



The Function of HLA-B*13:01 Involved in the Pathomechanism of Dapsone-Induced Severe Cutaneous Adverse Reactions

Wei-Ti Chen^{1,11}, Chuang-Wei Wang^{1,2,11}, Chun-Wei Lu^{1,3,4}, Chun-Bing Chen^{1,3,4}, Hua-En Lee^{1,3}, Shuen-lu Hung⁵, Siew-Eng Choon⁶, Chih-Hsun Yang^{1,3}, Ming-Tsan Liu⁷, Ting Jui Chen⁸, Wen-Lang Fan⁹, Shih-Chi Su^{1,9}, Yang Yu-Wei Lin¹, Ya-Ching Chang^{1,3,12} and Wen-Hung Chung^{1,2,3,9,10,12}, and Taiwan Severe Cutaneous Adverse Reaction Consortium

Dapsone-induced hypersensitivity reactions may cause severe cutaneous adverse reactions, such as drug reaction with eosinophilia and systemic symptoms (DRESS). It has been reported that HLA-B*13:01 is strongly associated with dapsone-induced hypersensitivity reactions among leprosy patients. However, the phenotype specificity and detailed immune mechanism of HLA-B*13:01 remain unclear. We investigated the genetic predisposition, HLA-B*13:01 function, and cytotoxic T cells involved in the pathogenesis of dapsone-induced severe cutaneous adverse reactions. We enrolled patients from Taiwan and Malaysia with DRESS and maculopapular eruption with chronic inflammatory dermatoses. Our results showed that the HLA-B*13:01 allele was present in 85.7% (6/7) of patients with dapsone DRESS (odds ratio = 49.64, 95% confidence interval = 5.89–418.13; corrected $P = 2.92 \times 10^{-4}$) but in only 10.8% (73/677) of general population control individuals in Taiwan. The level of granulysin, the severe cutaneous adverse reaction-specific cytotoxic protein released from cytotoxic T cells, was increased in both the plasma of DRESS patients (36.14 ± 9.02 ng/ml, $P < 0.05$) and in vitro lymphocyte activation test (71.4%, 5/7 patients) compared with healthy control individuals. Furthermore, dapsone-specific cytotoxic T cells were significantly activated when co-cultured with HLA-B*13:01–expressing antigen presenting cells in the presence of dapsone (3.9-fold increase, compared with cells with no HLA-B*13:01 expression; $P < 0.01$). This study indicates that HLA-B*13:01 is strongly associated with dapsone DRESS and describes a functional role for the HLA-restricted immune mechanism induced by dapsone.

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¹Department of Dermatology, Drug Hypersensitivity Clinical and Research Center, Chang Gung Memorial Hospital, Linkou, Taipei and Keelung, Taiwan; ²Chang Gung Immunology Consortium, Chang Gung Memorial Hospital and Chang Gung University, Taiwan; ³College of Medicine, Chang Gung University, Taoyuan, Taiwan; ⁴Graduate Institute of Clinical Medical Sciences, College of Medicine, Chang Gung University, Taoyuan, Taiwan; ⁵Department and Institute of Pharmacology, School of Medicine, Infection and Immunity Research Center, National Yang-Ming University, Taipei, Taiwan; ⁶Hospital Sultanah Aminah, Clinical School Johor Bahru, Jeffrey Cheah School of Medicine and Health Sciences, Monash University, Malaysia; ⁷Center for Diagnostics and Vaccine Development, Centers for Disease Control, Taiwan; ⁸Department of Dermatology, Taoyuan General Hospital, Ministry of Health and Welfare, Taiwan; ⁹Whole-Genome Research Core Laboratory of Human Diseases, Chang Gung Memorial Hospital, Keelung, Taiwan; and ¹⁰Department of Dermatology, Xiamen Chang Gung Hospital, China

¹¹These authors contributed equally as first authors.

¹²These authors contributed equally to this work.

Correspondence: Wen-Hung Chung, MD, PhD, Director, Department of Dermatology, Drug Hypersensitivity Clinical and Research Center, Chang Gung Memorial Hospital, Number 5, Fusing Street, Taoyuan, 333 Taiwan. E-mail: wenhungchung@yahoo.com

Abbreviations: CTLs, cytotoxic T cells; DRESS, drug reaction with eosinophilia and systemic symptoms; EBV, Epstein-Barr virus; ELISpot, enzyme-linked immunospot assay; HHV, human herpesvirus; LAT, lymphocyte activation test; MPE, maculopapular eruption; PBMC, peripheral blood mononuclear cell; P_c , corrected P-value; SCAR, severe cutaneous adverse reactions; SJS, Stevens-Johnson syndrome; TEN, toxic epidermal necrolysis
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INTRODUCTION

Dapsone (4,4'-diaminodiphenylsulfone), a type of sulfone, was first synthesized by Fromm and Wittmann in 1908 (Fromm and Wittmann, 1908). Concerning the mechanisms of action, it is characterized by dual functions, including its antibiotic and anti-inflammatory abilities (Zhu and Stiller, 2001). Dapsone was used alone or in combination with other drugs such as antibiotics for primary and prophylactic treatment of Hansen's disease, *Pneumocystis jiroveci* pneumonia (Goldie et al., 2002; Podzamczar et al., 1993), and toxoplasmosis (Girard et al., 1993; Torres et al., 1993) in patients with AIDS. It was further used as an anti-inflammatory drug for chronic inflammatory dermatoses with neutrophilic or eosinophilic infiltrates, such as linear IgA dermatosis, IgA pemphigus, subcorneal pustular dermatosis, and dermatitis herpetiformis (Wozel, 1989, 2010). In recent years, dapsone has also been used in a variety of non-dermatologic diseases such as immune thrombocytopenia (Damodar et al., 2005), cortisone-dependent bronchial asthma (Berlow et al., 1991), and eosinophilic fasciitis (Servy et al., 2010). With the increasing use of dapsone in clinical practice, numerous adverse effects have been reported, including methemoglobinemia (Ashurst et al., 2010), hemolysis (Grossman and Jollow, 1988), agranulocytosis (Coleman, 2001), and drug-induced hypersensitivity (Lorenz et al., 2012). Drug-induced delayed-type hypersensitivity

