control groups: (1) antibiotic-tolerant controls, patients who had tolerated at least 4 consecutive weeks of single antibiotic at therapeutic intra-venous or oral dosing, and (2) healthy random donors, patients with AA-SCARs tested against antibiotics that previously resulted in a positive IFN-g release ELISpot assay.

Peripheral blood mononuclear cell (PBMCs) were isolated from whole heparinized blood of patients with AA-SCARs, tolerant controls, and healthy donors, washed, and counted. PBMCs were stored at 80oC in 90% heat-inactivated FBS and 10% dimethyl sulfoxide until use for IFN-g release ELISpot assay. Patients were followed for adverse events and antibiotic prescribing for 90 days after testing. Ingestion challenge was not performed as routine after *ex vivo* and *in vivo* testing; rather, it was based on acute antibiotic requirements. This study was approved by the Austin Health Ethics Committee (HREC/15/Austin/75) and laboratories where this testing was performed had independent review board approvals (Institute for Immunology & Infectious Diseases, Murdoch [Murdoch University HREC 2011/056] University and Vanderbilt University Medical Center).

Skin testing (*in vivo*)

IDT and PT were performed for all implicated antibiotics at least 6 weeks after AA-SCAR onset using previously recommended nonirritating antibiotic concentrations.[17–19]. In patients in whom an intravenous formulation of the implicated antibiotic was not available or incompatible with IDT and/or in the setting of SJS/TEN, PT was performed in isolation. IDT was performed on the volar forearm of the skin with 0.02 mL of antibiotic reagent or normal saline (negative control) and read after 24 and 48 hours. A positive IDT result was considered when there was evidence of dermal induration and erythema at the injection site

that exceeded 5 mm from baseline. In PT, a patch was applied to the upper back and removed at 48 hours and re-read at 72 hours, using white petroleum jelly as drug carrier in all cases of the antibiotics tested and negative control (Figure 1).

IFN-g release ELISpot assay (*ex vivo)*

IFN-g release in response to overnight incubation with the implicated antibiotic(s) was performed by ELISpot assay in triplicate from thawed PBMCs (rested overnight) as previously described.[20] PBMCs (200,000 cells per well) were incubated with investigated drugs at concentrations representative of peak serum concentrations (Cmax) and a level 10-fold higher than Cmax,21 avoiding concentrations associated with T-cell cytotoxicity (data not shown). Testing was also performed with a negative (unstimulated) and positive control (anti-CD3 antibody; Mabtech, Victoria, Australia) in duplicate. The mean number of spots for the test and unstimulated wells were calculated. A positive response was defined as greater than 50 spot-forming unit (SFU)/million cells after background (unstimulated control) removal as per previously published definitions20,22 (Figure 1).

Indeterminate results were defined by failure of the positive CD3 control. In addition to IFN-g release ELISpot assay, in patients with a positive result (SFU/million cells >50) and available PBMCs from successive time points, T-cell stimulation was assessed via flow cytometry by measuring upregulation of the early activation marker CD137, a member of the TNF receptor family, on viable CD3þCD8þ and CD3þCD4þ T cells (Figure 1).

Statistical analysis

Categorical variables were summarized and compared between groups using the chi-square test or Fisher exact test. Continuous variables were compared using a student t test or Wilcoxon signed-rank test. A P value of less than .05 (2-tailed) was deemed statistically significant. IFN-g release ELISpot assay's diagnostic performance was expressed in terms of sensitivity and specificity, with indeterminate results included in the analysis. Statistical analyses were performed using Stata 13.0 (Statacorp, College Station, Texas).

RESULTS

Baseline demographic characteristics

Nineteen patients with AA-SCARs meeting the inclusion/exclusion criteria and 16 antibiotic-tolerant controls were recruited. There was no statistically significant difference (P > .05) in clinical characteristics between study patients and tolerant controls (Table I). The clinical characteristics, implicated antibiotics, and phenotypic scores are summarized in Table II. From the 19 study patients, there were 36 implicated antibiotics, with 12 patients (63%) with more than 1 implicated antibiotic. The most commonly implicated antibiotics were vancomycin (10 of 19 [52%]) and piperacillin-tazobactam (8 of 19 [42%]). The phenotypes encountered were DRESS (14 of 19 [73%]), SJS/TEN (4 of 19 [21%]), and AGEP (1 of 19 [5%]) (Table II).

Skin testing results (*in vivo*)

Seventeen patients (89%) underwent skin testing, PT only (21%; 4 of 19), IDT only (42%; 8 of 19), or combined PT/IDT (26%; 5 of 19). Two patients declined skin testing (S3 and S15), and of the 4 patients who underwent PT without IDT, 3 had SJS/TEN and 1 had multiorgan involvement DRESS. Fifty-two percent of patients (9 of 17) were positive on skin testing, 44% (4 of 9) on PT, and 100% (9 of 9) on IDT. All PT results were confirmed positive on follow-up IDT (4 of 4 [100%]) (Table III). All IDT positive results were positive within 24 hours of being administered and read. Of those skin tested, there was a higher rate of skin test positivity in DRESS compared with SJS/TEN phenotypes (8 of 13 [61%] vs 0 of 3 [0%], P .20). Of the skin test positive, beta-lactams predominated over non-ebeta-lactams (7 of 9 [77%] vs 2 of 9 [22%], P .05) and there were no positive *in vivo* test results to vancomycin, despite it being implicated in 58% (11 of 19) of the patients. There was a trend toward increased skin test positivity if performed more than 3 months after SCAR onset compared with less than 3 months (7 of 11 [63%] vs 2 of 6 [33%]; P .34). There was no difference in skin test positivity in immunocompromised versus nonimmunocompromised patients (6 of 11 [54%] vs 3 of 6 [50%]; P 1). The median time from SCAR onset to skin testing was longer in patients with positive skin test results than in patients with negative skin test results (301 days vs 100 days; P .07), even when patients who ultimately tested positive to vancomycin on *ex vivo* testing were excluded (301 days vs 146 days; P .33). There were no systemic events noted from PT or IDT in this cohort.

