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Original Article

The Role of *In Vitro* Detection of Drug-Specific Mediator-Releasing Cells to Diagnose Different Phenotypes of Severe Cutaneous Adverse Reactions

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ABSTRACT

Propose: The purpose of this study was to investigate panels of enzyme-linked immunospot assays (ELISpot) to detect drug-specific mediator releasing cells for confirming culprit drugs in severe cutaneous adverse reactions (SCARs).
Methods: Frequencies of drug-induced interleukin-22 (IL-22)-, interferon-gamma (IFN-γ)-, and granzyme-B (GrB)-releasing cells were measured by incubating peripheral blood mononuclear cells (PBMCs) from SCAR patients with the culprit drugs. Potential immunoadjuvants were supplemented to enhance drug-induced mediator responses.
Results: Twenty-seven patients, including 9 acute generalized exanthematous pustulosis (AGEP), 10 drug reactions with eosinophilia and systemic symptoms, and 8 Stevens-Johnson syndrome and toxic epidermal necrolysis (SJS/TEN) were recruited. The average frequencies of drug-induced IL-22-, IFN-γ-, and GrB-releasing cells were 35.5±16.3, 33.0±7.1, and 164.8±43.1 cells/million PBMCs, respectively. The sensitivity of combined IFN-γ/IL-22/GrB ELISpot was higher than that of IFN-γ ELISpot alone for culprit drug detection in all SCAR subjects (77.8% vs 51.9%, P < 0.01). The measurement of drug-induced IL-22- and IFN-γ

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There are no financial or other issues that might lead to conflict of interest.

releasing cells confirmed the culprit drugs in 77.8% of AGEP. The measurement of drug-induced IFN-γ- and GrB-releasing cells confirmed the culprit drugs in 62.5% of SJS/TEN. Alpha-galactosylceramide supplementation significantly increased the frequencies of drug-induced IFN-γ releasing cells.

Conclusion: The measurement of drug-induced IFN-γ-releasing cells is the key for identifying culprit drugs. The additional measurement of drug-induced IL-22-releasing cells enhances ELISpot sensitivity to identify drug-induced AGEP, while the measurement of drug-induced GrB-releasing cells could have a role in SJS/TEN. ELISpot sensitivity might be improved by supplementary alpha-galactosylceramide.

Trial Registration: ClinicalTrials.gov Identifier: NCT02574988

Keywords: Drug allergy, diagnosis, in vitro; T cell; cytokine; IFN-γ

INTRODUCTION

The identification and confirmation of culprit drugs in patients who experience drug-induced severe cutaneous adverse reactions (SCARs) is difficult. Most of the time, the culprit drugs suspected are not re-challenged, so the diagnostic value of the test cannot be validated. The sensitivity of skin tests to identify culprit drugs is limited, and there are risks of exacerbating the allergic reaction.[1,2] *In vitro* diagnostic tests have been introduced to identify culprit drugs, for example, the lymphocyte transformation test (LTT) and the measurement of drug-specific mediator release using enzyme-linked immunospot assays (ELISpot), which have been reported to be more sensitive than LTT.[3,4] Interferon-gamma (IFN-γ) is the cytokine most often used to study drug-specific T cell responses.[5,6] As several cytokines play roles in the pathogenesis of SCARs, the diagnostic values of IFN-γ ELISpot in different phenotypes of SCAR are still limited.

Several factors might affect the results of drug-specific mediator responses in a real-world situation, such as the elapsed time since the last exposure, systemic steroid administration before having blood drawn, and SCAR phenotypes.[7,8] It has been proposed that LTT should be performed during the recovery phase, since the assay may yield false-negative results during the acute drug allergic phase.[9,10] "However, ELISpot data showed that the measurement of drug-specific mediator- releasing cells would be helpful at both acute and recovery phases, although sensitivity would decrease after 2 years since the last reaction"[4,11] Taken together, the exploration of the appropriate panel of drug-specific mediators to yield the maximum sensitivity of the *in vitro* test is essential.