reactions may range from mild maculopapular eruption (MPE) to life-threatening severe cutaneous adverse reactions (SCAR), such as drug reaction with eosinophilia and systemic symptoms (DRESS)/drug-induced hypersensitivity syndrome, Stevens-Johnson syndrome (SJS), and toxic epidermal necrolysis (TEN) (Choudhary et al., 2013; Paulmann and Mockenhaupt, 2015; Roujeau and Stern, 1994). Dapsone often induced DRESS/drug-induced hypersensitivity syndrome, which is characterized by fever, skin rash, and systemic involvement and was first described by Lowe and Smith in 1949. Based on previous epidemiologic studies, the estimated prevalence and associated mortality rate of dapsone-induced DRESS are 1.4% and 9.9%, respectively (Lorenz et al., 2012).

The proposed pathomechanism of SCAR is known to involve the cytotoxic T lymphocytes (CTLs) that recognize culprit drugs presented by HLA class I molecules (Pichler et al., 2011; Su et al., 2016; Yun et al., 2016). The theory is supported by genetic association studies (e.g., the strong association between HLA-B*15:02 and carbamazepine-SJS/TEN [Chung et al., 2004], HLA-A*31:01 and carbamazepine-DRESS and MPE [Hsiao et al., 2014], HLA-B*58:01 and allopurinol-induced SCAR [Hung et al., 2005], and HLA-B*57:01 and abacavir hypersensitivity [Mallal et al., 2008] and by immune mechanism studies [Illing et al., 2012; Ko et al., 2011; Lin et al., 2015; Wang CW et al., 2013; Wei et al., 2012]). Furthermore, we previously reported that secretory granulysin, a cytotoxic protein released by CTLs/natural killer cells/natural killer T cells, could lead to disseminated keratinocyte apoptosis, detachment of the epidermis, and mucous membranes in SJS/TEN (Chung et al., 2008). These findings suggest that HLA predisposition and CTLs play important roles in the pathogenesis of drug-induced hypersensitivity.

Recent genome-wide association studies showed the HLA-B*13:01 allele to be a useful biomarker in predicting dapsone-induced hypersensitivity reactions among leprosy patients in the Chinese population (Wang H et al., 2013; Zhang et al., 2013). However, the roles of CTLs involved in an HLA-B*13:01-dependent mechanism for dapsone SCAR remain unclear. Therefore, we investigated the genetic predisposition, HLA-B*13:01 function, and CTLs involved in the pathogenesis of dapsone SCAR.

RESULTS

Demographic data

Nine patients with dapsone-induced hypersensitivity reactions were recruited, including eight with DRESS and one with MPE. There were seven DRESS patients enrolled from Taiwan and one DRESS patient from the Malaysian cohort. The underlying diseases caused by use of dapsone were mostly chronic inflammatory dermatoses, including linear IgA dermatosis, systemic lupus erythematosus, rosacea, pemphigus foliaceus, angiolymphoid hyperplasia with eosinophilia, eosinophilic folliculitis, and chronic spontaneous urticaria. There was one case of idiopathic thrombocytopenia (Table 1). The average initial daily drug dose was 90.0 ± 20.4 mg. The average onset time of cutaneous manifestation was 29.2 ± 15.7 days after exposure to dapsone.

Clinical and laboratory features

The representative clinical features of dapsone hypersensitivity are shown in Supplementary Figure S1 online. Eight patients were categorized as having dapsone DRESS, and all of them had a RegiSCAR diagnosis score for DRESS of more than 3. One patient was characterized as having MPE. The detection of human herpesvirus (HHV) 6 and Epstein-Barr virus (EBV) was determined by real-time quantitative PCR, and none of the HHV6 and EBV reactivations were identified with dapsone-induced hypersensitivity in these six patients (patients 3, 5, 6, 7, 8, and 9). The results of clinical manifestation, virus reactivation, and histopathologic characteristics are shown in Table 1. Hepatitis was the most commonly encountered systemic organ complication (5/8, 62.5%). All patients (5/5) with hepatitis had dapsone DRESS. Overall, 55.6% of the patients with dapsone hypersensitivity were managed with systemic corticosteroids, and there were no cases of mortality in any of the phenotype groups. Most patients had a good prognosis and clinical outcome during the follow-up period.

Association between the HLA-B*13:01 allele and dapsone DRESS

The details of HLA-A and HLA-B genotypes in patients with dapsone-induced hypersensitivity reactions are shown in Table 2. All participants from Taiwan were of Han Chinese ethnicity. Comparing the differences in the carrier rate of the HLA-A and -B alleles among the eight Han Chinese patients with dapsone-induced hypersensitivity reactions in the Taiwanese population, we identified HLA-A*11:01 (75.0%) and HLA-B*13:01 (75.0%) as the most frequent HLA genotypes (see Supplementary Table S1 online); however, only HLA-B*13:01 was significantly associated with dapsone SCAR when we compared 677 patients who never had drug hypersensitivity before ($P = 4.57 \times 10^{-5}$) (see Supplementary Table S1 online). One Myanmar patient from Malaysia with dapsone DRESS also carried the HLA-B*13:01 allele (Table 2). We then examined the association between HLA-B*13:01 and phenotype difference in dapsone hypersensitivity (Table 3). Subgroup analysis showed a strong association between HLA-B*13:01 and dapsone DRESS, which showed that 85.7% of DRESS patients carried the HLA-B*13:01 (6/7 DRESS patients; odds ratio = 49.64, 95% confidence interval = 5.89–481.13, corrected $P [P_c] = 2.92 \times 10^{-4}$). In contrast, HLA-B*13:01 was not present in only one patient with dapsone-induced MPE ($P > 0.99$) (Table 3).

