Ex vivo diagnostics using varied cellular inputs in drug-induced severe cutaneous adverse reactions

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Abstract

Background Drug-induced severe cutaneous adverse reactions (SCARs) are presumed T-cell-mediated hypersensitivities associated with significant morbidity and mortality. Traditional *in-vivo* testing methods, such as patch or intradermal testing, are limited by a lack of standardisation and poor sensitivity. Modern approaches to testing include measurement of IFN-γ release from patient peripheral blood mononuclear cells (PBMC) stimulated with the suspected causative drug. *Objective* We sought to improve *ex-vivo* diagnostics for drug-induced SCAR by comparing enzyme-linked immunospot (ELISpot) sensitivities and flow cytometry-based intracellular cytokine staining (ICS) and cellular composition of separate samples (PBMC or blister fluid cells (BFC)) from the same donor. *Methods* IFN-γ release ELISpot and flow cytometry analyses were performed on donor-matched PBMC and BFC samples from four SCAR patients with distinct drug-allergies. *Results* Immune responses to suspected drugs were detected in both PBMC and BFC samples of two donors (Case 1 in response to ceftriaxone and Case 4 to oxypurinol), with BFC eliciting stronger responses. For two other donors, only BFC samples showed a response to meloxicam(Case 2) or sulfamethoxazole and its 4-Nitro metabolite (Case 3). Consistently, flow cytometry revealed a greater proportion of IFN-γ-secreting cells in the BFC compared to PBMC. BFC cells from Case 3 were also enriched for memory/activation/tissue-recruitment markers over PBMC. *Conclusion* Analysis of BFC samples for drug-allergy diagnostics offers a higher sensitivity for detecting positive responses compared to PBMC. This is consistent with recruitment (and enrichment) of cytokine-secreting cells with a memory/activated phenotype into blisters.

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Short Title: *Ex vivo* diagnostics in SCAR

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Declaration of interests

The authors do not report any conflicts of interest.

Consent

All the subjects gave written informed consent for this study and this has been approved by the Ethics Review Committee (**Supplementary Methods**)

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ABSTRACT

Background

Drug-induced severe cutaneous adverse reactions (SCARs) are presumed T-cell-mediated hypersensitivities associated with significant morbidity and mortality. Traditional *in-vivo* testing methods, such as patch or intradermal testing, are limited by a lack of standardisation and poor sensitivity. Modern approaches to testing include measurement of IFN-γ release from patient peripheral blood mononuclear cells (PBMC) stimulated with the suspected causative drug.

Objective

We sought to improve *ex-vivo* diagnostics for drug-induced SCAR by comparing enzyme-linked immunospot (ELISpot) sensitivities and flow cytometry-based intracellular cytokine staining (ICS) and cellular composition of separate samples (PBMC or blister fluid cells (BFC)) from the same donor.

Methods

IFN-γ release ELISpot and flow cytometry analyses were performed on donor-matched PBMC and BFC samples from four SCAR patients with distinct drug-allergies.

Results

Immune responses to suspected drugs were detected in both PBMC and BFC samples of two donors (Case 1 in response to ceftriaxone and Case 4 to oxypurinol), with BFC eliciting stronger responses. For two other donors, only BFC samples showed a response to meloxicam(Case 2) or sulfamethoxazole and its 4-Nitro metabolite (Case 3). Consistently, flow cytometry revealed a greater proportion of IFN-γ-secreting cells in the BFC compared to PBMC. BFC cells from Case 3 were also enriched for memory/activation/tissue-recruitment markers over PBMC.

Conclusion

Analysis of BFC samples for drug-allergy diagnostics offers a higher sensitivity for detecting positive responses compared to PBMC. This is consistent with recruitment (and enrichment) of cytokine-secreting cells with a memory/activated phenotype into blisters.