IFN-g release ELISpot assay responses (*ex vivo*)

Ten patients (53%) exhibited a positive IFN-g release ELISpot assay to at least 1 implicated antibiotic (median SFU/ million cells, 102; interquartile range [IQR], 71.46-147.3), 8 patients were negative (median SFU/million cells, 0; IQR, 0-15.42), and 1 patient indeterminate (Table III). Seven patients (70%) were positive to more than 1 antibiotic concentration of the same drug and 9 patients (90%) were positive at the highest tested antibiotic concentration (see Figure E1 in this article's Online Repository at www.jaci-inpractice.org). No tolerant controls exhibited a positive result (median SFU/million cells, 0; IQR, 0-8.953) (Figure E1), nor did healthy random donors at highest tested antibiotic concentrations (data not shown). The IFN-g release ELISpot assay results for all tested antibiotics in patients with SCARs and controls are provided in this article's Online Repository at www.jaci-inpractice.org.

A summary of IFN-g release ELISpot assay results stratified for phenotype and timing is demonstrated in Table IV. From the 10 positive IFN-g release ELISpot assays, 40% (4 of 10) were to a beta-lactam and 60% (6 of 10) a non-ebeta-lactam (P .66). All the non-ebeta-lactam positive ELISpot assays were to a glycopeptide (6 of 6 [100%]). There was no difference in *ex vivo* positivity with DRESS compared with SJS/TEN phenotypes (8 of 13 [61%] vs 2 of 3 [75%]; P .60). The median time from SCAR onset to ELISpot assay testing was shorter in *ex vivo* positive than in *ex vivo* negative patients (115 days vs 140 days; P . 66). A trend toward a higher number of *ex vivo* positives was noted if performed within 1 year of SCAR onset (7 of 10 vs 3 of 10; P 0.17) and if the patient was immunocompetent compared with immunocompromised (8 of 10 [80%] vs 5 of 9 [55%]; P .34). In the 3 IFN-g release ELISpot assay positive patients with subsequent time point PBMCs available, 2 (2 of

3 [67%]) remained positive out to a median time of 552 days after AA-SCAR onset (IQR, 548-556) (Figure 2). In these 2 patients, CD137 activation on CD4 and/or CD8 T cells was also noted at the corresponding time points (Figure 2).

Correlation and utility of combined *in vivo* and *ex vivo* testing

Using combined testing (*in vivo* and *ex vivo*), 15 patients (79%) with AA-SCARs were positive to an implicated antibiotic (Table III). The proportion of patients who are positive on *ex vivo, in vivo*, or a combination of modalities is outlined in Figure 3. In patients who were positive on both ELISpot assay and skin testing, there was a 100% (4 of 4) correlation. On examining *in vivo* negative and *ex vivo* positive patients (n 5), we found that all patients were positive on IFN-g release ELISpot assay to a glycopeptide (vancomycin 4, teicoplanin 1) with a short median time from SCAR onset to ELISpot assay (20 days; IQR, 3.5-115). In those *in vivo* positive and *ex vivo* negative patients (n 5), a longer median time from SCAR onset to ELISpot assay was noted (301 days; IQR, 100.5-1443), longer than in the *in vivo* negative and *ex vivo* positive patients above (P .055).

Of the most commonly encountered phenotype, DRESS, 86% (12 of 14) had a positive *in vivo* or *ex vivo* test result (Table II): 66% (8 of 12) *in vivo*, 66% (8 of 12) *ex vivo*, and 33% (4 of 12) both. For those positive on both *in vivo* and *ex vivo* testing, they were all toward beta-lactams, with a 100% correlation (4 of 4). For those with a positive *in vivo* testing result, 50% (4 of 8) were toward piperacillin-tazobactam (2 piperacillin-tazobactam alone, 2 piperacillin-tazobactam with an additional penicillin) (Table III). Of the *in vivo* positives, 88% (7 of 8) were positive on IDT and 25% (2 of 8) on PT (1 trimethoprim, 1 ceftriaxone). In all patients who were *ex vivo* positive and *in vivo* negative, the IFN-g release ELISpot assay was positive for vancomycin (4 of 4). The sensitivity and specificity of IFN-g release ELISpot assay for patients with DRESS was 57.74% and 100%, respectively (Table V).

Overall sensitivity and specificity of IFN-g release ELISpot assay

Using the phenotypic causality assessments as the reference, the sensitivity and specificity for varied phenotypes and timing of *ex vivo* testing are outlined in Table V. The highest sensitivity and specificity was achieved from acute bleeds (<6 weeks after SCAR onset), 75% and 100%, respectively. When only including SCAR cases with a probable or definite phenotypic score (n ¼ 17), the sensitivity was 58.82% and specificity 100%.

AA-SCAR follow-up and posttesting prescribing

In the 90 days following combined testing, on comparing those with a positive *ex vivo* or *in vivo* test result to those without, we found that there was increased use of class-related antibiotics (9 of 15 [60%] vs 0 of 4 [0%]; P .086) and narrow-spectrum beta-lactams (10 of 15 [66%] vs 0 of 4 [0%]; P .032). In patients with DRESS and a positive *in vivo* or *ex vivo* test result to a beta-lactam (n 8), 75% (6 of 8) were able to tolerate an alternative beta-lactam posttesting. There were no adverse events reported or recurrent SCARs in the follow-up period, including in those who were prescribed an antibiotic that was implicated in causality but negative on *in vivo* or *ex vivo* testing (n ¼ 4).

