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Research paper

Guidelines for analysis of low-frequency antigen-specific T cell results: Dye-based proliferation assay vs ^{3}H-thymidine incorporation

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ABSTRACT

It is generally recognized that dysregulation of the immune system plays a critical role in many diseases, including autoimmune diseases and cancer. T cells play a crucial role in maintaining self-tolerance, while loss of immune tolerance and T cell activation can lead to severe inflammation and tissue damage. T cell responses have a key role in the effectiveness of vaccination strategies and immunomodulating therapies. Immunomonitoring methods have the ability to elucidate immunological processes, monitor the development of disease and assess therapeutic effects. In this respect, it is of particular interest to evaluate antigen (Ag)-specific T cells by determining their frequency, type and functionality in cellular assays. Nevertheless, Ag-specific T cells are detected infrequently in most diseases using current techniques. Many efforts have been made to develop more sensitive, reproducible, and reliable methods for Ag-specific T cell detection. It has been found that analysis of cellular proliferation can be a useful tool to determine the presence and frequency of Ag-specific T cell and to provides insight into modulation of the T cell response by a specific antigen or therapy. However, the selection of a cut-off value for a positive response and therefore a more accurate interpretation of the data, continues to be a major concern. Here, we provide guidelines to select a proper cut-off for monitoring of Ag-specific CD4^{+} T cell responses.

In vitro Ag-stimulation has been assessed with two methods; a dye-based proliferation assay and ^{3}H-thymidine-based assay. Two cut-off approaches are compared; mean and variance of control wells, and the stimulation index. By evaluating the proliferative response to the in vitro Ag-stimulation using these two methods, we demonstrate the importance of taking into consideration the variability of the control wells to distinguish a positive from a false positive response.

1. Introduction

Monitoring of antigen (Ag)-specific CD4^{+} T cells ex vivo from human samples is an essential part of translational immunology and is required for a deeper understanding of the biological processes underlying human immune-mediated diseases, Ag-specific diagnoses and development of novel therapies. (Phetsouphanh et al., 2015; Ten Brinke et al., 2017) The study of the Ag-specific T cell response can provide insight into the nature of the full immune responses of patients and may predict the outcomes of immunotherapy, leading to identification of the patients most likely to benefit from treatment. For instance, monitoring of T cell-specific immune responses has been used to develop new myelin-derived peptide-based therapy for the treatment of multiple sclerosis. T-specific responses have also been used to predict patient response to anti-PD1 therapy for the treatment of PDL1-expressing non-small-cell lung carcinoma. (Grau-Lopez et al., 2011; Kagamu et al., 2020).

Due to their relative accessibility for ex vivo study, peripheral mononuclear blood cells (PBMCs) are the most widely used cells to evaluate Ag-specific responses. Even though the study of Ag-specific CD4^{+} T cells is technically difficult, primarily due to their low frequency in peripheral blood (Barbey et al., 2007), several distinct assays have been developed and optimized to quantify and characterize these cells. Most methods use a functional response to a specific Ag as the read-out, including the induction of proliferation, (Mannering et al.,

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2003; Marits et al., 2014) the production of cytokines that are analyzed by intracellular cytokine staining, enzyme-linked immunosorbent assay (ELISA) or enzyme-linked immunospots (ELISpots) (Calarota and Baldanti, 2013; Mobs and Schmidt, 2016) and the induction of activation markers, which can be analyzed by flow cytometry. (Hartmann et al., 2019; Uchtenhagen et al., 2016) MHC class II tetramer staining can be used to detect Ag-specific $CD4^+$ T cells directly. This analysis enables characterization, quantification and sorting of Ag-specific T cells but it is limited by the high sample volume requirements, HLA epitope restrictions and the requirement for specific knowledge of the unique peptide sequence that elicits the response. (Boelen et al., 2016).

Immunomonitoring of the Ag-specific T cell response in most diseases is limited by the low frequency of these cells in PBMCs (Phetsouphanh et al., 2015; Ahmed et al., 2019) and therefore, cell proliferation assays are widely used for monitor Ag-specific $CD4^+$ T cells response. (Albert-Vega et al., 2018) Several assays are currently used for evaluating T cell proliferation. (Wallace et al., 2008; Sylvester, 2011; Nikbakht et al., 2019).

