Oxypurinol-Specific T Cells Possess Preferential TCR Clonotypes and Express Granulysin in Allopurinol-Induced Severe Cutaneous Adverse Reactions

Wen-Hung Chung[1,2,6], Ren-You Pan[3,6], Mu-Tzu Chu[4], See-Wen Chin[1,2], Yu-Lin Huang[1,2], Wei-Chi Wang[5], Jen-Yun Chang[5] and Shuen-Iu Hung[3,4]

Allopurinol, a first-line drug for treating gout and hyperuricemia, is one of the leading causes of severe cutaneous adverse reactions (SCARs). To investigate the molecular mechanism of allopurinol-induced SCAR, we enrolled 21 patients (13 Stevens–Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN) and 8 drug reaction with eosinophilia and systemic symptoms (DRESS)), 11 tolerant controls, and 23 healthy donors. We performed *in vitro* T-cell activation assays by culturing peripheral blood mononuclear cells (PBMCs) with allopurinol, oxypurinol, or febuxostat and measuring the expression of granulysin and IFN-γ in the supernatants of cultures. TCR repertoire was investigated by next-generation sequencing. Oxypurinol stimulation resulted in a significant increase in granulysin in the cultures of blood samples from SCAR patients ($n=14$) but not tolerant controls ($n=11$) or healthy donors ($n=23$). Oxypurinol induced T-cell response in a concentration- and time-dependent manner, whereas allopurinol or febuxostat did not. T cells from patients with allopurinol–SCAR showed no crossreactivity with febuxostat. Preferential TCR-V-β usage and clonal expansion of specific CDR3 (third complementarity-determining region) were found in the blister cells from skin lesions ($n=8$) and oxypurinol-activated T-cell cultures ($n=4$) from patients with allopurinol–SCAR. These data suggest that, in addition to *HLA-B*58:01*, clonotype-specific T cells expressing granulysin upon oxypurinol induction participate in the pathogenesis of allopurinol-induced SCAR.

Journal of Investigative Dermatology (2015) **135,** 2237–2248; doi:10.1038/jid.2015.165; published online 4 June 2015

INTRODUCTION

Allopurinol, a xanthine oxidase inhibitor, is currently a first-line drug for the treatment of gout and hyperuricemia (Braden *et al.*, 1994). Although generally well tolerated, allopurinol is associated with severe cutaneous adverse reactions (SCARs) that include drug rash with eosinophilia and systemic symptoms (DRESS), Stevens–Johnson syndrome (SJS), and toxic epidermal necrosis (TEN) (Espiritu *et al.*, 1976; Braden *et al.*, 1994; Ramasamy *et al.*, 2013). We first reported a strong association between the *HLA-B*58:01* allele and allopurinol-induced SCAR in Han Chinese, and this association was further validated in different populations (Hung *et al.*, 2005; Kaniwa *et al.*, 2008; Lonjou *et al.*, 2008; Tassaneeyakul *et al.*, 2009; Phillips and Mallal, 2010; Kang *et al.*, 2011). Although the strength of association was very strong, the positive predictive value of *HLA-B*5801* for allopurinol-SCAR was low (~2.7%), indicating that other factors also participate in the pathogenesis (Hung *et al.*, 2005). We proposed that, in addition to *HLA-B*58:01*, other immune receptors such as TCRs are involved in the induction of the catastrophic immune response seen in allopurinol–SCAR.

SCAR is thought to involve drug-specific T cells (Posadas *et al.*, 2002; Roujeau, 2006). The conventional lymphocyte transformation test (LTT) is the most widely used *in vitro* test to examine the causative drugs of T cell–mediated hypersensitivity (Pichler and Tilch, 2004). However, the sensitivity of LTT is very low for SCAR, and examination of

[1]*Department of Dermatology, Drug Hypersensitivity Clinical and Research Center, Chang Gung Memorial Hospital, Linkou, Taipei and Keelung, Taiwan;* [2]*College of Medicine, Chang Gung University, Taoyuan, Taiwan;* [3]*Program in Molecular Medicine, National Yang-Ming University and Academia Sinica, Taipei, Taiwan;* [4]*Department and Institute of Pharmacology, National Yang-Ming University, Taipei, Taiwan and* [5]*Health GeneTech Corporation, Taoyuan, Taiwan*

Correspondence: Shuen-Iu Hung, Institute of Pharmacology, National Yang-Ming University, 155, Linong Street, Section 2, Beitou, Taipei 11221, Taiwan. E-mail: sihung@ym.edu.tw

[6]*These authors contributed equally to this work*

Abbreviations: CDR3, third complementarity-determining region; DRESS, drug reaction with eosinophilia and systemic symptom; LTT, lymphocyte transformation test; NK cell, natural killer cell; PBMC, peripheral blood mononuclear cell; SCAR, severe cutaneous adverse reaction; SJS, Stevens–Johnson syndrome; TEN, toxic epidermal necrolysis; TRBJ, T-cell receptor β joining; TRBV, T-cell receptor β variable

Received 8 July 2014; revised 15 April 2015; accepted 20 April 2015; accepted article preview online 6 May 2015; published online 4 June 2015

the causative drugs of SJS/TEN usually yields negative results (Porebski *et al.*, 2011). We previously showed that granulysin, a secretory protein produced by cytotoxic T lymphocytes and natural killer (NK) cells, played a key role in the disseminated keratinocyte death in SJS/TEN (Chung *et al.*, 2008). It was recently suggested that a combination of different assays examining the expression of granulysin, granzyme B, and IFN-γ may improve the sensitivity of *in vitro* T-cell activation assays for SCAR (Porebski *et al.*, 2013).

Febuxostat, an alternative xanthine oxidase inhibitor, is recently introduced into the market, and it has been shown to be well tolerated in patients with hyperuricemia (Becker *et al.*, 2005; Goldfarb *et al.*, 2011). Currently, there is no report demonstrating tolerability or possible crossreactivity of allopurinol–SCAR T cells to febuxostat. In this study, we performed *in vitro* T-cell activation assays measuring the expression of granulysin and IFN-γ in the cultures to examine the drug antigen of allopurinol–SCAR and to assess the potential crossreactivity of T cells to febuxostat. Furthermore, we analyzed the TCR repertoire of blister cells directly from the skin lesions of allopurinol–SCAR patients and compared their characteristics with those of *in vitro* expanded T cells.

RESULTS

We enrolled 21 patients with allopurinol–SCAR (13 SJS/TEN and 8 DRESS), 11 tolerant controls, and 23 healthy donors; the demographic data are shown in Supplementary Table S1 online. The *HLA-B*58:01* allele was present in 19 (90.48%) of 21 patients with allopurinol–SCAR, 6 (54.55%) of 11 tolerant controls, and 15 (65.22%) of 23 healthy donors (Supplementary Table S1 online).

Oxypurinol-induced T cells to release granulysin in allopurinol–SCAR

We isolated peripheral blood mononuclear cells (PBMCs) from 14 patients with allopurinol–SCAR, 11 tolerant controls, and 23 healthy donors and performed *in vitro* T-cell activation assays by culturing the PBMCs with allopurinol (10 and 100 μM), oxypurinol (10 and 100 μg ml^{-1}), febuxostat (4 and 40 μg ml^{-1}), tolerant drugs, or solvent control for 1 to 3 weeks. The levels of granulysin and IFN-γ in the supernatants of cultures were measured by ELISA. No significant increase in granulysin levels was found in the PBMC cultures with drugs at the physiological concentrations (i.e., allopurinol: 10 μM; oxypurinol: 10 μg ml^{-1}; febuxostat: 4 μg ml^{-1}; Supplementary Figure S1 online). In comparison, robust expression of granulysin was induced in the cultures when a high concentration of oxypurinol (100 μg ml^{-1}, which is 10-fold higher than the physiological level) was incubated with the PBMCs from allopurinol–SCAR patients ($n=14$) for 2 weeks (Figure 1a–c and Supplementary Table S2 online). There was no statistical change in granulysin levels in the T-cell activation assays using PBMCs from 11 tolerant controls (6 of 11 carrying *HLA-B*58:01*) or 23 healthy donors (15 of 23 carrying *HLA-B*58:01*) (Figure 1d–f and Supplementary Table S2 and Supplementary Figure S2 online). Culturing PBMCs with oxypurinol (100 μg ml^{-1}) for 2 weeks in a granulysin-based T-cell activation assay showed a sensitivity of 86% and a specificity of 91% for allopurinol–SCAR (Figure 1g).

