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The predominant drug-specific T-cell population may switch from cytotoxic T cells to regulatory T cells during the course of anticonvulsant-induced hypersensitivity

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ABSTRACT

Background: Delayed hypersensitivity is responsible for severe cutaneous adverse drug reactions (cADRs), especially in Stevens-Johnson syndrome (SJS), toxic epidermal necrolysis, and drug-induced hypersensitivity syndrome (DIHS) (also known as drug rash with eosinophilia and systemic symptoms [DRESS] syndrome). The drug-induced lymphocyte stimulation test (DLST), or lymphocyte transformation test (LTT), is used to identify the culprit drug in severe cADR cases.
Objective: The aim of this study was to examine the immune reactions in cADR patients through the identification of the drug-specific proliferating cells by flow cytometric DLST (FCM-DLST).
Methods: The peripheral blood mononuclear cells of 16 anticonvulsant-induced cADR patients were investigated by conventional DLST and a FCM-DLST protocol in which CFSE dilution and BrdU incorporation were combined. FCM-DLST allowed for the identification of the drug-specific proliferating cells in six cases. Three of these cases were DIHS cases, whereas there was one case of SJS, one case of maculopapular rash (MP), and one case of erythema multiforme (EM) among the six cases.
Results: In FCM-DLST, drug-specific proliferating T cells were detected as $CFSE^{low}$ $BrdU^{high}$ cells. These cells corresponded to the cells incorporating ^{3}H-thymidine in conventional DLST. Although $CD4^{+}$ T-cell proliferation dominated the observed proliferation in most of the cases (in the recovery stage of the three DIHS cases, the MP case, and the EM case), drug-specific $CD8^{+}$ cytotoxic T lymphocytes (CTLs) were detected, especially in the acute stages of the SJS case and one of the DIHS cases. There was a dramatic switch in the predominant drug-specific proliferating T-cell population in the course of one of the cases of DIHS in which $CD8^{+}$ CTLs were predominant initially, whereas $CD4^{+}$ T cells were predominant later. Moreover, drug-specific $CD4^{+}$ $CD25^{+}$ $Foxp3^{+}$ regulatory T cells (Tregs) proliferated during the recovery stage in one DIHS case.
Conclusions: FCM-DLST revealed that the cell proliferation detected by conventional DLST is a heterogeneous proliferation of both $CD8^{+}$ CTLs and $CD4^{+}$ T cells that likely includes Tregs. However, the number of cADR cases in this study was limited, which limits the conclusions that can be drawn from it.

1. Introduction

T-cell-mediated delayed hypersensitivity is responsible for the pathogenesis of severe cutaneous adverse drug reactions (cADRs), including Stevens-Johnson syndrome (SJS), toxic epidermal necrolysis (TEN), and drug-induced hypersensitivity syndrome (DIHS), also called hypersensitivity syndrome (HSS) or drug rash with eosinophilia and systemic symptoms (DRESS) syndrome [1–4]. In severe cADR, the drug-induced lymphocyte stimulation test (DLST), or lymphocyte transformation test (LTT), is used to identify culprit drugs [5]. In conventional DLST, freshly isolated peripheral blood mononuclear cells (PBMCs) are incubated with the culprit drug for 5–7 days, and cell proliferation is measured by ^{3}H-thymidine incorporation. Because ^{3}H-thymidine incorporation occurs in all proliferating PBMCs, drug-specific proliferating cells cannot be individually assessed by conventional DLST.

Flow cytometry (FCM) is a well-established semiquantitative assay that can measure the cell surface and intracellular molecules expressed by individual cells in a heterogeneous population. It has also been used for analyzing the in vitro immune reaction that occurs in DLST [6]. Drug-specific T cells and their cytokine production were detected by a carboxyfluorescein diacetate succinimidyl ester (CFSE)-based proliferation assay [7–9]. CFSE is partitioned equally during cell division, resulting in the

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sequential halving of cellular fluorescent intensity with each successive generation. Although CFSE dilution is usually a useful technique for the measurement of cell proliferation, cells that have proliferated are sometimes indistinguishable from a non-specific peak of dead cells, especially when the cell proliferation is small in magnitude, which it often is in a DLST reaction. 5-bromo-2′-deoxyuridine (BrdU) is a non-radioactive thymidine analogue that becomes incorporated into DNA during the S-phase of the cell cycle (Fig. 1A). Here, drug-specific proliferating cells were identified by a flow cytometric DLST (FCM-DLST) protocol that combines CFSE dilution and BrdU incorporation and utilizes them as a substitute for ^{3}H-thymidine incorporation. The combination of the CFSE and BrdU assays allows for the clear identification of the very small proliferating cell population as $CFSE^{low}$ $BrdU^{high}$ cells. A FCM-DLST protocol that uses the combination of CFSE and BrdU assays can reveal the proliferating drug-specific cell population responsible for the proliferation found by conventional DLST. We took advantage of this feature and analyzed the drug-specific T cells of anti-convulsant hypersensitivity patients during the acute and recovery stages of the disease. Interestingly, drug-specific $CD8^{+}$ T cells were detected only in the acute stage of severe drug hypersensitivity, whereas drug-specific $CD4^{+}$ T cells were found to be dominant in the recovery stage. Moreover, the percentage of drug-specific $CD4^{+}$ T cells that were $Foxp3^{+}$ regulatory T cells (Tregs) was increased during the recovery stage in one of the DIHS cases, suggesting that different subsets of drug-specific T cells are induced during different disease stages of a cADR.