Key words : Severe cutaneous drug reactions, ex-vivo assays, PBMC, BFC

Abbreviations:

SCARs:	Severe cutaneous adverse reactions
PT:	Patch testing
DT:	Intradermal testing
AGEP:	Acute generalised exanthematous pustulosis
DRESS:	Drug reaction with eosinophilia and systemic symptoms
SJS:	Steven-Johnson syndrome
TENs:	Toxic epidermal necrolysis
ELISpot:	Enzyme linked immunospot
SFU:	Spot forming units
TRM:	Tissue resident memory CD8+ T cells
GBFDE: IFN: BFC: PBMC:	Generalised Bullous Fixed Drug Eruption Interferon Blister fluid cells Peripheral blood mono

INTRODUCTION

Delayed drug-induced hypersensitivities are a group of presumed conventional T-cell-mediated reactions that range from mild skin conditions (e.g. maculopapular exanthema) to severe cutaneous adverse reactions (SCARs), associated with significant morbidity and mortality[1]. Traditional *in vivo* skin testing techniques such as patch testing (PT) or intradermal testing (DT) are limited by an absence of standardisation, risk of disease-relapse, and ill-defined drug testing concentrations[2]. These limitations can impact the sensitivity of such tests, with published studies suggesting sensitivity ranging from 58-64% for acute generalised exanthe-

matous pustulosis (AGEP), 32-80% for drug reaction with eosinophilia and systemic symptoms (DRESS), and 9-24% for Steven-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN)[3]. There is also drug-associated variability in PT, with beta-lactams displaying higher sensitivities while allopurinol and its active metabolite, oxypurinol, exhibit very low sensitivities[4]. Evolving approaches include *ex-vivo*assays, such as the enzyme-linked immunospot (ELISpot), which detects interferon-γ (IFN) release following drug challenge. Traditionally ELISpot assays use the patient's peripheral blood mononuclear cells (PBMC) stimulated with the candidate drug to measure cytokine output. The ELISpot method is advantageous as patients are not subjected to additional risk through drug re-exposure. Our recent data suggests IFN- γ release ELISpot is an effective diagnostic tool with a 52-68% sensitivity and 100% specificity in SCAR patients[5, 6].

ELISpot assays detect cytokine (typically IFN-γ) release, which is presumed to be produced by CD4+ or CD8+ T cells, in patient PBMC or blister fluid cells (BFC) following *ex vivo* stimulation with the candidate drug. Cytokine secretion is measured as the number of spot-forming units (SFU)/million cytokine secreting cells. Previous case reports have suggested a diminished PBMC IFN-γ ELIspot response over time from SCAR onset, highlighting the importance of performing assays during the acute phase of drug reactions[7]. This diminished response in peripheral blood may be associated with the lack of a key cell population known as tissue resident memory CD8+ T cells (TRM), which reside within the dermal-epidermal and drug-reactive CD8+ T cells are gradually lost from peripheral blood during the recovery period[8]. In contrast, CD8+ TRM cells are more likely to be recruited into BFC in SCAR patients. One study compared cytokine production between PBMC and BFC, noting that there was a higher expression of perforin and granzyme B in BFC[9]. This could be due to localised skin TRM cells mediating the inflammatory response by recruiting memory CD8+ T cells from circulation and suggests that ELISpot assays conducted with PBMC from patients in the late stages of drug reaction could be less sensitive[6, 7, 10]. Here, we sought to find ways to improve *ex vivo* assay sensitivity in SCAR diagnostics by examining differences in ELISpot results between two different cellular sources: PBMC and BFC. This study aims to provide knowledge that will inform future SCAR testing strategies.

RESULTS/DISCUSSION

In this study, we included PBMC and BFC samples that had been cryogenically stored from four patients with confirmed SCAR (Cases 1-4) including SJS, TEN, DRESS, and generalised bullous fixed drug eruption (GBFDE) identified from previous prospective studies (**Supplementary Methods**). All patients had a Naranjo score of 4 or higher[11], a minimum Scorten score of 2 and a minimum Alden score of 4 for SJS/TEN[12, 13]. Cases 2 and 4 had one implicated drug while Cases 1 and 3 had three implicated drugs and all cases were receiving the implicated drug at time of rash onset (Table 2). The latency period for cases (defined as time between drug commencement and rash onset) ranged from 0-38 days with a median value of 18.5 days. PBMC and BFC collection delay for testing had means of 24 and 22 days, respectively. Case 4 had a delayed collection latency for PBMC and BFC of 48 and 49 days, respectively. Baseline demographics, clinical features and biological sampling are shown in **Tables 1 and 2** .

