

Enhancement of Drug-Specific Lymphocyte Proliferation Using CD25^{hi}-Depleted CD3⁺ Effector Cells

Yuttana Srinoulprasert^{a–c} Werner J. Pichler^{b, c}

^aDepartment of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand;

^bDivision of Allergology, Clinic for Rheumatology and Clinical Immunology/Allergology, Inselspital, Bern University Hospital, University of Bern, and ^cADR-AC GmbH, Bern, Switzerland

Key Words

Drug hypersensitivity · Lymphocyte transformation test · Regulatory T cells

Abstract

Background: The lymphocyte transformation test (LTT) is used for in vitro diagnosis of drug hypersensitivity reactions. While its specificity is over 90%, sensitivity is limited and depends on the type of reaction, drug and possibly time interval between the event and analysis. Removal of regulatory T cells (Treg/CD25^{hi}) from in vitro stimulated cell cultures was previously reported to be a promising method to increase the sensitivity of proliferation tests. **Objective:** The aim of this investigation is to evaluate the effect of removal of regulatory T cells on the sensitivity of the LTT. **Methods:** Patients with well-documented drug hypersensitivity were recruited. Peripheral blood mononuclear cells, isolated CD3⁺ and CD3⁺ T cells depleted of the CD25^{hi} fraction were used as effector cells in the LTT. Irrelevant drugs were also included to determine specificity. ³H-thymidine incorporation was utilized as the detection system and results were expressed as a stimulation index (SI). **Results:** SIs of 7/11 LTTs were reduced after a mean time interval of 10.5 months (LTT 1 vs. LTT 2). Removal of the CD25^{hi} fraction, which was FOXP3⁺ and had a suppressive effect on drug-induced proliferation, resulted in an

increased response to the relevant drugs. Sensitivity was increased from 25 to 82.35% with dramatically enhanced SI (2.05 to 6.02). Specificity was not affected. **Conclusion:** Removal of Treg/CD25^{hi} cells can increase the frequency and strengths of drug-specific proliferation without affecting specificity. This approach might be useful in certain drug hypersensitivity reactions with borderline responses or long time interval since the hypersensitivity reaction.

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Introduction

Drug hypersensitivity reactions represent a major public health problem. Among drug hypersensitivity reactions, adverse cutaneous drug reactions are the most common. The majority represent delayed hypersensitivity reactions and are related to reactivation of drug-specific immune responses. They can vary from mild to very severe reactions with organ involvement [1]. It was proposed to identify the relevant drug by in vivo as well as in vitro tests. In vivo skin tests were evaluated in many studies and were found to be useful and applicable for antibiotics, but rather low sensitivity and limited availability of suitable drugs for skin testing restrict their use [2]. Importantly, in case of severe reactions like drug reaction

with eosinophilia and systemic symptoms/drug-induced hypersensitivity syndrome, Stevens-Johnson syndrome/toxic epidermal necrolysis (SJS/TEN) and acute generalized exanthematous pustulosis (AGEP); skin tests as well as drug provocation tests should be omitted [3, 4]. In vitro tests for detecting drug-sensitized T cells have been proposed as an alternative to elucidate the cause of drug hypersensitivity reactions [3–5]. Among these, the lymphocyte transformation test (LTT) is the oldest and still most widely used assay. The specificity of the LTT has often been documented to be high (>90–95%) [3–5], while the determination of sensitivity was less clear, since provocation tests in delayed drug hypersensitivity reactions are not standardized (dose and test duration unclear). Thus the LTT was mostly compared to a combination of history and skin test as proof of true sensitization [3–5]. These studies yielded a sensitivity of 30–70%, depending on patient selection, type of reaction and drugs involved [3].

The LTT measures the proliferation of lymphocytes to an antigen in vitro. It is suitable for T-cell-mediated reactions, but can also be positive in anaphylactic reactions, if the involved drug, like e.g. amoxicillin, stimulated the T cell immune system as well. Lymphocyte proliferation is controlled by regulatory T cells (Treg) also if nonprotein antigens are used: Cavani et al. [6] have demonstrated that Treg cells can suppress both naïve and memory nickel-specific T cell responses in vitro. Our group recently found that removal of Treg cells by beads enhanced the CD4⁺ T cell response to drugs in patients with multiple drug hypersensitivity reactions [7]. However, removal of Treg cells did not enhance sensitivity or the magnitude of LTT in peripheral blood mononuclear cells (PBMCs) of patients with SJS/TEN caused by lamotrigine [8].

The aim of the present study was to evaluate the effect of Treg removal on the proliferative capacity to drugs in vitro. We compared patients with various drug hypersensitivity reactions with a standard LTT using PBMCs, or assays using isolated CD3⁺ T cells or CD3⁺ T cells depleted of CD3⁺CD25^{hi} T cells. Sensitivity, specificity, and magnitude of the modified investigations were compared with those of original LTT protocols.

Materials and Methods

Donor Characteristics

Patients with drug hypersensitivity reactions with a clear medical history but negative or borderline LTT results were recruited as shown in table 1. Results from in vivo skin tests as well as clinical

symptoms according to reexposure to culprit drugs were included as a criterion for clinical diagnosis of true drug allergy. The patients had anaphylaxis (1/11), SJS/TEN (3/11), AGEF (2/11) and maculopapular drug eruptions (5/11). Three healthy donors without drug allergy were recruited as well. Blood samples were taken in full clinical remission at least 2 months or later after the acute event. No immunosuppressive drug was used prior to in vitro testing. The study was approved by the local ethics committee and all participants gave informed consent.

Drugs and Antibodies

The concentrations of drugs used for all studies are summarized in online supplementary table S1 (for all online suppl. material, see www.karger.com/doi/10.1159/000358491). Three different nontoxic concentrations of each drug that showed no suppressive effect on phytohemagglutinin-induced PBMC proliferation (unpublished data) were employed. The antibodies were purchased from BioLegend Co. (San Diego, Calif., USA).