DISCUSSION

Delayed hypersensitivity reactions related to antibiotics are confounded by multiple drugs or classes of drug implicated in causality and high morbidity, not only related to the sequelae of the acute hypersensitivity reaction but due to uncertainty and constriction of future antibiotic choices.[1,2,23–27] At present, PT and delayed skin testing in isolation are hampered by uncertain availability and poor sensitivity.[23,24] *Ex vivo* techniques such as ELISpot assay have shown promise in small cohorts; however, their availability and performance have not been validated in large-scale high-throughput clinical laboratories against a clinical probability score.[7,10,11] Our pilot study notably demonstrated that the combination of a clinical causality algorithm in combination with *in vivo* and *ex vivo* diagnostics could aid causality in 79% of patients with AA-SCARs, higher in cases of the most predominate phenotype, DRESS. Furthermore, in these patients with a positive combined result, particularly to a beta-lactam antibiotic, this had a clinical impact by allowing the safe prescribing of both narrow-spectrum beta-lactams and drugs from within implicated antibiotic classes after SCARs. The use of alternative beta-lactams even in those with positive beta-lactam testing is likely to significantly aid safe and appropriate antibiotic prescribing in this cohort.

Neither IDT nor PT (*in vivo*) in our patients with AA-SCARs was associated with systemic events, supporting similar findings by Barbaud et al.[4] This is an important finding because the use of skin testing, in particular IDT in SCARs, often remains absent from local and international clinical guidelines due to safety concerns.[25,26]. *In vivo* testing was positive in 52% of patients, falling within the wide range reported in the literature (6.6%-100%),23 with greater success evident in beta-lactam DRESS.[27,28] Of interest was the persistence of skin testing responses beyond the acute period, where *ex vivo* diagnostics appeared to lack sensitivity. Pinho et al 29 have recently demonstrated the long-term reproducibility of positive patch test reactions in patients with delayed hypersensitivities to antibiotics, mainly maculopapular exanthems to beta-lactams.[29] This maintenance of skin test positivity at a time where antibiotic antigen-specific T cells are diminishing in the blood may be due to the sensitivity for some implicated drugs (ie, glycopeptides) may also relate to the absence of well-supported testing concentrations and constraints of locally induced mast cell activation, which have been suggested to independently upregulate IL-10 and suppress the local hypersensitivity reaction.[31].

The use of antigen-specific IFN-g production using ELISpot assay is a well-established principle in the diagnosis of latent tuberculosis,[32,33] yet the utility in AA-SCARs has been limited to small studies. Porebski34 recently reviewed the literature of in vitro and *ex vivo* testing in SCARs, which demonstrated (1) variations in clinical phenotyping methods and measurable in vitro cytokine outputs, (2) grouping of SCARs with other non-SCAR T-cellemediated hypersensitivity phenotypes, and (3) use primarily in nonantibiotic cases.[34]. In comparison, we present one of the largest single cohorts of AA-SCARs with extended patient follow-up, using strict phenotypic scoring algorithms and IFN-g release ELISpot assay. IFN-g release ELISpot assay was able to aid the diagnostic algorithm in 53% of patients, picking up an additional 5 cases that were missed with skin testing alone. The benefit of ELISpot assay was apparent in acute samples and glycopeptide-associated cases.

Tanvarasethee et al.[35] demonstrated improved responses if performed within 2 years of reaction, supporting our findings of increased positives within 1 year postonset. Although the overall sensitivity and specificity of 52% and 100%, respectively, is consistent with previous reports for *ex vivo* T-cell diagnostics (27%-70%),12,23 we postulate that improved sensitivity is likely to require early case ascertainment and PBMC collection and an understanding of the role of drug metabolites for antibiotics that proved problematic *ex vivo* (eg, sulfamethoxazole and trimethoprim).[36,37] Previous reports have demonstrated drug-specific T-cell responses for up to 20 years after drug exposure,9,38 and both long-lived patch test and *ex vivo* responses particularly in patients with a history of the HLA-B*57:01erestricted CD8 T-celledependent abacavir hypersensitivity reaction.[39–41] In the absence of reexposure to drugs or structurally unrelated drugs, the pathway to such long-lived memory T-cell responses and whether there are drug-specific memory T-cell responses that cross-react with a chronic prevalent pathogen is currently unclear.[42] Although we and others have clearly shown long-lived *ex vivo* positivity, we also highlight the apparent loss of reactivity in a teicoplanin TEN patient11 and the paucity of literature demonstrating robust peripheral T-cell responses to antibiotics over time. Furthermore, a trend was noted of less *ex vivo* positivity in immunocompromised hosts, and the role of costimulation (ie, IL-2, IL-7) in such patients may be worth exploring.[43]

There are limitations to this pilot study, including the small study numbers, single-center experience, use of frozen PBMCs, and absence of standardized testing concentrations (especially for *ex vivo*). Furthermore, for delayed hypersensitivity reactions, the criterion standard for testing sensitivity and specificity is multiple dose oral or ingestion challenge, which is rightly discouraged in clinical practice because it is neither evidence nor guideline based and causes potential harm to patients.[19,23,26] Our small study however suggests that improved patient outcomes may be obtained through the use of strict causality assessment (to ascertain pretest clinical probability) in combination with *in vivo* and *ex vivo* testing and postprescribing follow-up. Although laboratory testing is not readily available to many clinicians, the development of centralized *ex vivo* and in vitro testing centers could allow the transfer of acute patient PBMCs (frozen) for assessment, to supplement traditional PT and IDT, and progress toward a personalized approach to drug hypersensitivity and aid safe antibiotic prescribing after AA-SCARs. For the future development of such testing, a number of key questions remain including the following: (1) the ideal range of antibiotic concentrations used for both *in vivo* and *ex vivo* testing at and above the physiological Cmax concentration; (2) the ideal timing of *ex vivo* testing and how this relates to causal antibiotics; (3) the concentration-dependent role of parent antibiotic versus antibiotic metabolite; and (4) the utility of an enhanced spectrum of *ex vivo* tests that may include the use of flow cytometry with intracellular cytokine staining to examine for T-cell activation,44 and other mechanisms that may improve the sensitivity of *ex vivo* testing.[12].