T cells of allopurinol–SCAR showed no crossreactivity to febuxostat

The lack of a significant increase in granulysin expression in the PBMC cultures of allopurinol (10 or 100 μM) suggested that the parent drug allopurinol was an ineffective antigen (Figure 1a–c and Supplementary Table S2 and Supplementary Figure S1 online). In addition, febuxostat did not induce granulysin expression in the PBMC cultures, indicating that the T cells of allopurinol–SCAR patients could not crossreact to febuxostat (Figure 1a–c). We performed clinical follow-up observations of allopurinol–SCAR patients receiving febuxostat. Of the 21 patients with allopurinol–SCAR, 10 patients (4 SJS, 1 TEN, and 5 DRESS) received febuxostat after the SCAR episode. All 10 patients carried *HLA-B*58:01* genotype. Febuxostat was prescribed to these patients in order to control their severe hyperuricemia and gout. None of the 10 patients developed recurrence of SCAR (DRESS or SJS/TEN). Only one patient had pruritus at the beginning of febuxostat treatment, but became tolerant to it after continuous use.

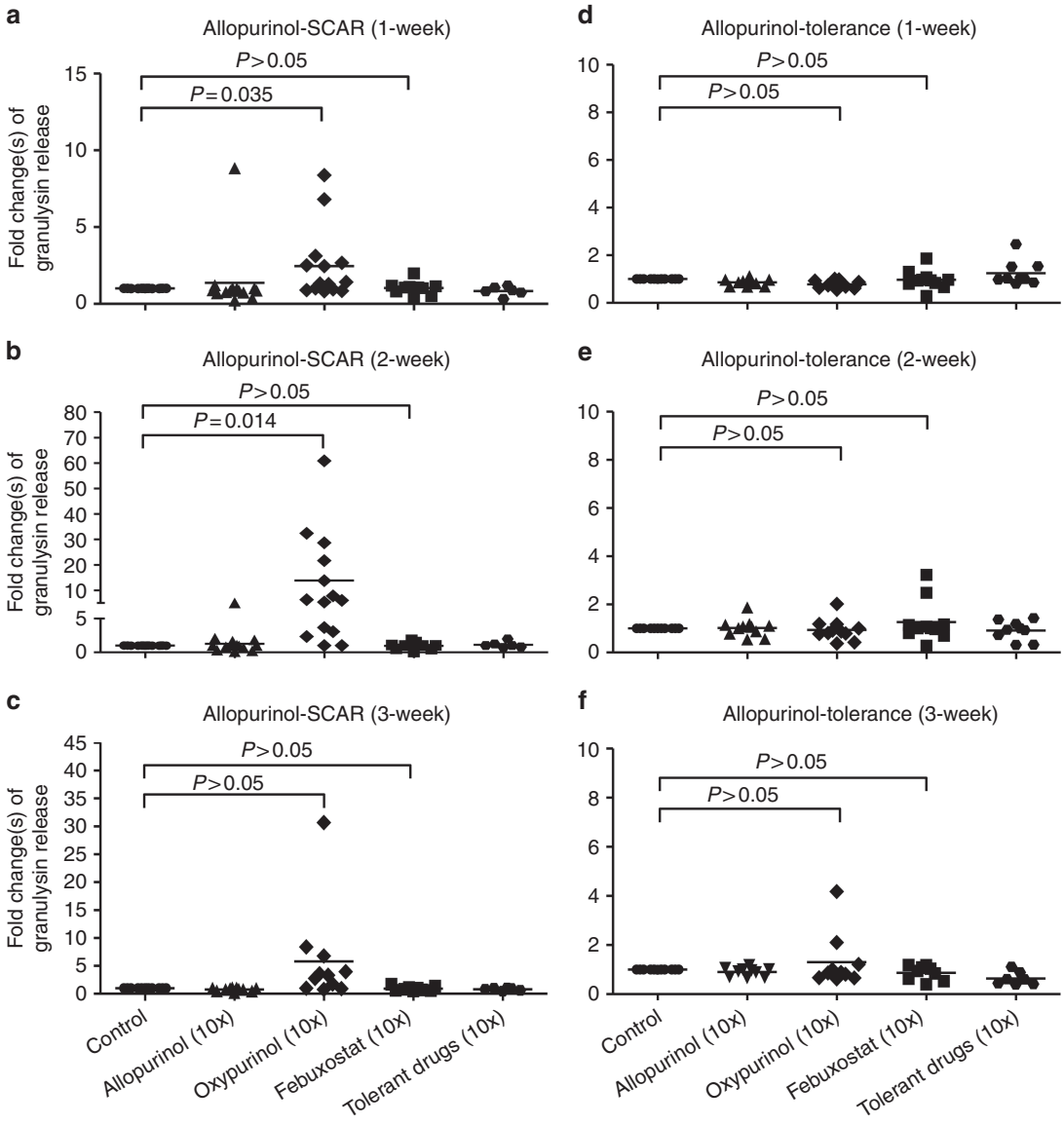
IFN-γ is not a sensitive marker for examining the drug antigen of allopurinol–SCAR

Expression of IFN-γ in the supernatants of PBMC cultures was determined by ELISA. Although IFN-γ expression increased in 5 (38%) of the 13 samples after 1 week of incubation, there was no statistical difference when compared with the solvent control (Figure 2a). In addition, the expression of IFN-γ did not reach significant difference in all of the tested conditions (Figure 2 and Supplementary Figure S3 online). These data suggested that IFN-γ is not a sensitive marker for identifying the drug antigen of allopurinol–SCAR in T-cell activation assays. We also evaluated T-cell proliferation by conventional LTT by measuring H^3-thymidine incorporation; however, the sensitivity of conventional LTT was too low for further analysis (Supplementary Table S3 and Supplementary Figure S4 online).

Immunophenotypes of oxypurinol-activated T cells

We investigated the immunophenotypes of *in vitro* expanded cells by flow cytometry and confocal microscopy. After 2 weeks of incubation with oxypurinol (100 μg ml^{-1}), the *in vitro* expanded CD45$^+$ PBMCs consisted of 73.1% of CD3$^+$ T lymphocytes and 5.8% of CD56$^+$ NK cells (Figure 3a). Oxypurinol stimulation resulted in an increase in CD3$^+$ T cells expressing granulysin (accounting for 5.03% of CD45$^+$ PBMCs), whereas allopurinol or febuxostat did not (Figure 3b and c). The oxypurinol-expanded cells were composed of 62.9% of CD4$^+$ T cells and 17.2% of CD8$^+$ T cells, and there were increased cell populations representing CD8$^+$ granulysin$^+$ cells (4.05%), CD4$^+$granulysin$^+$ cells (6.84%), and CD56$^+$granulysin$^+$ cells (27.21%) compared with the cultures of solvent controls (Figure 3d). Confocal microscopy