2. Materials and methods

2.1. Patients

Sixteen patients clinically diagnosed with anticonvulsant-induced cADR were enrolled in this study from July 2008 to July 2011. Conventional DLST was performed in all 16 cases, while FCM-DLST was performed in six of the cases. Our institutional review board approved this study, and informed consent for all diagnostic procedures and research was obtained from all patients and healthy controls.

2.2. Cell preparation and culture

Cell preparation and culture for DLST were performed in accordance with standard DLST protocols [6,10,11]. Briefly, PBMCs were isolated with Ficoll-Hypaque solution (Sigma–Aldrich), labeled with 6 mM CFSE (Invitrogen), and cultured at 2×10^{5} cells/well in two 96-well flat-bottomed plates for 7 days. After addition of the identified culprit drug, one plate was used for conventional DLST and one for FCM-DLST (Fig. 1B).

2.3. Preparation of culprit drugs

Culprit drugs were dissolved in phosphate-buffered saline (PBS), or PBS with 0.025% dimethyl sulfoxide (Wako) if the drug was PBS-insoluble, and added to the PBMC culture medium at the beginning of incubation (day 0). Sodium valproate was PBS-soluble, whereas phenytoin, zonisamide, and carbamazepine were PBS-insoluble. The final drug concentrations were 100, 10, and 1 μg/ml. PBMCs were also incubated without a drug (negative control) and with 10 μg/ml phytohemagglutinin (PHA, Sigma–Aldrich; positive control).

2.4. ^{3}H-thymidine incorporation assay for DLST

Conventional DLST that used ^{3}H-thymidine was performed as previously described [10,11]. The results are presented as the stimulation index (SI), which was the ratio of the highest count per minute of the samples cultured with diluted drug to that of the control cultured without a drug. A SI value >2.0 was interpreted as a positive result.

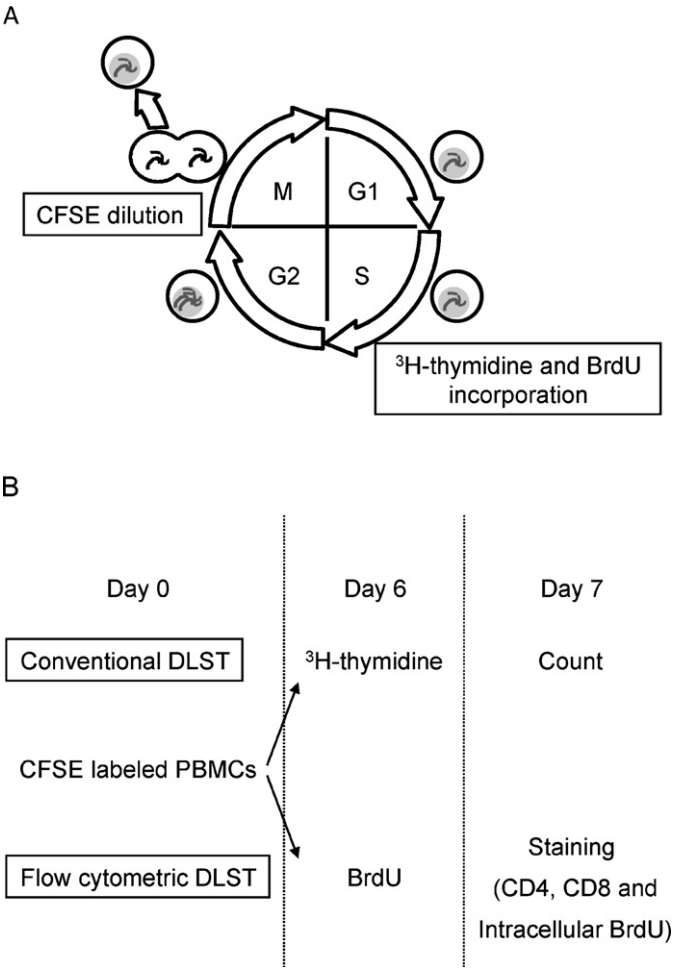

Fig. 1. (A) Cell cycle scheme. ^{3}H-thymidine and BrdU are incorporated into S-phase cells, while the CFSE intensity of a labeled cell is reduced by half every cell division. G1: gap 1 phase; S: synthesis phase; G2: gap 2 phase; M: mitotic phase; BrdU: 5-bromo-2′-deoxyuridine; CFSE: carboxyfluorescein diacetate succinimidyl ester. (B) Comparison of the conventional and flow cytometric drug-induced lymphocyte stimulation tests (DLST). A patient's peripheral blood mononuclear cells (PBMCs) were isolated and CFSE-labeled. They were cultured for 7 days in two culture plates with a culprit drug for conventional and flow cytometric DLST (one plate for conventional, one for flow cytometric). Six days after incubation, ^{3}H-thymidine or BrdU were added. After an additional 20–24 h of incubation, the cells were harvested, and ^{3}H-thymidine incorporation was measured for conventional DLST. For flow cytometric DLST, BrdU-pulsed cells were stained with anti-CD4 and anti-CD8 antibodies, fixed, permeabilized, and then intracellularly stained for BrdU.