The analysis of the incorporation of ^{3}H-thymidine during DNA synthesis remains the most commonly used method for measuring T cell proliferation despite its development in the early sixties. (Romar et al., 2016).

This assay requires the incubation of cells with ^{3}H-thymidine for a few hours to overnight. As a result, proliferating cells integrate the radioactive labeled thymidine into their nascent DNA during the incubation period, which is then measured using a scintillation counter. The ^{3}H-thymidine assay is a simple and easy method to perform and it has a relatively high throughput. Large numbers of antigens/peptides can be tested and a high number of replicates can be evaluated.

Over the past decades new methods have been developed to monitor cell proliferation using cell proliferation dyes. (Tario Jr et al., 2018) A wide variety of fluorescent dyes are nowadays available for monitoring T cell proliferation (e.g. CellTrace™ CFSE, CellTrace™ Violet (CTV), Violet Proliferation Dye (VPD)-450, CellTrace™ Far Red (CTFR), CytoTrack™ Yellow (CYY) and eFluor® 670). (Wallace et al., 2008).

Proliferation dyes diffuse easily into cells and bind covalently to the amino groups of intracellular proteins. The use of these dyes is based on the principle that during each cell division the fluorescent intensity is divided over the two daughter cells, resulting in the fluorescence being halved with each generation. Cells are labeled at the start of the culture and by analyzing the fluorescence at the end of the culture. The divisions during the whole culture can be visualized by flow cytometry, in which increasingly lower mean fluorescence intensities shows the cells have been through X-number of cell division rounds. In addition, proliferating cells can be further phenotypically characterized by multicolor flow cytometry, by using antibodies specific for surface markers and intracellular proteins, e.g. cytokines.

Correct data interpretation is a critical aspect in Ag-specific T cell immunomonitoring. In the simplest situation, these assays provide a result that can be expressed as positive or negative response. For quantitative tests it is necessary to identify a cut-off value which discriminates the results to be declared positive from the negative ones. The lack standardization in defining this cut-off value may result in different interpretation of obtained results and prevent direct laboratory and clinical trial comparisons of Ag-specific T cell responses.

In the current study, we aim to provide practical guidelines for defining the cut-off value and data interpretation for results of proliferation assays. Here we address two different frequently used approaches to set the cut-off for detecting the response of low frequency Ag-specific T cells, the mean and variance of control wells and the stimulation index (SI). Furthermore, we applied selected cut-offs to compare the applicability of ^{3}H-thymidine incorporation and dye-based proliferation assays to detect low frequency Ag-specific responses under the same experimental condition.

2. Material and methods

2.1. Study population

Peripheral blood was collected from four healthy volunteers 2 weeks after vaccination with the MF59-adjuvanted A (H1N1) pdm09 subunit vaccine in heparin tubes. (Rosendahl Huber et al., 2018).

The protocols were approved by the medical ethical reviewing committee of the Netherlands and the study was conducted in accordance with Good Clinical Practice and the principles of the Declaration of Helsinki. In addition, blood was collected from three multiple sclerosis (MS) patients treated with natalizumab in citrate tubes. MS patients were recruited at the at the MS center of the Amsterdam UMC. All patients had a current diagnosis of relapsing-remitting multiple sclerosis (RRMS) and were treated with natalizumab with a minimum of six consecutive infusions and participated in study extending the standard 4-week treatment interval based on individual natalizumab trough concentrations. The study was approved by the local institutional board of the Amsterdam UMC. All patients gave written informed consent. Blood samples were taken through the infusion needle before start of natalizumab treatment.

2.2. PBMCs isolation

Peripheral blood samples from healthy volunteers were collected in 9 ml Vacuette sodium heparin tubes (Greiner Bio-One, Kremsmünster, Austria) and peripheral blood mononuclear cells (PBMCs) were extracted within 8 h after blood collection by density gradient centrifugation using Lymphoprep (Axis- Shield, Norway) and subsequently cryopreserved in liquid nitrogen in 90% fetal bovine serum (FBS) (Nederlands vaccine institute, Netherlands) and 10% dimethyl sulfoxide (DMSO) (Sigma Aldrich, St. Louis, Missouri, United States) until use. Peripheral blood mononuclear cells (PBMCs) from MS patients were isolated from peripheral blood collected in 9 ml Vacuette tubes with 3.8% coagulation sodium citrate (Greiner Bio-One) within 4 h after blood collection by density gradient centrifugation with Lymphoprep (Axis- Shield) and were cryopreserved in liquid nitrogen in IMDM (Lonza Biowhittaker, Basel, Switzerland) + 42.5% fetal bovine serum (FCS) (Sigma Aldrich) and 10% dimethyl sulfoxide (DMSO) (J. T. Baker, Phillipsburg, New Jersey, United States) until use.