Genetic distribution of dapsone-metabolizing enzymes in patients with dapsone DRESS

In addition to HLA associations, the divergences in individual metabolism and drug clearance may contribute to SCAR development (Chung et al., 2014b). Two of the main phase I enzymes, CYP2C9 and NAT2, are responsible for dapsone metabolism (Hutzler et al., 2001; Zhu and Stiller, 2001), and thus we further examined the genetic variants of CYP2C9 and NAT2 in patients with dapsone SCAR (Table 2). For CYP2C9, the CYP2C9*3 variant (A1075C) is known to be important for drug clearance. Our results showed that one CYP2C9*3 carrier (12.5%) was found in eight patients with dapsone DRESS and in 5.2% of our general population control

Table 1. Demographic characteristics, clinical manifestations, and laboratory features of nine patients with dapsone-induced hypersensitivity reactions

Characteristics	Patient Number								
	1	2	3	4	5	6	7	8	9
Age in years/sex	38/F	21/F	82/M	34/F	44/M	26/F	37/M	35/M	26/F
Underlying disease	Linear IgA dermatosis	SLE	Idiopathic thrombocytopenia	Rosacea	PF	ALHE	Eosinophilic folliculitis	CSU	Eczema rule out LE
Delayed type	DRESS	DRESS	DRESS	DRESS	DRESS	DRESS	DRESS	DRESS	MPE
Biopsy result	NA	LCV	Interface dermatitis	NA	NA	Interface dermatitis	NA	NA	NA
Erythema, % TBSA	40	50	40	50	70	50	40	>90	55
Blood analysis									
GPT, U/L	180	485	37	305	465	426	31	17	19
Creatinine, mg/dl	NA	0.45	1.36	0.52	0.78	0.76	NA	0.94	NA
Eosinophil, %	NA	18	2.7	0	2	0	13.5	5.5	0
Atypical lymphocyte, %	0	0	0	27	0	15	0	0	0
Fever > 38.5°C	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes
Lymphadenopathy	No	No	Yes	No	No	No	Yes	No	No
EBV/HHV6	NA	NA	—/—	NA	—/—	—/—	—/—	—/—	—/—
DRESS score ¹	3	6	4	4	4	5	4	4	2

Abbreviations: ALHE, angiolymphoid hyperplasia with eosinophilia; CSU, chronic spontaneous urticaria; DRESS, drug reaction with eosinophilia and systemic symptoms; EBV, Epstein-Barr virus; F, female; GPT, glutamic pyruvic transaminase; HHV6, human herpesvirus 6; LE, lupus erythematosus; LCV, leukocytoclastic vasculitis; M, male; MPE, maculopapular eruption; NA, not available; PF, pemphigus foliaceus; SLE, systemic lupus erythematosus; TBSA, total body surface area.

¹Calculated according to RegiSCAR group diagnosis score for DRESS (<http://www.regiscar.org/index.html>).

individuals. Furthermore, the distributions of allele and acetylase genotypes of *NAT2* in individuals were also determined, as described previously (Hein et al., 2000). Of the eight patients with dapsone DRESS, only two patients (25.0%) were identified as having the slow acetylase genotype of *NAT2* (Table 2), which was detected in 24.2% of our healthy control individuals. Neither result was significantly different.

Plasma granulysin level and in vitro lymphocyte activation test (LAT) of dapsone DRESS

To examine whether CTLs are involved in the pathogenesis of dapsone SCAR, we first detected the granulysin level (a specific cytotoxic protein of SJS/TEN [Chung et al., 2008; Wang CW et al., 2013] and DRESS [Weinborn et al., 2016], mainly released from CTLs) in the plasma of dapsone DRESS patients. (Figure 1a). The up-regulated plasma granulysin level was observed in dapsone DRESS patients (38.34 ± 8.77 ng/ml) compared with the healthy control individuals (11.34 ± 0.99 ng/ml) ($P < 0.05$). We then performed an in vitro LAT in the presence of dapsone to measure granulysin expression and found that granulysin expression was increased in most dapsone DRESS patients after 7 days of culture; the sensitivity and specificity of granulysin-based LAT were 71.4% (5/7) and 100%, respectively (Figure 1b and 1c).

Identification of activated T cells from patients with dapsone DRESS

To further investigate the roles of CTLs in the pathogenesis of dapsone SCAR, we determined the population of activated T cells after in vitro LAT assay. Peripheral blood mononuclear cells (PBMCs) from four patients with dapsone SCAR were treated with dapsone for 1 week, and the expression of the degranulation/activated marker CD107a from T cells was

measured by flow cytometry (Figure 1d). Our results showed that the populations of activated CD4⁺ T cells and CD8⁺ CTLs were increased after dapsone stimulation in the assays of four patients (Figure 1e).

HLA-B*13:01—dependent activation of CTLs in patients with dapsone DRESS

To further confirm that HLA-B*13:01 and CTLs are involved in the pathogenesis of dapsone DRESS, we generated HLA-B*13:01—expressing cells (C1R-t13:01) (see Supplementary Figure S2 online) and performed in vitro co-culture assay. The dapsone-specific CTLs were expanded for 2 weeks and isolated by fluorescence-activated cell sorting. After that, dapsone-specific CTLs were co-cultured with C1R-t13:01 to detect the IFN- γ release by enzyme-linked immunospot (ELISpot) assay (Figure 2a and 2b). Our results showed that the IFN- γ —expressing spots released from dapsone-specific CTLs against antigen-presenting cells expressing HLA-B*13:01 were significantly activated after stimulation by dapsone (3.9-fold increase compared with cells with no HLA-B*13:01 expression, $P < 0.01$). We further performed HLA blocking assay (Figure 2c and 2d) and found that CTL activation could be blocked by anti-HLA class I or anti-HLA-B antibody (83% decrease, both $P < 0.05$, but not by isotype control) (Figure 2c and 2d). The data suggested that dapsone-specific CTLs were activated in an HLA-B*13:01—dependent manner.