2.5. Flow cytometric DLST

The other plate of CFSE-labeled PBMCs was incubated with or without culprit drug in the same manner (Fig. 1B). Six days after the start of incubation, 10 μm BrdU (Sigma–Aldrich) was pulsed into the wells instead of ^{3}H-thymidine. After 20–24 h of further incubation, the cells were collected and stained with the following antibodies and reagents: Peridinin chlorophyll protein (PerCP)-conjugated CD4, allophycocyanin (APC)-conjugated CD8, streptavidin–phycoerythrin (PE) (all BD Biosciences), and biotin-conjugated BrdU (Abcam). For intracellular BrdU staining, cells were labeled with CD4 and CD8, fixed and permeabilized with BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit (BD Biosciences), and treated with 0.3 mg/ml Deoxyribonuclease I

(Sigma–Aldrich) before BrdU staining [12]. In one case, cells were stained with the following antibodies and reagents: Pacific-Blue-conjugated CD4 (BioLegend), AmCyan-conjugated CD8, PE-conjugated CD45RA, APC-Cy7-conjugated CD25 (BD Biosciences), and APC-conjugated Foxp3 (eBioscience). For intracellular Foxp3 staining, cells were first incubated with anti-CD4, anti-CD8, anti-CD45RA, and anti-CD25 antibodies, then fixed, permeabilized with the Anti-Human Foxp3 Staining Set APC (eBioscience), and stained with anti-Foxp3 (eBioscience). All stained cells were analyzed with a FACS Calibur or FACS Canto II cytofluorometer (BD Biosciences). Subsequent analysis was performed with FlowJo software (TreeStar).

3. Results

3.1. Utility of conventional DLST in the clinical course of cADR

Positive results are not always obtained when conventional DLST is performed during a cADR case. However, in many cADR cases, conventional DLST is positive at certain times of the clinical course. Kano et al. previously reported that regardless of whether patients were treated with systemic prednisolone, positive DLST reactions were obtained in the acute, but not the recovery, stage of MP and SJS/TEN, while the exact opposite was observed in DIHS, where positive reactions were obtained in the recovery, but not the acute, stage [13]. Therefore, we analyzed 16 patients with anti-convulsant-induced delayed-type hypersensitivity to examine the correlation between the SI value of conventional DLST and the examination date after cADR onset. The clinical data of our 16 cADR patients are summarized in Table 1. The SI values dramatically changed in individual patients over the course of the disease, and some patients who were negative by conventional DLST during the acute stage were positive 30 days after disease onset. These results indicated that the drug-specific immune reactions detected by conventional DLST could vary during different clinical stages of the disease course. Therefore, we speculated that the variations in the SI value of conventional DLST might reflect alterations in the immune status and the magnitude of the drug-specific immunity. This led us to focus on the drug-reactive proliferating cells that lead to positivity on conventional DLST through the use of FCM.

3.2. Drug-specific proliferating T cells in conventional DLST are detected as $CFSE^{low}$ $BrdU^{high}$ cells in flow cytometric DLST

To visualize the proliferating cells that incorporate ^{3}H-thymidine in conventional DLST, samples were examined for both CFSE dilution and BrdU incorporation. To exclude the effects of CFSE labeling, CFSE-labeled PBMCs were divided into two samples before incubation, with one aliquot used for conventional DLST and the other for FCM-DLST. PBMCs used for FCM-DLST analysis were incubated in the same manner as for conventional DLST for 6 days and pulsed with BrdU for 24 h (Fig. 1B). Theoretically, CFSE dilution reflects the total number of divided and proliferated cells during the 7-day culture, whereas ^{3}H-thymidine and BrdU incorporation into cells during the synthesis phase of the cell cycle represent cells that proliferated during the last 24 h before cell harvesting.

PBMCs of a patient (Case 1) in the acute stage of phenytoin-induced maculopapular rash were used for FCM-DLST and conventional DLST (Fig. 2). In conventional DLST, PBMCs incorporated ^{3}H-thymidine (SI 5.0) after treatment with 10 μg/ml phenytoin, whereas PBMCs from healthy controls did not (SI 0.81).

Back-gating analysis revealed that the $CFSE^{low}$ population was distributed in the lymphocyte area, indicating that the cells that incorporated ^{3}H-thymidine in conventional DLST were lymphocytes,

Table 1
Summary of the 16 cADR cases examined by conventional DLST. M: male; F: female; cADR: cutaneous adverse drug reaction; d: days; MP: maculopapular rash; SJS: Stevens-Johnson syndrome; DIHS: drug-induced hypersensitivity syndrome; EM: erythema multiforme; TEN: toxic epidermal necrolysis; SI: stimulation index.