2.3. Peptides

Pools of 139 peptides, 15-mer peptides with 11-mer overlap, derived from Hemagglutinin (HA) (Swiss-Prot ID: C3W5X2) of Influenza A virus (A/California/07/2009(H1N1)) were purchased from JPT (Berlin, Germany). Peptide stocks (500 μg/ml) were made by dissolving freeze-dried peptides in DMSO (J. T. Baker).

Antigenic peptides derived from the myelin basic protein (Swiss-Prot ID: P02686) (MBP), myelin oligodendrocytes glycoproteins (Swiss-Prot ID: Q16653) (MOG) and proteolipid protein (Swiss-Prot ID: P60201) (PLP) were purchased from (Hemmo pharmaceutical, Maharashtra, India). Peptide stocks (100 μg/ml) were produced by dissolving freeze-dried peptides in MBP diluent (0.2 M Sodium Acetate pH 4) (Hemmo pharmaceutical) and kept at −80 °C.

Working solution was prepared from stock solution by dilution with IMDM (Sigma-Aldrich) supplemented with 5% human serum (HS) (Sanquin), 100 U/ml Penicillin, 100 μg/ml Streptomycin (Life technology Carlsbad, California, United States).

2.4. VPD-450 labeling

Fresh PBMCs were labeled with VPD-450 (BD Bioscience, New Jersey, United States). A total of $10*10^6$ cells/ml were stained with 1 μM VPD-450, 10 min at RT, in dark. After two washing steps, the first with IMDM with 5% fetal bovine serum (FBS) (Gibco - Thermo Fisher

Scientific Massachusetts, United States) and the second one with IMDM with 5% HS, PBMCs were resuspended in 1 ml of IMDM supplemented with 5% HS, 100 U/ml Penicillin, 100 μg/ml Streptomycin (Life technology). A total of $1.5*10^5$ PBMCs were seeded in 96-well round -bottom plates (12 wells, 200 μl/well) for 7 days at 37 °C in the presence of HA peptide pool (1 μg/ml) (California PepMix, JPT). Twelve wells without Ag served as negative control and three wells stimulated with Staphylococcus Enterotoxin B (SEB) (Sigma-Aldrich) as a positive stimulation control to demonstrate the functional integrity of each PBMC sample.

2.5. Flow cytometry

PBMCs were washed once with PBS (Fresenius Kabi, Huis ter Heide, the Netherland) and resuspended in LIVE/DEAD staining buffer (PBS with 0.001% LIVE/DEAD Fixable Near-IR dead Cell dye for 633/635 nm (Thermo fisher) for 30 min at room temperature, protected from light. The dead cells can then be identified and removed from the final analysis by gating on the unstained population (live cells). For cell surface staining, cells were subsequently washed in staining buffer (PBS containing 5% BSA (Sigma Aldrich) + 0.01% N_aN_3 (Sigma Aldrich) and stained with CD3 BUV496 (BD Bioscience), CD4 BUV395 (BD Bioscience), and CD8 BUV805 (BD Bioscience), CD14 APC-Cy7 (Biolegend, San Diego, California, United States), CD19 APC-Cy7 (Biolegend) for 30 min at 4 °C protected from light. After staining cells were subsequently washed twice in staining buffer. Samples were acquired with FACS Canto II (BD Bioscience) and flow cytometry data was analyzed using FlowJo v10.1 software (TreeStar, BD Bioscience).

Results are expressed as percentage of proliferating $CD3^+$ VPD-450^{dim} cells.

