Improvement of lymphocyte proliferation assessment in non-immediate drug hypersensitivity reactions using flow-cytometry

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Abstract

Background. Lymphocyte transformation test (LTT) has been widely used to evaluate non-immediate drug hypersensitivity reactions (NIDHRs). However, the lack of standardisation and the low sensitivity have limited its routine diagnostic use. The drug presentation by dendritic cells (DCs) and the assessment of proliferation on effector cells have shown promising results. Flow-cytometry-based methods can help apply these improvements. We aimed to assess the added value of using drug-primed-DCs and the determination of the proliferative response of different lymphocyte subpopulations in NIDHRs. Methods. Patients with confirmed NIDHR were evaluated by both conventional (C-LTT) and with drug-primed-DCs LTT (dDC-LTT) analysing the proliferative response in T-cells and other effector cell subpopulations by using the fluorescent molecule, carboxyfluorescein diacetate succinimidyl ester. Results. The C-LTT showed a significantly lower sensitivity (33.3%) compared with dDC-LTT (65.2%), which was confirmed analysing each particular clinical entity: SJS-TEN (62.5% vs 87.5%), MPE (14.3% vs 41.7%), and AGEP (33% vs 80%). When including the effector cell subpopulations involved in each clinical entity, $CD3^{+}$+$CD4^{+}T_h1$ cells in SJS-TEN, $CD3^{+}$+$CD4^{+}T_h1$+NK cells in MPE, and $CD3^{+}$+NK cells in AGEP, we could significantly increase the sensitivity of the in vitro test to 100%, 66.6%, and 100%, respectively. With an overall sensitivity of 87% and 85% of specificity in NIDHR. Conclusions. The use of a flow-cytometry-based test, DCs as drug presenting cells, and focussing on effector cell subpopulations for each clinical entity significantly improved the drug-specific proliferative response in NIDHRs with a unique cellular in vitro test.

KEYWORDS

Drug hypersensitivity reactions, in vitro tests, lymphocytes, non-immediate reactions, proliferation.

ABBREVIATIONS AND ACRONYMS

DHR: Drug hypersensitivity reactions

SJS: Stevens-Johnson Syndrome

TEN: Toxic epidermal necrolysis

DRESS: Drug reaction with eosinophilia and systemic symptoms

IDHR: Immediate drug hypersensitivity reaction

NIDHR: Non-immediate drug hypersensitivity reaction

MPE: Maculopapular exanthemas

ST: Skin test

PT: Patch test

IDT: Intradermal test

DPT: Drug provocation test

LTT: Lymphocyte transformation test

moDC: Monocyte-derived dendritic cell

APC: Antigen presenting cell

BL: Betalactam

RCM: Radio contrast media

CFSE: Carboxyfluorescein diacetate succinimidyl ester

EAACI: European Academy of Allergy and Clinical Immunology

ASPS: Spanish Pharmacovigilance System

ESCD: European Society of Contact Dermatitis

PBMC: Peripheral blood mononuclear cell

PHA: Phytohemaglutinin

C-LTT: Conventional lymphocyte transformation test

dDC-LTT: Drug-primed-moDCs lymphocyte transformation test

PI: Proliferation index

ROC curve: Receiver operating characteristic curve

AGEP: Acute generalized exanthematous pustulosis

FDR: Fixed drug reaction

AUC: Area under curve

INTRODUCTION

Drug hypersensitivity reactions (DHRs) are currently a burden on Healthcare Systems since, although not very frequent, 5-10% of all adverse drug reactions, they have shown a significant increase in prevalence over last years in adults and children.[1,2]Moreover, they can be severe, producing longer patients´ stays and higher rates of hospital associated infections, requiring the prescription of alternative drugs that may be less effective, more toxic, and expensive. It is therefore very important to establish a correct diagnosis of DHRs avoiding false label of allergy and of non-allergy, being the latter particularly important for severe DHRs, as anaphylaxis, Stevens-Johnson syndrome (SJS), toxic epidermal necrolysis (TEN), and drug reaction with eosinophilia and systemic Symptoms (DRESS).[3]

DHRs can be classified according to the time of onset of the symptoms after drug intake in both immediate (IDHRs) and non-immediate reactions (NIDHRs). NIDHRs appear more than 1 hour after drug administration. They show heterogeneous clinical manifestations, ranging from mild maculopapular exanthemas (MPEs), the most frequent (almost 90% of cases), to life-threatening as SJS-TEN, or DRESS.[4,5]The diagnostic procedure is complex including a detailed clinical history,[6] followed by skin tests (STs), patch tests (PTs) and delayed-reading intradermal tests (IDTs), tests that show low sensitivity.[4,7] Therefore, drug provocation test (DPT) is in many cases needed for confirming diagnosis; however, it is not allowed in the evaluation of severe reactions.[8] Given the limitations of STs and DPTs, there is a need for developing validated *in vitro* tests to correctly identify the responsible drug in NIDHRs.