This study encompassing one of the largest tested cohorts of AA-SCARs was carried out in a population of patients with high antibiotic needs. It demonstrates the potential utility of combined safe *in vivo* and novel *ex vivo* testing for patients with AA-SCARs, aiding the global causality assessment in a disease associated with significant morbidity, mortality, and high-risk prescribing. In the future, improving antibiotic appropriateness is likely to be aided by combined testing programs in patients with AA-SCAR. Personalization of such testing

based on phenotype, implicated antibiotic, and timing postonset may improve sensitivity, specificity, and the negative predictive value of such testing, leading to safer options for patients and overall improvement in their care.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

AA	Antibiotic-associated
AGEP	Acute generalized exanthematous pustulosis
DRESS	Drug reaction with eosinophilia and systemic symptoms ELISpot-Enzyme-linked immunospot
IDT	Intradermal testing IQR-Interquartile range PT-Patch testing
SCAR	Severe cutaneous adverse reaction SFU-Spot-forming unit
SJS	Stevens-Johnson syndrome TEN-Toxic epidermal necrolysis

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What is already known about this topic?

The individual use of *in vivo* skin testing and *ex vivo* IFN-g release enzyme- linked immunoSpot (ELISpot) assay for assigning drug causality in severe cutaneous adverse reactions (SCARs) shows promise, yet the joint utility in antibiotic-associated SCARs remains ill-defined.

What does this article add to our knowledge?

The combined use of *in vivo* and *ex vivo* diagnostics in antibiotic- associated SCARs assigned causality safely in 79% of cases, and IFN-g release ELISpot assay demonstrated good sensitivity and high specificity.

How does this study impact current management guidelines?

Skin testing (*in vivo*) and IFN-g release ELISpot assay (*ex vivo*) are complementary approaches that may prove safe and effective in ascertaining antibiotic causality and improve, often difficult antibiotic prescribing, after SCARs.

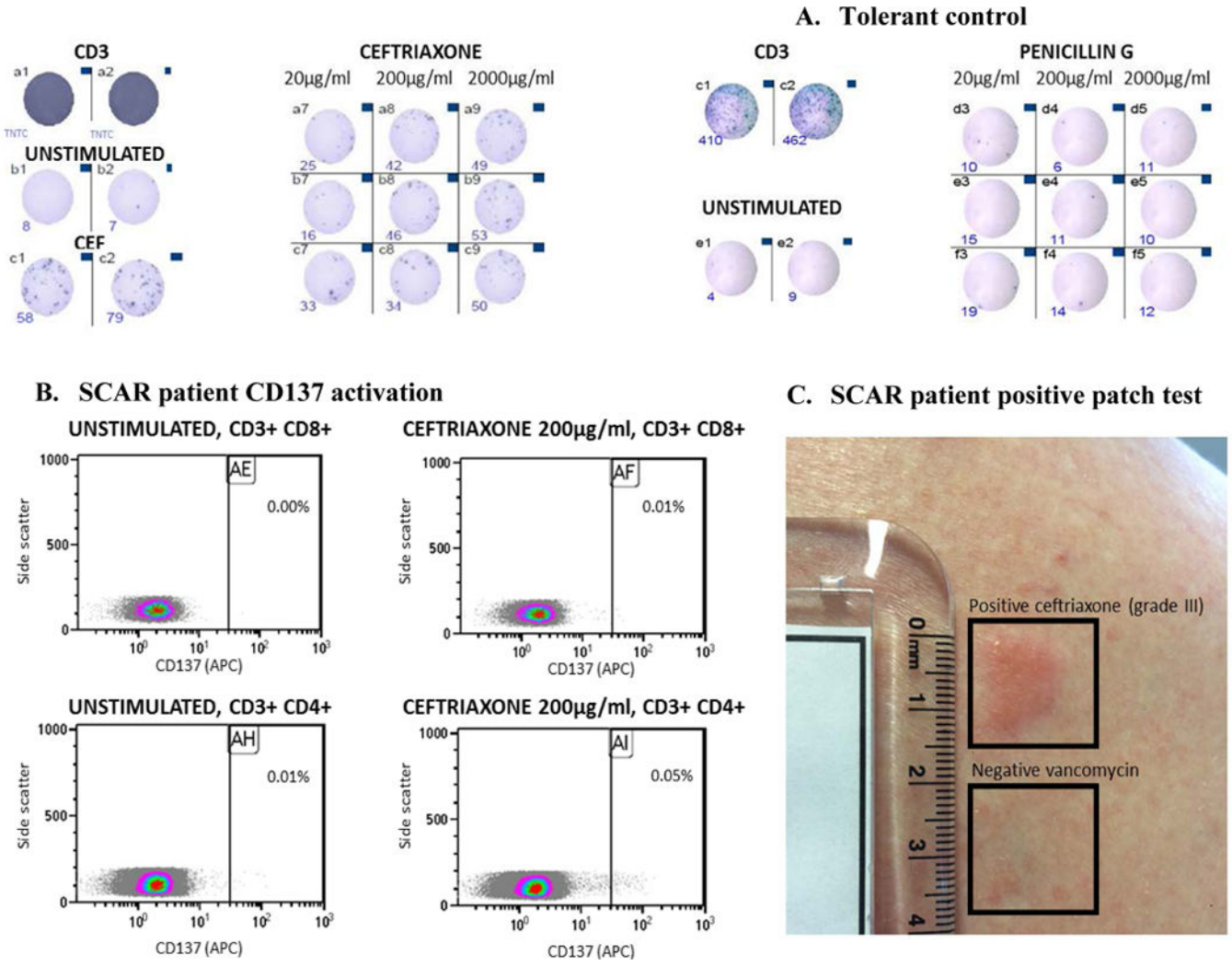


Figure 1.