2.6. ^{3}H-thymidine incorporation

PBMCs from vaccinated volunteers ($1.5*10^5$ PBMCs/well) labeled with VPD-450 were cultured in 96-well round-bottom plates in IMDM with 5% HS for 6 days at 37 °C in the presence of HA peptide pool (1 μg/ml). Twelve wells without Ag served as negative control. PBMCs from MS patients ($1.5*10^5$ PBMCs/well, 36 wells per Ag) were seeded in 96-well round-bottom plates and stimulated with a mixture of myelin peptides for 6 days at 37 °C. A pool of 7 myelin peptides was added at 10 μM each. Thirty-six wells stimulated with an equivalent concentration of MBP diluent without myelin peptides served as negative control. After 6 days, ^{3}H-thymidine was added at 1 μCi/well and the cells were harvested 18 h later. ^{3}H-thymidine incorporation was determined by harvesting cells using an automatic cell harvester (Harvester 96; Tomtec, Hamden, Connecticut, United States), and counting with a MicroBeta^2 Microplate Counter (Perkin Elmer, Waltham, Massachusetts, United States). Incorporated radioactivity is expressed as counts per minute (CPM).

2.7. Statistics

Analysis was performed using the GraphPad Prism 8.0 software. The results are expressed as the mean value of the unstimulated samples (CTRL), mean of CTRL + one time standard deviation (SD), mean of CTRL + two times SD (2SD), mean of CTRL + three times SD (3SD) and as SI. SI was calculated by dividing the percentage of $CD3^+$ VPD-450^{dim} cells or CPM ^{3}H-thymidine incorporation by the respective mean of the unstimulated cultures. A SI greater than 2 was considered a positive response. In all analyses, Wilcoxon signed rank test was used. The correlation between the different assays was evaluated by the non-parametric Spearman's rank correlation analysis. A $p \leq 0.05$ (95% confidence interval) is considered statistically significant.

3. Results

3.1. Ag-specific HA T cell responses detected in the ^{3}H-thymidine and VPD-450 dilution assays

A challenging aspect of monitoring the effects of a certain treatment on T cells is the low frequency of antigen-specific T cells in peripheral blood. (Pogorelyy et al., 2018; Jenkins and Moon, 2012) We compared two in vitro proliferation assays, either based on ^{3}H-thymidine incorporation or fluorescent dye dilution, to detect Ag-specific T-cell responses in individuals 2 weeks after vaccination with adjuvanted pH1N1 influenza vaccine. To compare the results of these two assays, all PBMCs have been fluorescently labeled before the start of the culture which is independent of the final read-out, in order to exclude any direct labelling effect on the proliferative response. These two proliferation assays require different critical steps. In the ^{3}H-thymidine assays, PBMCs are stimulated with the antigens for 6 days, after which ^{3}H-thymidine is added to each well for the last 18 h of incubation. Cell proliferation during this last 18 h is reflected by the incorporated ^{3}H radioactivity that is measured via scintillation counter. Alternatively, in the VPD-450 dilution assay fluorescently labeled PBMCs are stimulated with the antigens for 7 days. During cell proliferation the dye is divided equally between the daughter cells and cell divisions of the proliferating cells are visualized by flow cytometry at the end of the culture period (Fig. 1).

The proliferative response to overlapping HA peptide pool stimulation using both assays are shown in Fig. 2. In order to compare the responses to the HA-stimulation obtained with both assays, we have analyzed the VDP-450 dye dilution of all T cells ($CD3^+$) (Fig. 2A,B), since the subset of proliferating lymphocytes can not be discriminated using ^{3}H-thymidine incorporation (Fig. 2C). Both assays detected a positive proliferative response to HA peptide pool stimulation of PBMCs from vaccinated healthy volunteers in most cases.

3.2. Determination of cut-off value for positive response to specific Ag in proliferation assay by using mean and variation of control wells

It is considered challenging to correctly identify true positive or true negative responses, especially for analyzing low frequency Ag-specific T cell response in which the individual values are close to the background response.

Easily applicable guidelines for the correct selection of an objective cut-off are missing, and are required to set the cut-off value for the detection of the low-frequency Ag-specific T cell response. (Bielekova et al., 2004; Ten Brinke et al., 2017).

To determine an objective cut-off, the variation between the assays themselves and the variable responses of the unstimulated samples both need to be considered. Therefore, the same number of replicates of unstimulated and stimulated samples should be analyzed. To define a positive response, we calculated the percentage of wells with a proliferative response above the mean of proliferative response in the unstimulated samples (CTRL), mean of CTRL + one time standard deviation (SD), mean of CTRL + two times SD (2SD), mean of CTRL + three times SD (3SD) for all unstimulated wells in both read-outs, e.g. proliferation dye (Fig. 3A–D) and ^{3}H thymidine (Fig. 3E–H).