NIDHRs are mainly induced by T-cells through the involvement of different inflammatory mediators and effector cell subsets.[6,9] Although in most cases T-cells with a Th1 pattern are involved,[10] other cell subpopulations can participate, i.e. cytotoxic T-cells producing soluble Fas-ligand, perforin/granzyme, granulysin, and TNF-α in SJS-TEN, and other bullous manifestations[11] or Th2 $CD4^+$T-cells in DRESS[12]. This highlights the importance for assessing the effector cellular response to increase the sensitivity of*in vitro* tests.[13,14]

Lymphocyte transformation test (LTT), which determines lymphocyte proliferation upon drug stimulation, has been widely used to evaluate NIDHRs with a specificity of 93.9%.[15-17] However, the lack of standardization and the low sensitivity (around 56.1%) has limited its routine diagnostic use, being largely restricted to research field.[17] Different studies have shown that these data highly depend on the drug involved and the clinical symptoms, with higher sensitivity (57.9-88.8%) in mild/moderate reactions[18-20] and lower sensitivity (25-75%) for severe NIDHR as SJS-TEN[13,21-24]. This creates the need for improving the sensitivity of the tests, particularly in severe reactions with very limited diagnostic approaches available.[13,14,25]

Several attempts have been performed for improving LTT sensitivity. The inclusion of the drug metabolites has shown to be important for some drugs.[26] Other studies have demonstrated that the inclusion of monocyte-derived dendritic cells (moDCs) as antigen presenting cells (APCs) improves the drug-specific-cellular proliferation, and therefore LTT sensitivity, when evaluating patients with NIDHRs to betalactams (BL), heparins, or radio contrast media (RCM).[19,27,28]

The cell proliferative response has been classically measured via the genome incorporation of tritiated thymidine (^{3}H).[19] This method presents the disadvantage of using radioactive tracers and of not being able to discriminate the proliferating subpopulation. Nowadays, the use of flow-cytometry-based methods, determining the decrease on the content of fluorescent molecules, such as carboxyfluorescein diacetate succinimidyl ester (CFSE), into proliferating cells, has allowed us assess not only the proliferative response but also the possibility of identifying different cell subtypes, including the effector cells involved in the reaction.[13]

The aim of this study was to assess the added value of using drug-primed-moDCs as APCs and the determination of the proliferative response of different lymphocyte subpopulations in each clinical manifestation of NIDHRs using the new LTT approach based on flow-cytometry technology. To this end, patients with confirmed NIDHR were evaluated by both conventional and with drug-primed-moDCs LTT analysing the proliferative response in T-cells and other effector cells.

METHODS

Allergic patients and healthy controls selection

Patients with suggestive clinical history of NIDHR attending the Allergy Unit of the Hospital Regional Universitario de Málaga (HRUM) from 2013 until 2019 were prospectively evaluated. Only patients with confirmed diagnosis of NIDHRs following European Academy of Allergy and Clinical Immunology (EAACI) recommendations were included in the study.[6,7,29] The diagnosis was based on STs (positive delayed-reading IDT or PT) and, if negative, on DPT. In cases reporting severe reactions or with a risky medical background in which DPT was contraindicated, the diagnosis was based on the causality algorithm of the Spanish Pharmacovigilance System (ASPS),[17,30]which classified reactions as not related (improbable (<0 score) and conditional (1-3 score)) or related (possible (4-5 score), probable (6-7 score) or defined ([?]8 score)).

A group of healthy, sex- and age-matched subjects with no history of DHRs were included as controls. All subjects were correctly informed and those who decided to participate signed an informed consent. The study was conducted in accordance with the Declaration of Helsinki and it was approved by the Ethical Committee of Malaga.

Allergological workup

Skin tests

IDT was done with drugs in 0.9% NaCl as recommended by the EAACI.[31] Readings were immediate at 20 minutes and delayed at 24, 48, and 72 hours, and patients were advised to return to show any positive responses occurring after the 72 hours. It was considered a positive result an infiltrated erythema with diameter >5mm.[7] PT was performed according to the European guidelines[7,31] using a concentration of 30% of the commercialised culprit drug in petrolatum. Reading was performed according to the European Society of Contact Dermatitis (ESCD), 20 min after removal of the strips and 48, 72, and 96 hours later.[32]

Drug provocation tests

Placebo-controlled single blinded DPT with the culprit drug was only done in mild NIDHRs with negative ST and after a careful risk-benefit assessment.[8,33] The DPT was sequential and additive when symptoms remitted and laboratory parameters became normal, not earlier than 4 weeks. Initially 1/100 of the therapeutic dose was administered. If tolerated, a dose of 1/10 was given 3 days to 1 week later, depending on the drug and the time interval between drug intake and the reaction. If tolerated, the full therapeutic dose was given after the same interval. If symptoms suggestive of NIDHR appeared, the procedure was stopped and the symptoms were evaluated and treated. Medications were stopped before DPT according to international guidelines.[33]

Samples obtaining:

Forty mL of heparinised blood were obtained from NIDHRs patients and healthy controls. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation (Rafer SL, Zaragoza, Spain), frozen and stored in liquid nitrogen by the Biobank of IBIMA-HRUM until the performance of the tests.

