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Article type : Original Article: Drug Allergy, Insect Sting Allergy, and Anaphylaxis

Title: Dendritic cells inclusion and cell-subset assessment improve flow-cytometry-based proliferation test in non-immediate drug hypersensitivity reactions.

Manuscript Acceptance Date: 04-Jan-2021

Short Title: Flow-cytometry-based lymphocyte proliferation test

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/all.14755](#)

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Acknowledgements

We thank Claudia Corazza for her invaluable English language support and BIOBANK of the HRUM for sample storage. The present study has been supported by Institute of Health “Carlos III” of the Ministry of Economy and Competitiveness (grants cofunded by European Regional Development Fund (ERDF): PI15/01206, PI18/00095, RETICS RIRAAF RD12/0013/0001, RETICS ARADYAL RD16/0006/0001 and RD16/0006/0010, Biobank network RD09/0076/00112, and Biobank platform PT13/0010/0006). Andalusian Regional Ministry of Economy and Knowledge (grants cofunded by European Regional Development Fund (ERDF): CTS-06603); Andalusian Regional Ministry Health (grants PI-0241-2016 and PE-0172-2018).

CM and TDF hold a “Nicolas Monardes” research contract by Andalusian Regional Ministry Health: RC-0004-2016C and C1-0019-2019 respectively. GB and EB hold a “Juan Rodes” (JR18/00054 and JR18/00049, respectively), all research contracts by Institute of Health “Carlos III” of the Ministry of Economy and Competitiveness (grants cofunded by European Social Fund (ESF)).

Conflicts of interest

The author declare that they have no conflict of interest.

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 (CFSE).

Results. The C-LTT showed a significantly lower sensitivity (29.4%) compared with dDC-LTT (61.8%), which was confirmed analysing each particular clinical entity: SJS-TEN (62.5% vs 87.5%), MPE (15% vs 47.4%), and AGEP (33% vs 80%).

When including the effector cell subpopulations involved in each clinical entity, CD3⁺CD4⁺T_h1 or CD3⁺NK 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%, 68.4%, 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 focusing 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

AGEP: Acute generalised exanthematous pustulosis
APC: Antigen presenting cell
ASPS: Algorithm of the Spanish Pharmacovigilance System
AUC: Area under curve
BL: Betalactam
CFSE: Carboxyfluorescein diacetate succinimidyl ester
C-LTT: Conventional lymphocyte transformation test
DHRs: Drug hypersensitivity reactions
dDC-LTT: Drug-primed-mo-DCs lymphocyte transformation test
DPT: Drug provocation test SJS: Stevens-Johnson Syndrome
DRESS: Drug reaction with eosinophilia and systemic symptoms
EAACI: European Academy of Allergy and Clinical Immunology
ESCD: European Society of Contact Dermatitis
FDE: Fixed drug eruption
IDHR: Immediate drug hypersensitivity reaction
IDT: Intradermal test
LTT: Lymphocyte transformation test
mo-DC: Monocyte-derived dendritic cell
MPE: Maculopapular exanthemas
NIDHR: Non-immediate drug hypersensitivity reaction
PBMC: Peripheral blood mononuclear cell
PHA: Phytohemagglutinin
PT: Patch test
PI: Proliferation index
RCM: Radio contrast media
ROC curve: Receiver operating characteristic curve
SJS: Stevens-Johnson Syndrome
ST: Skin test
TEN: Toxic epidermal necrolysis

INTRODUCTION

Drug hypersensitivity reactions (DHRs) are currently a burden on Healthcare Systems accounting for 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 into 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), which 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 T_H1 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 manifestations¹¹ or CD4⁺ T_H2 cells in DRESS.¹² This highlights the importance of 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 around 94%.¹⁵⁻¹⁷ However, the lack of standardisation and the low sensitivity have 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 entity, with higher sensitivity (57.9-88.8%) in mild/moderate reactions¹⁸⁻²⁰ and lower sensitivity (25-75%) for severe NIDHR as SJS-TEN.^{13,21-24} Nevertheless, recent studies in well selected patients reported higher sensitivity in DRESS (73%-82%)²⁵ or SJS-TEN (85%)²⁶, mainly when LTT is performed during the resolution phase.

This creates the need for improving the sensitivity of the test, particularly in severe reactions with very limited diagnostic approaches available.^{13,14,27}

Several attempts have been performed for improving LTT sensitivity. The inclusion of the drug metabolites has shown to be important for some drugs.²⁸ Other studies have demonstrated that the inclusion of monocyte-derived dendritic cells (mo-DCs) 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).^{19,29,30}

The cell proliferative response has been classically measured via the genome incorporation of tritiated thymidine (³H).¹⁷ 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, 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-mo-DCs as APCs and the determination of the proliferative response of different lymphocyte subpopulations in each clinical manifestation of NIDHRs using the LTT approach based on flow-cytometry technology. To this end, patients with confirmed NIDHR were evaluated by both, conventional (C-LTT) and with drug-primed-mo-DCs (dDC-LTT) LTTs, analysing the proliferative response in different T-cell subsets and other cell subpopulations as NK cells.

METHODS

Allergic patients and healthy controls selection

From patients with suggestive clinical history of NIDHR attending the Allergy Unit of the Hospital Regional Universitario de Malaga (HRUM), prospectively evaluated between 2013 and 2019, 299 patients were confirmed as NIDHRs following European Academy of Allergy and Clinical Immunology (EAACI).^{6,7,31,32} 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)^{25,26,33} by evaluating the chronology, the degree of knowledge of the relationship between the drug and the reaction, the withdrawal and the rechallenge drug effect, and alternative causes. This algorithm classified drugs 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.³⁴ 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^{7,34} 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.³⁵

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.^{8,36} 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.³⁶

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. To maintain the homogeneity between methods, thawed cells were used for both LTT, and at the same run.

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 1.5×10^5 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 37°C and 5% of CO₂.³⁷ PBMCs without stimulus, only culture media and phytohemagglutinin (PHA) (Sigma, St. Louis, USA) at 20µg/mL were used as negative and positive control respectively.

Drug-primed-moDCs LTT (dDC-LTT). Immature mo-DCs were differentiated from monocytes (CD14⁺ cells) isolated from PBMCs by positive selection (MiltenyiBiotec, BergischGladbach, 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).

Change of cells morphology was assessed by inverted microscopy to ensure the correct differentiation from monocytes to mo-DCs. These immature mo-DCs were cultured with the culprit drug for 3 days at different concentrations (Table S1). After this, the cell culture supernatants were discarded, and 1.5x10⁴ drug-primed-mo-DCs were co-cultured with 1.5x10⁵ autologous monocyte-depleted PBMCs labelled with CFSE for 6 days at 37°C and 5% of CO₂.

Phenotypical analysis by flow cytometry

After the incubation period, specific proliferations were assessed in duplicate 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 γ ⁺ (CD4⁺T_h1); CD3⁺CD4⁺CRTH2⁺IL-4⁺ (CD4⁺T_h2)³⁵; NK cells including the subtypes, CD3⁺CD56⁺Perforin⁺ (NK_{Perf}) and CD3⁺CD56⁺IFN γ ⁺(NK_{IFN- γ}).¹¹Data were analysed by FlowJo software (BD Biosciences Milpitas, USA) following different gate strategies (Figure S1). Isotype controls were used for each marker to establish the correct gating selection (Figure S2). The inclusion of mo-DCs as APCs produces a little unspecific increase in the % of CFSE_{Dim} lymphocytes that could be due to the characteristic autofluorescence of mo-DCs. To avoid this interference with the LTT analysis, the results were expressed as Proliferation Index (PI)³⁸ calculated as:

$$PI = \frac{(\% \text{ CFSE}_{\text{Dim}} \text{ stimulated lymphocytes} + \text{moDCs}) - (\% \text{ CFSE}_{\text{Dim}} \text{ unstimulated lymphocytes} + \text{moDCs})}{\% \text{ CFSE}_{\text{Dim}} \text{ unstimulated lymphocytes}}$$

Both LTTs were carried out in duplicates for each cell population and condition. Arithmetic mean was calculated for each one. The highest PI obtained for each drug was selected.