Cell Sorting and Analysis

To obtain specific T cell subsets, the freshly isolated PBMCs were subjected to fluorescence-activated cell sorting (FACSaria, BD Biosciences, San Jose, Calif., USA). The PBMCs were stained for anti-CD3-PerCP/Cy5.5 and anti-CD25-PE to sort either CD3⁺CD25^{hi} or CD3⁺/CD25^{intermediate/low} (CD3⁺CD25^{int/low}) subsets. CD3⁺CD25^{int/low} cells were subjected to analysis by staining with anti-CD4-APC/Cy7 and intracellular staining with FOXP3 staining kit (eBioscience, San Jose, Calif., USA). All kits were used according to the manufacturer's protocols. For specific purposes, different subsets of PBMCs were isolated by immunomagnetic beads (StemCell Technologies, Grenoble, France). In brief, antigen-presenting cells were prepared by employing a human CD3 positive selection kit to deplete CD3⁺ from PBMCs. In order to prepare effector cells, CD3⁺ T cells were enriched using a human CD3⁺ T cell enrichment kit (negative selection). To prepare Treg cell-depleted CD3⁺ T cells, T cells expressing a high density of CD25 (CD25^{hi}) were removed from CD3⁺ T cells using anti-CD25 immunomagnetic beads. The CD3⁺ T cells depleted of CD25^{hi} cells were also termed CD3⁺CD25^{int/low} subsets. CD3⁺CD25^{int/low} cells were utilized as effector cells.

Peripheral Blood Mononuclear Culture and T Cell Proliferation Assay

PBMCs were used at 200,000 cells/well in LTT 1 and LTT 2 [3] and 100,000 cells/well in LTT 3. In the experiments using separated cell subsets, 100,000 cells/well were tested [7], which consisted of purified CD3⁺ or purified CD3⁺CD25^{int/low} T cell subsets (80,000 cells/well) and autologous γ-irradiated antigen-presenting cells (20,000 cells/well). CD3⁺CD25^{hi} T cells (16,000 cells/well) were added to study suppressive function of Treg cells. The cell cultures were performed in quintuplicate at 37°C in 5% CO₂ with tetanus toxoid (1 µg/ml) as positive control and the drugs at three to four concentrations. After 6 days 0.4 µCi ³H-thymidine (Perkin-Elmer, Boston, Mass., USA) was added for overnight incubation, followed by measuring the thymidine incorporation as counts per minute on a β-counter (Perkin-Elmer). Thymidine incorporation (counts per minute, cpm) was between 150 cpm and less than 3,000 cpm for unstimulated cultures, except some experiments as shown in online supplementary table S2. Proliferative responses were expressed as a stimulation index (SI); SI = counts per minute in the

Table 1. Profiles of drug hypersensitivity and results of investigative LTTs expressed as SIs

Patient	Gen-der	Age	Diagnosis	Culprit drug	Skin test	LTT 1 (2×10^5) SI	Interval ^a , months	LTT 2 (2×10^5) SI	LTT 3 (1×10^5) SI	CD3 ⁺ SI	CD3 ⁺ w/o CD25 ^{hi} SI
1	F	43	anaphylaxis	amoxicillin	positive	5.1	37	0.8	1.0	2.0	8
2	F	55	MPE late urticaria	amoxicillin sulfamethoxazole	not tested negative	5.4 2.5	2	4.3 1.3	3.1 1.4	3.9 6.8	3.6 16.9
3	M	31	MPE	amoxicillin clavulanic acid	negative negative	0.8 2.3	5	NA	1.0 1.2	3.3 2.0	2.0 0.6
4	M	68	MPE	sulfamethoxazole trimethoprim	negative negative	4.9 2.7	1	NA	5.0 3.8	1.3 1.9	5.0 13.7
5	M	56	MPE	Iomeron	reexposure positive	5.1	7	6.2	3.9	6.2	9.6
6	F	66	MPE	amoxicillin	positive	1.5	13	4.0	1.4	4.1	8.6
7	M	26	AGEP	amoxicillin clavulanic acid	positive positive	2.6 2.0	4	2.4 0.7	2.7 0.4	3.5 0.5	7.0 0.4
8	F	47	AGEP	amoxicillin	positive	3.4	6	NA	1.2	2.6	2.0
9	M	59	TEN	sulfamethoxazole trimethoprim	negative negative	1.7 1.2	2	1.7 1.8	2.1 1.2	1.4 1.0	10.4 3.4
10	M	65	SJS	carbamazepine	reexposure positive	2.4	23	1.4	1.4	1.1	1.0
11	M	24	SJS	metfenamic acid	reexposure positive	1.6	15	0.8	1.0	NA	1.0

Patient profiles demonstrate gender, age, drug hypersensitivity syndromes with their specific culprit drugs and results of diagnostic tests (in vivo skin test and in vitro LTT, LTT 1). Investigative LTTs in the present study included repeated LTT (LTT 2) and modified methods (LTT 3, CD3⁺, and CD3⁺ w/o CD25^{hi}). 200,000 cells/well were utilized in LTT 1 and LTT 2 while 1×10^5 cells/well

were utilized in other LTTs. Representative SIs were the highest SI of each test as shown in online suppl. table S2. MPE = Maculopapular exanthema; NA = not accessible; w/o = without. ^a Defined as time between first diagnostic LTT (LTT 1) and investigative LTTs.

cell cultures with culprit drug divided by counts per minute in the culture with medium alone. An SI of >2 was considered positive for non- β -lactams, of >3 for β -lactams [3].