Representative positive (**A.** SCAR patient, S1) and negative (**B.** tolerant control) IFN-γ release ELISpot. Representative CD137 T-cell activation (**C.**) and positive patch test of (**D.**) of SCAR patient S1.

Abbreviations: *CD3*, anti-CD3 antibodies (polyclonal T-cell activator); CEF, peptide pool consisting of 23 viral peptides (EBV, CMV and influenza) which stimulated human CD8+ T cells.

A, IFN-γ release ELISpot of positive SCAR patient. **B**, IFN-γ release ELISpot of antibiotic tolerant control. Representative images of IFN-γ release spots in 96 well plate in the presence of, (i) CD3 & 200,000 cells/well, media and 200,000 cells/well (unstimulated) and implicated drug (A. SCAR patient) and tolerate drugs (B. Tolerant Control) [left to right of image]. In **A**, the addition of a physiological control (CEF) is also demonstrated. **C**, Drug-induced T-cell activation (flow cytometry) from patient demonstrated in **A**. D, Representation of positive patch test to ceftriaxone 10% for patient represented in **1A** and **1C**

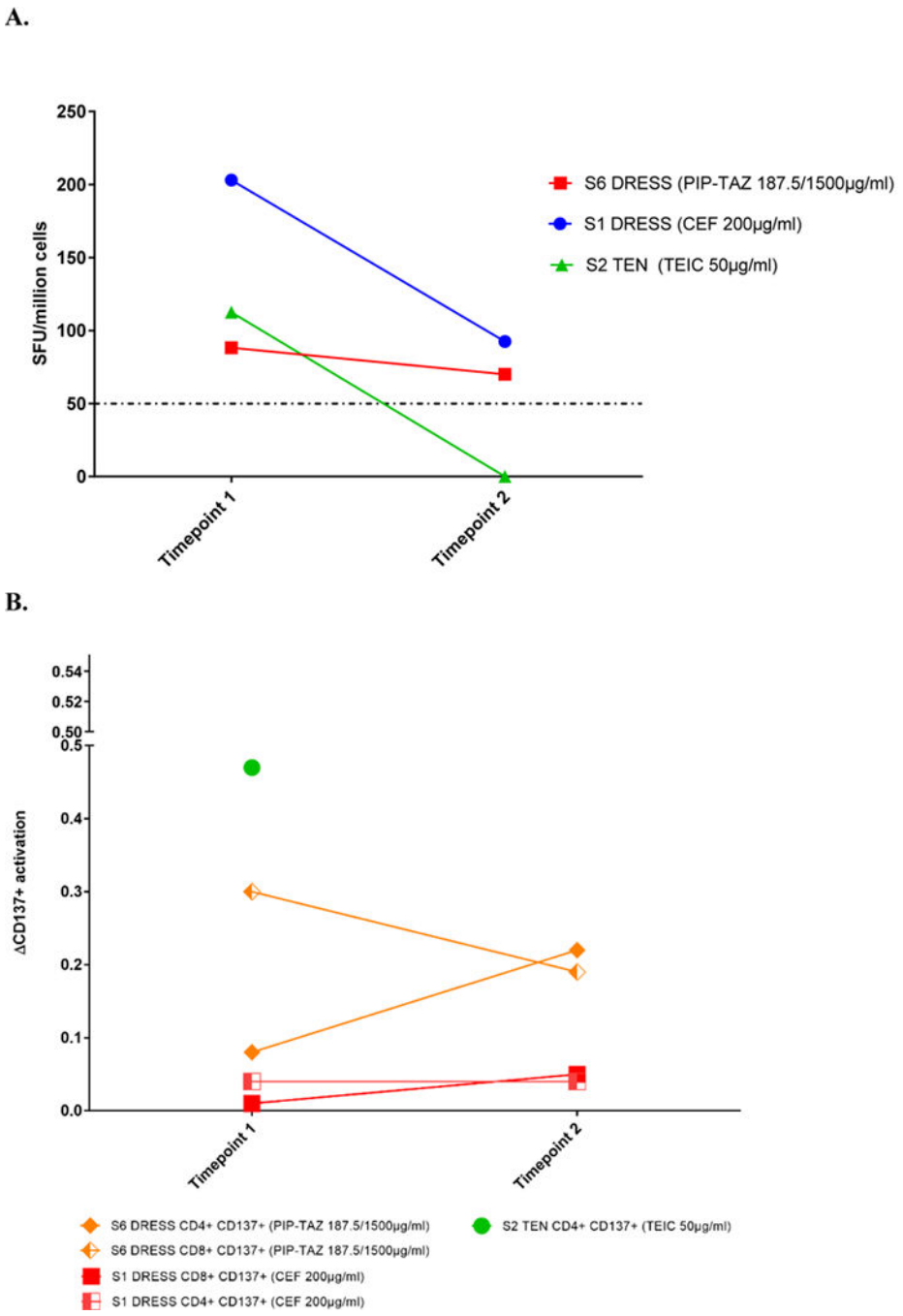


