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

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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. ^{3}H-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.

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

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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 be 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), AGEP (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% CO_2 with tetanus toxoid (1 μg/ml) as positive control and the drugs at three to four concentrations. After 6 days 0.4 μCi ^{3}H-thymidine (PerkinElmer, Boston, Mass., USA) was added for overnight incubation, followed by measuring the thymidine incorporation as counts per minute on a β-counter (PerkinElmer). 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	Gender	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 1and 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

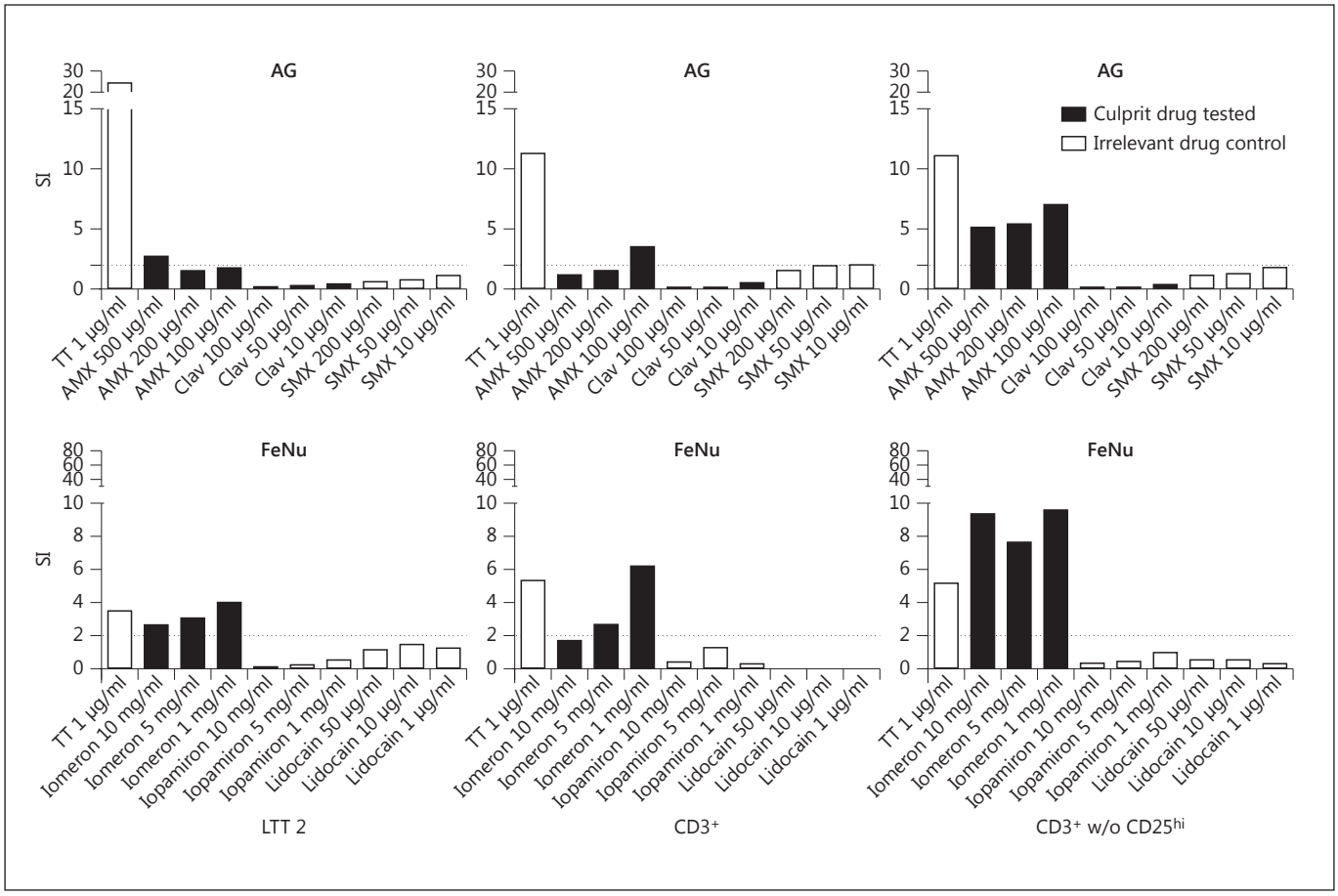


Fig. 1. Representative results of modified LTT. SIs of effector cells exposed to known culprit drugs and irrelevant drug controls. The cutoff line is set at SI = 2. TT = Tetanus toxoid; AMX = amoxicillin; Clav = clavulanic acid; SMX = sulfamethoxazole.

$CD25^{hi}$, 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%).

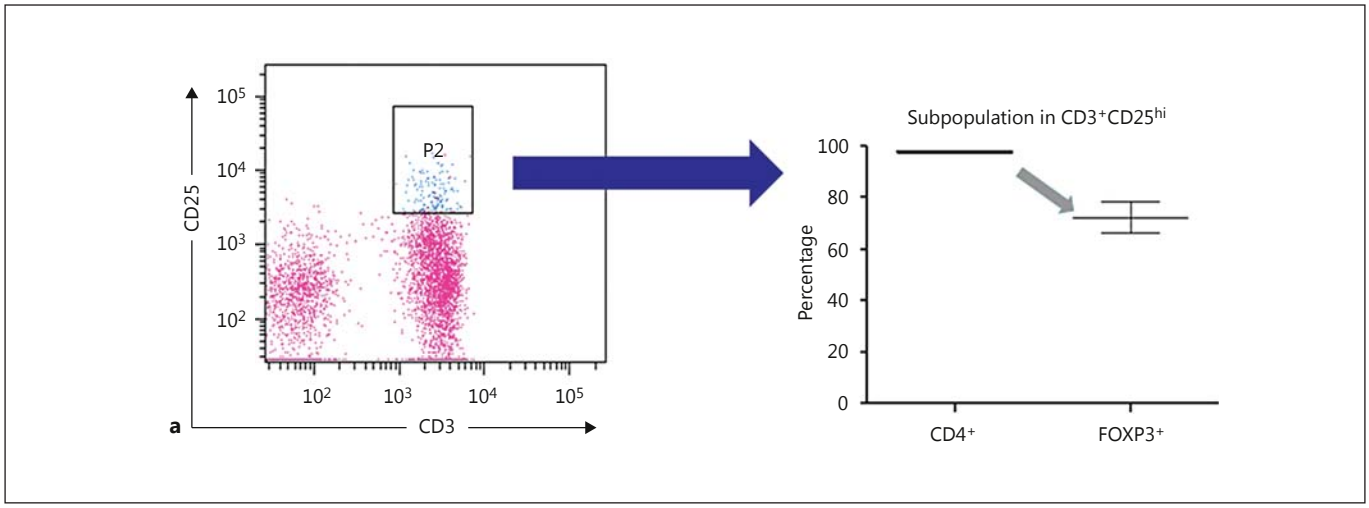
Sensitivity and Specificity

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. * $p < 0.05$.