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 X² 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 selected from 299 patients with a confirmed diagnosis of NIDHRs. Twenty-four were females (64.9%) and thirteen 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 generalised exanthematous pustulosis) in 3 cases (8.1%), DRESS in 2 cases (5.4%), and FDE (fixed drug eruption) 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 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%). Drug causality was assessed in 13 (35%) patients by ST (10 IDT and 3 PT), 13 (35%) by DPT, and 11 (30%) by the ASPS (in 4 of these patients ST was not done because they suffered from atopic dermatitis and severe reactions) (Table 1). Additionally, 21 sex- and age-matched healthy controls with no hypersensitivity reaction reported to any drugs were included.

Lymphocyte transformation test with CFSE

ROC curves were performed for getting the cut- off point to consider positive results on CD3⁺ cells for both C-LTT and dDC-LTT.

The area under curve (AUC) was 0.605 ($p=0.128$) and 0.783 ($p<0.0005$) respectively. Cut-offs of 2.22 and 1.28 for C-LTT and dDC-LTT were selected, respectively, based on the best balance between sensitivity and specificity. The specificity was of 85% in both LTTs (Figure 1A).

The C-LTT with PBMCs showed a significant lower sensitivity (29.4%) compared with dDC-LTT (61.8%) ($p=0.026$). When we combined the results of C-LTT and dDC-LTT, no increase in the sensitivity was observed (61.8%) compared with dDC-LTT alone. Nevertheless, its specificity reduced to 82.6% (Figure 2A).

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 2B). 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 61.8% to 73.9%, and to 82.6% when CD4⁺T_H1 and NK cells were respectively included in the analysis, and to 87% when the three cell subsets were analysed together (Figure 2C). 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 was reduced to 80% (Figure 2C).

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 (Figure 1B-D).

To build these curves, we included PI results from 21 randomly selected healthy controls that were tested with the culprit drugs used to stimulate patient cells for each clinical entity, in the same proportion for both groups.

In SJS-TEN patients, comparisons between both LTTs showed differences with an AUC of 0.76 ($p=0.02$) for C-LTT and of 0.95 ($p<0.0001$) for dDC-LTT (Figure 1B). 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 3B). In MPE patients, after ROC curve analysis, we established a cut-off of 2.1 (AUC=0.56, $p=0.50$) for C-LTT and 1.20 (AUC=0.60, $p=0.28$) for dDC-LTT, obtaining a specificity of 85% in both cases (Figure 1C). Results showed lower sensitivity in C-LTT, 15%, than in dDC-LTT, 47.4% (Figure 3B). In patients with AGEP, ROC curves showed an AUC of 0.56 ($p=0.09$) and 0.90 ($p=0.006$) 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 1D). The sensitivity of C-LTT was only 33%, whereas in dDC-LTT it increased to 80% (Figure 3B).

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 3A). 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 (47.4%) ($p<0.05$ in both cases) (Figure 3B).

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 4A). 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 γ ⁺ (Figure 4B). 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 4C).

According to the cut-off previously described for each clinical entity, we analysed the proliferative response in terms of positivity (Figure 4D-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 or NK cells without reducing LTT specificity (Figure 4D). 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 47.4% to 50% when including CD4⁺T_h1 cells and to 58.3% with NK cells (Figure 4E). Moreover, when we combined the results of CD3⁺, CD4⁺T_h1 and NK cells, the sensitivity increased to 68.4% 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 4F), but it increased to 100% after the inclusion of NK and CD8⁺ cells with a specificity of 85% and 80% respectively. Contingency tables for each clinical entity according the cut-off and cell subpopulations selected are shown in table S2.

The positivity based on the proliferation of CD4⁺T_h1 cells is higher in the three clinical entities studied (Figure 5A). 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 5B). 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 their 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.³⁹

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 ³H-thymidine and was measured by radioactivity, making it impracticable for routine laboratories.⁴⁰ The mean sensitivity of LTT with PBMCs ranges from 56.1% to 78%.⁴¹

In our study, including patients with different clinical manifestations and drugs involved, we obtained a low sensitivity (29.4%) in the C-LTT; result in agreement with Polak's study, which also includes a wide panel of clinical symptoms.²¹ However, other studies, also with different clinical symptoms, reported a higher sensitivity.^{18,23,42,43} 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.^{19,29,30} In our study, in which in most cases BLs, RCM as well as some non-BLs antibiotics were the culprit drugs included, comparisons between C-LTT with the test using mo-DCs (dDC-LTT) showed an important increase in general sensitivity from 29.4% to 61.8%.

An important issue is the capacity of *in vitro* tests 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 (15%) and better sensitivity in severe reactions as SJS-TEN (62.5%). These results are in accordance with recent studies which reported high sensitivity in severe reactions when the group of patients is well-defined.^{25,26} Moreover, the different drugs involved in the reactions in each study could be a factor for these heterogeneous results. Regarding this, 2 patients with SJS-TEN reactions to anticonvulsants: phenobarbital (P-29) and carbamazepine (P-30), and 2 with SJS-TEN and AGEP (P-31 and P-34 respectively) to allopurinol were included in the study. There is great evidence that these concrete drugs (and/or their metabolites) are not chemically reactive to carrier proteins, and their recognition occurs by non-covalent binding to MHC complex^{44,45} or directly to HLA molecules.^{46,47} In both cases, involvement of intracellular metabolism and antigen processing are not needed. In our study, we have obtained positive results in 3 of 4 patients with dDC-LTT, suggesting the importance of including mo-DCs as APCs, independently of the mechanism involved in each case. Moreover, although the complete removal of the drug after the APC pre-priming could abolish the T-cell reactivity, as it has been shown for allopurinol/oxypurinol,⁴⁷ in our study, we only discard the cell supernatants after priming mo-DCs with the culprit drug, so the drug which is non-covalently bound to the MHC complexes could be still bound, allowing the presentation to lymphocytes. Nevertheless, more studies are needed to completely understand the concrete recognition mechanism involved for each drug.

Moreover, the inclusion of mo-DCs in the LTT significantly increases the sensitivity to 87.5% in SJS-TEN and 47.4% 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 focusing 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, 40, 48} Therefore, since no *in vitro* test produces enough sensitivity, other authors recommend the combination of different assays to evaluate NIDHRs.^{21, 22, 40}

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-cells¹⁵ or cytokine production⁴⁰ 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 assessing the proliferation of different cell subpopulations involved, including those with low rates but important implications.⁴⁹ 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 NIDHRs.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_{IFN- γ}) in MPE as previously described¹¹, and cytotoxic NK (NK_{Perf}) in SJS-TEN according to the mechanism involved in these reactions⁵⁰. 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 specific cell subpopulations involved in each reaction, CD3⁺CD4⁺T_h1 cells or CD3⁺NK 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. This increase in sensitivity was more pronounced (68.4%) in MPE patients, in which sensitivity even with DC was very low (47.4%). Importantly, this increase in sensitivity was achieved with the performance of a unique *in vitro* test.

One possible limitation of this study is the heterogeneity in responsible drugs, with some of them underrepresented, and clinical symptoms of the patients included; however, this is a representative sample of the population with confirmed NIDHR. Moreover, as main differences in the immunological mechanism involved could be related to the clinical symptoms, we have tried to make comparisons in those with enough number of patients. Moreover, due to the low number of patients with AGEP, the interpretation of these results should be cautiously considered.