Conventional DLST patient number	Age·sex	Culprit drug	Underlying disease	Type of cADR	Days after cADR onset	SI	FCM-DLST patient number
1	38F	Phenytoin	Cerebral arteriovenous malformation	MP	25	5.0	Case 1
					38	24	
2	76M	Phenytoin	Post-operative subdural hematoma	SJS	3	6.4	Case 2
					14	7.5	
3	70M	Phenytoin	Brain metastasis of lung cancer	DIHS	13	3.8	Case 3
					27	6.5	
					68	3.3	
4	53M	Phenytoin	Glioblastoma	MP	16	1.6	
		Carbamazepine			16	1.5	
5	65M	Phenytoin	Brain metastasis of lung cancer	EM	27	2.6	
6	71F	Phenytoin	Glioblastoma	EM	9	16	
					17	24	
7	71F	Phenytoin	Cerebral aneurysm	MP	55	1.7	
8	77M	Phenytoin	Epilepsy	TEN	15	3.2	
9	61F	Phenytoin	Epilepsy	MP	42	1.4	
10	20F	Zonisamide	Epilepsy	DIHS	6	2.4	Case 4
					20	8.9	
					40	2.7	
					97	1.9	
11	30F	Sodium valproate	Migraine	EM	5	4.6	Case 5
					47	2.2	
					84	1.3	
12	74F	Carbamazepine	Peritoneal cancer	DIHS	4	1.4	Case 6
					51	11	
13	67F	Carbamazepine	Mononeuropathy multiplex	MP	11	1.9	
14	51F	Carbamazepine	Pituitary tumor	SJS	16	0.9	
					71	22	
15	29M	Carbamazepine	Herpes encephalitis	SJS	5	1.7	
					33	2.6	
16	24F	Carbamazepine	Epilepsy	SJS	4	2.5	
					11	1.3	
					60	2.5	

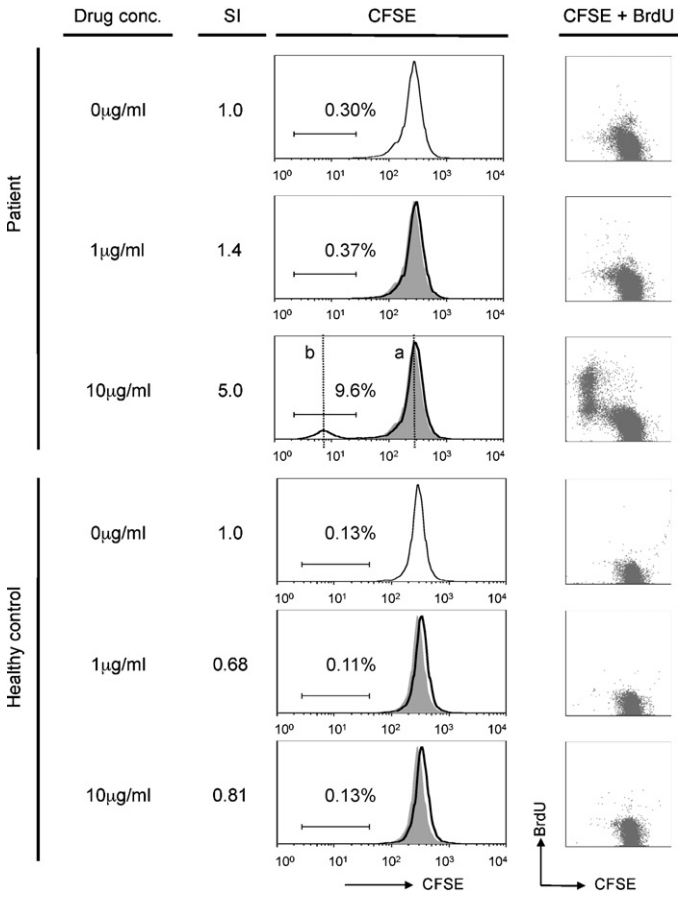

Fig. 2. Representative data of conventional DLST, the CFSE dilution assay alone, and the CFSE dilution assay combined with the BrdU incorporation assay (Case 1 and a healthy control). Drug conc: concentrations of culprit drugs, SI: stimulation index. Corresponding with a positive SI value, 9.6% of the cells were found to be drug-specific proliferating cells by the CFSE dilution assay in the culture treated with 10 μg/ml of a culprit drug. The $CFSE^{low}$ $BrdU^{high}$ population was detected by the dilution assay combined with the BrdU incorporation assay in PBMCs treated at the same concentration (10 μg/ml) of the culprit drug. On the other hand, no cell proliferation was detected at any concentration of culprit drug in healthy control PBMCs. The number of cell divisions was estimated as follows: *a* the value of the peak CFSE fluorescence intensity of the non-proliferating cell population, *b* the value of peak fluorescence intensity of the proliferating cell population. Since CFSE intensity is reduced by half per single cell division, the number of cell divisions can be roughly calculated by taking the binary logarithm of *a* by *b* times ($\log_2 a/b$).