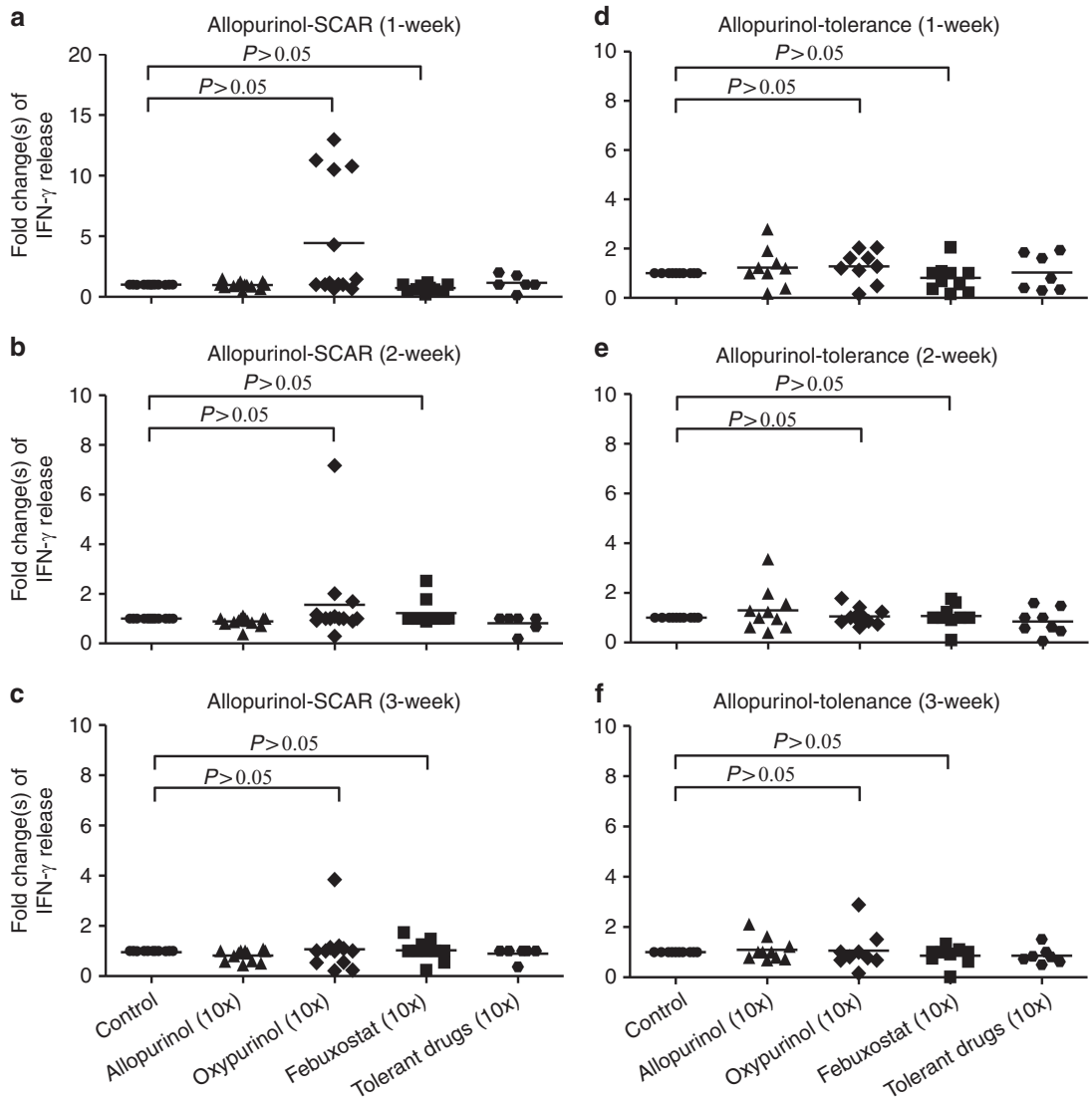
data further validated the finding that granulysin was expressed by $CD4^+$ T cells, $CD8^+$ T cells, or NK cells (Figure 3e). In addition, we examined the expression of CD107a (LAMP-1), which is a marker for activated T cells and NK cells (Betts *et al.*, 2003), and found that the cell populations representing $CD4^+CD107a^+$ T cells, $CD8^+CD107a^+$ T cells,



g

	Allopurinol		Oxypurinol		Febuxostat
	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)	Cross-reactivity (%)
1 week	7	100	43	100	0
2 week	14	100	86	91	0
3 week	0	100	64	80	0

Figure 1. Oxypurinol induced granulysin expression in peripheral blood mononuclear cell (PBMC) cultures from patients with allopurinol–severe cutaneous adverse reaction (SCAR). PBMCs were isolated from 14 patients with allopurinol–SCAR and 11 tolerant controls. (**a**–**f**) Data of T-cell activation assay obtained by incubating PBMCs with drugs at concentrations of 10-fold the therapeutic levels (i.e., allopurinol: 100 μM, oxypurinol: 100 μg ml^{-1}, and febuxostat: 40 μg ml^{-1}) for 1 to 3 weeks. Granulysin expression in the culture supernatant was determined by ELISA, and the fold change in each sample was normalized by that of solvent control. The bars represent the mean values of the fold changes of each group. A positive result of the assay was defined as a 2-fold increase in granulysin expression compared with the solvent control. The *P*-values were analyzed by one-sample *t*-test. (**g**) The sensitivity, specificity, and crossreactivity of granulysin-based T-cell activation assay for allopurinol–SCAR.



g

	Allopurinol		Oxypurinol		Febuxostat
	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)	Cross-reactivity (%)
1 week	0	89	38	78	0
2 week	0	90	15	100	10
3 week	0	90	8	89	0

Figure 2. IFN-γ expression in peripheral blood mononuclear cell (PBMC) cultures from patients with allopurinol–severe cutaneous adverse reaction (SCAR) or tolerant controls. PBMCs were isolated from 13 patients with allopurinol–SCAR and 11 tolerant controls. (**a**–**f**) Data of T-cell activation assay obtained by incubating PBMCs with drugs at concentrations of 10-fold the therapeutic levels (i.e., allopurinol: 100 μM, oxypurinol: 100 μg ml^{-1}, febuxostat: 40 μg ml^{-1}) for 1 to 3 weeks. The expression of IFN-γ in the culture supernatant was determined by ELISA, and the fold change of IFN-γ in each sample was normalized by the solvent control. The bars represent the mean values of fold changes of each group. A positive result of the assay was defined as a 2-fold increase in IFN-γ expression compared with the solvent control. The *P*-values were analyzed by one-sample *t*-test. (**g**) The sensitivity, specificity, and crossreactivity of the IFN-γ-based T-cell activation assay for allopurinol–SCAR.

and $CD56^+CD107a^+$ NK cells increased after oxypurinol stimulation (Supplementary Figure S5 online). These results support that oxypurinol activated specific T cells to produce granulysin in allopurinol–SCAR.

Preferential TCR clonotypes present in blister cells and oxypurinol-expanded T cells from allopurinol–SCAR

We characterized the TCR repertoire of allopurinol–SCAR using RNA isolated from (1) 8 samples of blister cells from the

skin lesions of allopurinol–SJS/TEN patients, (2) 8 samples of PBMCs from healthy donors, (3) 4 samples of oxypurinol-expanded PBMCs and 4 samples of solvent control-cultured PBMCs from the allopurinol–SCAR cases, and (4) 2 samples of oxypurinol-cultured PBMCs from tolerant controls (Supplementary Table S1 online). The data of *TRBV* (*T-cell receptor β variable*), *TRBJ* (*T-cell receptor β joining*) usages, and 10 most common TCR clonotypes in each sample are summarized in Figures 4 and 5 and Supplementary Tables S4 and S5 and Supplementary Figure S6 online. Compared with the *TRBV* usage of PBMCs from the 8 healthy donors, a significant increase in the frequencies of *TRBV*3-1, 5–1, 9, and 29-1 was noticed in the blister cells and oxypurinol-expanded T cells from the allopurinol–SCAR patients ($P<0.05$; Figure 4). Expansion of specific TCRβ CDR3 (third complementarity-determining region) clonotypes was found in blister cells or oxypurinol-cultured PBMCs from patients with allopurinol–SCAR (Figure 5). For example, the individual CDR3 clonotype "SVLIEGTQY" of TRBV29-1 accounted for 16.07% of all CDR3 clonotypes of case 1, "ASSQDLTGNTIY" of TRBV3-1 for 46.08% of case 4, and "ASSPRDFSYEQY" of TRBV9 for 18.83% of case 6 (Figure 5a). Table 1 lists the predominant TCRβ CDR3 clonotypes with frequencies >3%. Although the expanded repertoire differed from case to case, several CDR3 clonotypes identified from the blister cells of some patients showed high similarity (Supplementary Figure S7 online). In comparison, these CDR3 clonotypes were absent or detected at frequencies of <0.002% in the CDR3 clonotypes of the PBMCs from healthy donors (Table 1 and Figure 5). These data revealed that blister cells and oxypurinol-expanded T cells possessed preferential TCR clonotypes in allopurinol–SCAR.