In summary, although further research is needed, 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 mo-DCs and focusing on the subpopulations involved 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 family	Culprit drug	Interval drug-reaction	Interval reaction-study	STs	DPT	ASPS	C-LTT results	dDC-LTT results
P-1	F	14	MPE	BL	Amoxicillin	6 days	70 days	-	+	12	1.32	0.58
P-2	M	11	MPE	BL	Amoxicillin	4 days	90 days	-	+	12	0.81	1.11
P-3	F	27	MPE	BL	Amoxicillin	7 days	130 days	-	+	12	1.65	8.81
P-4	M	12	MPE	BL	Amoxicillin	1 days	305 days	+	ND	9	3	5.22
P-5	F	29	MPE	BL	Amoxicillin	1 days	140 days	-	+	11	1.23	1.20
P-6	F	43	MPE	BL	Amoxicillin	2 days	730 days	-	+	11	0.93	1.09
P-7	F	46	MPE	BL	Amoxicillin	2 hours	155 days	-	+	11	2	5.57
P-8	F	74	MPE	BL	Amoxicillin	2 days	120 days	+	ND	11	1.32	0
P-9	F	34	MPE	BL	Clavulanic acid	2 hours	150 days	+	ND	8	0.43	0.19
P-10	F	82	MPE	BL	Cefixime	5 days	14 days	-	+	11	0.58	12.6
P-11	F	70	MPE	RCM	Ibuprofen	10 hours	70 days	+	ND	11	1.29	0
P-12	F	69	MPE	RCM	Ibuprofen	1 days	152 days	-	+	11	1.36	3.35
P-13	F	63	MPE	RCM	Ibuprofen	12 hours	30 days	+	ND	12	1.53	0.14
P-14	F	66	MPE	RCM	Ibuprofen	2 days	640 days	-	+	11	0.86	0.23
P-15	F	75	MPE	RCM	Ibuprofen	12 hours	70 days	+	ND	11	0.29	0.54
P-16	M	79	MPE	RCM	Ibuprofen	2 hours	710 days	-	+	11	1.02	1.59
P-17	F	77	MPE	RCM	Ibuprofen	12 hours	365 days	-	+	11	0.87	ND
P-18	F	59	MPE	RCM	Ibuprofen	12 hours	70 days	-	+	11	3.43	10.1
P-19	M	75	MPE	RCM	Ibuprofen	10 hours	41 days	-	+	11	ND	10.5
P-20	M	83	MPE	RCM	Ibuprofen	1 days	10 days	+	ND	7	0.86	1.05
P-21	F	35	MPE	SULPH	Sulfamethoxazole	7 days	30 days	+	ND	9	12.41	ND
P-22	M	17	SJS/TEN	BL	Amoxicillin	5 days	220 days	ND	ND	8	3.97	4.46
P-23	F	75	SJS/TEN	RCM	Ibuprofen	1 days	40 days	-	ND	9	1.76	8.64
P-24	F	64	SJS/TEN	SULPH	Sulfamethoxazole	2 days	180 days	ND	ND	8	6.34	13.61
P-25	M	54	SJS/TEN	QNL	Ciprofloxacin	2 days	31 days	+	ND	8	1.21	0.93
P-26	F	44	SJS/TEN	QNL	Ciprofloxacin	13 days	30 days	-	ND	8	1.39	10.5
P-27	M	50	SJS/TEN	QNL	Ciprofloxacin	4 days	200 days	ND	ND	9	2.87	ND
P-28	F	91	SJS/TEN	QNL	Ciprofloxacin	19 days	400 days	-	ND	8	2.47	ND
P-29	F	38	SJS/TEN	ACV	Phenobarbital	20 days	50 days	+	ND	8	ND	2.19
P-30	F	57	SJS/TEN	ACV	Carbamazepine	20 days	150 days	+	ND	8	ND	1.47
P-31	M	55	SJS/TEN	XOI	Allopurinol	2 days	60 days	-	ND	10	2.29	21.53
P-32	M	53	AGEP	BL	Amoxicillin-Clavulanic acid	12 days	180 days	+	ND	8	16.86	10.10
								-	ND	8	ND	2.2
P-33	F	24	AGEP	BL	Amoxicillin-	8 hours	52 days	ND	ND	9	1.24	2.42

					Clavulanic acid			ND	ND	9	ND	3.19
P-34	M	66	AGEP	XOI	Allopurinol	3 days	86 days	-	ND	11	1.23	1.04
P-35	M	73	DRESS	BZD	Diazepam	6 days	30 days	-	ND	11	1.29	5.66
P-36	F	82	DRESS	Iron	Iron	3 days	86 days	-	ND	9	3.43	ND
P-37	M	75	FDE	BL	Cefepime	6 days	38 days	+	ND	9	0.96	0.77

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 generalised exanthematous pustulosis; FDE: fixed drug eruption; BL: Betalactam; RCM: Radiocontrast media; BZD: Benzodiazepine; QNL: Quinolone; SULPH: Sulphonamide; XOI: Xanthine oxidase inhibitors; ACV: Anticonvulsant; DPT: Drug provocation test; ST: skin test; ND: not done.

Figure Legends

Figure 1. ROC curves. ROC curve analysis of CD3⁺ cells in C-LTT and dDC-LTT in **A)** NIDHRs patients (14 tested to betalactams, 11 to radiocontrast media, 2 to sulfamethoxazole, 4 to quinolones, 2 to anticonvulsants, 2 to allopurinol, 1 to diazepam and 1 to iron) and healthy controls (8 tested to betalactams, 6 to radiocontrast media, 1 to sulfamethoxazole, 2 to quinolones, 1 to anticonvulsant, 1 to allopurinol, 1 to diazepam and 1 to iron); **B)** SJS-TEN patients (1 tested to betalactams, 1 to radiocontrast media, 1 to sulfamethoxazole, 4 to quinolones, 2 to anticonvulsants and 1 to allopurinol) and healthy controls (3 tested to betalactams, 2 to radiocontrast media, 2 to sulfamethoxazole, 8 to quinolones, 4 to anticonvulsants and 2 to allopurinol); **C)** MPE patients (10 tested to betalactams, 10 to radiocontrast media and 1 to sulfamethoxazole) and healthy controls (10 tested to betalactams, 10 to radiocontrast media and 1 to sulfamethoxazole); **D)** AGEP patients (2 tested to betalactams and 1 to allopurinol) and healthy controls (16 to betalactams and 5 to allopurinol). Arrows represent the Proliferation index value with the best balance between sensitivity and specificity.

Figure 2. General proliferative response. **A)** Bars represent percentage of positive tests assessed by proliferation of 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 tests using dDC-LTT combining different cell subpopulations in NIDHR patients and healthy controls. Comparisons in terms of positivity by χ^2 test and proliferation index by Kruskal-Wallis test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

Figure 3. Proliferative response of CD3⁺ cells in different clinical entities. **A)** Scatter plot (median with interquartile range) represents proliferation index of CD3⁺ cells on different clinical entities studied and healthy controls (HC) in both C-LTT and dDC-LTT; **B)** Bars represent percentage of positive tests on different clinical entities studied and healthy controls in both C-LTT and dDC-LTT. Differences in the proliferation index have been performed using Mann-Whitney U test. Comparisons in terms of positivity have been performed using χ^2 test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

Figure 4. 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 tests

using dDC-LTT combining different cell subpopulations in NIDHR patients in **D)** SJS-TEN; **E)** MPE; **F)** AGEP and healthy controls. Proliferation index comparisons between cell populations have been performed using Kruskal-Wallis test. Comparisons in terms of positivity have been performed using χ^2 test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

Figure 5. Cell subpopulations positivity based on the clinical entity. **A)** Bars represent percentage of positive tests of CD4⁺T_h1 and CD4⁺T_h2 cells in SJS (n=8, n=8), MPE (n=7, n=6), and AGEP (n=4, n=4) patients; **B)** Bars represent percentage positive tests of NK_{IFN- γ} :CD3⁻CD56⁺IFN- γ ⁺ and NK_{Perf}cells: CD3⁻CD56⁺Perforin⁺ in SJS (n=8, n=8), MPE (n=7, n=7), and AGEP (n=5, n=3) patients.

Figure S1. Flow cytometry strategy. **A)** Selection of different cell subpopulations strategy. **B)** Representative figure of the selection of proliferative CD3⁺ cells as those expressing CFSE_{Dim} in unstimulated cells, stimulated with PHA, and stimulated with the culprit drug.

Figure S2. Representative isotype controls. The isotype control for CD3 was selected from the lymphocyte population; CD4 from CD3⁺ cells; CXCR3, IFN- γ , CRTH2 and IL-4 from CD4⁺ cells; CD56 and Perforin from CD3⁻ cells.