IFN-γ ELISpot was performed in matched PBMC and BFC samples from Cases 1-4, as per previously published methods[7] and**Supplementary Methods** (**Figures 1 and 2** ,**Supplementary Figures 1 and 2)** . Two of these patients displayed positive ELISpot results (defined as SFU [?] 50U/million cells [7, 8]) upon *ex vivo* challenge with suspected drugs for both PBMC and BFC (Cases 1-ceftriaxone and 4-oxypurinol), while Cases 2 and 3 only displayed a positive result with BFC (**Figure 1).**

Case 1 BFC tested positive for both doses of ceftriaxone (200 and 2000μg/mL), whilst matched PBMC only tested positive to the highest dose, with half of the response elicited in BFC. Case 4 BFC tested positive at both concentrations (5 and 50μg/mL) of allopurinol in addition to its metabolite, oxypurinol, while PBMC only showed a positive response to oxypurinol. This suggests that BFC analysis can provide higher sensitivity to drug-allergy testing than PBMC. This is further supported by analysis of Cases 2 and 3 whereby positive IFN-γ release ELISpot responses were detected using BFC but not PBMC samples. Case 2 BFC displayed positivity to all doses of meloxicam (2, 20 and 200 μg/mL) and Case 3 only to the highest dose of sulfamethoxazole (SMX-500μg/mL), its metabolite 4-Nitro-SMX (100μg/mL), as well

as to the commercial product (Bactrim©; trimethoprim-sulfamethoxazole) at 50 and 250μg/mL of the sulfamethoxazole component, respectively.

Flow cytometry was used to investigate whether different cellular compositions of matched BFC and PBMC for Cases 1, 3 and 4 could account for differences in ELISpot sensitivities **(Figure 2, Supplementary Figure 1-2)** . We found that BFC samples were enriched for total T (CD3+) cells and for IFN-γ-secreting cells, relative to matched PBMC (**Figure 2A, Supplementary Figure 1 and 2**). The total proportions of CD4+, CD8+, or double negative (DN) T cells varied across individuals (**Figure 2A, Supplementary Figure 1**), likely reflecting differences in the pathology and/or treatments, with Case 1 displaying a strong bias for CD4+ T cells, which is typical of DRESS[14]. In Contrast, Case 4 BFC were enriched for CD8+ T cells relative to matched PBMC, which may be associated with a delayed BFC sampling, compared to other cases (**Table 2**), possibly reflecting CD8+ T cells egress from peripheral blood[8]. Cases 3 and 4 BFC showed an enrichment for T cell populations with a tissue residency/recruitment (CD69+CD103+) phenotype, which have been implicated in SCAR[8] **(Figure 2A, Supplementary Figure 1**). Case 3 BFC samples further displayed higher proportions of memory (CD45RO) and activated (CD69) T cells, relative to PBMC, whilst remaining similar for Cases 1 and 4, which may partly account for the differences in ELISpot sensitivities between the two samples (**Figure 1**). As unconventional T cells (not HLA-restricted) are also known to produce IFN-γ, and their role in SCAR remains unexplored[15], we assessed the proportions of γδ T cells, mucosal-associated invariant T (MAIT) cells and CD56-expressing T cells (T cells expressing natural killer (NK) markers, likely including natural killer T (NKT) cells)[16]. While MAIT cells and γδ T cells did not show preferential recruitment into BFC (**Figure 2A and Supplementary Figure 1**), they were found among IFN-γ+ populations (**Figure 2B, Supplementary Figure 2B**), representing a large proportion of Case 3 PBMC (38.4% and 11.4%, respectively). IFN-γ secreting cells also comprised NK-like T cells (CD56+CD3+), and NK cells (CD56+CD3-) - Case 3 PBMC. Overall, IFN-γ-secreting cells comprised CD4+, CD8+ and DN (CD4-CD8-) T cells, with preferential enrichment for CD8+ T cells in BFC from Cases 3 and 4 and displayed memory and activated phenotypes (CD45RO+/CD69+) (**Figure 2,Supplementary Figure 2B**). Overall BFC samples display T lymphocytes that have been recruited from the blood or adjacent tissue with an activated phenotype and cytokine secreting capacity. This leads to higher proportions of cells with an IFN-γ secreting capacity (when compared to blood), which may reflect higher representations of the drug-antigen-specific clones. Collectively, these results suggest a higher sensitivity for BFC samples in ELISpot-testing relative to PBMC, likely reflecting differences in their cellular composition.