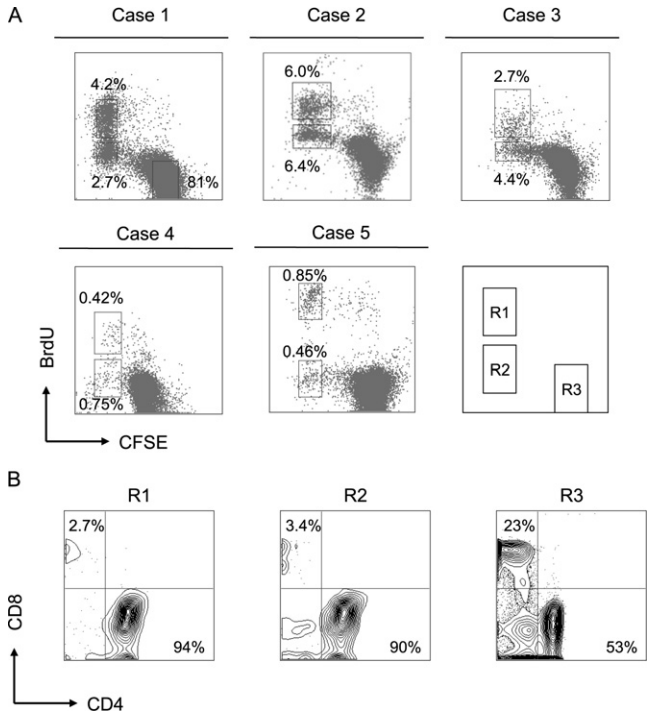

Fig. 3. (A) The results of flow cytometric DLST in five cases and the T-cell subsets found in the flow cytometric DLST of Case 1. Seven-day-cultured PBMCs were categorized into three subpopulations: R1, the $CFSE^{low}$ $BrdU^{high}$ drug-specific proliferating cells that had incorporated BrdU within the last 24 h of culture (days 6–7); R2, the $CFSE^{low}$ $BrdU^{low}$ drug-specific proliferating cell population that had divided until day 6 but did not incorporate BrdU within the last 24 h of culture; and R3, the $CFSE^{high}$ $BrdU^{low}$ non-proliferating cell population. The $CFSE^{low}$ $BrdU^{high}$ population, the population that corresponded to the cells that incorporated ^{3}H-thymidine, was composed entirely of $CD4^+$ and $CD8^+$ T lymphocytes even though the undivided cell population ($CFSE^{high}$ $BrdU^{low}$) contained $CD4^-$ $CD8^-$ non-T cells (R3).

which was consistent to what had been previously reported [13]. Although a few contaminating granulocytes in PBMCs showed greater BrdU incorporation (likely due to these cells being larger than lymphocytes), they never appeared as $CFSE^{low}$ cells, suggesting that granulocytes did not proliferate (Supplementary Fig. 1). Therefore, the lymphocyte gate was used for all further analysis. As shown in a histogram of CFSE fluorescence intensity (Fig. 2), the $CFSE^{low}$ proliferated population (9.6%), the cell population that led to the positive result by conventional DLST, appeared as a small peak when the cells were treated with 10 μg/ml phenytoin. However, cells cultured with 1 μg/ml phenytoin were found to be negative for proliferation by conventional and FCM-DLST. In FCM-DLST, the phenytoin-specific proliferating cells were detected as a $CFSE^{low}$ $BrdU^{high}$ population, which clearly correlated with the SI value determined by conventional DLST.

FCM-DLST allows for a detailed and precise analysis of the drug-specific proliferating cells that correspond to cells that incorporate ^{3}H-thymidine in conventional DLST. We examined CD4 and CD8 expression in the cells that proliferated in FCM-DLST. In a positive case of FCM-DLST, cultured PBMCs were categorized into three populations: R1, the $CFSE^{low}$ $BrdU^{high}$ drug-specific proliferating cells that incorporated BrdU within the last 24 h; R2, the $CFSE^{low}$ $BrdU^{low}$ drug-specific proliferating cell population that did not incorporate BrdU within the last 24 h; and R3, the $CFSE^{high}$ $BrdU^{low}$ non-proliferating cell population (Fig. 3A). The $CFSE^{low}$ $BrdU^{high}$ population, the population that incorporated ^{3}H-thymidine, was composed entirely of $CD4^+$ and $CD8^+$ T lymphocytes even though the undivided cell population ($CFSE^{high}$ $BrdU^{low}$) contained $CD4^-$ $CD8^-$ non-T cells (R3 in Fig. 3B). When four additional cases were analyzed, these three populations were identified in each case (Fig. 3A). Proliferating drug-specific T cells were better isolated in R1 than R2, suggesting that a FCM-DLST protocol that used both CFSE and BrdU was superior to DLST that used CFSE only (Supplementary Fig. 2). To date, we have not detected proliferation in any cells other than T cells.

Since CFSE intensity is reduced by half with every cell division, the number of cell divisions can be calculated (Fig. 2) [14]. The average number of divisions was 5.5 (range: 3.54–6.47; Table 2). Thus, the drug-specific proliferating lymphocytes divided approximately five or six times in the 7 days in DLST-culture medium.

3.3. $CD8^+$ T cells are the predominant proliferating population in a DLST culture of the PBMCs from a severe cADR patient

Drug-specific $CD4^+$ T cells produce cytokines, including interferon-gamma, and this production is related to the

Table 2
Summary of the six cADR cases examined by conventional and flow cytometric DLST concurrently. M: male; F: female; cADR: cutaneous adverse drug reaction; PBMC: peripheral blood mononuclear cell; d: days; MP: maculopapular rash; SJS: Stevens-Johnson syndrome; DIHS: drug induced hypersensitivity syndrome; EM: erythema multiforme; N.D: no data.