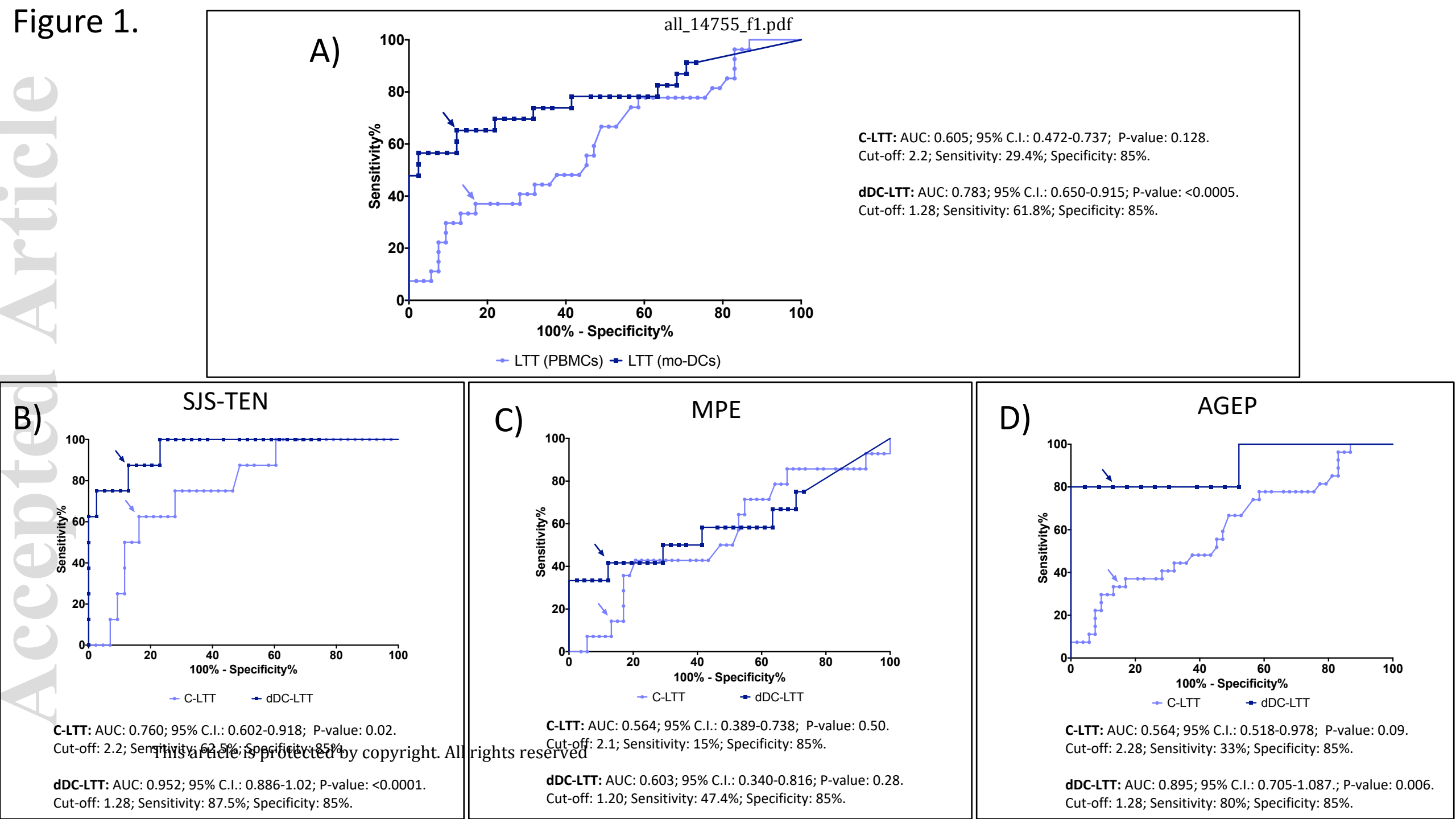


Figure 2.

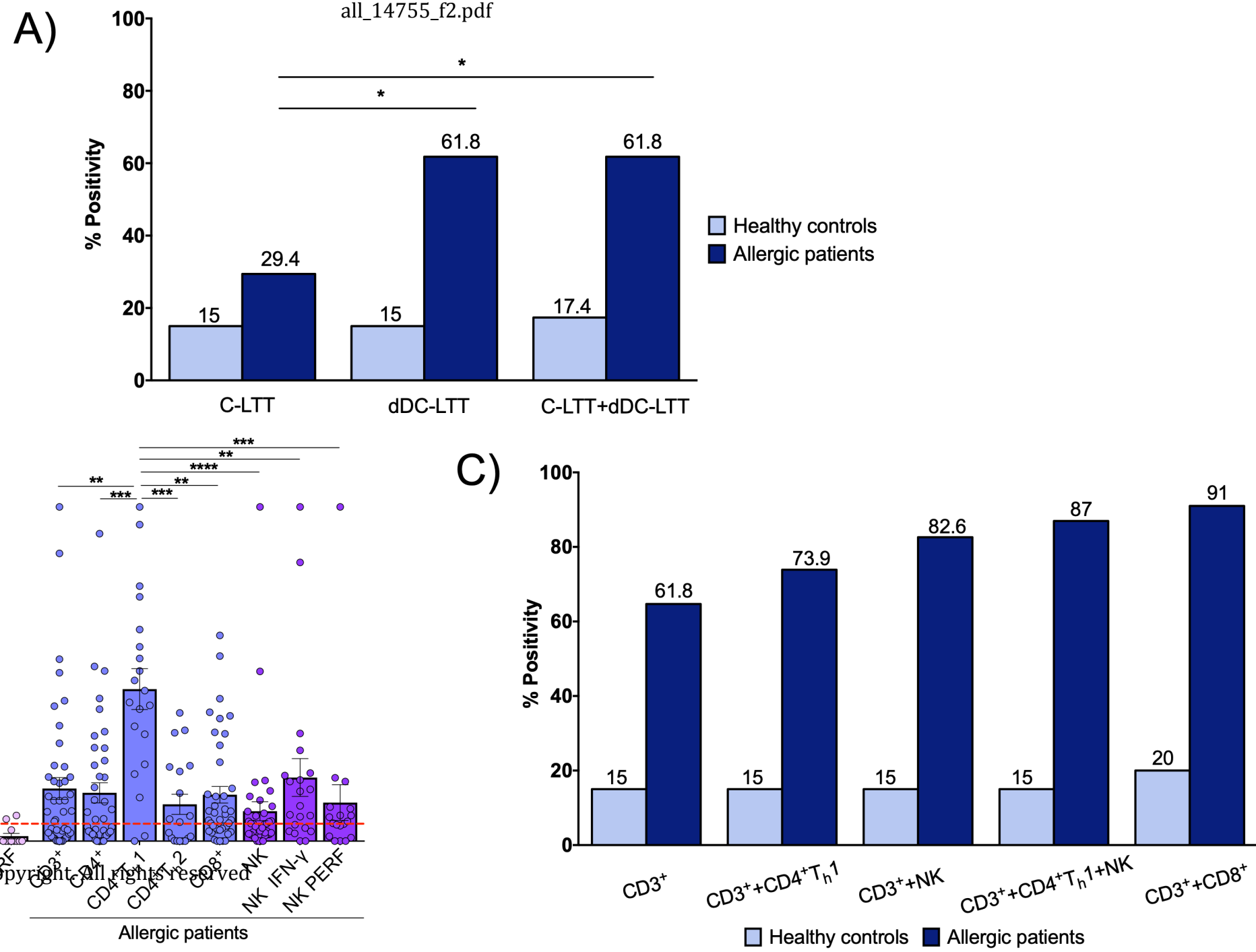
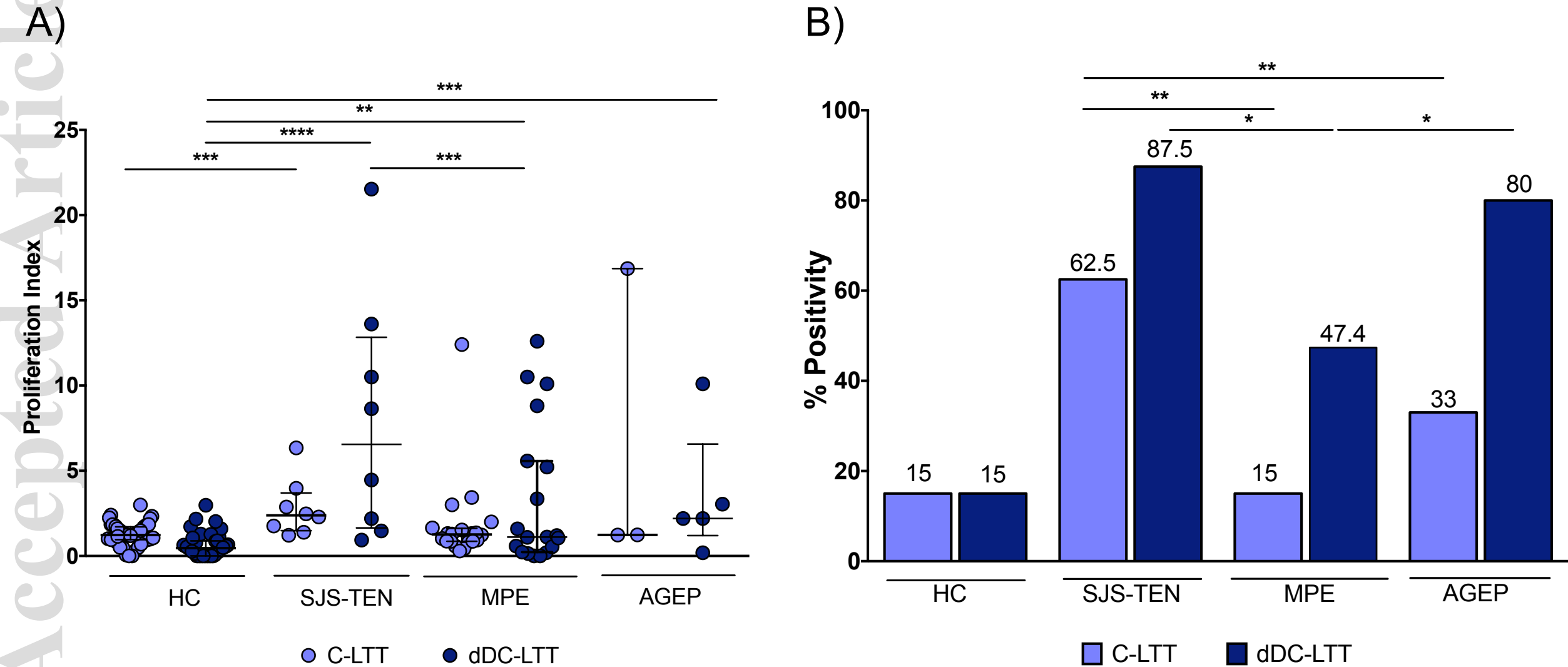