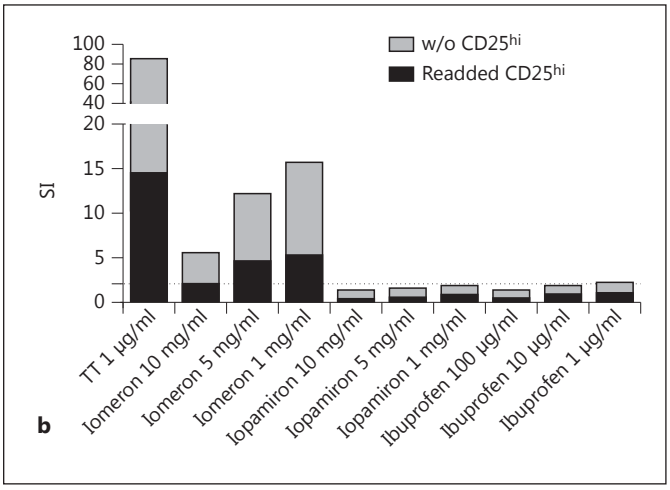


Fig. 2. Characterization of Treg cells. **a** Representative FACS sorting (left) of PBMCs from drug hypersensitivity patient and further analysis of the $CD3^+CD25^{hi}$ fraction with co-staining with fluorochrome-conjugated monoclonal antibodies against CD4 and FOXP3 (right). **b** Study of function 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

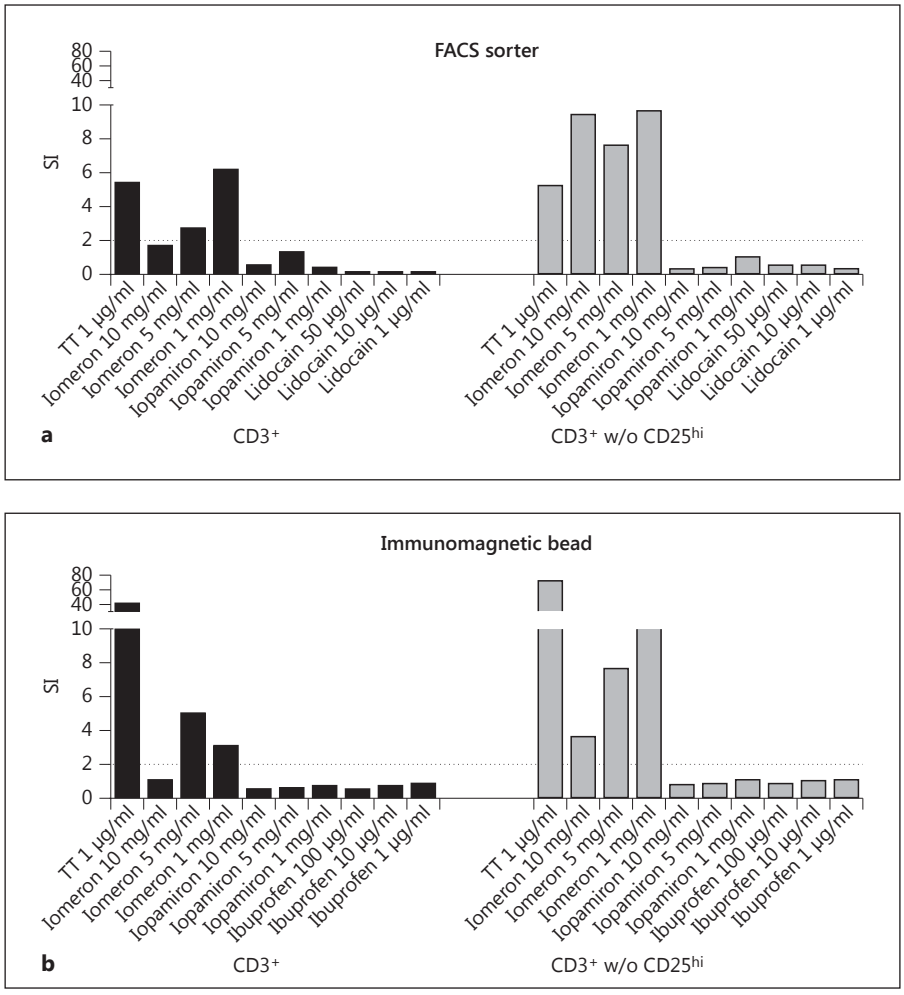


Fig. 3. Representative SIs of modified LTTs using effector cells from FACS sorter and immunomagnetic bead separation. **a** SIs of modified LTTs using $CD3^+$ (left) and $CD25^{hi}$-depleted $CD3^+$ (right) as effector cells. Both effector cells were isolated by FACS sorter. The cutoff line was set at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