DISCUSSION

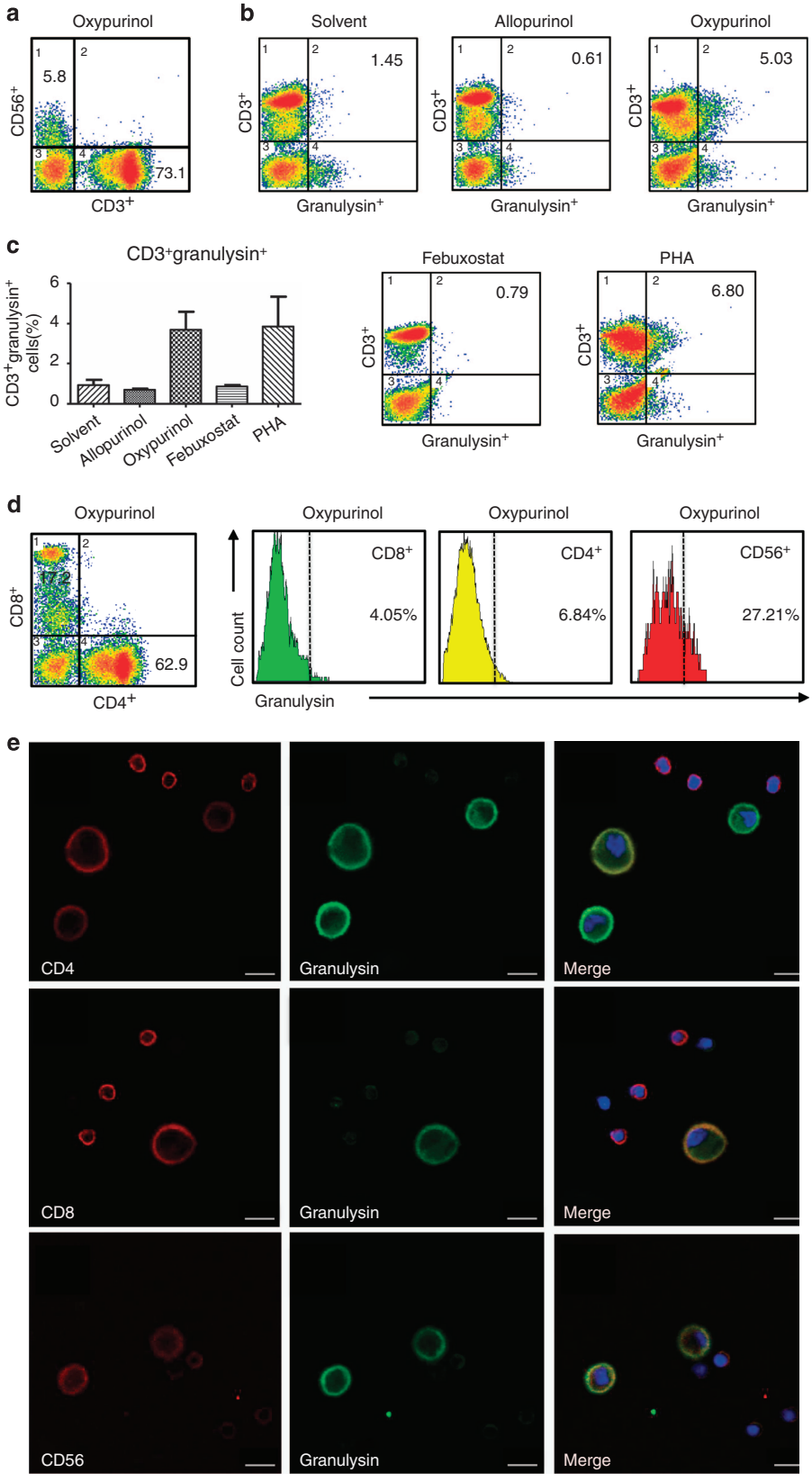
Despite low incidence of SCAR, the mortality rate is ~10% for SJS, 30% for SJS/TEN overlap, and 50% for TEN (Roujeau and Stern, 1994; Roujeau *et al.*, 1995; Sekula *et al.*, 2013). Different methods and techniques have been used to identify the causative drug and avoid the recurrent induction of SCAR. These include patch tests (Barbaud *et al.*, 2013), LTTs (Pichler and Tilch, 2004), cytokine release tests, and delayed reading of intradermal tests (Macías *et al*, 2007). However, the results of these tests were often negative for allopurinol-related SCAR (Porebski *et al.*, 2013). Here we show that a granulysin-based T-cell activation assay validated oxypurinol as the drug antigen in allopurinol–SCAR with high specificity and sensitivity. The oxypurinol-specific T cells could be expanded *in vitro* from the blood samples of patients with allopurinol–SCAR, possessed specific CDR3 clonotypes, and expressed granulysin upon induction. Oxypurinol-specific T cells might be very few in the blood samples of allopurinol–SCAR patients in the recovery stage, and a 2-week *in vitro* culture is required for detecting the secondary activation of oxypurinol-specific T cells.

The *HLA-B*58:01* is a genetic marker of allopurinol–SCAR and has been proposed to present drug antigen to T cells for the induction of drug hypersensitivity (Hung *et al.*, 2005; Pan *et al.*, 2014; Lin *et al.*, 2015). Recent studies using PBMCs mostly from healthy donors with *HLA-B*58:01* showed that oxypurinol directly activated naive T cells via the preferential use of *HLA-B*58:01* (Yun *et al.*, 2013, 2014). The *in vitro* expanded T cells reacted immediately to the drug antigen and bypassed intracellular antigen processing, consistent with the "pharmacological interaction with immune receptors" (p-i) concept (Yun *et al.*, 2013, 2014). In this study, we cultured the blood samples of allopurinol–SCAR patients and found that oxypurinol induced T-cell activation in a dose-dependent manner, consistent with a recent report (Yun *et al.*, 2013). However, our PBMCs from tolerant controls or healthy donors carrying *HLA-B*58:01* failed to activate T cells. We found that the TCR repertoire of PBMCs of healthy donors or oxypurinol-cultured cells from tolerant controls carrying *HLA-B*58:01* showed polyclonal and high diversity. Our results suggested that the presence of high concentrations of oxypurinol and the *HLA-B*58:01* risk allele was insufficient to induce specific T-cell activation in allopurinol–SCAR. These data were consistent with epidemiologic observations showing that the incidence of allopurinol–SCAR is very low, and most individuals carrying *HLA-B*58:01* are tolerant to allopurinol administration.

Different mechanisms of HLA-associated drug hypersensitivity have been described (Yun *et al.,* 2012; Pan *et al.*, 2014). Abacavir hypersensitivity is associated with the *HLA-B*57:01* allele with a positive predictive value of ~55% in Caucasians (Mallal *et al.*, 2002; Hetherington *et al.*, 2002). Abacavir hypersensitivity has been explained by the "altered peptide repertoire hypothesis," in which abacavir may change the shape and chemistry of the antigen-binding cleft of *HLA-B*57:01*, thereby altering the repertoire of endogenous peptides (Illing *et al.*, 2012; Ostrov *et al.*, 2012). The *in vitro* abacavir-activated T cells showed heterogeneous and polyclonal TCR usages, and the *HLA-B*57:01* allele, rather than TCR, has been proposed to have the major role in abacavir hypersensitivity (Adam *et al.*, 2012; Bharadwaj *et al.*, 2012; Illing *et al.*, 2012; Ostrov *et al.*, 2012). Another example is the association between carbamazepine-induced SJS/TEN and the *HLA-B*15:02* allele that has a very low positive predictive value (Chung *et al.*, 2004). Our previous study reported that HLA-B*15:02 possessed affinity toward carbamazepine or its metabolite and was able to present the drug antigens to T cells via "pharmacological interaction with immune receptors" (p-i) mechanism (Wei *et al.*, 2012). Shared and restricted TCR clonotypes were noted in the *in vitro* expanded T cells from PBMCs of carbamazepine–SJS/TEN patients (Ko *et al.*, 2011). In this study, we found preferential TCR usage, clonal expansion, and similar CDR3 clonotypes in the blister cells of patients with allopurinol–SCAR. However, we did not observe identical TCR clonotypes in the samples of allopurinol–SCAR. The TCR repertoire of allopurinol–SCAR seems more complex and different from that of carbamazepine–SJS/TEN (Ko *et al.*, 2011). In addition, the *in vitro* expansion of antigen-specific T cells may distort

the T-cell repertoire (Koning *et al.*, 2014). More experiments and samples directly isolated from the skin lesions are needed to investigate the mechanism of SCAR.

In addition to the predisposition of susceptible *HLA* alleles and the induction of specific TCR clonotypes, deficiency of drug metabolism or impaired drug clearance



has been linked to SCAR (Chung *et al.*, 2014a). The risk factors for allopurinol–SCAR include *HLA-B*58:01* allele, renal insufficiency, and delayed clearance of oxypurinol (Chung *et al.*, 2014b). Chronic renal diseases may delay the clearance of oxypurinol and increase the plasma levels of the drug antigen. In this study, we reported that supraphysiological concentrations of oxypurinol induced specific T-cell activation in PBMC cultures from patients with allopurinol–SCAR. However, this does not reflect physiological conditions, where oxypurinol may work with a number of other cofactors in a synergistic manner to become more immunogenic for low-avidity T cells in susceptible subjects.

In summary, our data demonstrated that granulysin-based T-cell activation assay is a sensitive method for examining the causative drug of allopurinol–SCAR. We propose that the mechanism underlying the pathogenesis of allopurinol-induced SCAR involves both *HLA-B*58:01* and clonotype-specific T cells with a dose-dependent response to oxypurinol. T cells from patients with allopurinol–SCAR did not crossreact to febuxostat. These data suggest that clonotype-specific T cells expressing granulysin upon oxypurinol induction participate in the pathogenesis of allopurinol-induced SCAR.