The measurement of drug-specific IFN-γ-releasing cells has most commonly been used with the ELISpot technique to identify culprit drugs. However, other mediators also play a role in T-cell-mediated drug reactions.[12] There is evidence that cytotoxic signals, such as granulysin, perforin/granzyme B (GrB), and Fas/Fas ligand, are elevated in not only Stevens-Johnson syndrome/toxic epidermal necrolysis (SJS/TEN), but also in drug reaction with eosinophilia and systemic symptoms (DRESS).[13,14] Furthermore, the roles of interleukin (IL)-17 family cytokines, such as IL-17 and IL-22, have been demonstrated in inflammatory skin diseases such as pustular psoriasis and acute generalized exanthematous pustulosis (AGEP).[15-17] The measurement of these mediators released from peripheral blood mononuclear cells (PBMCs) in patients with SCAR would probably be helpful in the identification of culprit drugs.

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In real-world practice, several factors could affect the sensitivity of drug-specific mediator measurements. Many regulatory molecules, particularly immune checkpoint inhibitors, have influences on the magnitude of immune responses. T-cell immunoglobulin and mucin domain 3 (TIM3) is known as a checkpoint receptor and exhaustion marker with both positive and inhibitory functions; reduced expression of TIM3 has been demonstrated in Th1 cells in drug-induced maculopapular exanthema.[18,19] The administration of immune checkpoint inhibitors, such as cytotoxic T-lymphocyte-associated protein 4 (CTLA4), programmed cell death protein 1 (PD-1), and programmed death-ligand 1 (PD-L1), could activate cytotoxic T cell responses in malignancies, but also lead to the development of immune-mediated cutaneous reactions.[20,21] There is a report that blockade of the PD-1/PD-L1 pathway could induce IFN-γ secretion from drug-specific T- cells.[22] Additionally, alpha-galactosylceramide (α-GalCer) has been shown to enhance cellular immune responses, mainly via increasing the functions of natural killer T (NKT) cells.[23] It would be interesting to explore whether the manipulation of these molecules could enhance the sensitivity of *in vitro* testing for drug allergy diagnosis.

The purpose of this study was to evaluate the diagnostic values of IFN-γ, IL-22, and GrB ELISpot assay as a confirmatory test in patients with a history of drug-induced SCARs and the potential benefit of *in vitro* adjuvant supplementation.

MATERIALS AND METHODS

Twenty-seven patients with a history of drug-induced SCARs were enrolled in the study. These patients were part of the Thailand Severe Cutaneous Adverse Reactions (ThaiSCARs) cohort. The diagnosis of SCARs was confirmed as probable or definite SJS/TEN, DRESS, or AGEP according to the RegiSCAR diagnostic criteria.[24-26] The culprit drugs suspected were selected based on those with the highest Naranjo score. [27]

Culprit drug preparations

The tested drugs for ELISpot assay were prepared from the intravenous drugs available in King Chulalongkorn Memorial Hospital, Chulalongkorn University, Bangkok, Thailand. Ten mg of each culprit drug powder was measured, and dissolved in 1 ml of sterile distilled water, then allowed to settle for 1 hour before adjusting for ELISpot assay concentrations (**Supplementary Table S1**). The solutions prepared were used immediately after preparation, and the remaining should not be kept for further use. Pure drug substances for which intravenous preparation was not available were purchased from Sigma-Aldrich (St Louis, MO, USA).

ELISpot

PBMCs were separated by Ficoll-Hypaque density gradient centrifugation. The frequencies of drug-induced mediator releasing cells were determined using ELISpot assay kits (human IL-22 single-color ELISpot [Mabtech, Stockholm, Sweden] and human IFN-γ/GrB double-color ELISpot [Cellular Technology Limited, Cleveland, OH, USA]). Briefly, 96-well polyvinylidene fluoride membrane plates were coated for 16 hours at 4°C with 5 μg/mL anti-IFN-γ antibody, anti-GrB antibody, and anti-IL-22 antibody provided in the kit and blocked with R10 medium (RPMI1640 supplemented with 100 U/mL penicillin, 100 U/mL streptomycin, and 10% heat-inactivated fetal bovine serum) for 1 hour at room temperature. PBMCs ($2.0–2.5 \times 10^5$ in 100 μL) were incubated for 48 hours at 37°C in 5% CO_2 with the culprit drugs in the presence or absence of α-GalCer (100 ng/mL; Abcam, Cambridge, UK), anti-CTLA4 (10 μg/mL; Merck Millipore, Burlington, MA, USA), anti-TIM-3 (10 μg/mL; Merck Millipore), or anti-PD-1 (5