Fig. 3 shows the percentage of positive wells in unstimulated wells and HA peptide pool-stimulated samples compared to the respective calculated cut-off values. All the samples with a value greater than this cut-off were considered as positive response.

From our data it becomes clear that using mean + 2SD or a lower cut-off might lead to incorrect assignment of a positive response, since using mean of CTRL +2SD the unstimulated condition itself would be classified positive in 1 out of 4 or 2 out of 4 donors, depending on the assay used. All donors tested here had a positive response to HA peptide pool using mean + 3SD as cut-off value in the VPD-450 assay. The number of positive wells slightly decreased between mean of CTRL +2SD and mean of CTRL +3SD (Fig. 3C-D). Using the ^{3}H thymidine incorporation as

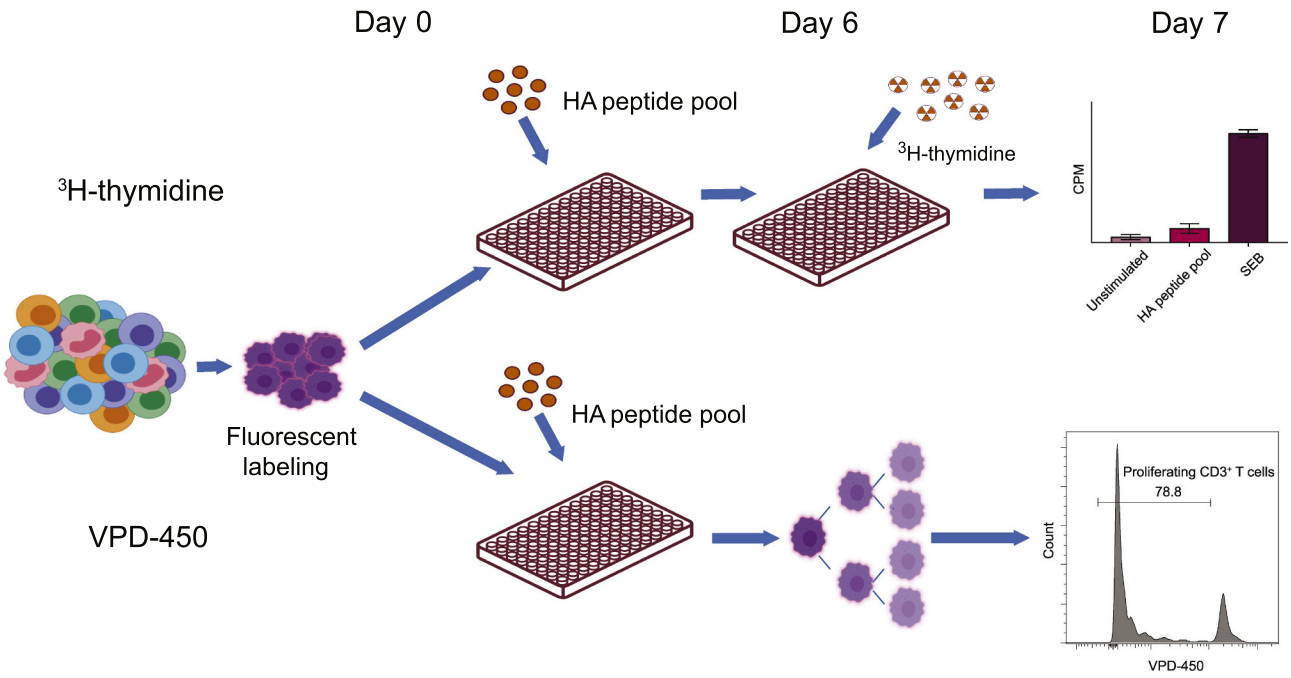

Fig. 1. Schematic representation of ^{3}H-thymidine assay and proliferation dye assay. PBMCs from donors 2 weeks after vaccination with adjuvanted pH1N1 influenza vaccine were seeded at the concentration of $1.5*10^5$ cell/well. PBMCs were fluorescently labeled with the proliferation dye (VPD-450), cultured and simulated with HA peptide pools for 7 days (lower part). For ^{3}H-thymidine read-out (upper part) PBMCs were stimulated with HA peptide pools at day 0 and after 6 days ^{3}H-thymidine was added to be incorporated in the DNA of the cells proliferating for the subsequent 18 h. At day 7 the read out of both assays was performed.

read-out, 3 out of 4 donors were classified as positive responding to HA peptide pool. Donor D2 was considered negative, but this was independent of the cut-off determined mean of CTRL+ 2SD vs mean of CTRL +3SD respectively (Fig. 3G–H).