MATERIALS AND METHODS

Patient enrollment and sample collection

Patients with allopurinol–SCAR were recruited at the Chang Gung Memorial Hospital Health System in Taiwan between 2011 and 2013. SJS/TEN or DRESS diagnoses were based on the definition of the RegiSCAR study group (Auquier-Dunant *et al.*, 2002; Kardaun *et al.*, 2007). Only patients with a probable or a definite diagnosis of SJS/TEN or DRESS were enrolled in this study. The Naranjo algorithm (Naranjo *et al.*, 1981) and the algorithm of drug causality assessment for SJS/TEN (ALDEN) (Sassolas *et al.*, 2010) were applied to identify the offending drug as allopurinol. In addition, we enrolled 23 healthy donors and 11 tolerant subjects who had received allopurinol for >6 months without any adverse reactions. We collected the information, including sex, age, indications for allopurinol use, duration and dose of allopurinol exposure, and *HLA-B* genotyping results of the enrolled subjects. *HLA-B* genotypes were determined by SeCore HLA Sequence–based typing (Invitrogen, Life Technologies, Carlsbad, CA). Approval for the study was obtained from the institutional review board, and written informed consent was obtained from each participant.

T-cell activation assay

PBMCs were isolated from the whole blood samples using Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden) density gradient centrifugation. PBMCs (1.0×10^6 per well) were cultured in 96-well microplates in RPMI-1640 medium (GIBCO Invitrogen, Life Technologies, Carlsbad, CA) supplemented with 10% autologous serum, IL-7 (1 ng ml^{-1}; Invitrogen), and the drugs for examination at 37 °C in 5% CO_2 for 1 to 3 weeks. Allopurinol, oxypurinol (Sigma-Aldrich, Munich, Germany), febuxostat (Teijin Pharma, Tokyo, Japan), or tolerant drugs were diluted in the medium to obtain a concentration reflecting the physiological therapeutic level (noted as 1-fold or 10-fold; Brunton *et al.*, 2011). Thus, cells were incubated in the medium containing allopurinol (10 and 100 μM), oxypurinol (10 and 100 μg ml^{-1}), febuxostat (4 and 40 μg ml^{-1}), or tolerant drugs that had been used by the patients for >3 months without any adverse reactions. In addition, the solvent was added to the medium as the negative control, and phytohemagglutinin at a concentration of 10 μg ml^{-1} was used as the positive control. Culture supernatants were collected on days 7, 14, and 21 for measuring the levels of granulysin using ELISAs (Chung *et al.*, 2008). Samples were analyzed in triplicate. The assay sensitivity for granulysin was 1.56 ng ml^{-1}. In addition, the levels of IFN-γ in samples were measured using IFN-γ ELISA kits (Invitrogen, Carlsbad, CA) with a sensitivity of 1.56 pg ml^{-1}.

Lymphocyte transformation test

The culprit drugs of SCAR patients were tested by conventional LTT using H^3-thymidine incorporation (Pichler and Tilch, 2004). Briefly, PBMCs (1.0×10^6 cells per well) were cultured in 96-well U-bottom plates containing medium in the presence of allopurinol, oxypurinol, or febuxostat. After 6 days of culture, 1 μCi H^3-thymidine was added for an overnight incubation. The cells were harvested and H^3-thymidine incorporation was measured as counts per min (c.p.m.) on a Top-Count. The proliferative response was calculated as the stimulation index: c.p.m. of drug-treated cultures/c.p.m. of cultures with solvent control. Stimulation index of >2 was considered as a positive response.

Flow cytometry, immunofluorescence analysis, and confocal microscopy

Flow cytometry was carried out using distinct fluorochrome-conjugated mAbs that recognize human CD3, CD4, CD8, CD45, CD56 (Beckman Coulter, Fullerton, CA), CD107a (Ebioscience, San Diego, CA), or intracellular granulysin (RB1) (BD Biosciences, Bedford, MA). These mAbs were labeled with Alexa Fluor 488, phycoerythrin-Texas Red (ECD), phycoerythrin-cyanine 5 (PC5), or phycoerythrin-cyanin 7 (PC7). The cells were examined by means of multicolor flow cytometry on the Cytomics FC500 flow cytometer

Figure 3. Oxypurinol-expanded T cells express granulysin. Peripheral blood mononuclear cells (PBMCs) from three patients (cases 13, 16, and 20) with allopurinol–severe cutaneous adverse reaction (SCAR) were cultured with allopurinol (100 μM), oxypurinol (100 μg ml^{-1}), febuxostat (40 μg ml^{-1}), solvent control, or phytohemagglutinin (PHA; 10 μg ml^{-1}) for 2 weeks. The immunophenotype of the cultured PBMCs was determined by flow cytometry. (**a**) Oxypurinol-expanded CD45$^+$ PBMCs comprised CD3$^+$ T lymphocytes (73.1%) and CD56$^+$ natural killer (NK) cells (5.8%). (**b** and **c**) Frequencies of CD3$^+$ granulysin$^+$ T cells in the PBMC cultures with different drugs and controls. Data of **c** are expressed as mean ± SD of three independent experiments (cases 13, 16, and 20). (**d**) The oxypurinol-cultured CD3$^+$ T cells from case 20 comprised CD4$^+$ T lymphocytes (62.9%) and CD8$^+$ T cells (17.2%). When comparing with the solvent controls, there were increases in 4.05% in CD8$^+$granulysin$^+$ cells, 6.84% in CD4$^+$granulysin$^+$ cells, and 27.21% in CD56$^+$granulysin$^+$ cells. (**e**) Photographs depicting oxypurinol-expanded cells expressing granulysin, CD4, CD8, and CD56. The blue color represented 4′,6-diamidino-2-phenylindole-stained nuclei of cells. Scale bar = 10 μm.