Lymphocyte Transformation Test:

Conventional LTT (C-LTT) . This was performed with PBMCs directly labelled with CFSE (Thermo Fisher Scientific, Waltham, USA). PBMCs were cultured in plates at $1.5x10^5$ cells per well in complete RPMI medium (supplemented with 10% FBS, 2mM L-Glutamine, 50ng/mL Streptomycin and 5mg/mL Gentamycin (Normon, Madrid, Spain)) and with the culprit drug at different concentrations (Table S1) for 6 days at 37degC and <5% of CO_2.[34]PBMCs without stimulus, only culture media and phytohemaglutinin (PHA) (Sigma, St. Louis, USA) at 20µg/mL were used as negative and positive control respectively.

Drug-primed-moDCs LTT (dDC-LTT) . Immature moDCs were transformed from monocytes ($CD14^+$ cells) isolated from PBMCs by positive selection (Miltenyi Biotec, Bergisch Gladbach, Germany) and cultured for 5 days in complete RPMI medium supplemented with 100ng/mL of IL-4 and 200ng/mL of GM-CSF (both from R&D Systems Inc, Minneapolis, USA). These immature moDCs were cultured with the culprit drug for 3 days at different concentrations (Table S1). After this, $1.5x10^4$ drug-primed-moDCs were co-cultured with $1.5x10^5$ autologous CFSE-labelled-lymphocytes for 6 days at 37°C and 5% of CO_2.

Phenotypical analysis by flow cytometry

After the incubation period, specific proliferations were assessed by flow cytometry in a FACS Canto II cytometer (BD Biosciences Milpitas, USA) analysing the $CFSE_{dim}$ expression in $CD3^+$, $CD4^+$, $CD8^+$and NK cells and in different subpopulations: $CD3^+CD4^+CXCR3^+IFN\gamma^+$($CD4^+$Th1); $CD3^+CD4^+CRTH2^+IL\text{-}4^+$($CD4^+$Th2)[35]; NK cells including the subtypes, $CD3^-CD56^+Perforin^+$(NK_{Perf}) and $CD3^-CD56^+IFN\gamma^+$($NK_{I\Phi N\text{-}\gamma}$).[11] Data were analysed by FlowJo software (BD Biosciences Milpitas,

USA) following different gate strategies (Figure S1B). Results were expressed as Proliferation Index (PI) calculated as (%$CFSE_{dim}$ stimulated lymphocytes+moDCs-%$CFSE_{dim}$ unstimulated lymphocytes+moDCs)/ %$CFSE_{dim}$lymphocytes.[35]

Statistical analysis:

Data normality was assessed by Kolmogorov-Smirnov test. Quantitative comparisons without a normal distribution were carried out using Mann-Whitney and Kruskal Wallis-tests. Comparisons of qualitative variables were performed using the X^2 test. Receiver operating characteristic curves (ROC curves) were performed for getting cut-off point to indicate positive results. P-values less than 0.05 were considered statistically significant.

RESULTS

The study included 37 patients, 24 were females (64.9%) and 13 males (35.1%) (mean age of 55.2±22.4 years). The time interval between drug administration and onset of symptoms was 103.3±132.8 hours, and between onset of symptoms and sample collection was 160±186 days. The most frequent clinical entities were MPE, in 21 (56.8%), and SJS-TEN, in 10 (27%), followed by AGEP (acute generalized exanthematous pustulosis) in 3 cases (8.1%), DRESS in 2 cases (5.4%), and FDR (fixed drug reaction) in 1 case (2.7%). The most frequent drugs implicated in the reactions were BLs in 14 (37.8%), RCM in 11 (29.73%), quinolones in 4 cases (10.81%) and in a lower degree xanthine oxidase inhibitors, anticonvulsants and sulphonamides in 2 cases each (5.4%), and benzodiazepines and ferrous supplement in 1 case each (2.7%). Thirteen (35%) cases were diagnosed by ST (10 IDT and 3 PT), 13 (35%) by DPT, and 11 (30%) by the ASPS (Table 1). Additionally, 21 sex- and age-matched healthy controls with tolerance to drugs were included.

Lymphocyte transformation test with CFSE

ROC curves were performed in $CD3^+$cells for both C-LTT and dDC-LTT. The area under curve (AUC) was 0.6045 (p=0.1283) and 0.7826 (p<0.0005) and we selected a cut-off of 2.22 and 1.28 for C-LTT and dDC-LTT respectively, both with a specificity of 85% (Figure S2A).