Case	Age sex	Days after onset	Days after drug withdrawal	Type of cADR	Culprit drug	SI	Drug-specific T cells in FCM-DLST		Calculated the number of cell division times	CD4(%)/CD8(%) in PBMC
							CD4(%)/CD8(%) (CFSE BrdU)	CD4(%)/CD8(%) (CFSE only)		
1	38F	25d	23d	MP	Phenytoin	5.0	94/2.7	88/3.3	5.38	N.D.
		38d	36d			24	78/0.1	73/2.3	6.21	N.D.
2	76M	14d	12d	SJS	Phenytoin	7.5	41/57	47/47	5.58	N.D.
3	70M	13d	1d	DIHS	Phenytoin	3.8	5.8/86	11/78	4.52	N.D.
		27d	15d			6.5	100/0	85/2.6	6.45	N.D.
4	20F	6d	1d	DIHS	Zonisamide	2.4	N.D.	N.D.	N.D.	15/48
		20d	15d			8.9	82/0	49/4.9	3.54	25/26
		40d	35d			2.7	89/0	77/2.6	5.43	40/26
5	30F	47d	33d	EM	Sodium valproate	2.2	100/0	66/4.1	6.00	N.D.
6	74F	51d	52d	DIHS	Carbamazepine	11	N.D.	83/5.4	6.47	N.D.

pathogenesis of cADR [9]. However, recent reports suggested that $CD8^+$ CTLs are the major effector cells in SJS/TEN [15] and are involved in DIHS development [16]. Therefore, we evaluated the percentage of $CD4^+$ and $CD8^+$ drug-specific T cells in FCM-DLST. PBMCs from six cADR patients who were conventional DLST-positive were analyzed by conventional DLST and FCM-DLST concurrently (Table 2). As previously reported, the drug-specific proliferated cells were mainly $CD4^+$ T cells in four of the six cases. Interestingly, the $CFSE^{low}$ $BrdU^{high}$ population was predominantly $CD8^+$ CTLs in the PBMCs of an SJS patient (Case 2) and those from a patient in the acute stage of DIHS (Case 3). Drug-specific CTLs were preferentially detected in these cases soon after the withdrawal of the culprit drug, demonstrating that ^{3}H-thymidine incorporation in conventional DLST actually represents a complex immune reaction against a drug antigen that could be classified into at least two subgroups according to the type of drug-specific proliferating T cell.

3.4. The predominant drug-specific proliferating cell population in DLST dramatically changes from $CD8^+$ CTLs to $CD4^+$ T lymphocytes in the clinical course of DIHS

Conventional DLST is sometimes measured several times during the course of a cADR, and SI values in the acute stage differ considerably from those in the recovery stage. To study differences in DLST during the clinical course of DIHS, conventional and FCM-DLST were concurrently examined at different time points in two cases of DIHS (Cases 3 and 4). In Case 3, a case of phenytoin-induced DIHS, the human herpes virus-6 immunoglobulin G (HHV-6-IgG) titer increased from 40 × (day 0 after the withdrawal of the culprit drug) to 2560 × (day 14), indicating that HHV-6 was reactivated (Fig. 4). The percentage of the $CFSE^{low}$ $BrdU^{high}$ population (R1) increased from 2.7% (day 1) to 5.4% (day 15) in accordance with the SI. Surprisingly, the major drug-specific proliferating cell population dramatically changed from $CD8^+$ CTLs on day 0 (86%) to $CD4^+$ T lymphocytes on day 14 (100%), indicating that the drug-specific T-cell subsets may play different roles in the pathogenesis of DIHS at different clinical stages.

In Case 4, a case of zonisamide-induced DIHS, the HHV6-IgG titer increased from 20× to 1280×, confirming a reactivation of HHV-6. From days 15 to 35, $CD4^+$ T lymphocytes were the predominant population that exhibited drug-specific proliferation (Fig. 5). However, drug-specific T cells were not detected in the acute stage of this DIHS case, likely because this case was only weakly positive on conventional DLST.