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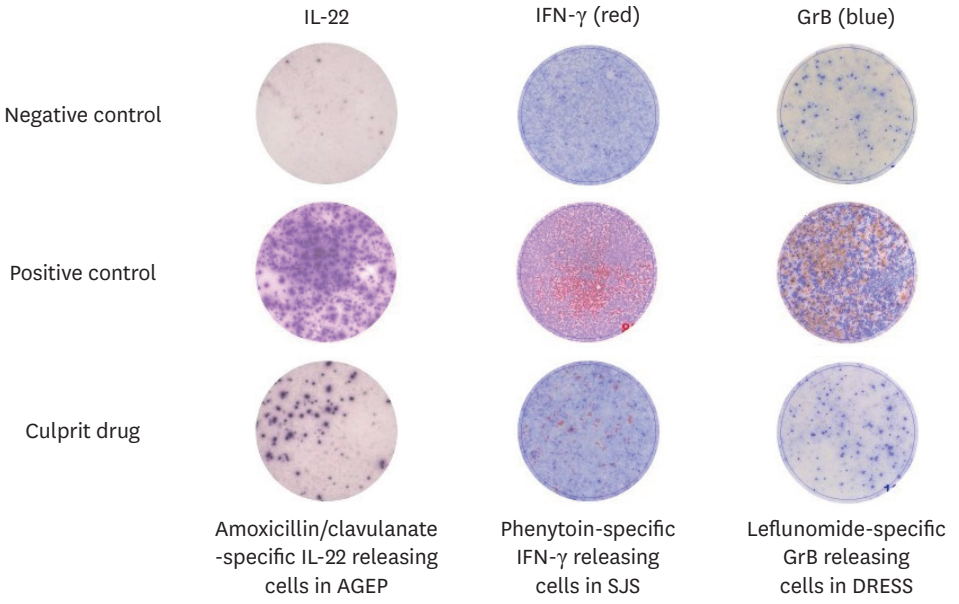

Fig. 1. Representative figures of drug-induced IL-22, IFN-γ, and GrB releasing cells as demonstrated by ELISpot assay after stimulating PBMCs with the suspected drugs in patients with a history of amoxicillin/clavulanate-induced AGEP (patient No. 4), phenytoin-induced SJS (patient No. 25), and leflunomide-induced DRESS (patient No. 14).
IL, interleukin; AGEP, acute generalized exanthematous pustulosis; IFN-γ, interferon-gamma; SJS, Stevens-Johnson syndrome; GrB, granzyme B; DRESS, drug reaction with eosinophilia and systemic symptoms.

μg/mL; Biolegend, San Diego, CA, USA). Plates were later washed 6 times with phosphate-buffered saline/Tween 0.05%, and incubated for 2 hours at room temperature with the corresponding biotinylated antibody, and then washed extensively. Spot-forming units (SFU) were developed using streptavidin-alkaline phosphatase, incubated for 1 hour at room temperature, and washed extensively before adding the substrate. The numbers of spots were analyzed using an ImmunoSpot S6 Ultimate Analyzer (Cellular Technology Limited) as shown in **Fig. 1**. The results were expressed as the highest numbers of SFU/10^6 PBMCs on stimulation with 2 concentrations of the culprit drugs tested, after subtracting the value obtained from PBMCs cultured without drugs (unstimulated control). The drug concentrations used for the ELISpot assay listed in **Supplementary Table S1** were generally in the same range as therapeutic serum concentrations, as mentioned in our previous study.[28]

The frequencies of IL-22-, IFN-γ-, and GrB-releasing cells when incubating PBMCs with the irrelevant non-culprit drugs were also measured in 18 out of the 27 SCAR subjects to evaluate non-specific mediator responses as a control group shown in **Fig. 2**. The irrelevant non-culprit drugs used in the control group were the culprits suspected in other SCAR subjects, but were not the drugs implicated in the particular SCAR subjects. The ELISpot assay was considered positive if the frequencies of drug-induced IL-22-, IFN-γ-, and GrB-releasing cells in 27 SCAR subjects upon incubating PBMCs with the culprit drugs were higher than the upper limit of the 95% confidence interval (CI) for mean mediator-releasing cells in the non-allergic control group (18.7, 17.2, and 97.7 SFU/10^6 PBMCs, respectively).