Ex vivo drug-allergy diagnostics have an increasing evidence base and clinical demand[3]. By analysing samples from four SCAR patients with distinct drug-allergies and clinical manifestations that are presumed to be T-cell-mediated, this study provides impetus for further work to explore alternative sampling sources for drug-allergy diagnostics. At present there is no gold-standard diagnostic for causality assessment in SCAR, and previous studies, whilst showing promising sensitivity[3], remain limited. Our results suggest higher sensitivity for BFC analysis relative to matched PBMC using *ex vivo* IFN-γ release ELISpot [3]. Whilst limited by low numbers and cell viability, the rare nature of both blister fluid capture and SCAR cases that have been accurately phenotyped provides a unique insight into the diagnostic potential for this IFN-γ release ELISpot assay.

Our results are consistent with recruitment of known populations involved in the pathology (T cells with a memory/activated phenotype and cytokine-secreting capacity) into blisters. We further reveal that, relative to BFC, PBMC may have lower representation of cells with an IFN-γ-secretion capacity[7, 8]. How much IFN-γ detection by ELISpot is due to direct activation of drug-specific cells or bystander secretion of non-specific cells remains to be understood, and it may vary with the drug causing SCAR. It is possible that some drug-specific cells may produce cytokines other than IFN-γ (such as TNF, IL-4, IL-17) upon activation that have not been tested. Whilst we also assessed IL-17-secretion using flow cytometry, our results do not seem to suggest that this could be a key contributor for the responses studied (**Supplementary Figure 3**). This may require ELISpot assays for other cytokines or markers yet to be identified, or even more generic activation assays using cellular activation markers like CD69, CD107a. Thus, we recommend that clinicians

sample BFC, whenever available, for testing with ELISpot assays in drug-allergy diagnostics, whilst retaining correlation with PBMC results. This may prove to be an invaluable resource for future studies aiming at characterising the immunopathogenesis and HLA (or HLA-like) restriction of these drug-induced allergies, including drug-presentation pathways, cell populations involved, and cytokine-outputs. Such knowledge may ultimately lead to improved diagnostics for SCAR patients, improving efforts to lower the significant morbidity and mortality associated with SCAR.

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TABLES

Table 1. Baseline demographics, biological sampling and testing of cohort

Case number	**Case 1**	**Case 2**	**Case 3**	**Case 4**
Age-Sex	88M	67F	38F	67M
Ethnicity	Caucasian	East-Asian	South-East Asian	Indo-Asian
Prior drug allergy	Nil	Cefalexin (Unknown reaction)	Nil	Nil
Charlson Comorbidity index (Age adjusted)	6	2	0	7
Immunosuppression +	Nil	Nil	Prednisolone 25mg daily	Splenectomy
SCAR phenotype	DRESS	GBFDE	TEN	TEN
Phenotypic score ++	RegiScar: 4	N/A	Alden: 4-5	Alden: 6
Skin test results §	IDT positive: BP, AMP, FLX, CFT	NP	NP	NP
HLA results	**HLA results**	**HLA results**	**HLA results**	**HLA results**
HLA-A	01:01:01G	24:02:01G	11:01:01G	33:03:01G
	03:01:01G	24:07:01G	24:02:01G	33:03:01G
HLA-B	07:02:01G	35:05:01G	40:01:01G	44:03:02G
	18:01:01G	40:02:01G	44:03:02G	58:01:01G
HLA-C	07:01:01G	03:04:01G	03:04:01G	03:02:01G
	07:02:01G	04:01:01G	07:01:01G	07:01:01G