The C-LTT with PBMCs showed a significant lower sensitivity (33.3%) compared with dDC-LTT (65.2%) (p=0.026). When we combined the results of C-LTT and dDC-LTT, no increase in the sensitivity was observed (65.2%) compared with dDC-LTT alone. Nevertheless, its specificity reduced to 82.6% (Figure 1A).

As dDC-LTT showed higher sensitivity than C-LTT, we focused on this test to evaluate the different cell subpopulations proliferation. Significant higher proliferations were obtained for all cell subpopulations from allergic patients comparing with healthy controls (Figure 1B). Moreover, in allergic patients, we observed significant higher proliferation in $CD4^+T_h1$ cells, compared with other cell subpopulations including $CD4^+T_h2$, $CD8^+$ and NK cells.

When we analysed the results in terms of positivity, the sensitivity of dDC-LTT in $CD3^+$cells increased from 65.2% to 73.91%, and to 82.6% when $CD4^+T_h1$ and NK cells were respectively included in the analysis and to 86.9% when the three cell subsets were analysed together (Figure 1C). Regarding specificity, it was similar for all cell subpopulations mentioned above (85%). Moreover, although the sensitivity increased to 91% with the inclusion of $CD8^+$ cells, the specificity reduced to 80% (Figure 1C).

No correlation was observed between proliferation results and the time interval between drug administration and onset of symptoms or the time interval between the onset of symptoms and the performance of LTT (data not shown).

LTT in different clinical manifestations

ROC curves were performed for both C-LTT and dDC-LTT for the most frequent clinical entities, SJS-TEN, MPE, and AGEP to select the cut-off for positivity. In SJS-TEN patients, comparisons between both LTTs showed differences with an AUC of 0.78 (p=0.01) for C-LTT and of 0.96 (p<0.0001) for dDC-LTT (Figure S2B). Using a cut-off 2.2 for C-LTT and 1.28 for dDC-LTT, the sensitivity was 62.5% and 87.5% respectively,

with 85% of specificity for both (Figure 2). In MPE patients, after ROC curve analysis, we stablished a cut-off of 1.71 (AUC=0.559, p=0-50) for C-LTT and 1.28 (AUC=0.603, p=0-28) for dDC-LTT, obtaining a specificity of 85% in both cases (Figure S2C). Results showed lower sensitivity in C-LTT, 14.3%, than in dDC-LTT, 41.7% (Figure 2). In patients with AGEP, ROC curves showed an AUC of 0.602 (p=0.45) and 0.854 (p=0.012) for C-LTT and dDC-LTT respectively. A cut-off of 2.28 for C-LTT and 1.28 for dDC-LTT was selected to a specificity of 85% in both LTT (Figure S2D). The sensitivity of C-LTT was only 33%, whereas in dDC-LTT it increased to 80% (Figure 2).

Taken into account the results obtained with dDC-LTT, we observed a significant higher proliferation in SJS-TEN patients compared with MPE patients (p=0.001) (Figure 2A). We also obtained a significant higher percentage of positive cases in SJS-TEN and AGEP patients, 87.5% and 80% respectively, compared with MPE patients (41.6%) ($p<0.05$ in both cases) (Figure 2B).

Afterwards, we analysed if the results in the proliferative response using dDC-LTT vary between different cell-subsets in these clinical entities. In SJS-TEN patients, the analysis of proliferation of different cell subpopulations showed significant higher levels of $CD4^+T_h1$ compared with $CD4^+T_h2$ cells, NK cells, mainly for the inflammatory subpopulation ($p<0.0001$, $p<0.01$, and $p<0.01$ respectively), but not compared with cytotoxic NK cells. Moreover, the proliferation of $CD3^+$, $CD4^+$, and $CD8^+$ cells was also significantly higher than $CD4^+T_h2$ cells proliferation ($p<0.01$) (Figure 3A). Regarding MPE patients, the proliferation of $CD4^+T_h1$ cells was again significantly higher ($P<0.001$) than the rest of cell subpopulations analysed except for $CD4^+T_h2$ cells and NK with inflammatory pattern, $CD3^-CD56^+IFN\gamma^+$(Figure 3B). Regarding AGEP patients, we observed a significant higher proliferation of $CD4^+T_h1$ cells compared with the general population of $CD4^+$($P<0.01$), $CD4^+T_h2$ ($P<0.01$) and cytotoxic NK cells ($P<0.01$) (Figure 3C).