Figure 3.



D)

Proliferation Index

CD3⁺ CD4⁺ CD4⁺T_h1 CD4⁺T_h2 CD8⁺ NK NK IFN-γ NK PERF

E)

Proliferation Index

CD3⁺ CD4⁺ CD4⁺T_h1 CD4⁺T_h2 CD8⁺ NK NK IFN-γ NK PERF

F)

Proliferation Index

CD3⁺ CD4⁺ CD4⁺T_h1 CD4⁺T_h2 CD8⁺ NK NK IFN-γ NK PERF

G)

% Positivity

CD3⁺ CD3⁺ + CD4⁺T_h1 CD3⁺ + NK CD3⁺ + CD8⁺

Healthy controls Allergic patients

H)

% Positivity

CD3⁺ CD3⁺ + CD4⁺T_h1 CD3⁺ + NK CD3⁺ + CD8⁺ CD3⁺ + CD4⁺T_h1 + NK

Healthy controls Allergic patients

I)

% Positivity

CD3⁺ CD3⁺ + CD4⁺T_h1 CD3⁺ + NK CD3⁺ + CD8⁺

Healthy controls Allergic patients

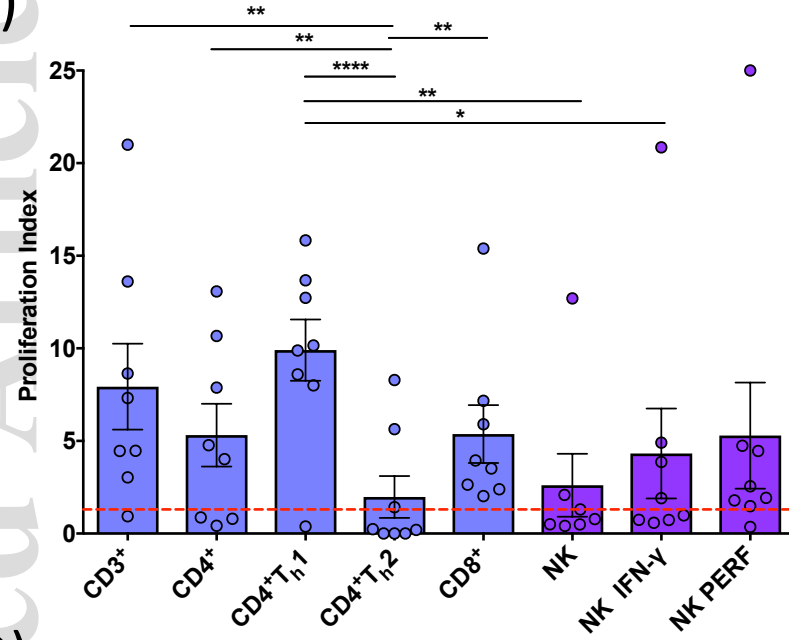
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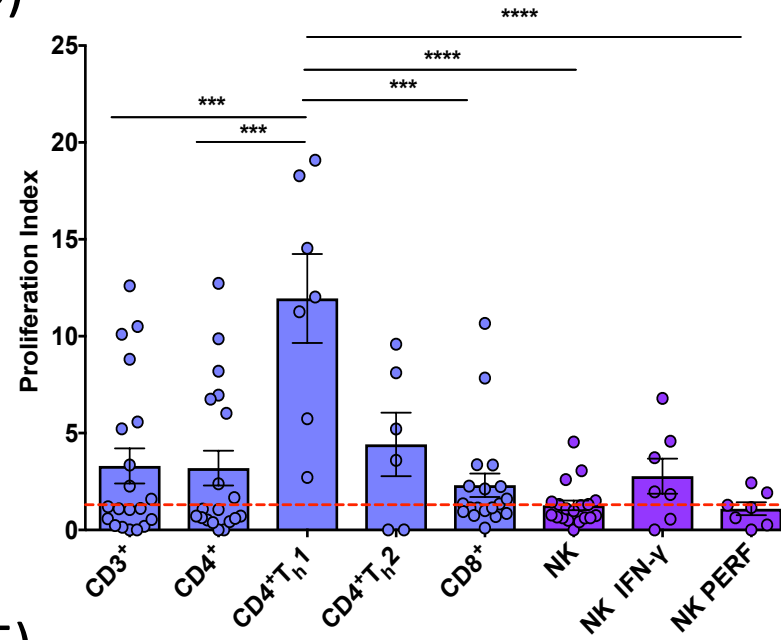
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AGEP