Statistical analysis

The average frequencies of drug-induced mediator-releasing cells are expressed as means and 95% CI. Student's *t*-test was used to compare the frequencies of drug-induced mediator-releasing cells among different phenotypes. McNemar's test was used to comparatively analyze the sensitivity between different types of ELISpot assays. All statistical calculations

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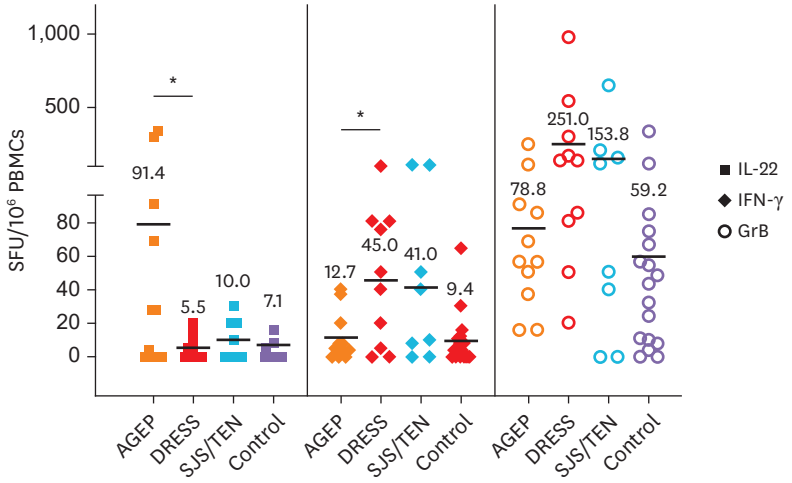

Fig. 2. Frequencies of drug-induced mediator releasing cells in different phenotypes of severe cutaneous adverse reactions. The average frequencies of drug-induced IL-22 releasing cells in AGEP subjects were significantly higher than those in non-AGEP SCAR subjects, while those of drug-induced IFN-γ releasing cells were significantly lower. SFU, spot-forming units; PBMCs, peripheral blood mononuclear cells; IL, interleukin; IFN-γ, interferon-gamma; GrB, granzyme B; AGEP, acute generalized exanthematous pustulosis; DRESS, drug reaction with eosinophilia and systemic symptoms; SJS, Stevens-Johnson syndrome; TEN, toxic epidermal necrolysis.
*P values < 0.05.

were analyzed using Prism version 8 software (GraphPad Prism, San Diego, CA, USA). P values < 0.05 were considered statistically significant.

Ethics

PBMCs employed in this experiment were cryopreserved specimens from patients enrolled in the ThaiSCAR registry. The registry was approved by the Ethics and Research Committee of the Faculty of Medicine, Chulalongkorn University, and the study was conducted in accordance with the Declaration of Helsinki. The informed consent for both study participation and publication of identifying information in an online open-access publication was obtained from all participants. The ThaiSCAR study is registered at ClinicalTrials.gov (NCT02574988).

RESULTS

A total of 27 patients who developed drug-induced SCARs (9 AGEP, 10 DRESS, and 8 SJS/TEN) were included in this study. Seventeen patients (63.0%) were female, with an average age of 54.1 ± 4.8 years. The average ELISpot assay latency (the time from drug reaction onset to PBMC collection for ELISpot assay) was 10.6 ± 2.3 days as shown in **Table**.

The average frequencies of drug-induced IL-22-, IFN-γ-, and GrB-releasing cells upon incubating PBMCs with the culprit drugs in all SCAR phenotypes were 35.5 ± 16.3, 33.0 ± 7.1, and 164.8 ± 43.1 SFU/10^6 cells, respectively. The average frequencies of drug-induced IL-22-releasing cells were significantly higher in AGEP subjects than in non-AGEP SCAR subjects, while those of drug-induced IFN-γ-releasing cells were significantly lower (P = 0.01 and 0.04, respectively) as shown in **Fig. 2**. A significant difference was demonstrated in the frequencies of drug-induced IFN-γ-releasing cells between AGEP and DRESS (12.7 ± 5.3 SFU/10^6 cells vs 45.0 ± 11.9 SFU/10^6 cells, P = 0.03).