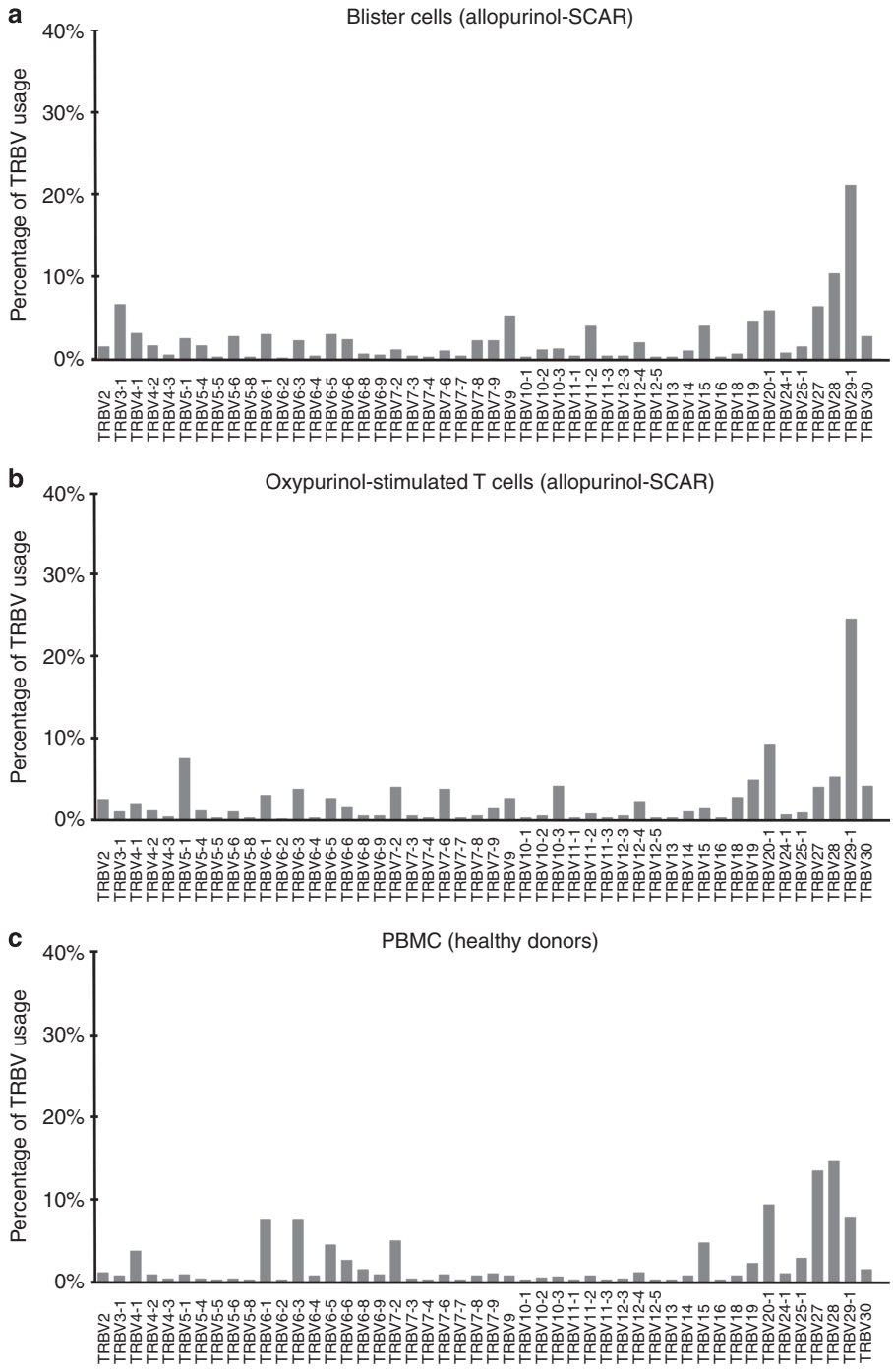


Figure 4. The *T-cell receptor β variable* (*TRBV*) gene usage in allopurinol–severe cutaneous adverse reaction (SCAR). TCR repertoire was analyzed using RNA extracted from (**a**) eight samples of blister cells from skin lesions of allopurinol–Stevens–Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN) patients, (**b**) oxypurinol-cultured T cells from four patients with allopurinol–SCAR, and (**c**) peripheral blood mononuclear cells (PBMCs) from eight healthy donors. Bars represent the mean values of the percentages of respective *TRBV* gene usage in the group of samples. The *P*-values were analyzed by Student's *t*-test. When compared with the *TRBV* usage of PBMCs from 8 healthy donors, the frequencies of *TRBV*3-1, 5-1, 9, and 29-1 were significantly increased in the blister cells and oxypurinol-cultured T cells from the allopurinol–SCAR patients ($P<0.05$).

(Beckman Coulter), and data were analyzed with the CXP software (Beckman Coulter). Immunofluorescence staining was performed using mouse anti-CD4 (RPA-T4, Ebioscience), mouse anti-CD8 (RPA-T8, Ebioscience), mouse anti-CD56 (MEM-188, BioLegend, Uithoorn, The Netherlands), rabbit anti-granulysin (H-130, Santa Cruz Biotechnology, Santa Cruz, CA), and specific fluorescence-conjugated secondary antibodies (Molecular Probes, Eugene, OR). The nuclei of cells were stained by 4′,6-diamidino-2-phenylindole.

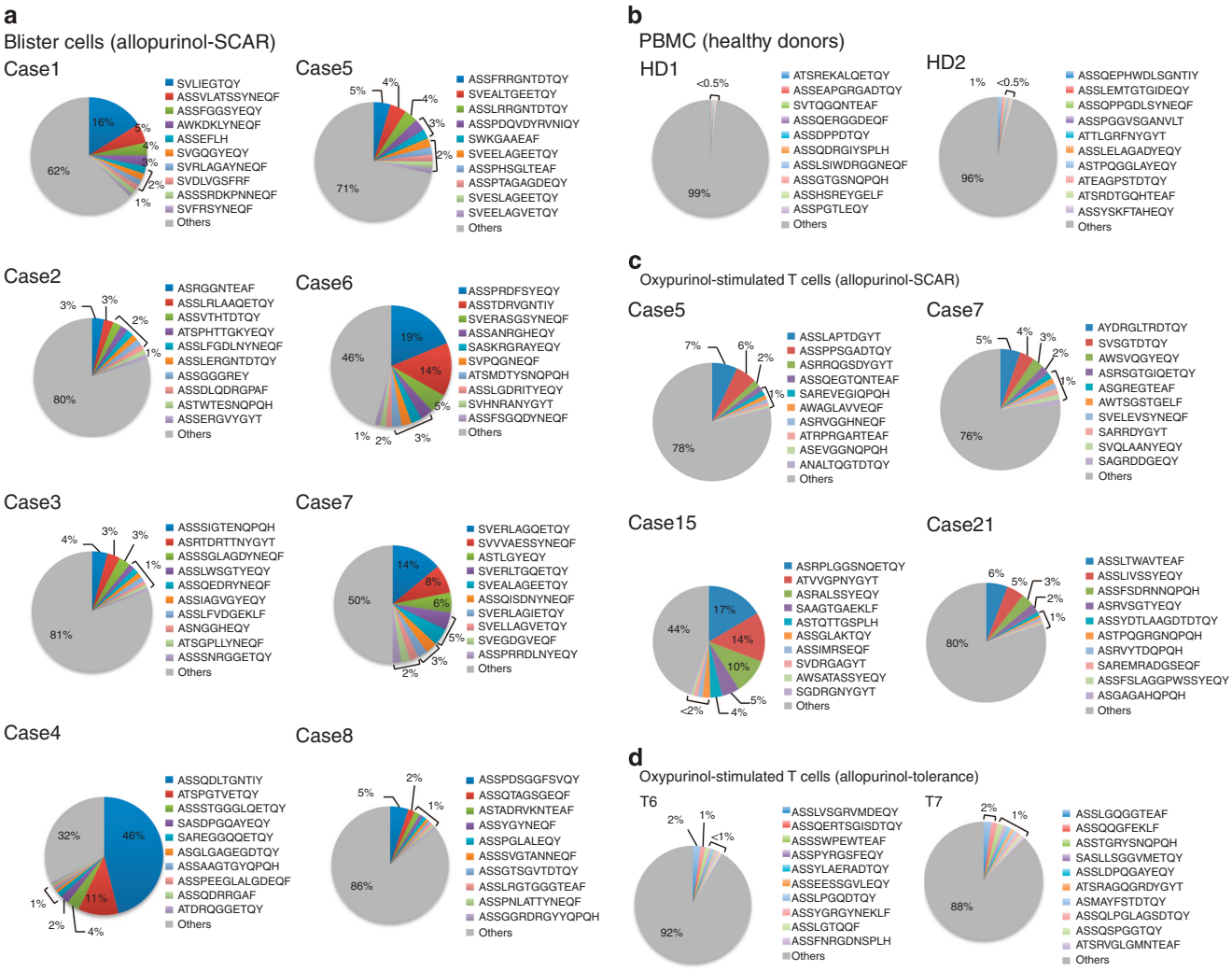


Figure 5. TCRβ CDR3 (third complementarity-determining region) clonotypic analysis of T cells in allopurinol–severe cutaneous adverse reaction (SCAR). Pie charts indicate the TCRβ CDR3 clonotypes of T cells from (**a**) blister cells of eight patients with allopurinol–Stevens–Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN), (**b**) peripheral blood mononuclear cells (PBMCs) of two representative healthy donors, (**c**) oxypurinol-cultured PBMCs from four patients with allopurinol–SCAR, and (**d**) oxypurinol-cultured PBMCs from two tolerant controls. The amino-acid sequences of the 10 most common TCRβ CDR3 clonotypes of each sample are listed, and the frequencies of the individual clonotypes are shown in the pie charts. Gray indicates other CDR3 clonotypes found in the sample, in which the frequency of the individual CDR3 clonotype is <1%. Clonal expansion of specific CDR3 was noted in the samples of blister cells (**a**) and the oxypurinol-cultured PBMCs from patients with allopurinol–SCAR (**c**) but not the PBMCs from healthy donors (**b**) or oxypurinol-cultured PBMCs from tolerant controls (**d**).

The Olympus FV1000 confocal microscope (Tokyo, Japan) and FV10-ASW 3.1 viewer software were used for image analysis.

TCR repertoire investigation by next-generation sequencing

PCR amplification and next-generation sequencing of the VDJ junction and the rearranged CDR3 regions of TCR were performed as previously described (Wang *et al.*, 2010). Briefly, RNA was isolated from the blister cells and the oxypurinol-stimulated cultures of PBMCs from the patients with allopurinol–SCAR. Multiple PCR amplifications were carried out using a panel of TCR primers specifically targeted to all the V and J regions of the TCRβ (iRepertoire, Huntsville, AL). The complementary DNA library was sequenced using Illumina Miseq system (San Diego, CA) or Roche 454 junior system (Branford, CT). The CDR3 interval of TCR transcripts was identified as comprising all the amino acids between the Y[YFLI]C at the 3′ end of the V gene segment and [FW]GXGT (X represents any amino acid) within the J segments (Wang *et al.*, 2010). The TRBV, TRBJ, and CDR3 clonotypes were defined according to the ImMunoGeneTics (IMGT) information database (www.imgt.org), as previously described (Yousfi Monod *et al*, 2004).