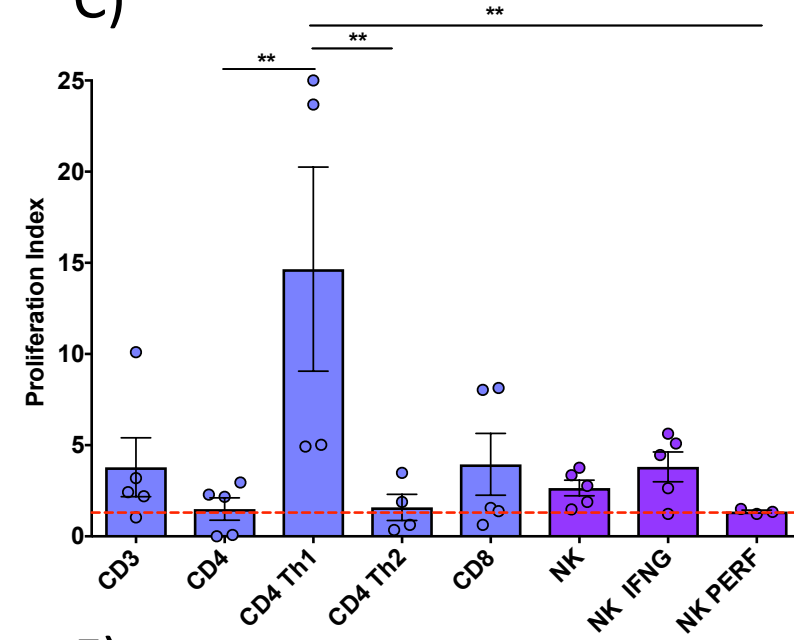
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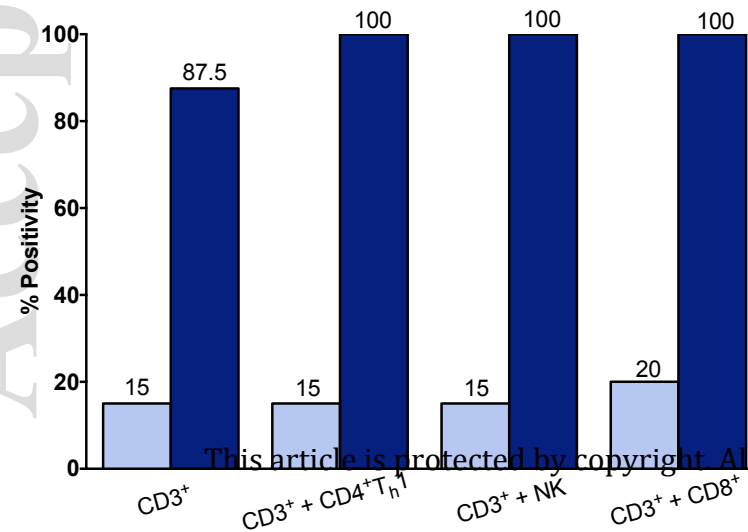
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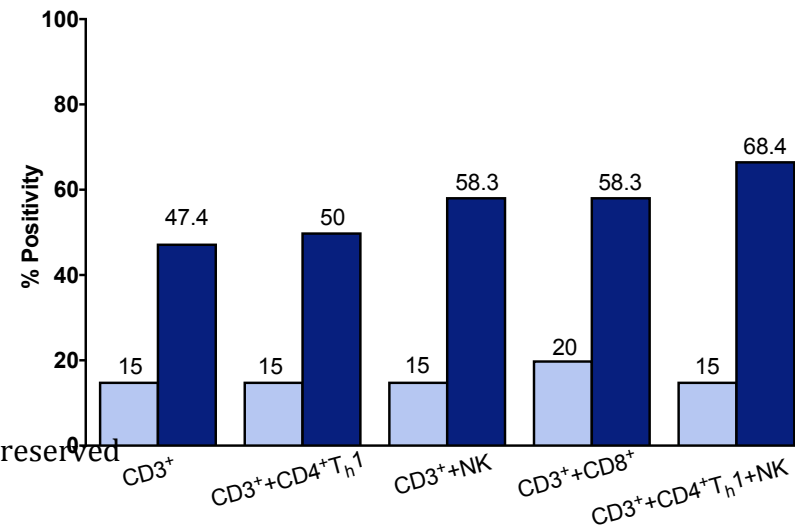
c)



D)



E)



F)

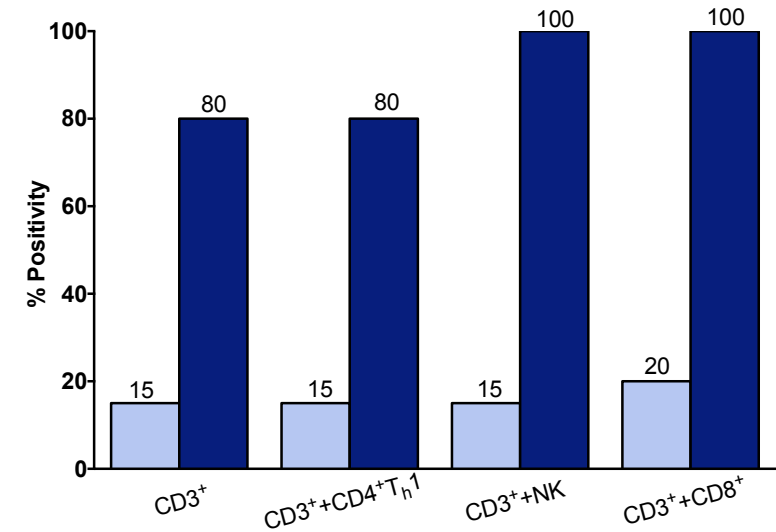


Figure 5.