According to the cut-off previously described for each clinical entity, we analysed the proliferative response in terms of positivity (Figure 3D-F). In SJS-TEN patients, despite the high sensitivity in $CD3^+$cells (87.5%), when we included the results from other cell subpopulations, we were able to detect all patients (100%), concretely, when including $CD4^+T_h1$ and NK cells without reducing LTT specificity (Figure 3D). When we analysed the sensitivity of dDC-LTT in MPE patients combining $CD3^+$ with the results of the most relevant cell subsets, there was an increase from 41.6% to 50% when including $CD4^+T_h1$ cells and to 58.3% with NK cells (Figure 3E). Moreover, when we combined the results of $CD3^+$, $CD4^+T_h1$ and NK cells, the sensitivity increased to 66.7% without reducing the general specificity of 85%. In case of AGEP patients, the inclusion of $CD4^+T_h1$ in the analysis with $CD3^+$ cells, did not improve the sensitivity (80%) (Figure 3F), but it increased to 100% after the inclusion of NK and $CD8^+$ cells with a specificity of 85% and 80% respectively. The positivity based on the proliferation of $CD4^+T_h1$ cells is higher in the three clinical entities studied (Figure 4A). Nevertheless, the involvement of $CD4^+T_h2$ cells was higher in MPE patients (67%) compared with SJS-TEN (37.5%) and AGEP (50%). We also found differences in the percentage of positivity between inflammatory and cytotoxic NK cells, being higher for cytotoxic NK cells in SJS-TEN patients (87.5% versus 50%) (Figure 4B). On the contrary, in MPE patients the positivity of inflammatory NK cells was higher (71.4%) compared with the cytotoxic ones (28.6%). AGEP patients showed a more balanced proliferative response between inflammatory (80%) and cytotoxic (66%) NK cells.

DISCUSSION

The diagnosis of NIDHRs involves a great complexity due to the existence of different clinical manifestations related to the involvement of many pathomechanisms and the existence of severe reactions which difficult the application of clinical procedures. Moreover, *in vivo* tests such as STs have a doubtful value to evaluate NIHDR, due to their low sensitivity and because for some drugs its use is not recommended or not available. For this reason, DPT is the gold-standard, although it is not risk-free, and for most severe clinical entities it is not allowed.[36]

The implementation of *in vitro* tests with good sensitivity in the clinical practice would be a crucial step in the improvement of NIDHR diagnosis, especially for severe cases. Among others, LTT is a widely used tool for assessing specific proliferation of cell populations in response to a concrete drug. Traditionally, this

proliferation has been measured by the uptake of ^{3}H-thymidine and was measured by radioactivity, making it impracticable for routine laboratories.[37] The mean sensitivity of LTT with PBMCs ranges from 60% to 70%.[38] Considering different clinical manifestations, it has been observed to be higher in mild-moderate reactions (65.1%) than in severe ones (39.9%).[24]

In our study, including patients with different clinical manifestations and drugs involved, we obtained a low sensitivity (33%) in the C-LTT, which agrees with previous studies that also include a wide panel of clinical symptoms.[18,21] However, other studies, also with different clinical symptoms, reported a higher sensitivity.[23,39,40] The main difference could be that, in these latter works, the responsible drugs are mainly BLs and anticonvulsants, which have shown a higher sensitivity in LTT.[13] All of this indicates that conventional LTT with PBMCs does not show optimal sensitivity.

It has been reported that the inclusion of professional APCs could improve the sensitivity of LTT as shown in NIDHRs to BLs, heparins, and RCM.[19,27,28] In our study, comparisons between C-LTT with the test using DCs (dDC-LTT) showed an important increase in general sensitivity from 33.3% to 65%.

An important issue is the capacity of *in vitro* test for evaluating different clinical manifestations, since in previous studies LTT reported higher sensitivity in mild-moderate NIDHR reactions than in severe ones.[13,14,20,22] However, in our study we found different results with lower sensitivity in MPE (14.5%) and better sensitivity in severe reactions as SJS-TEN (62.5%). Although we do not know the real reasons, the different drugs involved in the reactions in each study could be a factor for these discrepant results. Moreover, the inclusion of moDCs in the LTT significantly increases the sensitivity to 87.5% in SJS-TEN and 41.6% in MPE, with no changes in specificity (85%). These data strongly show the beneficial effect of including mo-DCs for amplifying the specific immunological response and specially for improving the results when evaluating patients with severe reactions as SJS-TEN or AGEP for which LTT has classically shown a low sensitivity.

On the other hand, different studies have stated that focussing on the effector response will help increase the sensitivity of *in vitro*cellular tests.[13,14] This has been analysed by determining different inflammatory mediators by flow-cytometry and ELISpot, however with heterogeneous results.[20,21,23,37,41] Therefore, since no *in vitro* test produces enough sensitivity, other authors recommend the combination of different assays to evaluate NIDHRs.[21,22,37]