DISCUSSION

In addition to its use as an antibiotic in patients with leprosy and as primary prevention and prophylaxis in patients with AIDS, dapsone was also used for the treatment of chronic inflammatory dermatoses (see Supplementary Table S2 online). With the increasing use of dapsone in clinical

Table 2. The genotypes of HLA and drug metabolism enzymes in patients with dapsone-induced hypersensitivity reactions

Patient	Phenotype	HLA				Drug Metabolism Enzymes	
		HLA-A		HLA-B		CYP2C9*3 ¹	NAT2 Activity
1	DRESS	A*11:01	A*26:01	B*13:01	B*40:02	–	NAT2*4/*4 (F)
2	DRESS	A*11:01	A*24:02	B*13:01	B*40:01	–	NAT2*6A/*6A (S)
3	DRESS	A*30:01	A*33:03	B*58:01	B*58:01	+	NAT2*6A/*7B (S)
4	DRESS	A*11:01	A*11:01	B*15:02	B*13:01	–	NAT2*7B/*4 (F)
5 ²	DRESS	A*33:03	A*68:01	B*13:01	B*52:01	–	NAT2*4/*4 (F)
6	DRESS	A*02:01	A*02:03	B*13:01	B*46:01	–	NAT2*4/*4 (F)
7	DRESS	A*11:01	A*11:01	B*13:01	B*46:01	–	NAT2*4/*4 (F)
8	DRESS	A*11:02	A*24:02	B*13:01	B*51:02	–	NAT2*6A/*4 (F)
9	MPE	A*02:03	A*11:01	B*15:12	B*15:18	–	NAT2*6A/*4 (F)

Abbreviations: DRESS, drug reaction with eosinophilia and systemic symptoms; F, fast acetylator genotype of NAT2; MPE, maculopapular eruption; S, slow acetylator genotype of NAT2.

¹The CYP2C9*3 variant (as known as A1075C; Ile359Leu) was determined by the single-nucleotide polymorphism of rs1057910 (<https://www.snpedia.com/index.php/Rs1057910>). The allele frequencies of NAT2*4 were defined as the fast acetylator genotype of NAT2, whereas NAT2*5B, NAT2*6A, and NAT2*7B were defined as the slow acetylator genotype of NAT2, as described previously (Hein et al., 2000).

²One Myanmar patient from Malaysia with dapsone DRESS also carried the HLA-B*13:01 allele.

practice, studies showed that the Asian population has a higher risk of developing dapsone-induced hypersensitivity reactions, with an incidence rate of more than 1% (Wang et al., 2017). In this study, we analyzed the clinical manifestations of dapsone SCAR in a cohort of eight patients who presented with clinical features characteristic of DRESS. In our patients with dapsone DRESS, the liver was the most frequently (80%) involved systemic organ, which is consistent with previous findings (Wang H et al., 2013; Zhang et al., 2013). The average initial daily drug dose was 90.0 ± 20.4 mg. Among these patients, the average onset time of cutaneous manifestation was 29.2 ± 15.7 days after exposure to dapsone. There was no significant difference among the initial daily dose and onset time in dapsone hypersensitivity subgroup phenotypes (data not shown). This suggests that the initial dosage of dapsone may not affect risk of dapsone hypersensitivity, which is known to be an idiosyncratic adverse effect.

We investigated the genetic susceptibility of HLA-A and HLA-B in dapsone hypersensitivity and found that only HLA-B*13:01 reached statistical significance in dapsone hypersensitivity when compared with healthy control individuals who had never had drug hypersensitivity before. The frequency of the HLA-B*13:01 genotype in dapsone

hypersensitivity was higher than that in healthy control individuals (75.0% vs. 10.8%; odds ratio = 24.82, 95% confidence interval = 4.92–125.26, $P_c = 1.05 \times 10^{-3}$), and subgroup analysis of the phenotypes showed that 85.7% of DRESS patients carried HLA-B*13:01 (odds ratio = 49.64, 95% confidence interval = 5.89–418.13, $P_c = 2.92 \times 10^{-4}$). Our findings showed that HLA-B*13:01 was strongly associated in dapsone DRESS patients with chronic inflammatory dermatoses, which is consistent with results of two genome-wide studies of leprosy patients in China (Wang H et al., 2013; Zhang et al., 2013). Because DRESS is a rare disease, the sample size is still limited. Further HLA association studies in dapsone DRESS patients will need to be investigated. In addition, the frequencies of HLA-B*13:01 risk alleles were different among various ethnic groups (see Supplementary Figure S3 online) (Clemens et al., 2016; Middleton et al., 2003). Current information indicates that the frequency of the HLA-B*13:01 allele is higher in India, southeastern Asia, the western Pacific (Indonesia), and northern Australia but lower in the United States and Europe, according to the Allele Frequency Net Database (<http://www.allelefrequencies.net/>). This may explain why a high prevalence of patients with dapsone DRESS was observed in southeastern Asia.

Table 3. The association study of the HLA-B*13:01 allele with phenotypes of dapsone-induced hypersensitivity reactions in a Taiwanese population¹

Phenotype	HLA-B*13:01, n (%)	n	OR (95% CI)	P	P_c
All patients	6 (75.0)	8	24.82 (4.92–125.26)	4.57×10^{-5}	1.05×10^{-3}
DRESS	6 (85.7)	7	49.64 (5.89–418.13)	1.27×10^{-5}	2.92×10^{-4}
MPE	0 (0)	1	2.74 (0.11–67.92)	1	–
Control ²	73 (10.8)	677			

Boldface indicates statistical significance ($P < 0.05$).

Abbreviations: CI, confidence interval; DRESS, drug reaction with eosinophilia and systemic symptoms; MPE, maculopapular eruption; OR, odds ratio; P_c , corrected P -value.

¹Only patients from Taiwan (n = 8) were enrolled for HLA-B*13:01 analysis. ORs were calculated using Haldane modification, and P -values were measured using Fisher exact test.

²The control population comprised healthy individuals without drug hypersensitivity history.