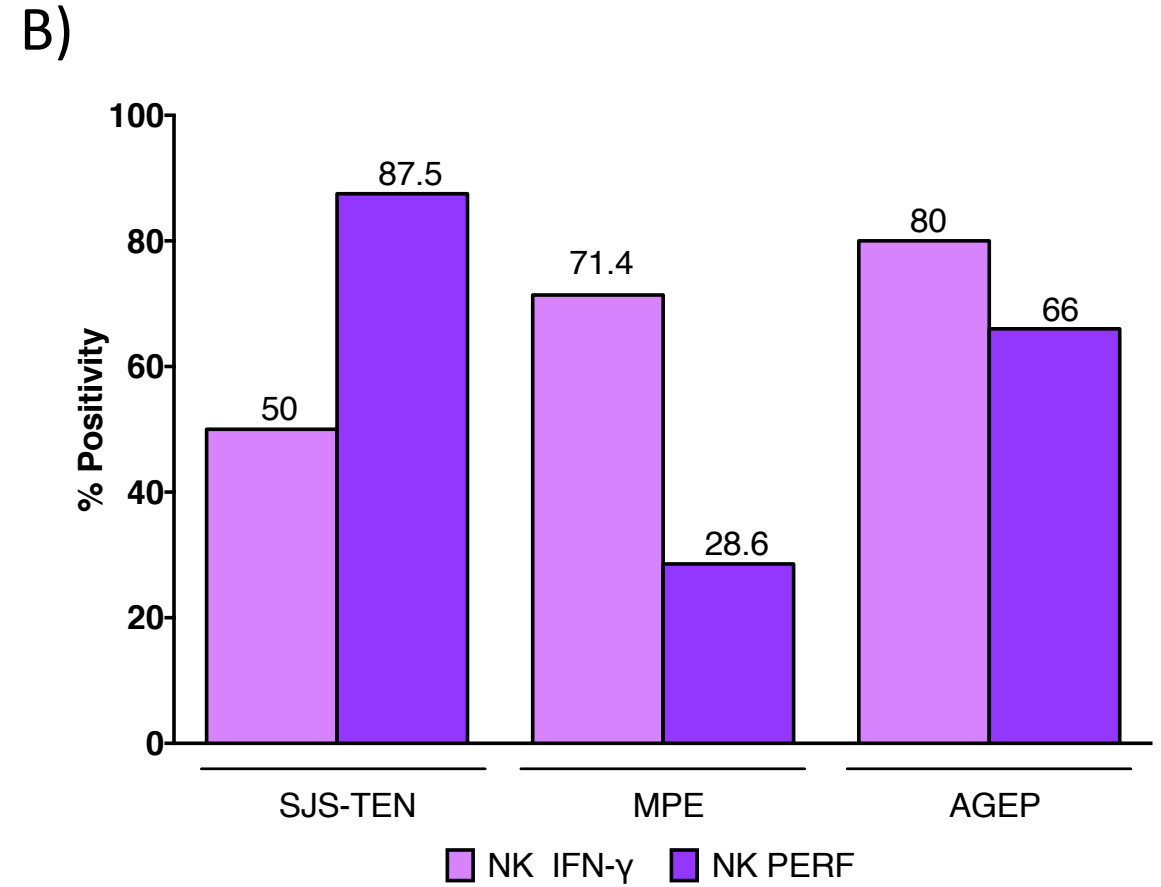
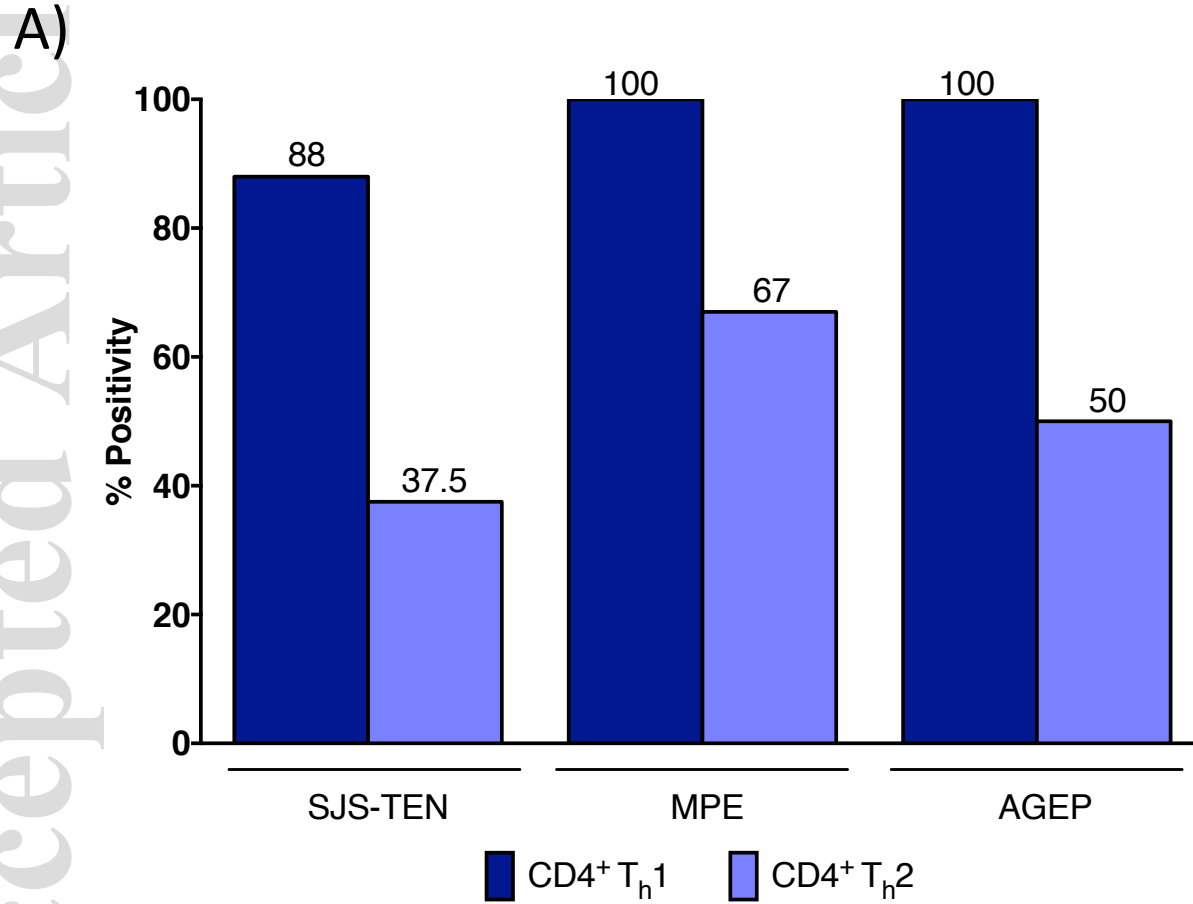


Figure S1. A)

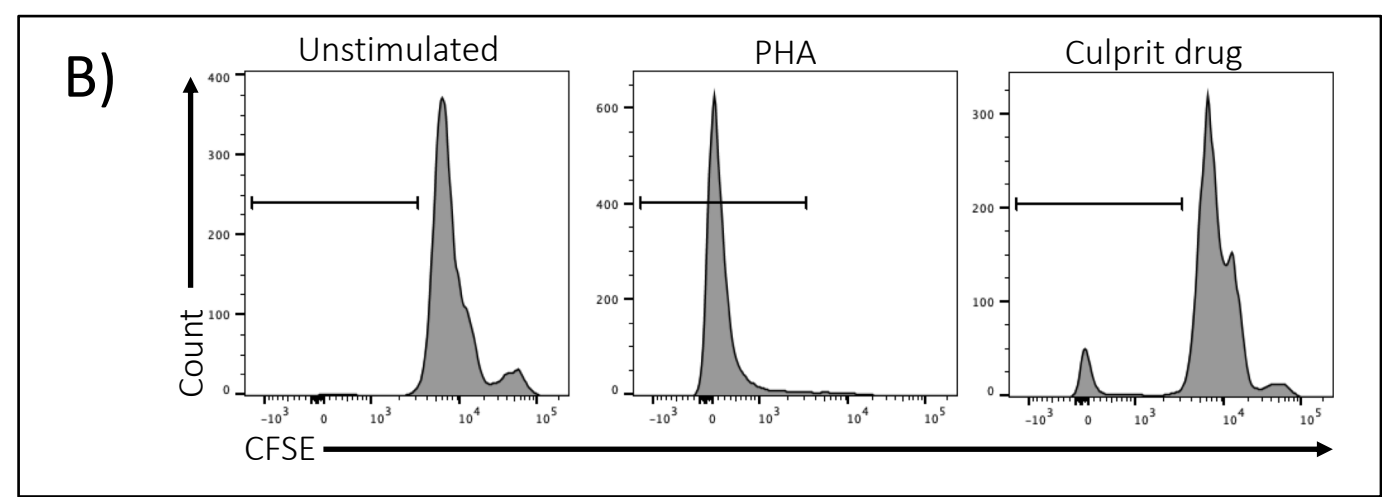
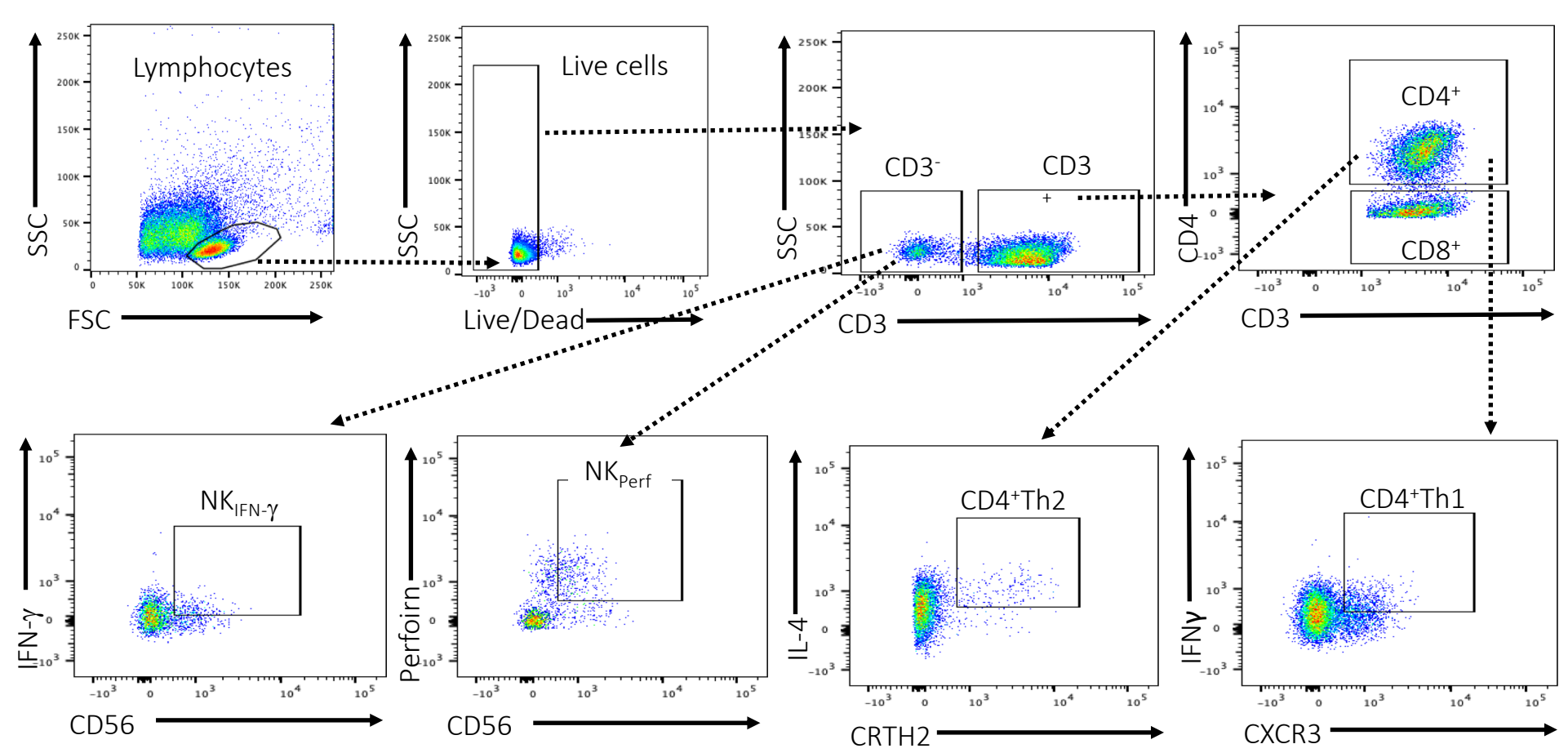