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Table. Clinical characteristics of 27 patients with severe cutaneous adverse reactions in this study and results of drug-induced mediator-releasing cell measurement by ELISpot (SFU/10^6 PBMCs)

Patient No.	Sex	Age (yr)	Phenotype	Culprit drugs	Underlying diseases	ELISpot assay latency* (days)	Concurrent steroid	IL-22	IFN-γ	GrB
1	F	87	AGEP	Vancomycin	DM	5	None	**90**	0	56
2	F	69	AGEP	Vancomycin	Cellulitis, HT	6	None	**68**	8	16
3	F	65	AGEP	Meropenem	None	3	Dexa 20 mg/day for 1 day	0	0	**110**
4	F	50	AGEP	Amoxicillin/ clavulanate	Psoriasis	5	None	**297**	4	**249**
5	M	62	AGEP	IRZE	Tuberculosis	25	None	**28**	0	16
6	F	88	AGEP	Ciprofloxacin	HT	4	None	0	5	85
7	F	82	AGEP	Piperacillin/ tazobactam	DM, HT	2	Pred 60 mg/day for 2 days	0	**37**	37
8	M	88	AGEP	Ciprofloxacin	DM, HT	6	None	**340**	**20**	30
9	F	76	AGEP	Omeprazole	None	7	None	0	**40**	90
10	M	36	DRESS	Co-trimoxazole	HIV infection	10	None	**20**	5	**545**
11	F	63	DRESS	Allopurinol	Hyperuricemia	5	Dexa 5 mg/d for 3 days	15	**20**	**980**
12	M	18	DRESS	Co-trimoxazole	None	48	Dexa 10 mg/day for 5 days then Pred 60 mg/day for 1 day	5	0	80
13	F	65	DRESS	Allopurinol	HT, gout	2	Dexa 24 mg/d for 1 day	0	**75**	50
14	F	35	DRESS	Leflunomide	Rheumatoid arthritis	39	Pred 20 mg/d for 90 days†	0	**80**	**300**
15	M	17	DRESS	Sulfadiazine	Toxoplasmosis	14	None	0	0	85
16	F	15	DRESS	Co-trimoxazole	Systemic vasculitis	4	None	5	**50**	**170**
17	M	29	DRESS	Co-trimoxazole	HIV infection	36	None	10	**80**	**140**
18	F	17	DRESS	Phenytoin	Ruptured AVM	5	Pred 60 mg/d for 5 days	0	**100**	**140**
19	M	19	DRESS	Phenytoin	Seizure	5	Dexa 15 mg/d for 5 days	0	**40**	20
20	F	65	SJS	Allopurinol	Gout	1	None	0	8	0
21	M	87	SJS	Allopurinol	Gout	7	Dexa 20mg/d for 1 day	**30**	**40**	**160**
22	F	67	SJS	Allopurinol	Gout	5	None	**20**	**110**	**650**
23	M	74	TEN	Meropenem	Brain abscess	7	Dexa 5mg/d for 7 days	0	10	**210**
24	F	72	SJS	Levofloxacin	MCTD, lymphoma	7	None	10	0	50
25	F	74	SJS	Phenytoin	Intracranial hemorrhage	2	Dexa 24mg/d for 1 day	**20**	**50**	40
26	M	36	SJS	Co-trimoxazole	HIV infection, chronic hepatitis C	16	Pred 40 mg/d for 3 days	0	**110**	**120**
27	F	51	SJS	Phenytoin	Breast cancer with brain metastasis	10	Pred 40 mg/d for 14 days then Pred 20 mg/d for 14 days†	0	0	0

Bold texts indicate positive ELISpot assays, ELISpot assay latency.
ELISpot, enzyme-linked immunospot assays; SFU, spot-forming units; PBMCs, peripheral blood mononuclear cells; IL, interleukin; IFN-γ, interferon-gamma; GrB, granzyme B; AGEP, acute generalized exanthematous pustulosis; DM, diabetes mellitus; HT, hypertension; Dexa, dexamethasone; IRZE, isoniazid/rifampicin/ pyrazinamide/ethambutol (anti-tuberculosis agents); DRESS, drug reaction with eosinophilia and systemic symptoms; HIV, human immunodeficiency virus; Pred, prednisolone; AVM, arteriovenous malformation; SJS, Stevens-Johnson syndrome; TEN, toxic epidermal necrolysis; MCTD, mixed connective tissue disease.
*Days: The time from drug reaction onset (the index date) to PBMC collection for ELISpot assay, †Systemic steroid was prescribed for the underlying disease before a drug hypersensitivity reaction developed.

Univariate and multivariate linear regressions were conducted to evaluate whether drug groups (antibiotics vs. non-antibiotics) and SCAR phenotypes (AGEP vs. non-AGEP) could influence cytokine release patterns. The results in **Supplementary Fig. S1** demonstrate that the frequencies of drug-induced IL-22-releasing cells were significantly higher in AGEP subjects than in non-AGEP subjects by 83.9 (95% CI, 20.2–147.7) SFU/10^6 PBMCs. After adjusting for the antibiotic group, the difference in IL-22 levels between patients with AGEP and those with non-AGEP remains statistically significant, with the mean difference of 77.2 (95% CI, 5.4–148.9) SFU/10^6 PBMCs (P< 0.05).