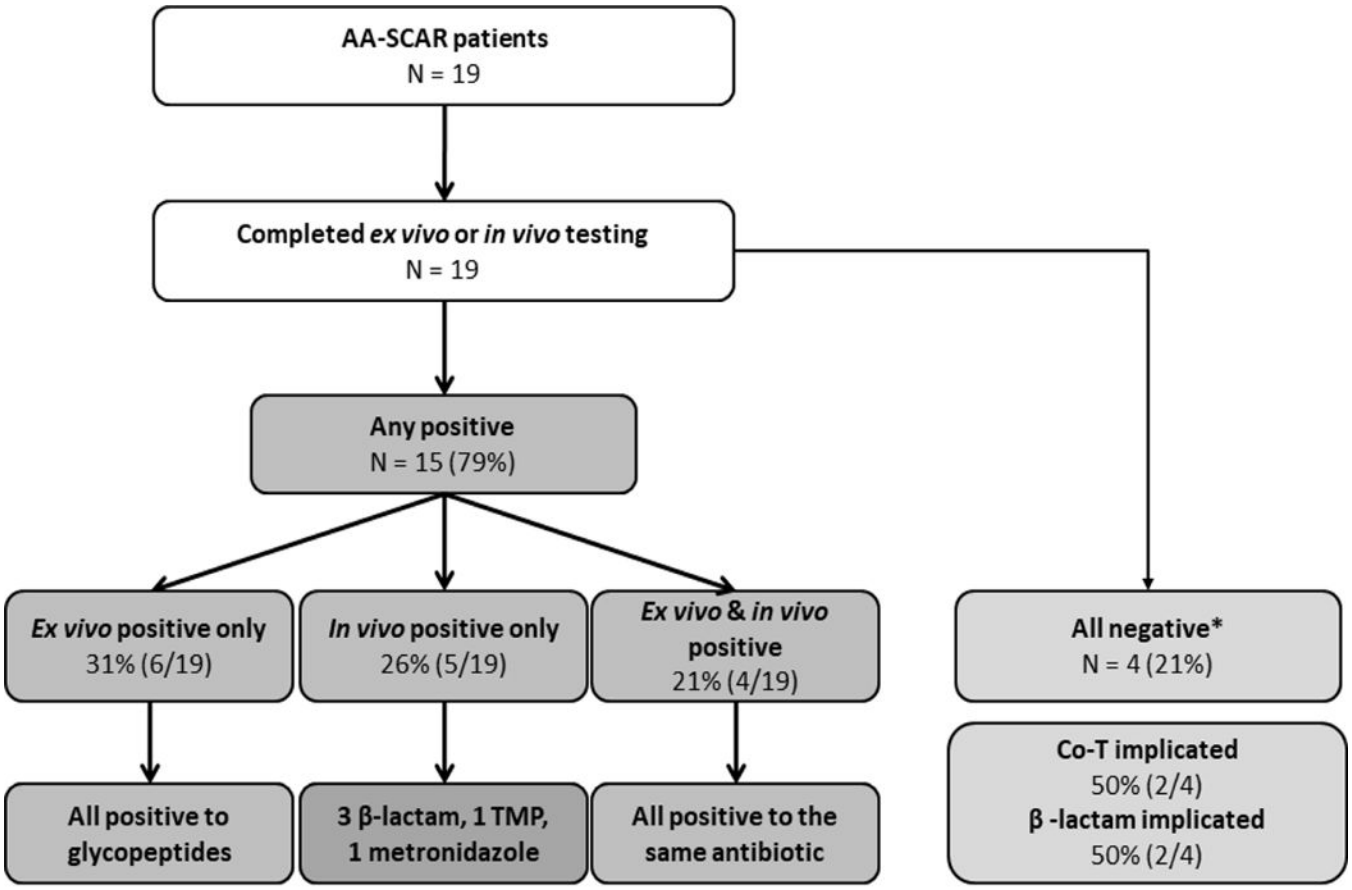
Figure 2. IFN-γ release ELISpot and CD137 T-cell activation over time in SCAR patients

A. IFN-γ release ELISpot for three patients with positive timepoint 1 results and subsequent timepoints.

B. CD137 T cell activation by flow cytometry of the three patients with positive timepoint 1 results above.

Abbreviations: PIP-TAZ, piperacillin-tazobactam; CEF, ceftriaxone; TEN, toxic epidermal necrolysis; SFU, spot forming units; DRESS, drug reaction with eosinophilia system symptoms.

Demonstration of IFN-γ release responses in patients with an initial positive result (> 50 SFU/million cells) with a subsequent bleed at least 9 months' post SCAR onset.
Timepoints post SCAR onset: S1 DRESS (Timepoint 1, 66 days; Timepoint 2, 548 days)
S2 DRESS (Timepoint 1, 312 days; Timepoint 2, 556 days)
S6 DRESS (Timepoint 1, 4 days; Timepoint 2, 286 days)



Abbreviations: AA-SCAR, antibiotic-associated severe cutaneous adverse drug reactions; TMP, trimethoprim; Co-T, trimethroprim sulfamethoxazole
* Included a patient that refused *in vivo* testing and patient with indeterminate *ex vivo*.