Statistical analysis

The fold changes of granulysin or IFN-γ expression in the culture supernatant of PBMCs stimulated by allopurinol, oxypurinol, febuxostat, or tolerant drugs were calculated by dividing the data by the solvent control. Significant difference between the groups was analyzed by one-sample *t*-test or Student's *t*-test. The sensitivity and specificity were calculated according to the standard definitions. All

Table 1. The preferential TCRβ CDR3 clonotypes in blister cells and oxypurinol-expanded T cells from patients with allopurinol-induced SCAR

			Preferential CDR3 clonotypes in the blister cells of 8 patients with allopurinol-SJS/TEN					Frequencies of the specific CDR3 in PBMCs of 8 healthy donors	
Case no.	**Symptom**		**CDR3 clonotypes**	***TRBV***	***TRBJ***	**Frequency (%)**	**Specific CDR3 reads/total reads**	**Frequency (%)**	**Specific CDR3 reads/ sum of the total reads**
Case 1	TEN	1	SVLIEGTQY	29-1	2-5	16.07	419/2,608	0	0/6,369,051
		2	ASSVLATSSYNEQF	5-6	2-1	5.45	142/2,608	0	0/6,369,051
		3	ASSFGGSYEQY	5-6	2-7	3.60	94/2,608	0.000109	88/6,369,051
		4	AWKDKLYNEQF	12-4	2-1	3.08	80/2,608	0	0/6369051
Case 2	TEN	1	ASRGGNTEAF	6-1	1-1	3.38	24,870/736,671	0.000188	155/6,369,051
Case 3	TEN	1	ASSSIGTENQPQH	28	1-5	4.20	27,778/661,129	0	0/6,369,051
		2	ASRTDRTTNYGYT	4-2	1-2	3.45	22,790/661,129	0	0/6,369,051
		3	ASSSGLAGDYNEQF	28	2-1	3.12	20,641/661,129	0	0/6,369,051
Case 4	TEN	1	ASSQDLTGNTIY	3-1	1-3	46.08	327,103/709,797	0	0/6,369,051
		2	ATSPGTVETQY	15	2-5	11.03	78,292/709,797	0	0/6,369,051
		3	ASSSTGGGLQETQY	28	2-5	3.51	24,881/709,797	0	0/6,369,051
Case 5	TEN	1	ASSFRRGNTDTQY	11-2	2-3	4.65	298/6,419	0.0007	7/6,369,051
		2	SVEALTGEETQY	29-1	2-5	4.52	290/6,419	0	0/6,369,051
		3	ASSLRRGNTDTQY	11-2	2-3	3.61	231/6,419	0	0/6,369,051
		4	ASSPDQVDYRVNIQY	6-6	2-4	3.36	215/6,419	0	0/6,369,051
Case 6	SJS/TEN	1	ASSPRDFSYEQY	9	2-7	18.83	1,027/5,456	0.0005	5/6,369,051
		2	ASSTDRVGNTIY	19	1-3	14.29	779/5,456	0	0/6,369,051
		3	SVERASGSYNEQF	29-1	2-1	5.11	279/5,456	0	0/6,369,051
		4	ASSANRGHEQY	7-6	2-7	3.46	188/5,456	0	0/6,369,051
Case 7	SJS/TEN	1	SVERLAGQETQY	29-1	2-5	14.00	166/1,187	0	0/6,369,051
		2	SVVVAESSYNEQF	29-1	2-1	7.85	93/1,187	0	0/6,369,051
		3	ASTLGYEQY	10-2	2-7	5.55	66/1,187	0.0016	15/6,3690,51
		4	SVERLTGQETQY	29-1	2-5	4.80	57/1,187	0	0/6,369,051
		5	SVEALAGEETQY	29-1	2-5	4.74	56/1,187	0	0/6,369,051
		6	ASSQISDNYNEQF	14	2-1	3.22	38/1,187	0	0/6,369,051
Case 8	SJS	1	ASSPDSGGFSVQY	5-4	2-7	5.09	30,640/601,764	0	0/6,369,051

			Preferential CDR3 clonotypes in the oxypurinol-expanded T cells from PBMCs of 4 patients with allopurinol–SCAR					Frequencies of the specific CDR3 in the solvent-incubated control	
Case no.	**Symptom**		**CDR3 clonotypes**	***TRBV***	***TRBJ***	**Frequency (%)**	**Specific CDR3 reads/ total CDR3 reads**	**Frequency (%)**	**Specific CDR3 reads/ total CDR3 reads**
Case 5	TEN	1	ASSLAPTDGYT	5-1	1-2	6.94	456/6,576	0	0/4,125
		2	ASSPPSGADTQY	18	2-3	5.76	379/6,576	0	0/4,125
Case 7	SJS/TEN	1	AYDRGLTRDTQY	30	2-3	5.42	572/10,560	0	0/2,415
		2	SVSGTDTQY	29-1	2-3	3.95	417/10,560	0	0/2,415
		3	AWSVQGYEQY	30	2-7	3.10	327/10,560	1.2008	29/2,415
Case 15	DRESS	1	ASRPLGGSNQETQY	7-6	2-5	16.50	975/5,909	0	0/4,080
		2	ATVVGPNYGYT	10-3	1-2	14.25	842/5,909	0.0490	2/4,080
		3	ASRALSSYEQY	5-1	2-7	10.31	609/5,909	0	0/4,080
		4	SAAGTGAEKLF	20-1	1-4	5.09	300/5,909	0	0/4,080
		5	ASTQTTGSPLH	19	1-6	3.46	204/5,909	0	0/4,080
Case 21	DRESS	1	ASSLTWAVTEAF	27	1-1	5.80	34,484/594,957	0.0006	4/650,727
		2	ASSLIVSSYEQY	7-2	2-7	4.73	28,153/594,957	0.0007	5/650,727
		3	ASSFSDRNNQPQH	28	1-5	3.37	20,051/594,957	0	0/650,727

Abbreviations: CDR3, third complementarity-determining region; DRESS, drug reaction with eosinophilia and systemic symptom; PBMC, peripheral blood mononuclear cell; SCAR, severe cutaneous adverse reaction; SJS, Stevens–Johnson syndrome; TEN, toxic epidermal necrolysis; *TRBJ, T-cell receptor β joining*; *TRBV, T-cell receptor β variable*.

P-values were two tailed, and a *P*-value of <0.05 was considered statistically significant. Statistical analyses were performed using the SPSS software Version 18.0 (SPSS, Chicago, IL). The phylogenetic tree of TCRβ CDR3 clonotypes was analyzed by the software MATLAB version 8.1 (MathWorks, Natick, MA).

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by grants from the National Science Council, Taiwan (NSC101-2325-B-182A-012, NSC101-2320-B-010-072-MY3, NSC101-2321-B-010-027, NSC101-2628-B-182-001-MY3, NSC101-2321-B-182-008, and NSC102-2314-B-010-014-MY3), and grants from Chang Gung Memorial Hospital (BMRPG290011, CMRPG-290051–3, OMRPG2C0011, OMRPG2C0021, and CLRPG340599).

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Adam J, Eriksson KK, Schnyder B *et al.* (2012) Avidity determines T-cell reactivity in abacavir hypersensitivity. *Eur J Immunol* 42:1706–16

Auquier-Dunant A, Mockenhaupt M, Naldi L *et al.* (2002) Correlations between clinical patterns and causes of erythema multiforme majus, Stevens-Johnson syndrome, and toxic epidermal necrolysis: results of an international prospective study. *Arch Dermatol* 138:1019–24

Barbaud A, Collet E, Milpied B *et al.* (2013) A multicentre study to determine the value and safety of drug patch tests for the three main classes of severe cutaneous adverse drug reactions. *Br J Dermatol* 168:555–62

Becker MA, Schumacher HR Jr., Wortmann RL *et al.* (2005) Febuxostat compared with allopurinol in patients with hyperuricemia and gout. *N Engl J Med* 353:2450–61

Betts MR, Brenchley JM, Price DA *et al.* (2003) Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. *J Immunol Methods* 281:65–78

Bharadwaj M, Illing P, Theodossis A *et al.* (2012) Drug hypersensitivity and human leukocyte antigens of the major histocompatibility complex. *Annu Rev Pharmacol Toxicol* 52:401–31

Braden GL, Warzynski MJ, Golightly M *et al.* (1994) Cell-mediated immunity in allopurinol-induced hypersensitivity. *Clin Immunol Immunopathol* 70: 145–51