Diagnostic values of the measurement of drug-specific mediator-releasing cells to identify suspected culprit drugs

According to the data in **Table**, the measurement of drug-induced IL-22-, IFN-γ-, and GrB-releasing cells yielded sensitivities of 33.3%, 51.9%, and 44.4%, respectively, for culprit drug identification in all SCAR subjects if each mediator was separately analyzed. It is worth

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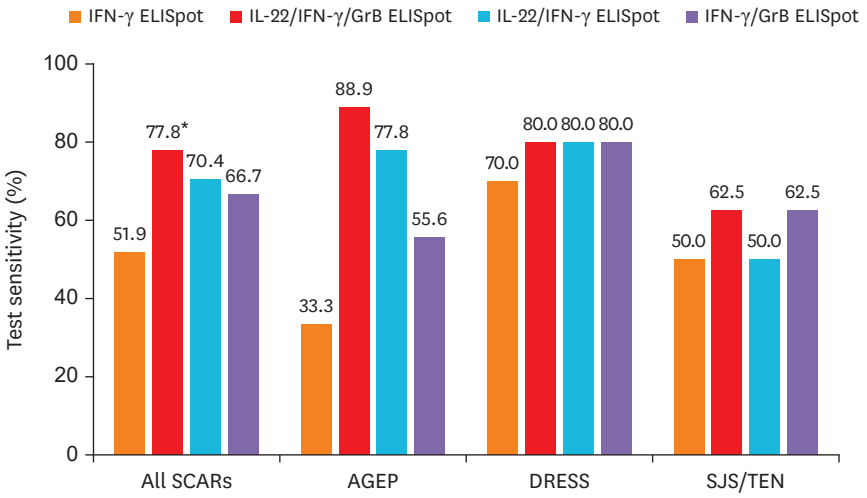

Fig. 3. The sensitivity of different ELISpot assays to identify the culprit drugs in severe cutaneous adverse reactions (n = 27). The sensitivity of the three-mediator combination to confirm the culprit drugs in overall SCAR subjects was significantly higher than that of IFN-γ measurement alone (77.8% versus 51.9%, respectively). ELISpot, enzyme-linked immunospot assays; SCARs, severe cutaneous adverse reactions; AGEP, acute generalized exanthematous pustulosis; DRESS, drug reaction with eosinophilia and systemic symptoms; SJS, Stevens-Johnson syndrome; TEN, toxic epidermal necrolysis; IFN-γ, interferon-gamma; IL, interleukin; GrB, granzyme B.
*P value < 0.01.

noting that almost all GrB-positive subjects (except for patients number 3 and 23) could be diagnosed by measuring other mediators as well.

The results in **Fig. 3** indicate that the sensitivity of the 3-mediator combination to confirm the culprit drugs in overall SCAR subjects was significantly higher than that of IFN-γ measurement alone (77.8% vs 51.9%, respectively, P value < 0.01). The culprit drugs in AGEP, DRESS, and SJS/TEN could be identified in 33.3%, 70.0%, and 50.0% of patients, respectively, by using the IFN-γ ELISpot assay, while 88.9%, 80.0%, and 62.5% could be identified using a combination of IL-22, IFN-γ, and GrB ELISpot assays.

The combined measurement of drug-induced IL-22- and IFN-γ- releasing cells identified the culprit drugs in 77.8%, 80.0%, 50.0%, and 70.4% of AGEP, DRESS, SJS/TEN, and all SCAR patients, respectively. The combined measurement of drug-induced IFN-γ- and GrB-releasing cells identified the culprit drugs in 55.6%, 80.0%, 62.5%, and 66.7% of AGEP, DRESS, SJS/TEN, and all SCAR patients, respectively. The combined measurement of drug-induced IL-22 and GrB identified the culprit drugs in only 48.1% of all SCAR subjects (data not shown).