Statistical Analysis

Sensitivity and specificity were calculated according to the numbers of positive and negative reactions to culprit and irrelevant drugs, respectively. To compare sensitivity and specificity, the paired Student's t test or Wilcoxon matched-pairs signed-ranks test was used to measure significance as appropriate. Values of $p < 0.05$ were statistically significant.

Results

Repetition of LTT as a Function of Time

As outlined in the 'Materials and Methods' section, the patients were first analyzed with an LTT using 2×10^5

cells per well (LTT 1). As shown in table 1 and 2, 7 of the patients gave a positive result to the incriminated drugs (11 patients, mean SI 2.13). To investigate the effect of the time interval on drug reaction and tests, we reevaluated the drug-induced proliferation after a time laps of 1–37 months. The results of the second LTT (LTT 2) were obtained with 11 LTTs (8 patients) and a mean time interval of 10.5 months: 7/11 had reduced proliferation, 3 SIs were increased. The overall mean of SIs was slightly increased, the SIs, however, were in the majority of LTTs quite low (SI 2.13–2.2, not statistically significantly different; tables 1, 2).

Comparison between Conventional LTT and Modified LTT

The third LTT (LTT 3) was done with 1×10^5 cells and with separated cell fractions (CD3⁺ and CD3⁺ without

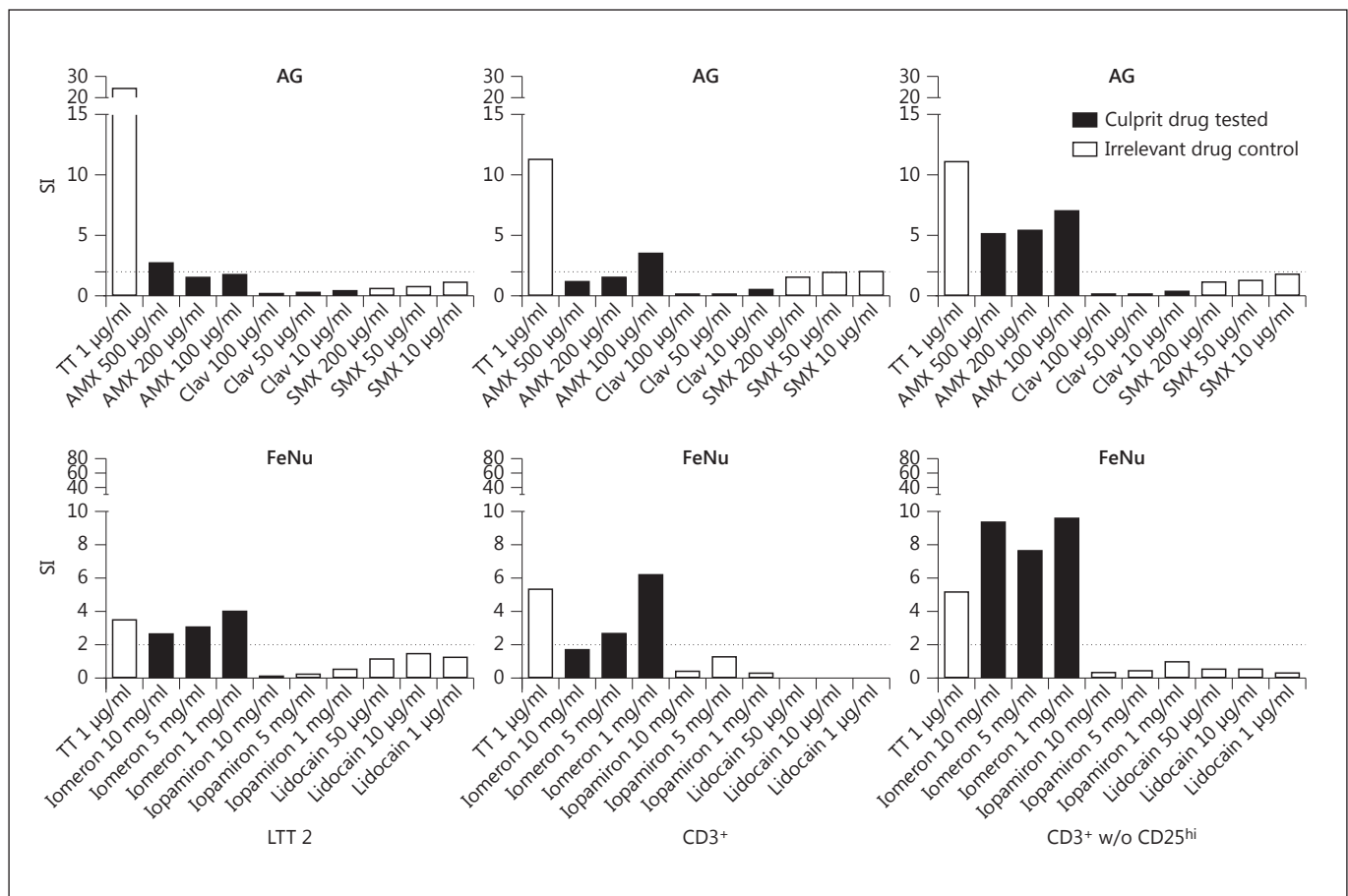


Fig. 1. Representative results of modified LTT. SIs of effector cells exposed to known culprit drugs and irrelevant drug controls. The cutoff line is set at SI = 2. TT = Tetanus toxoid; AMX = amoxicillin; Clav = clavulanic acid; SMX = sulfamethoxazole.

CD25^{hi}, table 1). The LTT 3 yielded substantially less positive results than LTT 1. Only 5 of the 16 analyses (31.25%) gave a positive result for LTT 3, while in LTT 1, 10 of the 16 analyses (62.5%) were positive. However, when the CD3-enriched cells were used (positive in 7 of 15, 46.67%), or when the CD25^{hi} fractions were depleted, 10 of 16 analyses were positive (62.5%).