Figure S2.

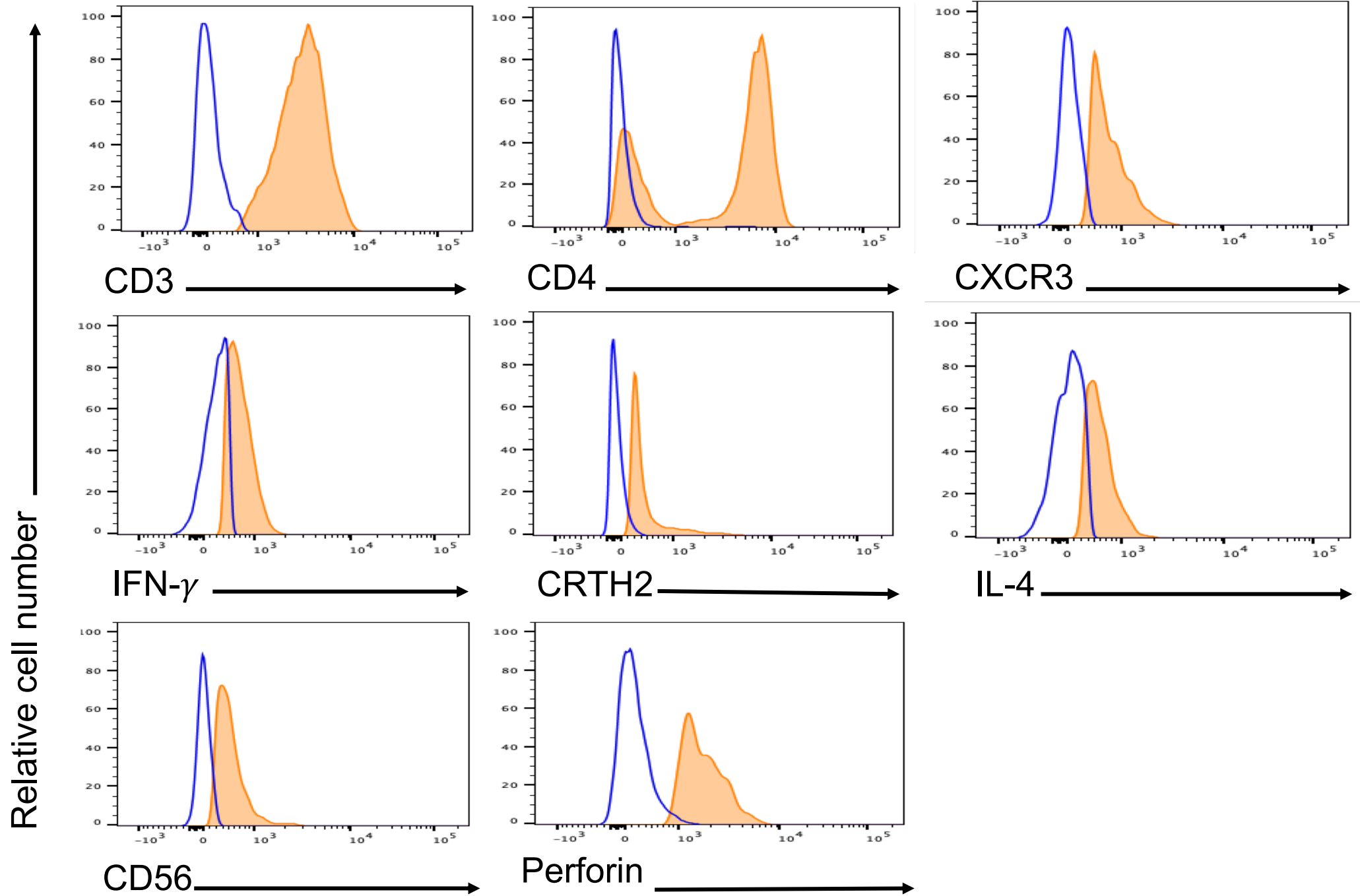


Table S1. Drugs, sources, forms, and concentrations tested in LTT.

Drug	Source	Form	Concentrations
Amoxicillin	Sigma-Aldrich (San Luis, USA)	Powder	250µg/mL, 125µg/mL, 25µg/mL
Allopurinol	Normon (Madrid, Spain)	Pill	
Cefepime	Fresenius (Bad Homburg, Germany)	Powder	
Cefixime	Merck (Darmstadt, Germany)	Oral capsule	
Clavulanic acid	Sigma-Aldrich (San Luis, USA)	Powder	
Diazepam	Roche (Basel, Switzerland)	Injectable solution	
Sulfamethoxazole	Almofarma S.L. (Barcelona, Spain)	Powder	
Trimetoprim	Almofarma S.L. (Barcelona, Spain)	Injectable solution	
Iobitridol	Guebert (Villepinte, France)	Injectable solution	5mg/mL,
Iodixanol	GE Healthcare (Chicago, USA)	Injectable solution	1mg/mL,
Iomeron	Bracco (Milan, Italy)	Injectable solution	0.1mg/mL
Ciprofloxacin	Sigma-Aldrich (San Luis, USA)	Powder	100µg/mL, 10µg/mL, 1µg/mL
Iron supplement	Medix (Madrid, Spain)	Oral solution	250µg/mL, 125µg/mL, 25µg/mL, 5µg/mL
Carbamazepine	Ken Pharma (Barcelona, Spain)	Pill	50µg/mL,
Phenobarbital	Normon (Madrid, Spain)	Injectable solution	10µg/mL, 1µg/mL

The solvent used to dilute each drug was culture medium.

Table S2. Contingency tables for positive and negative results of CD3⁺ cells in C-LTT and dDC-LTT for each clinical entity according to the cut-off selected in each case.

General (C-LTT)		
	+	-
Allergic patients	10	24
Healthy controls	3	18

General (dDC-LTT)		
	+	-
Allergic patients	21	13
Healthy controls	3	18

SJS-TEN (C-LTT)		
	+	-
Allergic patients	5	3
Healthy controls	3	18

SJS-TEN (dDC-LTT)		
	+	-
Allergic patients	7	1
Healthy controls	3	18

MPE (C-LTT)		
	+	-
Allergic patients	3	17
Healthy controls	3	18

MPE (dDC-LTT)		
	+	-
Allergic patients	9	10
Healthy controls	3	18

AGEP (C-LTT)		
	+	-
Allergic patients	1	2
Healthy controls	3	18

AGEP (dDC-LTT)		
	+	-
Allergic patients	4	1
Healthy controls	3	18