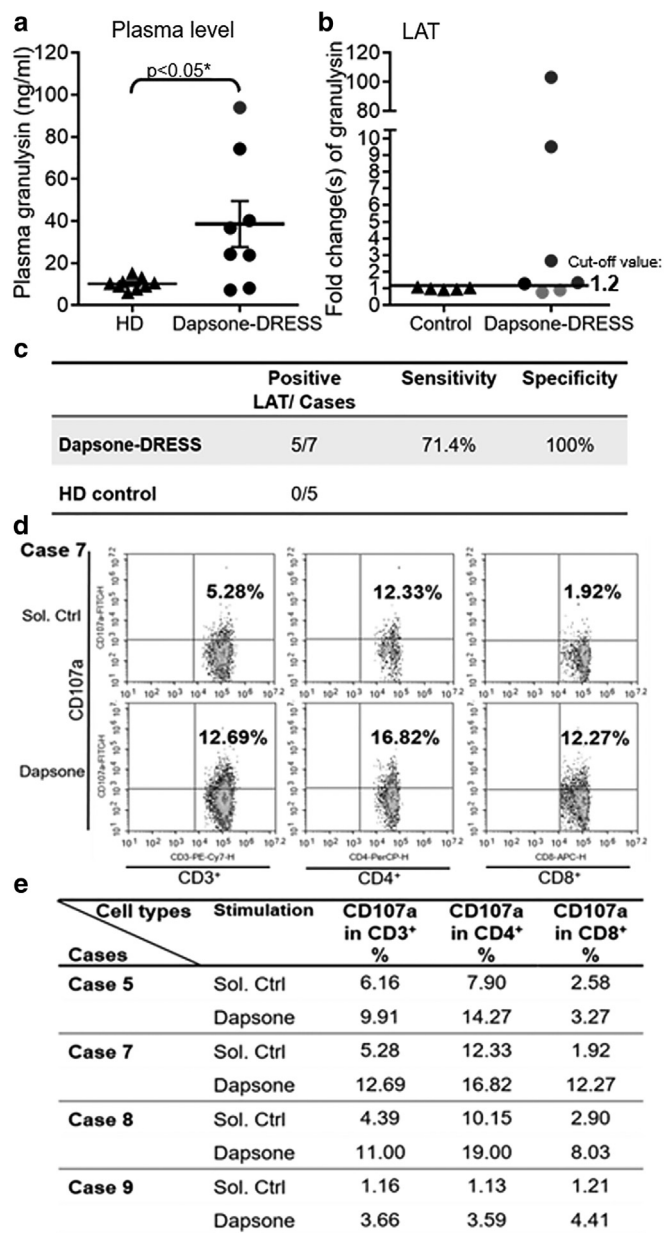


Figure 1. Cytotoxic T cells involved in the pathogenesis of dapsone DRESS. (a) Plasma granulysin expression was measured in eight patients with dapsone DRESS and nine healthy individuals. (b, c) Lymphocyte activation test (LAT) was performed in seven patients with dapsone DRESS and five healthy individuals. A positive result was defined as a 1.2-fold increase in granulysin release compared with the control individuals (solid line). Black dot indicates true positive result in granulysin-based LAT, gray dot indicates false negative result, and black triangle indicates healthy control results for comparison. (d, e) After in vitro culture assay, the populations of CD107a⁺ activated T cells were measured in four dapsone DRESS patients in the presence or absence of dapsone treatment. CD107a⁺CD3⁺ cells were defined as activated T cells, CD107a⁺CD8⁺ cells were defined as activated CD8⁺ CTLs, and CD107a⁺CD4⁺ cells were defined as activated CD4⁺ T cells. * $P < 0.05$ by two-tailed Student *t* test. Ctrl, control; DRESS, drug reaction with eosinophilia and systemic symptoms; HD, healthy donor; LAT, lymphocyte activation test; Sol., solvent.

In addition to HLA association, the divergences in individual metabolism and drug clearance contributing to development of SCAR have been identified previously. Our recent study showed that the genetic variants of

CYP2C are strongly associated with phenytoin-related SCAR (Chung et al., 2014a). For dapsone metabolism, CYP2C9 and NAT2 are two of the main phase I enzymes for dapsone (Hutzler et al., 2001; Zhu and Stillier, 2001). In this study, no significant association was observed among genotypes of CYP2C9*3 or NAT2 with occurrence of dapsone DRESS, which is consistent with results of a previous study (Wang H et al., 2013). Although no significant association of CYP2C9*3 and NAT2 was found in our study, we can speculate why patient 4 with DRESS had no risk of the HLA-B*13:01 allele but carried both lower metabolizing enzyme activity genotypes of CYP2C9*3 and NAT2. The association of dapsone-metabolizing enzymes or other risk factors in dapsone DRESS without existing HLA-B*13:01 needs further exploration.

The pathomechanism of SCAR is known to involve the CTLs that recognize culprit drugs presented by HLA class I molecules (Pichler et al., 2011; White et al., 2015; Yun et al., 2016). We previously reported that granulysin was highly expressed in blister fluids of SJS/TEN patients and served as a key mediator responsible for their disseminated keratinocyte death (Chung et al., 2008). However, an elevated granulysin level has also been reported in the plasma and skin lesions of DRESS patients (Saito et al., 2012; Weinborn et al., 2016). Weinborn et al. showed that granulysin is expressed in skin lesions from six types of patients with delayed-type hypersensitivity (including MPE, fixed drug eruption, acute generalized exanthematous pustulosis, DRESS, SJS, and TEN) but mainly in the keratinocytes of patients with TEN and strongly by the inflammatory cells of patients with DRESS. In their immunohistochemical study, Weinborn et al. found that granulysin is mainly expressed around the detached necrotic area of the epidermis in SJS/TEN patients but is expressed in the dermis or upper dermis of DRESS patients. Our recent study found that plasma granulysin level was elevated in both DRESS and SJS/TEN patients compared with healthy donors but that the increased level was lower in DRESS patients than in SJS/TEN patients (Su et al., 2017). Because granulysin is located at the dermis of skin lesions in DRESS patients compared with epidermis of SJS/TEN patients, these results may explain why extensive blistering epidermal necrosis does not develop in patients with DRESS as in those with SJS/TEN. The certain expression level of granulysin in the dermis of DRESS patients may also explain the erythema multiforme-like dyskeratosis observed in some DRESS patients (Chi et al., 2014).