Figure 3.
Flow chart of testing results in antibiotic-associated SCAR patients

Table 1

Baseline characteristics of patients and controls

Variable	Patients (*n* = 19)	Tolerant Controls (*n* = 16)	P value
Age, years, median (IQR)	58 (51,71)	67.5 (55,76)	0.93
Sex (M: F)	12:7	10:6	>0.99
Immunocompromised[a]	6 (32)	6 (38)	0.73
Caucasian	17 (89)	13 (81)	0.64
Age-adjusted CCI, median (IQR)	3 (1,4)	4 (2,5.75)	0.23
Lymphopenia[b]	3 (16)	2 (11)	>0.99
Implicated antibiotic(s) present at time of blood draw[n]	1 (5)	15 (94)	0.001
Multiple implicated antibiotics	12 (63)	NA	-
Multiple implicated drugs	14 (74)	NA	-
Skin test latency[d], days, median, (IQR) [range]	193 (69, 470) [53-3650]	NA	-
ELISpot latency[d], days, median (IQR) [range]	138.5 (62, 504) [3, 3650]	NA	-

Values are given as No. (%), unless otherwise stated.

Abbreviations: IQR, interquartile range; M, male; F, female; CCI, Charlson comorbidity index.

[a]Immunocompromised– transplant recipient, haematological or oncological malignancy (last 5 years), steroids > 10 mg prednisolone equivalent per day, connective tissue or autoimmune condition.

[b]Lymphopenia defined as a total white blood cell count < 1 units

[c]A point within at least 5 drug half-lives of the last drug administration in patients (implicated antibiotic[s]) and controls (tolerated antibiotic).

[d]This was taken as the time from onset of SCAR phenotype to skin testing being performed (“skin test latency”) or PBMC collection (“ELISpot latency”).

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Table 2

Summary of cohort clinical characteristics, SCAR history and outcomes post testing

Study No.	Age-Sex	ICH[a]		SCAR Phenotype	Culprit antibiotics(s)		*Ex vivo* or *in vivo* positive	Antibiotic use post testing
		Status	Immunosuppression		Antibiotic(s)	Phenotypic Score[b]		
S1	72F	N		DRESS	CEF, VAN	6	Yes	VAN, AMP, FLU
S2	20M	N		TEN	TEIC	4	Yes	FLU
					MER	4		
					VAN	4		
S3	43M	N		SJS	TMP-SMX[c]	5	No	NIL
S4	48M	N		DRESS	TMP-SMX	4	No	DOX
S5	83M	Y	ITP/Prednisolone	SJS	CEF	4	No	MER, DOX, CIP, VAN
					PIP-TAZ	3		
S6	38F	N		DRESS	PIP-TAZ, VAN	7	Yes	CEFU
S7	74M	N		DRESS	CEFZ, MER, PENG	5	Yes	PENV, FLU
S8	62M	Y	NHL	DRESS	PIP-TAZ	4	Yes	CEPH
S9	57M	Y	AML/chemotherapy	DRESS	VAN, PIP-TAZ, MER	6	Yes	PIP-TAZ, MER
S10	51F	N		DRESS	VANC, CEFZ	6	Yes	NIL
S11	59F	N		DRESS	AMP	4	Yes	NIL
S12	52F	N		DRESS	VAN, PIP-TAZ, ADF	4	Yes	ADF, MER
S13	53F	N		$AGEP_1$	MET	8	Yes	PENV
				$AGEP_2$	VAN, CIP			
S14	60M	Y	AML/chemotherapy	DRESS	VAN, PIP-TAZ	4	No	CIP
S15	58M	N		DRESS	VAN, CIP	6	Yes	PENV
S16	75M	Y	DRESS/Prednisolone	DRESS	VAN, PIP-TAZ	2	Yes	CEPH
S17	66M	Y	HCL	DRESS	TMP-SMX[c]	4	Yes	NIL
S18	71M	N		DRESS	PIP-TAZ, AMP	2	Yes	AMP
S19	54F	N		SJS	VAN	4	Yes	PENG

Abbreviations: ICH, immunocompromised host; SCAR, Severe Cutaneous Adverse Drug Reaction; N, no; Y, yes; ITP, immune mediated thrombocytopenia; NHL, non-Hodgkin lymphoma; AML, acute myeloid leukemia; DRESS, drug reaction with eosinophilia and systemic symptoms; HCL, hairy cell leukemia; M, male; F, female; AMP, ampicillin; ADF, amoxicillin clavulanate; CEF, ceftriaxone; CEFZ, cefazolin, CEFU, cefuroxime; CEFT, ceftazidime; CEPH, cephalexin; CIP, ciprofloxacin; FLU, flucloxacillin; MER, meropenem; MET, metronidazole; PENG, benzylpenicillin; PENV, penicillin VK; PIP-

TAZ, piperacillin-tazobactam; TEIC, teicoplanin; VAN, vancomycin; 4NO, 4-Nitro sulfamethoxazole; TMP, trimethoprim, SMX, sulfamethoxazole; TMP-SMX, trimethoprim-sulfamethoxazole; FLU, flucloxacillin; Nil, no antibiotics required; DOX, doxycycline

[a]ICH: Transplant recipient, haematological or oncological malignancy (last 5 years), steroids > 10mg prednisolone equivalent per day, connective tissue or autoimmune condition. Immunosuppression was defined as that the patient was receiving at time of PBMC collection.

[b]Phenotypic scores as per published guidelines for SJS/TEN (ALDEN), DRESS (RegiSCAR) and AGEP.

[c]Patient S3 had ibuprofen implicated in causality, ALDEN score (3) was lower than that of TMP-SMX. Patient S18 had cladribine and allopurinol implicated, also with lower ALDEN scores (3) that TMP-SMX.