The average frequency of drug-induced IL-22-releasing cells upon incubating PBMCs with the culprit drugs was 35.5 ± 16.3 SFU/10^6 cells, while that upon incubating the culprit drugs with potential adjuvants ranged from to 22.4 ± 8.9 to 36.6 ± 14.1 SFU/10^6 cells as shown in **Fig. 4**. The average frequency of drug-induced IFN-γ releasing cells upon incubation with the culprit drugs alone was 33.0 ± 7.1 SFU/10^6 cells, while supplementation with α-GalCer, anti-TIM-3, and anti-PD-1 increased the frequency to 72.3 ± 18.5*, 63.4 ± 18.5, and 83.9 ± 38.2 SFU/10^6 cells, respectively (P = 0.02 compared to no adjuvant supplementation). The average frequency of drug-induced GrB-releasing cells upon incubating PBMCs with the culprit drugs was 164.8 ± 43.1 SFU/10^6 cells, while that upon incubating the culprit drugs with various potential adjuvants ranged from 77.9 ± 16.9 to 153.3 ± 53.1 SFU/10^6 cells.

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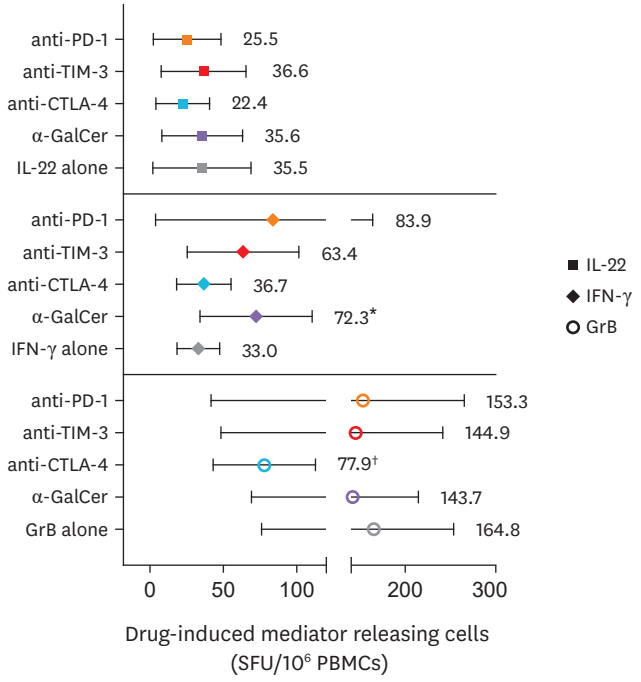

Fig. 4. The effects of potential immunoadjuvants on the frequencies of drug-induced mediator releasing cells in severe cutaneous adverse reactions (n =27). The average frequencies of drug-induced IFN-γ releasing cells upon incubation with the culprit drugs alone were 33.0 ± 7.1 SFU/10^6 cells, while supplementation with α-GalCer significantly increased the frequencies to 72.3 ± 18.5 SFU/10^6 cells.
IL, interleukin; IFN-γ, interferon-gamma; GrB, granzyme B; PD-1, programmed cell death protein 1; TIM3, T-cell immunoglobulin and mucin domain 3; CTLA4, cytotoxic T-lymphocyte-associated protein 4; α-GalCer, alpha-galactosylceramide; SFU, spot-forming units; PBMCs, peripheral blood mononuclear cells.
*P value < 0.05 compared to IFN-γ alone, †P value < 0.05 compared to GrB alone.

DISCUSSION

Culprit drug confirmation is a difficult task, particularly in drug-induced SCARs. Since drug provocation is generally contraindicated in SCARs and the sensitivity of skin tests to identify the culprit drugs is inadequate, the roles of *in vitro* diagnostic tests are currently being explored. IFN-γ is the cytokine most frequently measured from T-cells in patients with a history of drug allergy. However, the sensitivity of the IFN-γ ELISpot assay is not yet satisfactory. Many obstacles limit the roles of *in vitro* testing; for example, different SCAR phenotypes have variable mediator expression patterns, and in certain phenotypes, especially DRESS, it is difficult to identify the culprit drugs during the acute allergic phase. Therefore, this study was designed to explore a combination of mediator panels to improve the sensitivity for identifying the culprit drugs in real-world practice in various SCAR phenotypes.