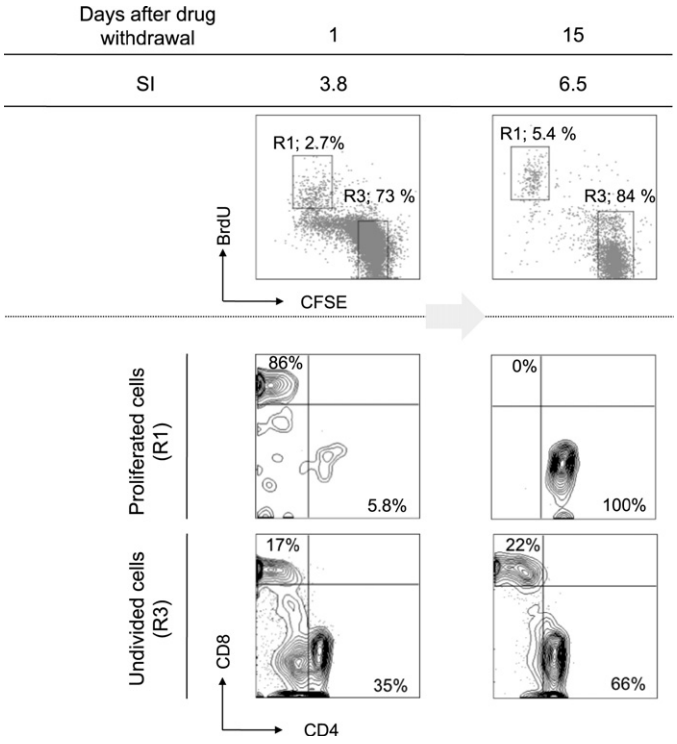

Fig. 4. The predominant drug-specific proliferating cell population changed from $CD8^+$ CTLs to $CD4^+$ T lymphocytes during the clinical course of DIHS (Case 3). When conventional and flow cytometric DLST were examined on day 0 after the withdrawal of phenytoin, the major drug-specific proliferating cell population was $CD8^+$ CTLs (86% of proliferating cells). However, on day 14, the proliferating population was composed entirely of $CD4^+$ T lymphocytes (100%). The undivided cell population remained predominantly $CD4^+$ T lymphocytes.

3.5. Drug-specific Tregs increase in DLST during the recovery stage

Next, we evaluated drug-specific Tregs by FCM-DLST in a case (Fig. 6) of carbamazepine-induced DIHS (Case 6). DLST was performed at the recovery stage (52 days). A BrdU incorporation assay was not performed in this case because intracellular Foxp3 staining is not compatible with the BrdU staining protocol. We compared the $CFSE^{low}$ population with the $CFSE^{high}$ population and used PHA-stimulated $CFSE^{low}$ proliferated $CD4^+$ T cells as a positive control (Fig. 6). The $CFSE^{low}$ drug-specific proliferating cells were mainly $CD4^+$ T cells. Almost all drug-specific $CD4^+$ T cells highly expressed CD25, and the ratio of $CD4^+$ $CD25^+$ $Foxp3^+$ drug-specific

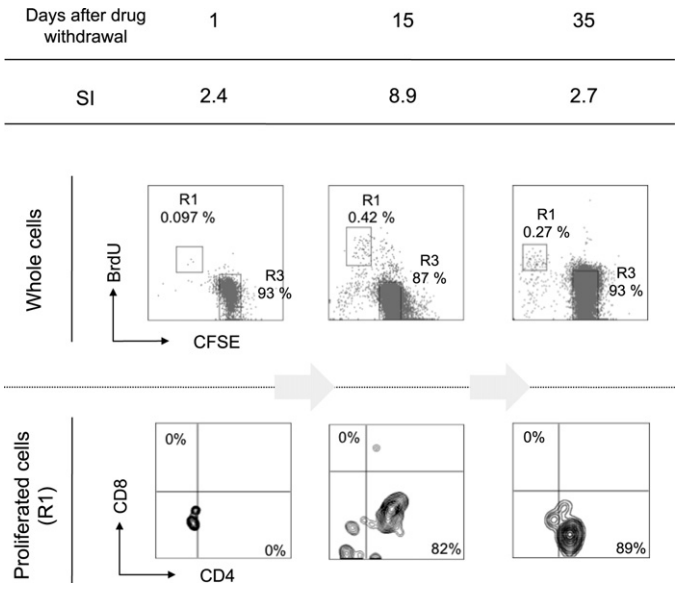

Fig. 5. The predominant drug-specific proliferating cell population remained CD4$^+$ T lymphocytes during the recovery course of DIHS (Case 4). From day 15 to day 35 after the withdrawal of zonisamide, the major drug-specific proliferating cell population remained the CD4$^+$ T lymphocyte population. Drug-specific T cells were not detected on day 1, perhaps because the SI was only weakly positive in this case (2.4).