Brunton LL, Chabner BA, Knollman BC (eds). (2011) *Goodman & Gilman's The Pharmacological Basis of Therapeutics*. 12th edn. Macmillan: New York

Chung WH, Hung SI, Hong HS *et al.* (2004) Medical genetics: a marker for Stevens-Johnson syndrome. *Nature* 428:486

Chung WH, Hung SI, Yang JY *et al.* (2008) Granulysin is a key mediator for disseminated keratinocyte death in Stevens-Johnson syndrome and toxic epidermal necrolysis. *Nat Med* 14:1343–50

Chung WH, Chang WC, Lee YS *et al.* (2014a) Genetic variants associated with phenytoin-related severe cutaneous adverse reactions. *JAMA* 312: 525–34

Chung WH, Chang WC, Stocker SL *et al.* (2014b) Insights into the poor prognosis of allopurinol-induced severe cutaneous adverse reactions: the impact of renal insufficiency, high plasma levels of oxypurinol and granulysin. *Ann Rheum Dis* advance online publication, 12 August 2014; pii: annrheumdis-2014-205577. doi:10.1136/annrheumdis-2014-205577 [e-pub ahead of print]

Espiritu CR, Alalu J, Glueckauf LG *et al.* (1976) Allopurinol-induced granulomatous hepatitis. *Am J Dig Dis* 21:804–6

Goldfarb DS, MacDonald PA, Hunt B *et al.* (2011) Febuxostat in gout: serum urate response in uric acid overproducers and underexcretors. *J Rheumatol* 38:1385–9

Hetherington S, Hughes AR, Mosteller M *et al.* (2002) Genetic variations in HLA-B region and hypersensitivity reactions to abacavir. *Lancet* 359: 1121–2

Hung SI, Chung WH, Liou LB *et al.* (2005) HLA-B*5801 allele as a genetic marker for severe cutaneous adverse reactions caused by allopurinol. *Proc Natl Acad Sci USA* 102:4134–9

Illing PT, Vivian JP, Dudek NL *et al.* (2012) Immune self-reactivity triggered by drug-modified HLA-peptide repertoire. *Nature* 486:554–8

Kang HR, Jee YK, Kim YS *et al.* (2011) Positive and negative associations of HLA class I alleles with allopurinol-induced SCARs in Koreans. *Pharmacogenet Genomics* 21:303–7

Kaniwa N, Saito Y, Aihara M *et al.* (2008) HLA-B locus in Japanese patients with anti-epileptics and allopurinol-related Stevens-Johnson syndrome and toxic epidermal necrolysis. *Pharmacogenomics* 9:1617–22

Kardaun SH, Sidoroff A, Valeyrie-Allanore L *et al.* (2007) Variability in the clinical pattern of cutaneous side-effects of drugs with systemic symptoms: does a DRESS syndrome really exist? *Br J Dermatol* 156:609–11

Ko TM, Chung WH, Wei CY *et al.* (2011) Shared and restricted T-cell receptor use is crucial for carbamazepine-induced Stevens-Johnson syndrome. *J Allergy Clin Immunol* 128:1266–76

Koning D, Costa AI, Hasrat R *et al.* (2014) In vitro expansion of antigen-specific CD8(+) T cells distorts the T-cell repertoire. *J Immunol Methods* 405: 199–203

Lin CH, Chen JK, Ko TM *et al.* (2015) Immunologic basis for allopurinol-induced severe cutaneous adverse reactions: HLA-B*58:01-restricted activation of drug-specific T cells and molecular interaction. *J Allergy Clin Immunol* 135:1063–5

Lonjou C, Borot N, Sekula P *et al.* (2008) A European study of HLA-B in Stevens-Johnson syndrome and toxic epidermal necrolysis related to five high-risk drugs. *Pharmacogenet Genomics* 18:99–107

Macías E, Ruiz A, Moreno E *et al.* (2007) Usefulness of intradermal test and patch test in the diagnosis of nonimmediate reactions to metamizol. *Allergy* 62:1462–4

Mallal S, Nolan D, Witt C *et al.* (2002) Association between presence of HLA-B*5701, HLA-DR7, and HLA-DQ3 and hypersensitivity to HIV-1 reverse-transcriptase inhibitor abacavir. *Lancet* 359:727–32

Naranjo CA, Busto U, Sellers EM *et al.* (1981) A method for estimating the probability of adverse drug reactions. *Clin Pharmacol Ther* 30:239–45

Ostrov DA, Grant BJ, Pompeu YA *et al.* (2012) Drug hypersensitivity caused by alteration of the MHC-presented self-peptide repertoire. *Proc Natl Acad Sci USA* 109:9959–64

Pan RY, Wu YC, Chung WH *et al.* (2014) HLA and TCR recognition of medications in severe cutaneous adverse reactions. *Curr Immunol Rev* 10:51–61

Phillips EJ, Mallal SA (2010) Pharmacogenetics of drug hypersensitivity. *Pharmacogenomics* 11:973–87

Pichler WJ, Tilch J (2004) The lymphocyte transformation test in the diagnosis of drug hypersensitivity. *Allergy* 59:809–20

Posadas SJ, Padial A, Torres MJ *et al.* (2002) Delayed reactions to drugs show levels of perforin, granzyme B, and Fas-L to be related to disease severity. *J Allergy Clin Immunol* 109:155–61

Porebski G, Gschwend-Zawodniak A, Pichler WJ (2011) In vitro diagnosis of T cell-mediated drug allergy. *Clin Exp Allergy* 41:461–70

Porebski G, Pecaric-Petkovic T, Groux-Keller M *et al.* (2013) In vitro drug causality assessment in Stevens-Johnson syndrome - alternatives for lymphocyte transformation test. *Clin Exp Allergy* 43:1027–37

Ramasamy SN, Korb-Wells CS, Kannangara DR *et al.* (2013) Allopurinol hypersensitivity: a systematic review of all published cases, 1950-2012. *Drug Saf* 36:953–80

Roujeau JC, Stern RS (1994) Severe adverse cutaneous reactions to drugs. *N Engl J Med* 331:1272–85

Roujeau JC, Kelly JP, Naldi L *et al.* (1995) Medication use and the risk of Stevens-Johnson syndrome or toxic epidermal necrolysis. *N Engl J Med* 333:1600–7

Roujeau JC (2006) Immune mechanisms in drug allergy. *Allergol Int* 55:27–33

Sassolas B, Haddad C, Mockenhaupt M *et al.* (2010) ALDEN, an algorithm for assessment of drug causality in Stevens-Johnson Syndrome and toxic epidermal necrolysis: comparison with case-control analysis. *Clin Pharmacol Ther* 88:60–8

Sekula P, Dunant A, Mockenhaupt M *et al.* (2013) Comprehensive survival analysis of a cohort of patients with Stevens-Johnson syndrome and toxic epidermal necrolysis. *J Invest Dermatol* 133:1197–204

Tassaneeyakul W, Jantararoungtong T, Chen P *et al.* (2009) Strong association between HLA-B*5801 and allopurinol-induced Stevens-Johnson syndrome and toxic epidermal necrolysis in a Thai population. *Pharmacogenet Genomics* 19:704–9

Wang C, Sanders CM, Yang Q *et al.* (2010) High throughput sequencing reveals a complex pattern of dynamic interrelationships among human T cell subsets. *Proc Natl Acad Sci USA* 107:1518–23

Wei CY, Chung WH, Huang HW *et al.* (2012) Direct interaction between HLA-B and carbamazepine activates T cells in patients with Stevens-Johnson syndrome. *J Allergy Clin Immunol* 129:1562–9

Yousfi Monod M, Giudicelli V, Chaume D *et al.* (2004) IMGT/JunctionAnalysis: the first tool for the analysis of the immunoglobulin and T cell receptor complex V-J and V-D-J JUNCTIONs. *Bioinformatics* 20:i379–85

Yun J, Adam J, Yerly D *et al.* (2012) Human leukocyte antigens (HLA) associated drug hypersensitivity: consequences of drug binding to HLA. *Allergy* 67: 1338–46

Yun J, Mattsson J, Schnyder K *et al.* (2013) Allopurinol hypersensitivity is primarily mediated by dose-dependent oxypurinol-specific T cell response. *Clin Exp Allergy* 43:1246–55

Yun J, Marcaida MJ, Eriksson KK *et al.* (2014) Oxypurinol directly and immediately activates the drug-specific T cells via the preferential use of HLA-B*58:01. *J Immunol* 192:2984–93