Sensitivity and Specificity

As no gold standard for the diagnosis of delayed drug reactions is available, we based the diagnosis of a 'true' drug hypersensitivity reaction on a combination of history (typical symptoms known for the drug), typical time course, use of only selected drugs, positive skin tests and accidental reexposure with symptoms. Table 2 summarizes the sensitivity and specificity, as well as the mean SI of the different cell analyses.

Table 2. Sensitivity and specificity of conventional LTT and modified methods

	LTT 1	LTT 2	LTT 3	CD3 ⁺	CD3 ⁺ w/o CD25 ^{hi}
Sensitivity, %	61.11	36.36	25.00	62.50	82.35*
Specificity, %	100.00	100.00	100.00	90.00	100.00
Mean SI	2.13	2.2	2.05	2.78	6.02*

LTT 1 and LTT 2 were run according to a similar protocol except for the investigation date as shown in table 1. LTT 3, CD3⁺ and CD3⁺ without (w/o) CD25^{hi} were run on a similar date of investigation with difference of effector cells as described in the 'Materials and Methods' section. * $p < 0.05$.

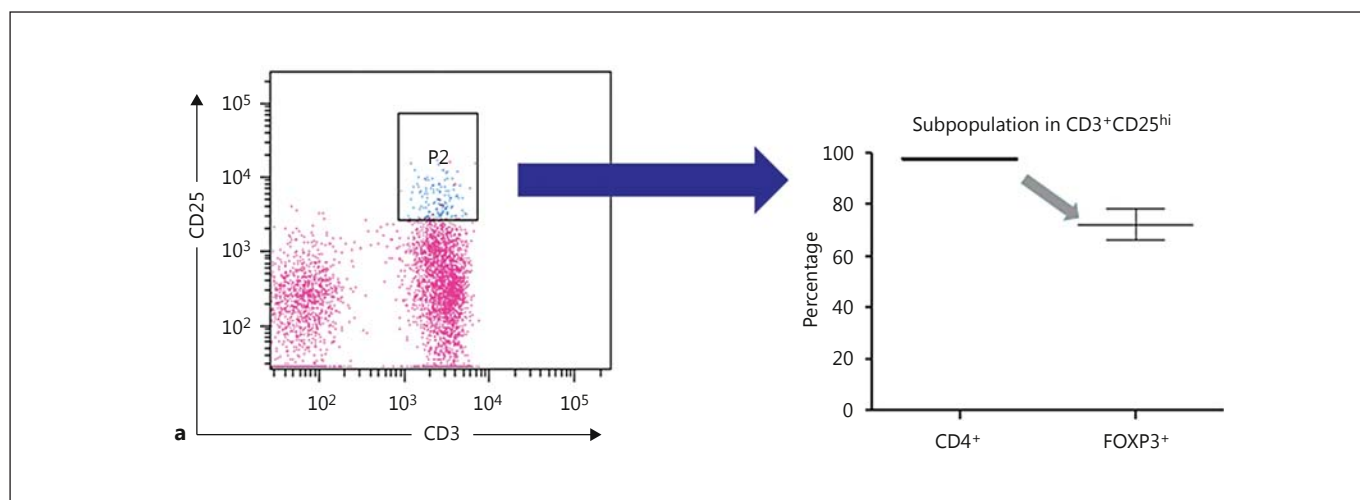
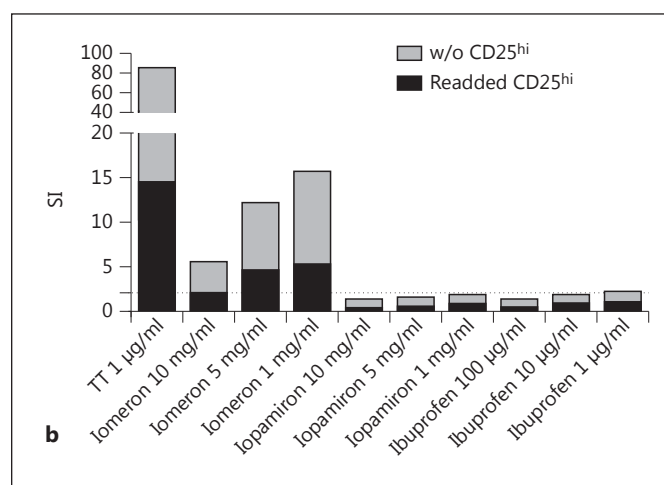


Fig. 2. Characterization of Treg cells. **a** Representative FACS sorting (left) of PBMCs from drug hypersensitivity patient and further analysis of the CD3⁺CD25^{hi} fraction with co-staining with fluorochrome-conjugated monoclonal antibodies against CD4 and FOXP3 (right). **b** Study of function of the CD3⁺CD25^{hi} fraction. Modified LTT using CD25^{hi}-depleted CD3⁺ as effector cells was added back with CD3⁺CD25^{hi} fraction at a ratio of 10:1 (effector:suppressor). In this study, Iomeron was the specific culprit drug; a structurally related radiocontrast medium (Iopamiron) and an irrelevant drug (ibuprofen) were used to prove test specificity.



The sensitivity of normal LTT was best at the start, soon after the event, and dropped at the repetitions. The lower sensitivity of LTT 2 might be due to decreased reactivity over time and/or due to the lower cell number used (LTT 3). Specificity, on the other hand, did not change with time, e.g. LTT 2 yielded lower sensitivity at 36.36% with 100% specificity.