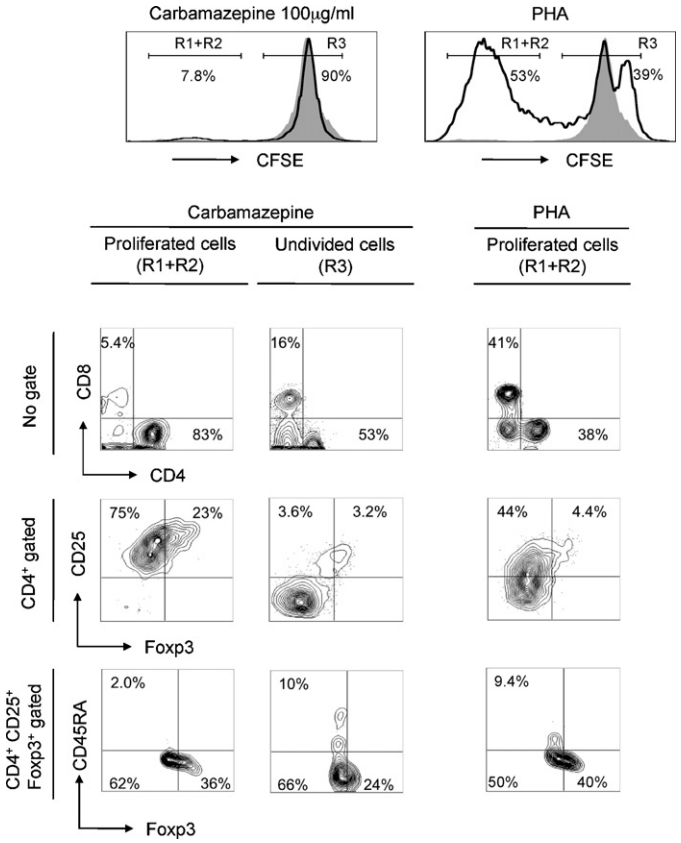

Fig. 6. Drug-specific Tregs were increased in DLST at the recovery stage. A case of a patient with a carbamazepine-induced DIHS was examined by CFSE dilution assay combined with Foxp3 staining. On day 52 after drug withdrawal, the major CFSE^{low} drug-specific proliferating cell population was CD4$^+$ T lymphocytes (83%). Almost all of the drug-specific CD4$^+$ T cells highly expressed the activation marker CD25, the CD4$^+$ CD25$^+$ Foxp3$^+$ drug-specific Treg population was expanded in the proliferated population compared to the CFSE^{high} non-proliferated population and a CFSE^{low} PHA-stimulated population, and the CD45RA$^+$ Foxp3^{low} resting Treg population almost disappeared. R1 + R2: the CFSE^{low} drug-specific or PHA-stimulated proliferated cell population; R3: the CFSE^{high} non-proliferated cell population.

Tregs was increased in this population compared to the CFSE^{high} population and controls. In contrast, the CD45RA$^+$ Foxp3^{low} resting Treg population almost disappeared in the CFSE^{low} population. These results indicate that drug-specific Tregs expand during the recovery stage of drug hypersensitivity.

4. Discussion

DLST, a widely used in vitro diagnostic tool for drug hypersensitivity, is used irrespective of the effector mechanism and clinical phenotype of the hypersensitivity reaction [10]. However, the sensitivity and specificity of conventional DLST is sometimes problematic, particularly when the SI value is not that high [10]. In our study, the SI values of conventional DLST dramatically changed in individual patients over the course of the disease. FCM-DLST determined that the percentage of drug-specific proliferating cells was very small even when the SI value was much higher than the current standard cut-off value. These results indicated that conventional DLST might be useful for the screening of the causative drug in a cADR case and that FCM-DLST, due to its ability to provide more detailed information about the drug-specific T-cell population, could be a suitable method for the determination of the culprit drug.

In vitro detection of drug-specific cytokine production by PBMCs appears to be an adequate alternative for the detection of drug hypersensitivities [6,9,17–19]. In many reports, the total T-cell population in the cultures, including the non-proliferating T cells, was analyzed. However, a few reports focused on the drug-specific proliferating T cells. In our FCM-DLST, the proliferating CD4$^+$ lymphocytes and CTLs, the cells that take up ^{3}H-thymidine in conventional DLST, are clearly visualized. Hashizume et al. previously reported that when DLST was performed with CFSE alone, the CFSE^{low} proliferated population appears even in the absence of the culprit drug. However, the CFSE^{low} population in a CFSE dilution assay not coupled to a BrdU assay might include non-specific dead cells as described above. Our FCM-DLST has the advantage of differentiating the overlapping dead cells as well as any cells with non-specific CFSE dilution from the proliferated population through BrdU labeling.

In previous clinical reports, CD4$^+$ T cells were the predominant population that infiltrated into maculopapular rash skin lesions [20], and most drug-specific T cells were CD4$^+$ T cells. In contrast, recent reports suggested that SJS and TEN result from HLA class I-restricted drug hypersensitivity. CTLs were the predominant population that infiltrated into the epidermis of skin lesions of SJS and TEN patients, and HLA B1502 was found to be fully associated with carbamazepine-induced SJS in Han-Chinese [21–23]. In addition, we reported that epidermal antigen-specific CTLs in Treg-depleted mice induce severe epidermal damage that mimics human TEN, suggesting the effector cells of SJS and TEN are CTLs [24–26].

Interestingly, although the number of patients is limited in this study, FCM-DLST revealed that drug-specific CTLs predominantly proliferated during the acute stages of SJS and DIHS, indicating that this proliferation corresponded to the administration of the culprit drug. On the other hand, drug-specific CD4$^+$ T cells, which likely included suppressive Foxp3$^+$ Tregs, were detected during the recovery stage of a DIHS patient after the withdrawal of the culprit drug. Moreover, unlike the previous report [13], positive DLST reactions were clearly observed during the acute stage of DIHS. This is likely because drug administration had been continued for

12 days after the onset of cADR, which could have led to the drug-specific CTLs becoming greatly expanded.

In conclusion, FCM-DLST demonstrated that the cell proliferation detected by conventional DLST is a heterogeneous proliferation of both $CD8^+$CTLs and $CD4^+$ T cells that likely includes Tregs. However, the conclusions that can be drawn from this study are limited due to the limited number of cases. As these T-cell populations recognize antigen on different MHC molecules, it will be interesting to test how a single drug antigen presented on MHC class I and class II independently primes and activates drug-specific T cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jdermsci.2011.12.002.

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