Abbreviations : NP, not performed; IDT, Intradermal testing; DRESS, Drug Rash with Eosinophilia and Systemic Symptoms; GBFDE, Generalised bullous fixed drug eruption; TEN, Toxic epidermal necrolysis; IDT, Intradermal Testing; M, male; F, female; HLA, Human leukocyte antigen; BP, Benzylpenicillin; AMP, Ampicillin; FLX, Flucloxacillin; CFT, Ceftriaxone.

+ The immunocompromised category includes patients who are known for any of the following conditions: transplant recipient, haematological or oncological malignancy (in the last 5 y), corticosteroid use of more than 10 mg prednisolone equivalent per day, connective tissue or autoimmune condition, and acquired immunodeficiency syndrome.

++ Phenotypic scores used as per previously published criteria for SJS/TEN (Alden)[13], DRESS (RegiSCAR) [17]

§ **Concentrations** : Benzylpenicillin 1mg/ml and 10mg/ml, Ampicillin 25mg/ml, Flucloxacillin 2mg/ml, Ceftriaxone 2.5mg/ml, Cephazolin 1mg/ml

Table 2. Implicated drugs, predictive scores and latency

Case number	Drugs implicated	Indication	SCORTEN Score +	Alden Score
1	Benzylpenicillin	Bacteraemia	N/A	N/A
	Ceftriaxone			
	Vancomycin			
2	Meloxicam	Joint pain	N/A	N/A
3	Trimethoprim/ Sulfamethoxazole	PJP prophylaxis	2	5
	Pantoprazole	Gastric ulcer prophylaxis		4
	Atorvastatin	Nephrotic syndrome		4
4	Allopurinol	Gout	4	6
	Ibuprofen			5

+ SCORTEN Score to predict mortality in patients with SJS/TEN [18]

++ Naranjo adverse reaction score for determining the likelihood of whether an ADR (adverse drug reaction) is actually due to implicated drug [11]

§Latency: Time between drug commencement and rash onset (Days)

¶ Receiving implicated drugs at onset of rash

FIGURE LEGENDS

Figure 1 . IΦN-γ release enzyme-linked immunospot (ELISpot) assay release for peripheral blood mononuclear cells (PBMC) and blister fluid cells (BFC). Data is for cryogenically stored PBMC and BFC samples from Cases 1-4 (Supplementary Table 1). A positive result is defined by greater than or equal to 50 spot forming units (SFU) per million cells (green dotted line). The maximum doses for each drug were shown to not elicit responses and cell death on a healthy control sample, using flow cytometry (7-AAD staining) or Lactate Dehydrogenase (LDH) viability assay ([19] and Supplementary Figure 4). SMX, Sulfamethoxazole; TMP, Trimethoprim.

Figure 2. Lymphocyte composition of blood and blister samples. Donor-matched BFC and PBMC were analysed by flow cytometry. **A.**Graphs show percentages of total IFN-γ+ and CD3+ lymphocytes (left of red line) among total live lymphocytes (gated as per Supplementary Figure 1i). T cells (CD3+) (gated after exclusion of CD14 (monocytes) and CD19 (B cells) as per Supplementary Figure 1ii) were subsequently analysed for: CD4 and CD8 co-receptors (CD4/CD8 double-negative cells are indicated as DN), CD45RO (memory), CD69 (activation), CD69 and CD103 co-expression (egress/tissue residency/memory), γδ T cell receptor (TCR), binding to MR1 5-OP-RU tetramers[20, 21] (MAIT cells), or expression of the NK receptor CD56 (NK-like T cells) (right of red line). **B.** Graphs show proportions of CD4, CD8 and CD4/CD8 DN T cells, γδ T cells, MAIT cells and CD56+ T cells amongst IFN-γ-secreting cells, gated as per Supplementary Figure 2.