Granulysin is mainly generated from CTLs and is also known to exhibit broadly cytolytic effects against microbial pathogens, including bacteria, fungi, yeast, and parasites (Krensky and Clayberger, 2009). Because HHV or EBV reactivation is observed during the acute phase of DRESS in some patients (Chung et al., 2016; Ichiche et al., 2003; Picard et al., 2010), Saito et al. (2012) suggested that activation of virus-specific CTLs resulted in granulysin release in the sera of DRESS patients (Saito et al., 2012). In this study, the increased granulysin level was observed in patients with dapsone DRESS compared with healthy control individuals (3.38-fold increase, $P < 0.05$); however, none of HHV or EBV reactivations was identified in any dapsone DRESS patients. Furthermore, the granulysin-based in vitro LAT assay under dapsone stimulation

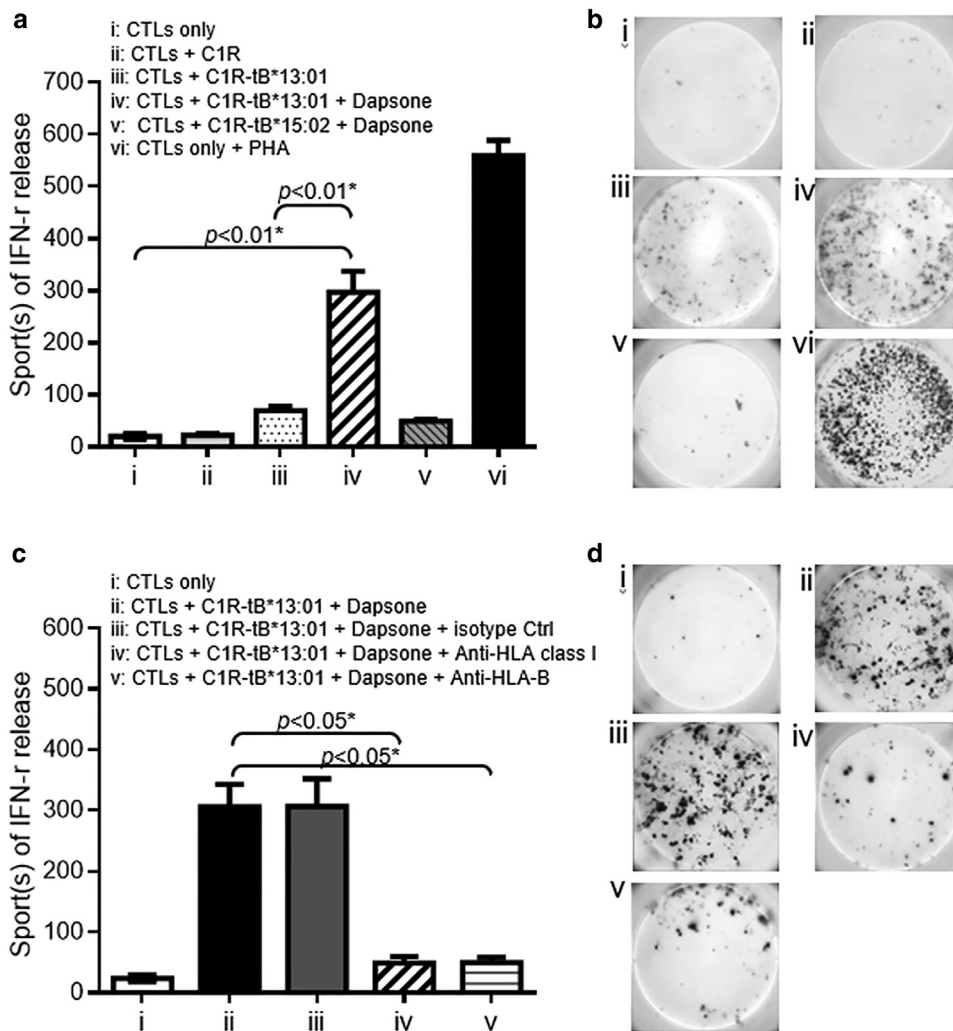


Figure 2. HLA-B*13:01—dependent activation of dapsone-specific CTLs obtained from patients with dapsone DRESS. (a) Dapsone-specific CTLs were expanded from PBMCs of dapsone DRESS patients ($n = 3$) and then were in vitro cultured with C1R (non-HLA-B*13:01 carrier), C1R-*t13:01* (HLA-B*13:01 carrier), C1R-*t15:02* (HLA-B*15:02 carrier), or dapsone. (b) The spot results of IFN- γ release. CTLs alone treated with phytohaemagglutinin (PHA) served as a positive control. (c) Dapsone-specific CTLs were cultured in vitro with C1R-*t13:01* and dapsone in the presence of isotype or anti-HLA antibodies, and (d) the spot results are shown. Values are mean \pm standard error of the mean. * $P < 0.05$, ** $P < 0.01$, by two-tailed Student *t* test. CTL, cytotoxic T cell; DRESS, drug reaction with eosinophilia and systemic symptoms; PBMC, peripheral blood mononuclear cell; PHA, phytohaemagglutinin.

showed high sensitivity and specificity in dapsone DRESS (sensitivity = 71.4%, specificity = 100%). We suggested that dapsone can induce drug-specific CTL activation, resulting in granulysin release in the plasma of dapsone DRESS patients. Because CTLs from dapsone DRESS patients were activated by dapsone stimulation and granulysin is known to be mainly released from CTLs, we interpret this to mean that CTLs are involved in the pathogenesis of dapsone DRESS.