The use of flow-cytometry technology could represent a novel approach that allows the evaluation in routine laboratories. Preliminary studies have shown the possibility of evaluating DHRs by measuring the upregulation of CD69 by T-cells[15] or cytokine production[37] after stimulation with the suspected drug. However, little is known about the utility of measuring the proliferation response by using CFSE. One important advantage of measuring the $CFSE_{dim}$ for the proliferative response by flow-cytometry is the direct possibility of measuring the proliferation of different cell subpopulations involved, including those with low rates but important implications.[42] In our study, we tried to evaluate the effect response by analysing the differential proliferative response of different cell subpopulations, showing that CD4 T-lymphocytes with a T_h1 pattern are strongly involved in NIDHRs and their evaluation increases the sensitivity of the*in vitro* test compared with the evaluation of general T cell, $CD3^+$cells. This was also observed for the different clinical entities included in this study, SJS-TEN, MPE, and AGEP, indicating the participation of this cell subset in the pathomechanism involved in NIHDRs.[9] The other important cell subpopulation was NK cells, which have shown to be involved in all clinical manifestations although with differences regarding the NK subpopulations with higher proliferation of inflammatory NK ($NK_{I\Phi N\text{-}\gamma}$) in MPE as previously described[11], and cytotoxic NK (NK_{Perf}) in SJS-TEN according to the mechanism involved in these reactions[43]. Interestingly, $CD4^+$T-lymphocytes with a T_h2 pattern have shown to be involved mainly in MPE as previously described.[9,21] With all these data and including the effector cell subpopulations involved in each reaction, $CD3^+$+$CD4^+T_h1$ cells in SJS-TEN, $CD3^+$+$CD4^+T_h1$+NK cells in MPE and $CD3^+$+NK cells in AGEP, we could significantly increase the overall sensitivity of the *in vitro*test to 87% with 85% of specificity. Importantly, this increase in sensitivity was achieved with the performance of a unique *in vitro* test.

In summary, these data indicate that *in vitro* test analysing the proliferative response to drugs in NIDHRs can be highly improved by presenting the drug by professional APC as moDCs and focussing on the subpopulations participating in the immunological mechanism for each clinical manifestation in a specific manner. This can be easily achieved thanks to the use of flow-cytometry-based tests. Further advances on the knowledge of the mechanism and the identification of specific biomarkers that will be included in the test will increase the *in vitro*diagnosis of NIDHRs.

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Table 1. Demographics and clinical data from the allergological workup of patients with NIDHRs

Patients	Sex	Age	Clinical entity	Drug	Time interval	STs	DPT	ASPS
P-1	F	14	MPE	BL	144	-	+	ND
P-2	M	11	MPE	BL	96	-	+	ND
P-3	F	69	MPE	RCM	24	-	+	ND
P-4	M	73	DRESS	BZD	144	-	ND	11
P-5	F	27	MPE	BL	168	-	+	ND
P-6	F	75	SJS/TEN	RCM	24	-	ND	9
P-7	M	53	AGEP	BL	288	+	ND	ND
P-8	M	75	FDR	BL	144	+	ND	ND
P-9	M	17	SJS/TEN	BL	120	ND	ND	8
P-10	F	82	MPE	BL	120	-	+	ND
P-11	M	12	MPE	BL	24	+	ND	ND
P-12	M	54	SJS/TEN	MET	48	+	ND	ND
P-13	F	64	SJS/TEN	SULPH	48	ND	ND	8
P-14	F	82	DRESS	Iron	60	-	ND	9
P-15	M	66	AGEP	XOI	72	-	ND	11
P-16	F	24	AGEP	BL	8	ND	ND	9
P-17	F	44	SJS/TEN	QNL	312	-	ND	8
P-18	F	63	MPE	RCM	12	+	ND	ND
P-19	F	66	MPE	RCM	24	-	+	ND
P-20	F	75	MPE	RCM	12	+	ND	ND
P-21	M	79	MPE	RCM	2	-	+	ND
P-22	F	77	MPE	RCM	12	-	+	ND
P-23	F	35	MPE	SULPH	168	+	ND	ND
P-24	F	29	MPE	BL	24	-	+	ND
P-25	M	83	MPE	RCM	24	+	ND	ND
P-26	M	55	SJS/TEN	XOI	48	-	ND	10
P-27	F	59	MPE	RCM	12	-	+	ND
P-28	F	34	MPE	BL	2	+	ND	ND
P-29	M	75	MPE	RCM	10	-	+	ND

Patients	Sex	Age	Clinical entity	Drug	Time interval	STs	DPT	ASPS
P-30	F	70	MPE	RCM	10	+	ND	ND
P-31	M	50	SJS/TEN	QNL	96	ND	ND	9
P-32	F	91	SJS/TEN	QNL	465	-	ND	8
P-33	F	43	MPE	BL	48	-	+	ND
P-34	F	46	MPE	BL	2	-	+	ND
P-35	F	38	SJS/TEN	ACV	480	+	ND	ND
P-36	F	57	SJS/TEN	ACV	480	+	ND	ND
P-37	F	74	MPE	BL	48	+	ND	ND

F: Female; M: Male; MPE: Maculopapular Exanthema; DRESS: drug reaction with eosinophilia and systemic symptoms; Steven-Johnson Syndrome/Toxic Epidermal Necrolysis (SJS/TEN); AGEP: Acute generalized exanthematous pustulosis; FDR: fixed drug reaction; BL: Betalactam; RCM: Radiocontrast media; BZD: Benzodiazepine; QNL: Quinolone; SULPH: Sulphonamide; XOI: Xanthine oxidase inhibitors; ACV: Anti-convulsant; DPT: Drug provocation test; ST: skin test; Time interval: Hours between drug administration and onset of symptoms; ND: not done

Table S1. Drugs and concentrations tested in LTT.