3.3. Determining the cut-off using the SI

Next, we evaluated the use of the SI to determine the objective cut-off for positive response. SI was calculated by dividing the percentage of $CD3^+$ VPD-450^{dim} cells (Fig. 4A) or CPM ^{3}H-thymidine incorporation (Fig. 4B) by the respective mean of the unstimulated cultures. In our study, we defined a response as positive if the SI was greater than 2, which was an empirically selected value based on other similar studies that have defined their SI cut-off value between 1.5 and 3. (Schultz et al., 2017; Mascarenhas et al., 2006; Karle et al., 2016).

For donor D4 one of the unstimulated wells was also classified positive using SI > 2 in the proliferation dye assay. In addition, using SI > 2 as a cut-off value, we found that both ^{3}H-thymidine incorporation and VPD-450 dilution assay detect a positive response in three of the four donors. Both read-outs do not recognize a positive response in Donor D2 using this cut-off value. Of note, donor D2 is the only donor tested in 6 instead of 12 replicates of stimulated and unstimulated cells due to the limited amount of PBMCs (Fig. 4C,D).

A drawback of using a SI-determined cut-off value is that the variation in the unstimulated control wells is not taken into account, because only the mean of the unstimulated control wells is used in the analysis. Therefore, this method was combined with a statistical analysis that determines statistically significant differences between non-stimulated and stimulated conditions, so that fluctuations in all measurements are also considered. To distinguish positive from a negative response we used a combination of two criteria; SI and a statistical difference. In this instance a positive response was defined by SI value >2 and $p < 0.05$ (Fig. 4E). In our experiment this results in only 2 out of 4 donors being positive responder in ^{3}H thymidine assay and no statistically significant difference was observed between stimulated and unstimulated samples for D3. Furthermore, it is important to realize that if the frequency of Ag-specific T cells is very low, only a small percentage of wells would respond to the Ag. Hence, we could expect that the difference between the means of unstimulated and stimulated samples would not become statistically significant and therefore making this approach less applicable.

3.4. Comparison of VPD-450 and ^{3}H-thymidine sensitivity in determining Ag-specific T cell responses

In order to test whether the proliferative response assessed using the ^{3}H-thymidine and VPD-450 dilution assay would yield similar results and if the sensitivity is comparable or not, responses from both assays were expressed in terms of percentage of positive wells. We first evaluated the response to HA peptide pool for each donor using mean + 3SD cut-off value or the SI > 2 cut-off value, and we plotted the percentage of positive wells above cut-off values. A significant correlation was observed between the percentage of positive wells assessed using mean + 3SD ($r = 0.815$, $p = 0.02$) using both assays (Fig. 5A).

A similar correlation has been found using SI > 2 cut-off ($r = 0.83$, $p = 0.01$) for ^{3}H-thymidine and VPD-450 dilution assay (Fig. 5B). Therefore, though differences were observed in assigning these low-frequency HA-specific responses as positive or not, overall ^{3}H-thymidine incorporation and proliferation dye assay are equally sensitive at population level (see Figs. 3, 4, and 6).

3.5. Cut-off selection as the critical step for the detection of very low-frequency T cell response in MS patients

Furthermore, we investigated whether our approaches for selection of the cut-off are both equally suitable to detect very low-frequency Ag-specific T-cell response such as to be found in an autoimmune environment. For this purpose, we study very low-frequency myelin-specific T cells responses in patients with MS. PBMCs collected from patients at consecutive natalizumab infusions were stimulated with a pool of myelin peptides and cell proliferation was determined by ^{3}H thymidine assay (Fig. 6A). This assay was selected because we increased the