According to our study, the measurement of drug-induced IFN-γ-releasing cells is still the key to culprit drug identification in SCARs. The results of this study support the previous literature that the measurement of drug-induced IFN-γ-releasing cells can identify the culprit drugs in at least half of patients with drug-induced SCARs, particularly in those with DRESS or SJS/TEN.[4,29] However, the sensitivity of the test is rather low in those with AGEP.[28]

Our study demonstrates different patterns of drug-induced mediator-releasing cells in patients with various SCAR phenotypes, and shows that the combination of 3 mediators (IL-22, IFN-γ, and GrB) can enhance the sensitivity of ELISpot to identify the culprit drugs in 77.8% of all SCAR

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subjects. Interestingly, we found that the measurement of drug-induced IL-22-releasing cells played a predominant role in identifying drug-induced AGEP. In fact, the sensitivity of combined IFN-γ and IL-22 ELISpot for culprit drug identification (70.4%) in SCARs was almost as high as the measurement of 3 mediators (77.8%), but it was slightly lower in SJS/TEN. Our study confirms earlier findings that not only SJS/TEN but also DRESS involve GrB in disease pathogenesis.[14] However, drug-induced GrB-releasing cells were often co-expressed with other mediators in this study. As a result, the additional advantage of GrB measurement together with IFN-γ for culprit drug confirmation was only modest, compared to the measurement of drug-specific IFN-γ alone. The IFN-γ/GrB combination might be worth considering if the test was focused on SJS/TEN.

According to this study, the supplementation of various potential adjuvants augmented drug-induced IFN-γ release, but had minimal effect on IL-22 and GrB production. Interestingly, we found that the α-GalCer supplement significantly enhanced the frequencies of drug-induced IFN-γ-releasing cells, followed by anti-TIM-3 and anti-PD-1 supplementation, while supplementation with anti-CTLA4 appeared to have little benefit. The fact that the immune checkpoint inhibitors, other than α-GalCer, failed to substantially enhance drug-specific *in vitro* immune responses probably due to the small number of tested subjects in each phenotype. Besides, further experiments are needed to explore the appropriate incubation time and the concentrations of the adjuvants of interest to achieve optimal responses for each specific cytokine.

It is well documented that various T cell subsets, such as cytotoxic T-cells, Th1, and Th2 cells, participate in the pathogenesis of SCARs.[30] According to our study, several cell types capable of producing IL-22, such as Th17 cells, Th22 cells, γδ T cells, NK cells, NKT cells, and innate lymphoid cells, might play a role in AGEP as well.[31,32] Nonetheless, the clinical benefit of α-GalCer supplement to increase the sensitivity of IFN-γ ELISpot assay for culprit drug identification needed to be confirmed in further studies. Since α-GalCer can stimulate IFN-γ production from NKT cells,[33] the roles of NKT cells in the pathogenesis of SCARs, particularly in DRESS and SJS/TEN, should also be explored.

There are several limitations to this preliminary study. Further studies with a larger sample size are needed to confirm the results. Additional research should be performed to measure the frequencies of these mediators at various time points after drug reaction onset to find the most appropriate timeline yielding a maximum sensitivity of each cytokine in different SCAR phenotypes. The measurement of other cytokines in the IL-17 family may also be helpful for AGEP diagnosis. Granulysin indeed plays roles in the pathogenesis of SCARs, particularly in SJS/TEN and DRESS.[34] However, the incubation time for granulysin expression is too long for a conventional ELISpot assay to detect drug-specific granulysin release.[35] High GrB background levels in the control subjects were observed which diminished the sensitivity of the test. The analyses of IFN-γ and GrB ELISpot assays in separated wells may be required to optimize the measurement of drug-induced GrB releasing cells. Whether supplementary adjuvants could increase the sensitivity of the IFN-γ ELISpot assay for culprit drug confirmation without reducing the specificity of the test is yet to be determined.

In conclusion, our study demonstrated differential mediator expressions among 3 main SCAR phenotypes, and supported the diagnostic value of IFN-γ ELISpot assay as the major tool to identify culprit drugs. While an additional GrB measurement might be helpful in SJS/TEN, the measurement of drug-specific IL-22-releasing cells should be encouraged for culprit drug identification in AGEP. The beneficial effects of *in vitro* tests for enhancing the detection of drug-induced IFN-γ-releasing cells in SCARs need further verification.

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SUPPLEMENTARY MATERIALS

Supplementary Table S1

Drug concentrations used for ELISpot in this study

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Supplementary Fig. S1

The impacts of SCAR phenotypes and drug groups on the mean differences of drug-induced mediator releasing cells. The frequencies of drug-induced IL-22 releasing cells in AGEP subjects were significantly higher than those in non-AGEP subjects.

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