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Table 3

Summary of number of *in vivo*, *ex vivo* and combined testing results in patients with a history of antibiotic-associated SCAR

Patient study No.	Skin test results (*in vivo*)			IFN-γ ELISpot results (*ex vivo*)[b]			Combined results
	Modality positive: Antibiotic(s)[a]	Concentration (mg/ml)	Interpretation	Antibiotics positive	Concentrations (μg/ml)[c]	Interpretation	*In vivo* or *ex vivo*
S1	*PT/IDT:* CEF	10%/2.5	Positive (CEF)	CEF	20,200,2000	Positive (CEF)	Positive
S2	Nil		Negative	TEIC	25, 50, 100	Positive (TEIC)	Positive
S3	NP		NP	Nil	-	Negative	Negative
S4	Nil		Negative	Nil	-	Negative	Negative
S5	Nil		Negative	Nil[d]	-	Indeterminate	Negative
S6	*IDT:* AMP, PIP-TAZ	25, 4.5	Positive (PIP-TAZ, AMP)	PIP-TAZ	37.5/300, 375/3000	Positive (PIP-TAZ)	Positive
S7	*IDT:* CEFZ, MERO	1, 2.5	Positive (CEFZ, MERO)	CEFZ MERO	20, 200, 200 20, 200	Positive (CEFZ, MERO)	Positive
S8	*IDT:* AMP, FLU, PENG, PIP-TAZ	25, 2, 10, 4.5	Positive (AMP, FLU, PENG, PIP-TAZ)	AMP FLUC PENG PIP-TAZ	200, 2000 200 20, 200, 2000 18.75/150, 187.5/1500	Positive (PENICILLINS)	Positive
S9	Nil		Negative	VAN	5, 50, 500	Positive (VAN)	Positive
S10	Nil		Negative	VAN	5, 50, 500	Positive (VAN)	Positive
S11	*IDT:* AMP	25	Positive (AMP)	Nil	-	Negative	Positive
S12	Nil		Negative	VAN	5, 50, 500	Positive (VAN)	Positive
S13	*PT/IDT:* MET	3	Positive (MET)	Nil	-	Negative	Positive
S14	Nil		Negative	Nil	-	Negative	Negative
S15	NP		NP	VAN	5, 50, 250	Positive	Positive
S16	*IDT:* PIP-TAZ	4.5	Positive (PIP-TAZ)	Nil	-	Negative	Positive
S17	*PT:* TMP	5%	Positive (TMP)	Nil	-	Negative	Positive
S18	*IDT:* PIP-TAZ	4.5	Positive (PIP-TAZ)	Nil	-	Negative	Positive
S19	Nil		Negative	VAN	5, 50, 500	Positive	Positive

Abbreviations: IDT, delayed intradermal testing; PT, patch testing; NP, not performed; Nil, nil positive. AMP, ampicillin; CEF, ceftriaxone; CEFZ, cefazolin; FLU, flucloxacillin; MER, meropenem; MET, metronidazole; PIP-TAZ, piperacillin-tazobactam; TEIC, teicoplanin; VAN, vancomycin; TMP, trimethoprim.

[a] In patients beta-lactam implicated in SCAR causality a standard panel employed for IDT (DAP-major, DAP -minor, PENG, FLU, AMP, PIP-TAZ, CEFX, CEF) in addition to implicated beta-lactam at recommended concentrations[1]. For all other drugs the implicated antibiotic was tested via either patch testing (PT) or delayed intradermal testing (IDT). For PT, expressed as %. In patient S3 ELISpot testing was also performed to ibuprofen and for S18 PT was also performed to allopurinol.

[b] A SFU/million cells of > 50 was interpreted as positive result by subtracting the spot count in the negative control (no drug) from the spot count in the wells with drugs.

[c] Performed in triplicate (or duplicate if thawed PBMC numbers low).

[d] Negative CD3 control.

Table 4

Summary of results for IFN-γ release ELISpot for patients and tolerant controls

Result of IFN-γ release ELISpot	Patients						Tolerant controls, n = 16
	SJS/TEN, *n* = 4	DRESS, *n* = 14	AGEP, *n* = 1	Acute patients[a], *n* = 4	Convalescent patients[b], *n* = 15	All patients, *n* = 19	
Positive	2 (50)	8 (57)	0 (0)	3 (75)	7 (47)	**10 (53)**	**0 (0)**
Negative	1 (25)	6 (43)	1	1 (25)	7 (47)	**8 (42)**	**16 (100)**
Indeterminate[c]	1 (25)	0 (0)	0 (0)	0 (0)	1 (7)	**1 (5)**	**0 (0)**

Values are given as No. (%)

Abbreviations: SJS, Stevens-Johnson syndrome; TEN, toxic epidermal necrolysis; DRESS, drug reaction with eosinophilia and systemic symptoms; AGEP, acute generalized exanthematous pustulosis.

[a] Acute patients were defined as within 6 weeks of SCAR onset

[b] Convalescent patients were defined as > 6 weeks' post SCAR onset

[c] In an indeterminate results being the CD3 response was not present; this was included in analysis as a 'false negative'.

Table 5

Sensitivity and specificity of IFN-γ release ELISpot

Result (N = 19)	Phenotype			Timing		Phenotype scoring[a]	All patients
	SJS/TEN	**DRESS**	**AGEP**	**Acute**	**Convalescent**	**≥ Probable or Definitive only**	
Sensitivity, % (CI)	50 (6.76-93.24)	57.14 (28.86-82.34)	0 (0 – 97.50)	75 (19.41-99.37)	50 (23.04-76.96)	58.82 (39.92-81.56)	**52 (28.86-75.55)**
Specificity, % (CI)	100 (79.41-100)	100 (79.41-100)	100 (79.41-100)	100 (79.41-100)	100 (79.41-100)	100 (79.41-100)	**100 (79.41-100)**

Abbreviations: PPV, Positive Predictive Value; NPV, Negative Predictive Value

[a]Patients were stratified based on their phenotypic score - “Possible” or “Probable or Definitive” (n = 17).