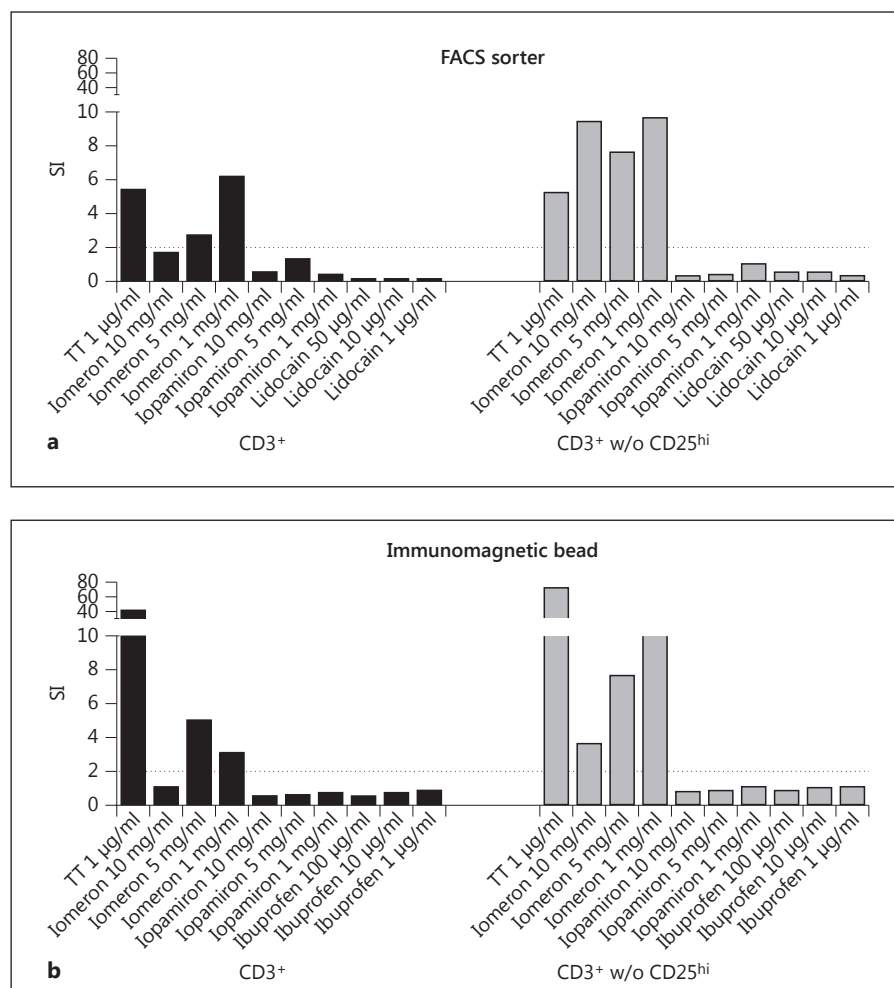
Interestingly, when we used the CD3⁺ subset after removal of the CD25^{hi} fraction as effector cells, sensitivity was higher than 80%. Figure 1 shows the data of PBMCs No. 7 and 5. It shows that an enhanced proliferation and responsiveness to different concentrations of incriminated drugs (amoxicillin and Iomeron) could be observed in Treg-depleted cell fractions. It also shows that in spite of removal of Treg cells, no unspecific proliferation to irrelevant drugs in these cell cultures from drug-allergic patients occurred.

To further substantiate this lack of Treg depletion on proliferation of PBMCs to drugs, PBMCs of healthy donors were tested with the drugs commonly utilized in this study, e.g. a group of antibiotics (amoxicillin, sulfamethoxazole, trimethoprim), nonsteroidal anti-inflammatory drugs (ibuprofen), and radiocontrast medium (Iomeron) with and without Treg removal. As compared to the results of the original LTT (LTT 2), no proliferation of PBMCs was observed with modified methods with or without Treg removal (online suppl. fig. S1).

Characterization and Function of the CD3⁺CD25^{hi} Fraction

To confirm that the CD3⁺CD25^{hi} cells contained the regulatory cell phenotype characterized by FOXP3 expression, we analyzed the phenotype of CD3⁺CD25^{hi} cell subsets (fig. 2a). It was almost 100% CD4⁺-positive, and up

Fig. 3. Representative SIs of modified LTTs using effector cells from FACS sorter and immunomagnetic bead separation. **a** SIs of modified LTTs using CD3⁺ (left) and CD25^{hi}-depleted CD3⁺ (right) as effector cells. Both effector cells were isolated by FACS sorter. The cutoff line was set at SI = 2. **b** SIs of modified LTTs using CD3⁺ (left) and CD25^{hi}-depleted CD3⁺ (right) as effector cells. Both effector cells were isolated by immunomagnetic bead separation. The cutoff line was set at SI = 2.



to 75% of the cells were indeed FOXP3-positive, confirming a regulatory phenotype (online suppl. fig. S2).

To prove the regulatory function of the CD3⁺CD25^{hi} fraction, the effect of readdition of the isolated Treg cells (CD25^{hi}FOXP3⁺ cells) on drug-specific proliferation was evaluated: it resulted in decreased proliferative responses to the relevant drug (Iomeron, fig. 2b). In spite of the structural similarity of different radiocontrast media, the radiocontrast medium Iopamiron could not induce any reactivity with or without Treg cells, confirming the high specificity of the in vitro test.

Comparison of Treg Removal by FACS Sorting or Immunomagnetic Beads

FACS sorting is a rather expensive and complicated procedure to isolate cell fractions. To evaluate whether sorting by immunomagnetic beads gives similar results, we analyzed the reaction to Iomeron with FACS-sorted

or bead-sorted cells: as shown in figure 3a, b, depleting CD25^{hi} cells resulted with both methods in enhanced proliferation without affecting the reactivity to other compounds.