Drug	Concentrations
Amoxicillin Diazepam Cefepime Cefixime Trimetoprim Sulfametoxazol Allopurinol Clavulanic acid	250µg/mL, 125µg/mL, 25µg/mL
Iobitridol Iomeron Iodixanol	5mg/mL, 1mg/mL, 0.1mg/mL
Ciprofloxacin	100µg/mL, 10µg/mL, 1µg/mL
Iron supplement	250µg/mL, 125µg/mL, 25µg/mL, 5µg/mL
Phenobarbital Carbamazepine	50µg/mL, 10µg/mL, 1µg/mL

Figure Legends

Figure 1. General proliferative response. A) Bars represent percentage of positive cases in $CD3^+$cells in both conventional LTT (C-LTT) and drug-primed-moDCs LTT (dDC-LTT) in NIDHR patients and healthy controls; **B)** Dots and bars represent proliferation index in dDC-LTT in different cell subpopulations in NIDHR patients and healthy controls; **C)** Bars represent percentage of positive cases using dDC-LTT combining different cell subpopulations in NIDHR patients and healthy controls. Comparisons in terms of positivity by X^2 test and proliferation index by Kruskal-Wallis test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

Figure 2. Proliferative response of $CD3^+$cells in different clinical entities. A) Box plot represents proliferation index of $CD3^+$ cells on different clinical entities studied in both C-LTT (in white) and dDC-LTT (in grey); **B)** Bars represent percentage of positive cases on different clinical entities studied in both C-LTT (in white) and dDC-LTT (in grey). Differences in the proliferation index have been performed using Mann–Whitney U test. Comparisons in terms of positivity have been performed using X^2 test (*$p < 0.05$; ***$p < 0.001$).

Figure 3. Proliferative response of the different cell subpopulations based on the clinical entity. Dots and bars represent proliferation index in dDC-LTT in different cell subpopulations in NIDHR patients with **A)** SJS-TEN; **B)** MPE; **C)** AGEP. Bars represent percentage of positive cases using dDC-LTT combining different cell subpopulations in NIDHR patients (in grey) in **D)**SJS-TEN; **E)** MPE; **F)** AGEP and healthy controls (in white). Proliferation index comparisons between cell populations have been performed

using Kruskal-Wallis test. Comparisons in terms of positivity have been performed using X^2 test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

Figure 4. Cell subpopulations positivity based on the clinical entity. A) Bars represent percentage of positive cases of $CD4^+T_h1$ (in white) and $CD4^+T_h2$ cells (in grey) in SJS, MPE, and AGEP patients; **B)** Bars represent percentage positive cases of $NK_{I\Phi N\text{-}\Upsilon}$ (in white) and NK_{Perf} cells (in grey) in SJS, MPE, and AGEP patients.

Figure S1. Flow cytometry strategy. A) Selection of proliferative $CFSE_{dim}CD3^+$ cells in unstimulated cells, cells stimulated with PHA, and cells stimulated with the culprit drug: **B)** Selection of cell subpopulations strategy.

Figure S2. ROC curves. ROC curve analysis of $CD3^+$ cells in C-LTT and dDC-LTT in **A)** NIDHRs patients and healthy controls; **B)** SJS-TEN patients; **C)**MPE patients; **D)** AGEP patients. Arrows represent the Proliferation index value with the best balance between sensitivity and specificity.

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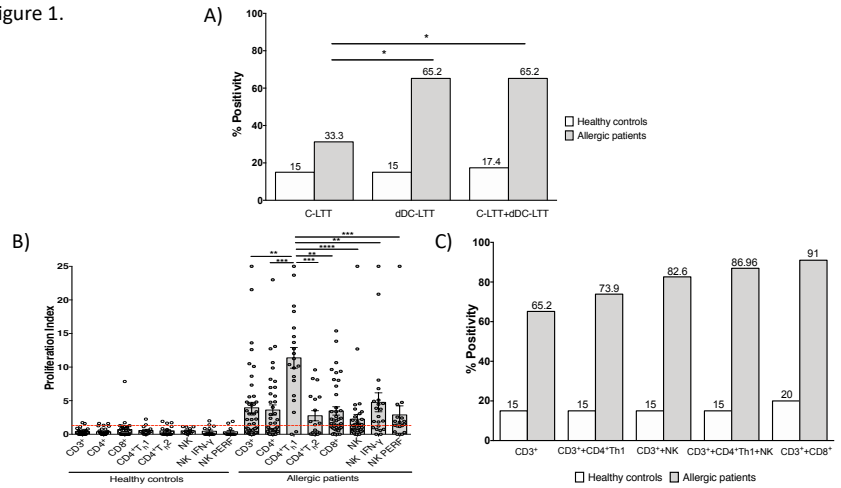
Figure 1.