There are several hypotheses about the interaction between HLA and drug antigens presented to T-cell receptors on CTLs, such as hapten/prohapten theory, pharmacological interaction model, altered peptide repertoire model, and altered T-cell receptor repertoire model (Chung et al., 2016; White et al., 2015). For example, carbamazepine-induced SJS/TEN is one of the examples for the pharmacological interaction model, in which carbamazepine was found to directly interact with the HLA-B*15:02 protein without the involvement of any antigen-processing pathway (Wei et al., 2012). Furthermore, the interaction between abacavir and HLA-B*57:01 is the well-known example for the altered peptide repertoire model, in which abacavir can trigger the conformational changes of endogenous peptides presented

by HLA-B*57:01 (Illing et al., 2012). However, to our knowledge there is no experimental evidence to support an interaction between dapsone and HLA-B*13:01. In this study, we generated HLA-B*13:01—expressing cells (C1R-*t13:01*) in HLA-B—deficient B cells (Hmy2.C1R cells) and obtained dapsone-specific CTLs from dapsone SCAR patients by in vitro expansion assay. We further performed in vitro co-culture assay and found that dapsone-specific CTLs against antigen-presenting cells expressing HLA-B*13:01 were significantly activated after stimulation by dapsone (3.9-fold increase compared with cells with no HLA-B*13:01 expression, $P < 0.01$). We also analyzed one patient with dapsone-induced SJS who carried HLA-B*13:01 (see Supplementary Figure S4 online). The same immune pathomechanism was elucidated by in vitro LAT and co-culture assay (data not shown), suggesting that HLA-B*13:01 and CTLs may also be involved in the pathogenesis of dapsone-induced SJS. Although the detailed interaction between HLA-B*13:01 and dapsone still needs to be further investigated, our results show that dapsone-specific CTLs were activated in an HLA-B*13:01—dependent manner.

In conclusion, our findings show that HLA-B*13:01 is strongly associated with dapsone DRESS in Taiwanese

and Malaysian populations. CTLs were found to be involved in the pathogenesis of dapsone SCAR and could be activated in an HLA-B*13:01–dependent manner. This study provides a functional role of an HLA-B*13:01–restricted immune mechanism induced by dapsone.

MATERIALS AND METHODS

Participants

From 2006 to 2017, we collected clinical data, blood and plasma samples, and blister cells from nine patients with dapsone-induced hypersensitivity reactions from the Taiwan-SCAR consortium (including Chang Gung Memorial Hospitals; National Taiwan University Hospital; Taichung Veterans General Hospital; National Cheng Kung University Hospital; and Kaohsiung Medical University, Chung-Ho Memorial Hospital, Taiwan) and Hospital Sultanah Aminah Johor Bahru in Malaysia. One of these patients (patient 2) had been discussed in an epidemiology study of DRESS in Taiwan (Chiou et al., 2008). We also collected clinical data and DNA samples of 677 individuals in the general population without history of adverse drug reactions as the control group. All 677 control individuals were recruited from the same hospitals in Taiwan and were of Han Chinese ethnicity.

Standard protocol approvals, registrations, and patient consents

Each participant enrolled in this study gave written informed consent, and the study was approved by the institutional review board and ethics committee of Chang Gung Memorial Hospitals based on Taiwan law (No. 97-0509B and No. 100-4657A3). Informed consent was obtained from all participants. One participant was enrolled from Malaysia, and a declaration from the Malaysian hospital based on relevant local laws, regulations, and guidelines for the use of human subjects was also obtained.

Disease assessment

All patients were assessed by at least two dermatologists. For drug causality, the culprit drug of DRESS was determined by the Naranjo algorithm and assessment of drug causality published by the RegiSCAR group (Kardaan et al., 2013; Naranjo et al., 1981; Sassolas et al., 2010). Only patients with probable or definite cases caused by dapsone (algorithm of drug causality for epidermal necrolysis [i.e., ALDEN] score > 4 or Naranjo algorithm result > 5) were recruited. Phenotypes were classified using the consensus definition (Bastuji-Garin et al., 1993; Cacoub et al., 2011). DRESS is characterized by an extensive erythematous maculopapular rash with periorbital edema and desquamation. The diagnosis of DRESS using the criteria and scoring system of the RegiSCAR group include cutaneous involvement with typical skin eruptions (e.g., exfoliative dermatitis, generalized maculopapular exanthema), fever ($\geq 38.5^{\circ}\text{C}$), enlarged lymph nodes (two or more sites, ≥ 1 cm), presence of atypical lymphocytes and eosinophilia, systemic involvement (e.g., liver, kidney, and lung), time of resolution, and the evaluation of other potential causes. The clinical course, dosage and duration of dapsone, systemic involvement, and mortality were analyzed in this study.

HHV and EBV detection

Patients' blood samples during acute stages were collected, and DNA was extracted. Real-time PCR was used to detect the presence of HHV6 and EBV in specimens with the Roche LightCycler 480 Probes Master (Roche, Penzberg City, Germany), using primers and probes as described in previous studies (Niesters et al., 2000; Sugita et al., 2008).

HLA genotyping

HLA-A and HLA-B genotypes were determined by SeCore HLA sequence-based typing (Invitrogen, Life Technologies, Carlsbad, CA) or HLA next-generation sequencing genotyping, which was carried out using the Hologate HLATM X2-96/7 (no. 1056733; Omixon Biocomputing, Budapest, Hungary) on MiniSeq System (Illumina, San Diego, CA) with HLA Twin software (Omixon) as described in the manufacturer's protocol. Statistical analysis of the differences in HLA frequencies among the patients with dapsone hypersensitivity and the general population were performed for the Han Chinese patients from Taiwan.

Genetic analysis in CYP2C9 and NAT2

For genetic analysis of dapsone-metabolizing enzymes, the allele of CYP2C9*3 (rs1057910) was determined by TaqMan quantitative PCR assay (Thermo Fisher Scientific, Waltham, MA). The primers and probes for detecting CYP2C9*3 were as follows: forward primer, 5'-GCCACATGCCCTACACAGATG-3'; reverse primer, 5'-CCTTGGGAATGAGATAGTTTCTGAA-3'; probe 1, 5'-TCCAGAGATACCTTGAC-3'; probe 2, 5'-TCCAGAGATACATTGAC-3'. For detection of the acetylator genotype of NAT2, Sanger sequencing was performed. The primers for detecting NAT2 were as follows: forward primer, 5'-GCCTCAGGTGCCTTGCATT-3'; reverse primer, 5'-CGTGAGGGTAGAGAGGA TAT-3'.