Discussion

The LTT is an in vitro diagnostic test to identify the culprit drugs in drug hypersensitivity reactions. Its sensitivity varies from 30 to 70% depending on drug type, type of drug hypersensitivity reaction [9], patient selection and optimal in vitro conditions [3]. Unclear is the effect of the time interval between the drug hypersensitivity event and analysis. From case records, one might conclude that some patients keep their drug hypersensitivity for years, while others loose it over time [10, 11]. The study presented here addresses the LTT in well-defined

patients with rather low LTT results and addresses the question if one can improve the sensitivity of this proliferation-based test by removing Treg cells.

The repetition of the LTT yielded somewhat lower but not statistically significant responses (in 7/11 LTTs compared). Previous studies have shown that in severe reactions about 2–5/10,000 drug-specific precursor T cells can be detected [10]. This number may slowly decrease over time, and explain our finding of lower sensitivity of LTT if we used only 10^6 cells/ml. Due to exponential growth of the few drug-specific precursor cells, the starting cell number in the cultures is important.

Removal of $CD3^+CD25^{hi}$ cells was achieved by FACS sorting. Similar results were obtained by immunomagnetic bead separation, making this approach more applicable. The latter method allowed confirming the immunosuppressive potential of Treg cells, demonstrating their suppressive potential by readding the isolated Treg cells to the culture.

Removing Treg from the $CD3^+$ T cell fractions resulted in two outcomes: (a) It revealed a previously not detected sensitization in 3/11 patients, as they were negative in normal LTT (patients 6, 7, 9). However, in 4 of the 11 patients the test remained negative even after Treg cell removal (patients 3, 7, 10, 11). (b) In 7 patients, the Treg depletion induced a stronger and thus clearer reaction by increasing the SI substantially. Actually, our data suggest that Treg depletion is effective only if a preexisting (borderline) proliferative reactivity was already present, and that Treg removal boosts this marginal proliferative response to drugs to higher levels. If the drug hypersensitivity reaction is mainly based on cytotoxic $CD8^+$ T cells, as it is assumed to be the case in patients with SJS/TEN [13], Treg removal appears to have a lesser effect, as illustrated by the 3 patients with SJS/TEN analyzed in this study. Indeed, in only 1/3 patients with SJS/TEN we were able to detect a clear sensitization to the incriminated drug after Treg depletion, which fits the low incidence of positive LTTs in SJS/TEN [9]. This finding supports the data by Tang et al. [8] show-

ing that Treg depletion is of limited value in identifying the causative drug in lamotrigine in SJS/TEN.

A very important aspect of LTTs is a rather high specificity. This excellent specificity somewhat compensates for the poor sensitivity of this assay, as a positive LTT can be linked to a relevant sensitization [12, 14]. In previous assays and also in this study, the enhanced reactivity after Treg cell depletion did not affect specificity as the negative results remained negative and no unspecific proliferation was observed (online suppl. fig. S1) [7].

The cutoff point for a positive LTT is normally 2, but in patients tested with β -lactams, the cutoff point is put at $SI > 3$ [3]. In practical terms, while a highly positive LTT ($SI > 5$) is easy to interpret, the interpretation of a borderline LTT result ($SI 2.5$ – 3) is difficult and less convincing. In this regard, a test procedure which can enhance sensitivity without inducing false positive reactions may be quite helpful.

In conclusion, removal of Treg cells is a possibility to increase the proliferative potential of drug-reactive T cells in LTTs. However, Treg removal had no effect on LTTs in patients with SJS/TEN, possibly because the involved T cells were not strongly proliferating and thus not regulated by Treg cells. As the specificity of the LTT is not affected by removal of Treg cells, assays with prior Treg cell removal might be helpful in selected cases of drug hypersensitivity reactions.

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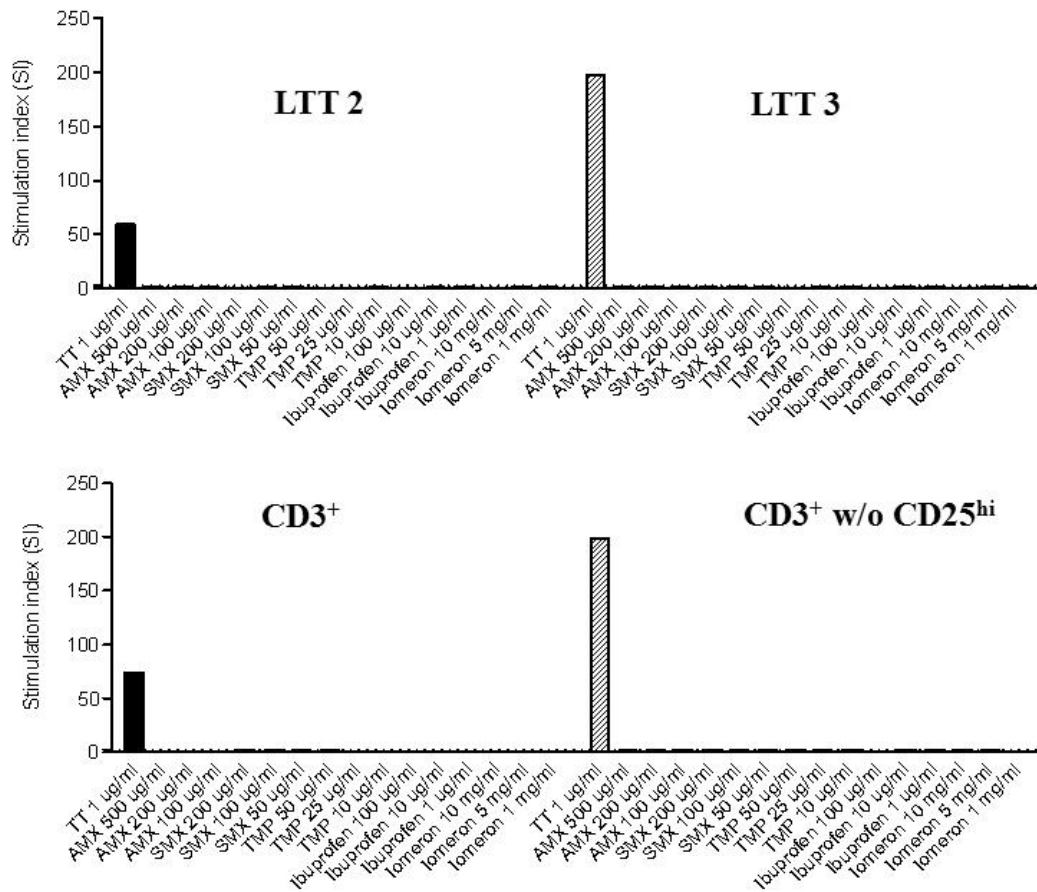