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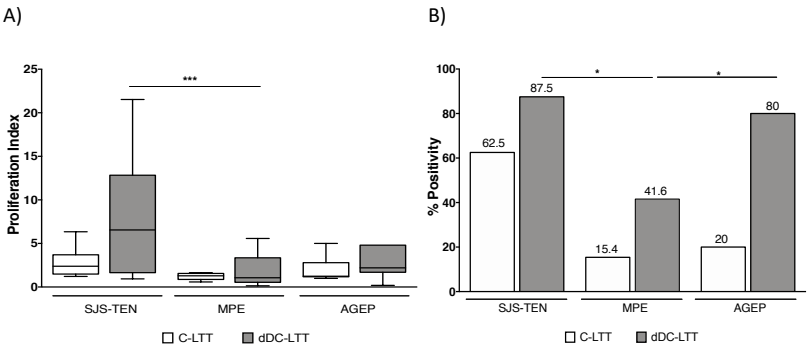

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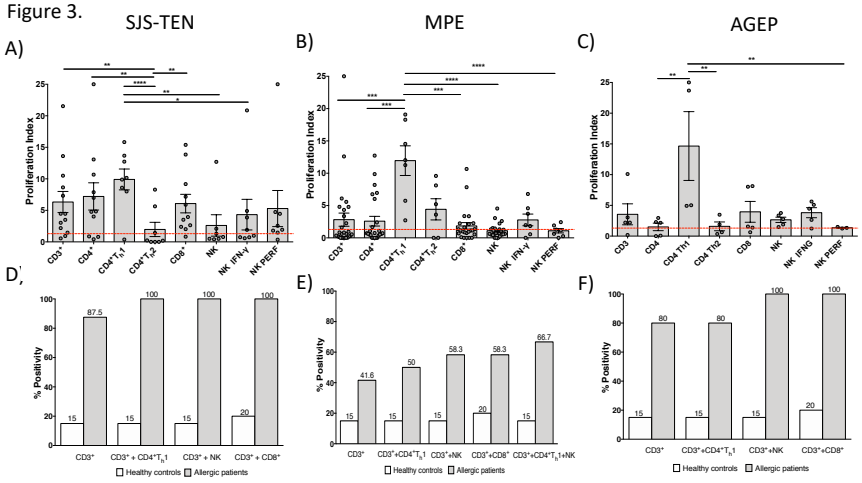

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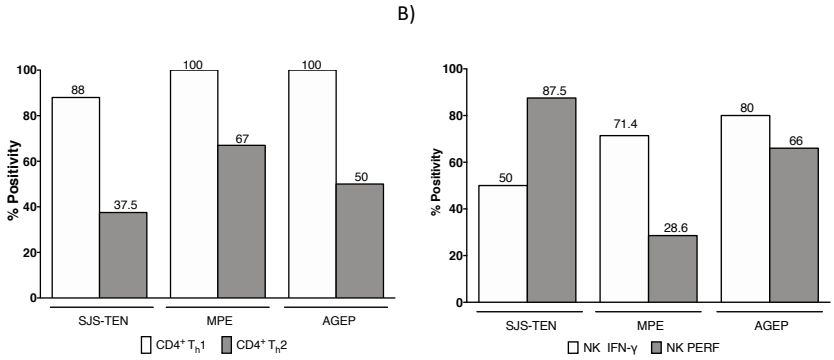

Figure S1.

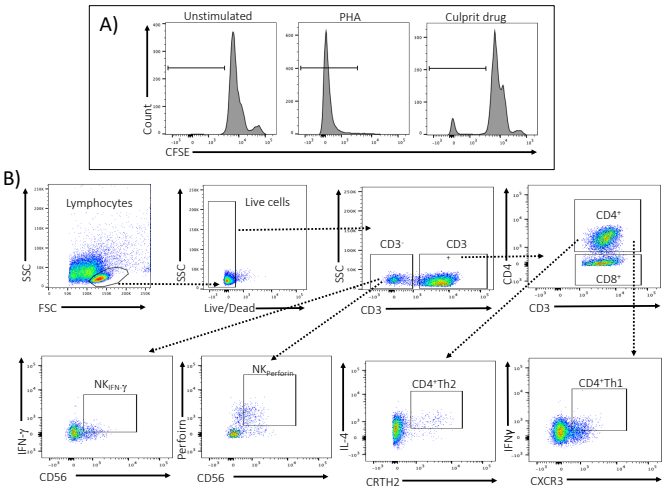

Figure S2.