The most common NAT2 single nucleotide polymorphisms in the Han Chinese population are rs1801280, rs1799929, rs1799930, rs1208, rs1799931, and rs1041983. We detected these variants to determine the acetylator genotypes of NAT2 according to a previously published study (Hein et al., 2000). Homozygotes (NAT2*4/NAT2*4) or heterozygotes (NAT2*4/NAT2*5B, NAT2*4/NAT2*6A, and NAT2*4/NAT2*7B) for the dominant NAT2*4 wild-type allele were classified as fast acetylator genotypes, and homozygotes of the mutant alleles (NAT2*5B, NAT2*6A, and NAT2*7B) were classified as slow acetylator genotypes.

Lymphocyte activation test

PBMCs were isolated from whole blood samples using Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden) density gradient centrifugation. The PBMCs (1.0×10^6 per well) of patients with dapsone SCAR and healthy control individuals were cultured in 96-well microplates in RPMI-1640 medium (Gibco Invitrogen, Life Technologies, Carlsbad, CA) supplemented with 10% human AB serum (Sigma-Aldrich, Darmstadt, Germany), IL-7 (Invitrogen), and dapsone (Sigma-Aldrich, St. Louis, MO) and were tested at 37°C in 5% CO_2 for 1 week. Dapsone was diluted in the medium to obtain a concentration reflecting a 10-fold physiological therapeutic level (50 $\mu\text{g}/\text{ml}$). In addition, dimethyl sulfoxide was added to the medium as the solvent control, and phytohemagglutinin (i.e., PHA) at a concentration of 10 $\mu\text{g}/\text{ml}$ was used as the positive control. Culture supernatants were collected on day 7 to measure the secretions of granulysin, which served as the high specific cytotoxicity protein in patients with SJS/TEN (Chung et al., 2008; Wang CW et al., 2013) and DRESS (Weinborn et al., 2016), by ELISA (using anti-granulysin antibodies, H3- and B04 biotin-labeled, both of which were generated by our laboratory). The sensitivity of these tests for granulysin was 1.56 ng/ml. The fold change in each sample was normalized by the solvent control. A positive result of the test was defined as a 1.2-fold increase in granulysin expression compared with the healthy control individuals. This cutoff value was calculated by using the values of the mean and 2-fold standard deviation from the healthy control individuals.

Flow cytometric analysis of activated T cells of dapsone DRESS patients

In vitro LAT assay with PBMCs from patients with dapsone DRESS was performed in the presence or absence of dapsone for 1 week, and the cells were then incubated with Alexa Fluor 488-labeled anti-human CD107a (FITC channel), phycoerythrin-cyanine dye 7 (PE-Cy7)-labeled anti-human CD3, allophycocyanin (APC)-labeled anti-human CD8, peridinin chlorophyll protein complex (PerCP)-labeled anti-human CD4, and APC-Cy7-labeled anti-human CD45 (eBioscience, San Diego, CA) at 4°C for 30 minutes. After washing, the stained cells were processed through a NovoCyte flow cytometer (ACEA Bioscience, San Diego, CA), and data were analyzed using NovoExpress 1.1.0 software (ACEA Bioscience). CD107a is known to be used as a marker for activated T cells as described previously (Aktas et al., 2009).

Construction and generation of C1R-transmembrane HLA-B*13:01 stable clones

The full length of HLA-B*13:01 cDNA was achieved from the homologous HLA-B*13:01 allele carrier by means of PCR amplification with the forward primer (5'-ATGCGGGTACGCGCCCCGA-3') and the reverse primer (5'-TCAAGCTGTGAGAGACATCAGAG-3') and then cloned into pGEM-T Easy vector (Promega, Madison, WI). After that, the full length of HLA-B*13:01 cDNA was further cloned into a pcDNA3.1 vector (Invitrogen, Carlsbad, CA) by using the HindIII and EcoRI cutting sites. This plasmid DNA was transfected to Hmy2.C1R cells (HLA-B-deficient cells) and selected with 600 mg/ml G418 (Sigma-Aldrich) for 3 weeks. The C1R-transmembrane HLA-B*13:01 (C1R-t13:01) stable clone was determined by detecting protein expression using anti-HLA antibody (w6/32, eBioscience) with flow cytometry.

ELISpot assay

PBMCs from patients were isolated and incubated with dapsone for 1 week. The dapsone-specific T cells were then expanded by adding 50 µg/ml dapsone, 1 ng/ml IL-7, 30 ng/ml OKT3 (the clone name of anti-CD3 antibody), and 6,000 U/ml IL-2 for 1 week. The expanded T cells were reset for 3 days, and CTLs were isolated by anti-human CD45, CD3, and CD8 using FACS. After that, the dapsone-specific CTLs were detected with the IFN- γ release by ELISpot assay according to the instructions of the Mabtech human IFN- γ ELISpot basic kit (Mabtech, Nacka Strand, Sweden). To perform ELISpot analysis, 1×10^4 dapsone-specific CTLs were incubated with 5×10^4 C1R or C1R-t13:01 antigen presenting cells at 37°C for 24 hours. Data were analyzed using the AID ELISpot Reader (AID, Strassberg, Germany). For the antibody blockage assay, cells were pretreated with HLA class I antibody (w6/32; BioLegend, San Diego, CA) or class II antibody (B1.23.2).

Statistical analysis

Statistical analyses were performed using SPSS for Windows, version 21.0 (IBM, Armonk, NY). Comparisons of genotype frequencies between groups were performed using Fisher exact tests. To achieve sufficient power to identify HLA associated with different phenotypes, the P_c -values were adjusted using Bonferroni correction for multiple comparisons. Odds ratios (ORs) were calculated according to Haldane modification, which added 0.5 to all fields to accommodate possible zero counts. Confidence intervals and P -values for rate ratio estimates were calculated using a two-sided test. For analysis of plasma granulysin levels and ELISpot results, statistical comparison between two variables was performed by two-tailed

Student t tests. Differences were considered statistically significant at P -values of less than 0.05.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at <https://doi.org/10.1016/j.jid.2018.02.004>.

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