Fig. S1. Specificity of lymphocyte transformation test (LTT) and its modified methods. Representative stimulation indices (SI) of lymphocyte transformation test (LTT) and its modified methods obtained from blood of healthy donors, who clearly have no history of any adverse drug reactions (one of three). Isolated PMBCs were subjected to test with drugs commonly used in the present study in order to emphasize the specificity of the modified protocols. Cut off line of SI was set at stimulation index (SI) = 2. AMX = amoxicillin, SMX = sulfamethoxazole, and TMP = trimethoprim.

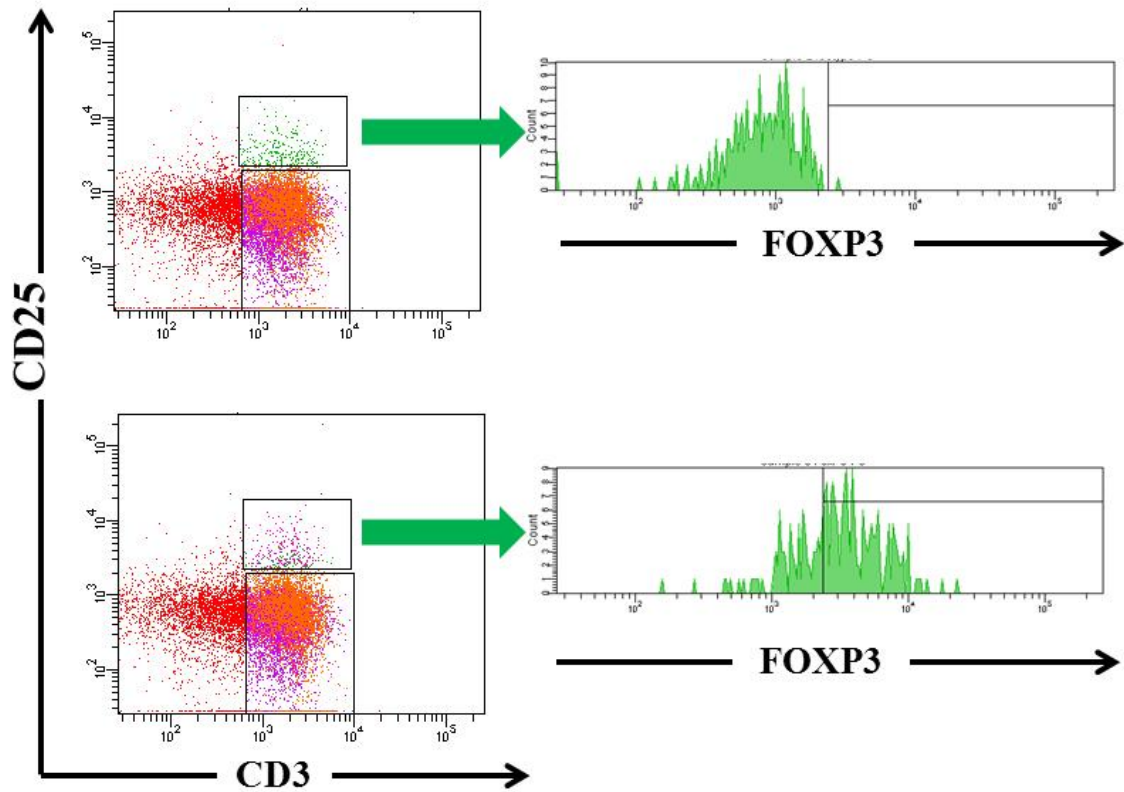


Fig. S2. Determination of FOXP3⁺ in CD3⁺CD25^{hi} subpopulation. Representative FOXP3 staining of CD3⁺CD25^{hi} fraction from blood of drug allergy donors, isolated PBMCs were stained with fluorescence labeled CD3, CD25, FOXP3 staining kit and the isotype control (manufacturer's guideline). After CD3⁺ and CD25^{hi} gating, CD3⁺CD25^{hi} subpopulation was determined as FOXP3 positive when its fluorescence intensity was higher than that of isotype control in upper panel. Average percentage of FOXP3 positive subpopulation (lower panel) from all drug-allergic donors was presented in right panel of Fig 2a.

Table S1. Sources and working concentrations of pure drugs utilized in lymphocyte transformation test and its modified methods

Drug	Concentration (µg/ml)			Company
	100	200	500	
Amoxicillin	100	200	500	Sigma, Basel, Switzerland
Budesonide	10	50	100	Sigma, Basel, Switzerland
Carbamazepine	10	50	10	Sigma, Basel, Switzerland
Cefuroxime	1	10	100	Sigma, Basel, Switzerland
Ciprofloxacin	1	10	50	Sigma, Basel, Switzerland
Clavulanic acid	10	100	200	GlaxoSmithKline, UK
Gabapentin	1	10	100	Sigma, Basel, Switzerland
Ibuprofen	1	10	100	Sigma, Basel, Switzerland
Iomeron	1 mg/ml	5 mg/ml	10 mg/ml	Bracco, Geneva, Switzerland
Iopamiron	1 mg/ml	5 mg/ml	10 mg/ml	Bracco, Geneva, Switzerland
Lidocaine	1	10	50	Sigma, Basel, Switzerland
Mefenamic acid	10	50	100	Sigma, Basel, Switzerland
Sulfamethoxazole	50	100	200	Sigma, Basel, Switzerland
Trimethoprim	10	25	50	Sigma, Basel, Switzerland

The drugs used in the present study were obtained from Sigma Company, Switzerland, except clavulanic acid and radio contrast medium. The in vitro concentrations used were previously determined for each drug by analyzing toxicity level and suppressive effect on PHA-induced PBMC proliferation (see ref 3-5, unpublished data). Most of these concentrations were employed at microgram per microliter except for radiocontrast medium, which were utilized at higher levels (milligram per microliter).

Table S2. Overall stimulation indices (SI) of lymphocyte transformation test (LTT) and its modified methods (LTT 3, CD3⁺, and CD3⁺ without (w/o) CD25^{hi}) using different effector cells from drug hypersensitivity patients

No.	Pt	Culprit drug	Conc. (µg/ml)	LTT 1 (2x10 ⁵) SI	LTT 2 (1x10 ⁵) SI	LTT 3 (1x10 ⁵) SI	CD3 ⁺ SI	CD3 ⁺ w/o CD25 ^{hi} SI
1	SR	Amoxicillin	500	5.1	0.4	0.5	0.5	8.0
			200	3.2	0.6	0.6	0.3	1.4
			100	2.6	0.8	1.0	2.0	3.1
2	TS	Amoxicillin	500	2.2	error	1.0	0.4	1.8 ⁺
			200	5.4	1.8	0.8	1.0	1.6 ⁺
			100	2.7	4.3	1.6	3.9	3.6 ⁺
		Sulfamethoxazole	200	2.5	1.3	2.5	0.8	3.6 ⁺
			100	1.2	0.5	1.8	6.2	9.0 ⁺
			50	1.4	0.6	2.9	6.8	16.9 ⁺
3	PM	Amoxicillin	500	0.5	NA	0.9	2.5	2.0
			200	0.7		1.0	1.1	1.5
			100	0.8		1.0	3.8	1.1
		Clavulanic acid	50	2.3	NA	0.8	0.1	0.1
			10	2.3		1.2	0.2	0.1
			1	1.3		0.9	1.1	0.6
4	FB	Sulfamethoxazole	200	4.9	NA	3.4	0.8	5.0 ⁺
			100	3.2		5.0	0.8	1.5 ⁺
			50	1.6		4.6	1.3	1.6 ⁺
		Trimethoprim	50	2.4	NA	1.0	1.1	0.8 ⁺
			20	2.7		2.4	1.0	1.6 ⁺
			10	1.9		3.8	1.9	13.7 ⁺
5	FeNu	Iomeron	10 mg/ml	2.4	2.8	2.6	1.7	9.4
			5 mg/ml	5.1	4.7	3.0	2.7	7.6
			1 mg/ml	4.0	6.2	3.9	6.2	9.6
6	RM	Amoxicillin	500	1.5	4.0	1.3	0.4	8.2
			200	1.1	2.5	0.9	2.1	8.6
			100	0.9	2.5	1.4	4.1	2.6
7	GA	Amoxicillin	500	2.0	2.4	2.7	1.2	5.1
			200	2.6	2.4	1.5	1.5	5.4
			100	1.2	2.0	1.7	3.5	7.0
		Clavulanic acid	50	2.0	0.3	0.1	0.2	0.1
			10	1.1	0.3	0.2	0.1	0.1
			1	1.2	0.7	0.4	0.5	0.4

8	AL	Amoxicillin	500	3.4	NA	1.0	1.4 ⁺	0.5
			200	2.8		1.2	2.6 ⁺	2.0
			100	1.0		1.0	2.0 ⁺	0.4
9	RW	Sulfamethoxazole	200	1.7	0.7	2.1	0.5	2.7
			100	1.7	0.8	1.6	0.2	4.5
			50	1.4	1.7	1.2	1.4	10.4
		Trimethoprim	50	1.1	1.2	1.2	1.0	2.0
			25	1.1	1.8	0.4	0.5	1.6
			10	1.2	1.3	0.6	0.2	3.4
10	AP	Carbamazepine	100	2.4	0.2	0.2	0.6 ⁺	0.5 ⁺
			50	-	0.4	0.4	1.0 ⁺	1.1 ⁺
			10	0.6	1.4	1.4	0.9 ⁺	1.0 ⁺
11	JoSc	Mefenamic acid	100	-	0.0	0.1	NA	0.0
			50	1.6	0.1	0.1		0.5
			10	1.3	0.8	1.0		1.0

LTT 1 was defined as the test at first diagnosis. LTT 2 was done later, together with the modified methods. Both LTT 1 and LTT 2 utilized 2×10^5 PBMCs/test well. LTT 3 was done with decreased numbers of effector cells (PBMCs) (1×10^5 cells/test well). $CD3^+$ and $CD3^+$ w/o $CD25^{hi}$ were tested to compare the proliferation with $CD3^+$ or $CD25^{hi}$ -depleted $CD3^+$ as effector cells, respectively (1×10^5 cells/test well).

All SI was calculated from ratio of count per minute (cpm) as described in material and method section. Range of cpm control was between more than 150 and less than 3000 except some tests as marked with +, their cpm control of which was between more than 70 and less than 150. NA: not accessible.