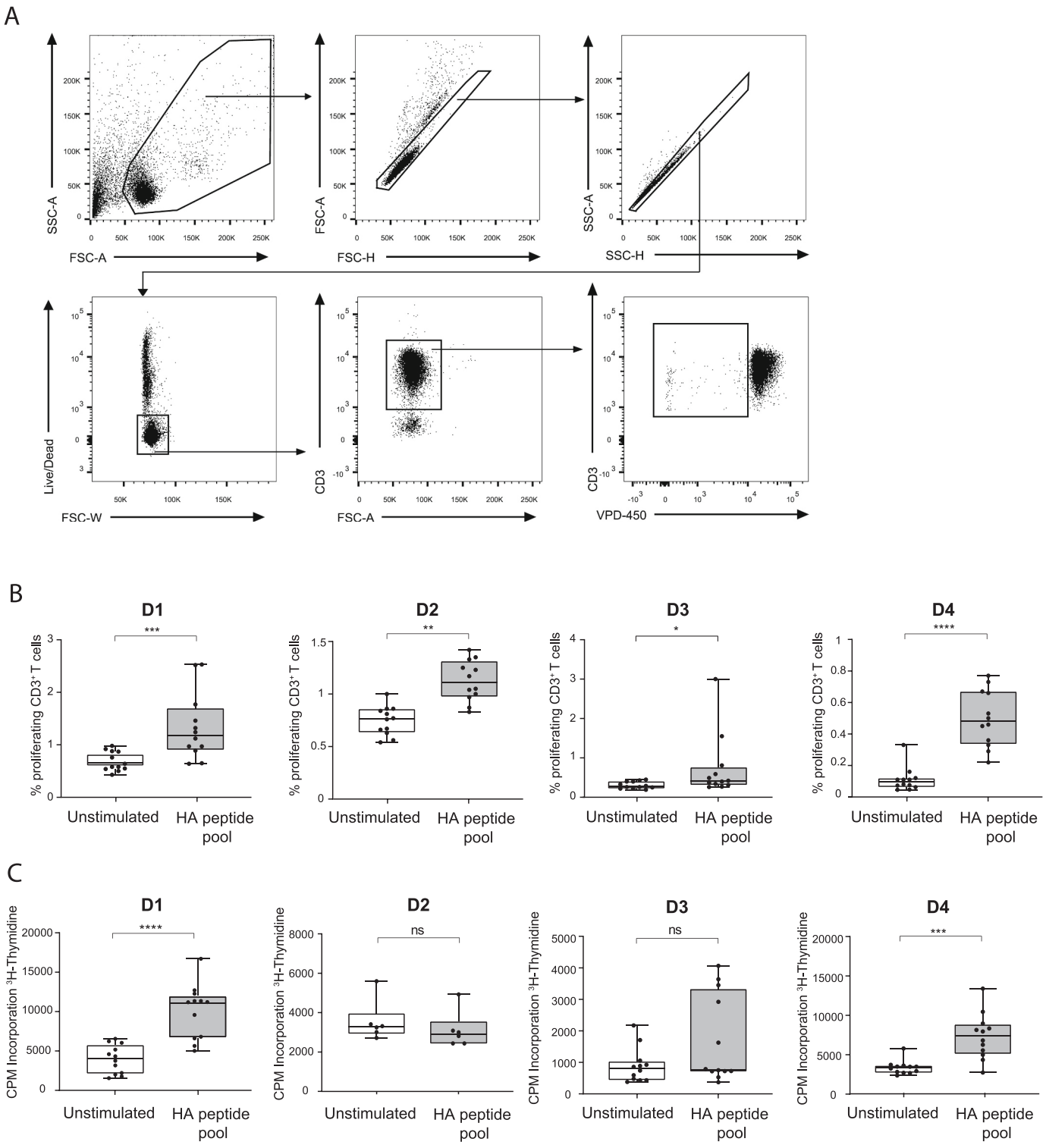

Fig. 2. Proliferative response of Ag-specific T cell response. The proliferation was evaluated by VPD-450 assay as percentage of proliferating $CD3^+$ T cells (A - B) or ^3H thymidine assay as CPM ^3H-thymidine incorporation (C).

Representative gating strategy to identify $CD3^+$ proliferating T cells in PBMCs from healthy volunteers (A). Box plots (B - C) show the data distribution: each dot represents one of the twelve replicates of PBMCs (except donor D2 which is the only donor tested in 6 replicates), either unstimulated or stimulated with HA peptide pool.

Differences in means were evaluated with nonparametric Mann-Whitney *U* tests based on the distribution levels. $^*p \leq 0.05$; $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$, ns: not significant.

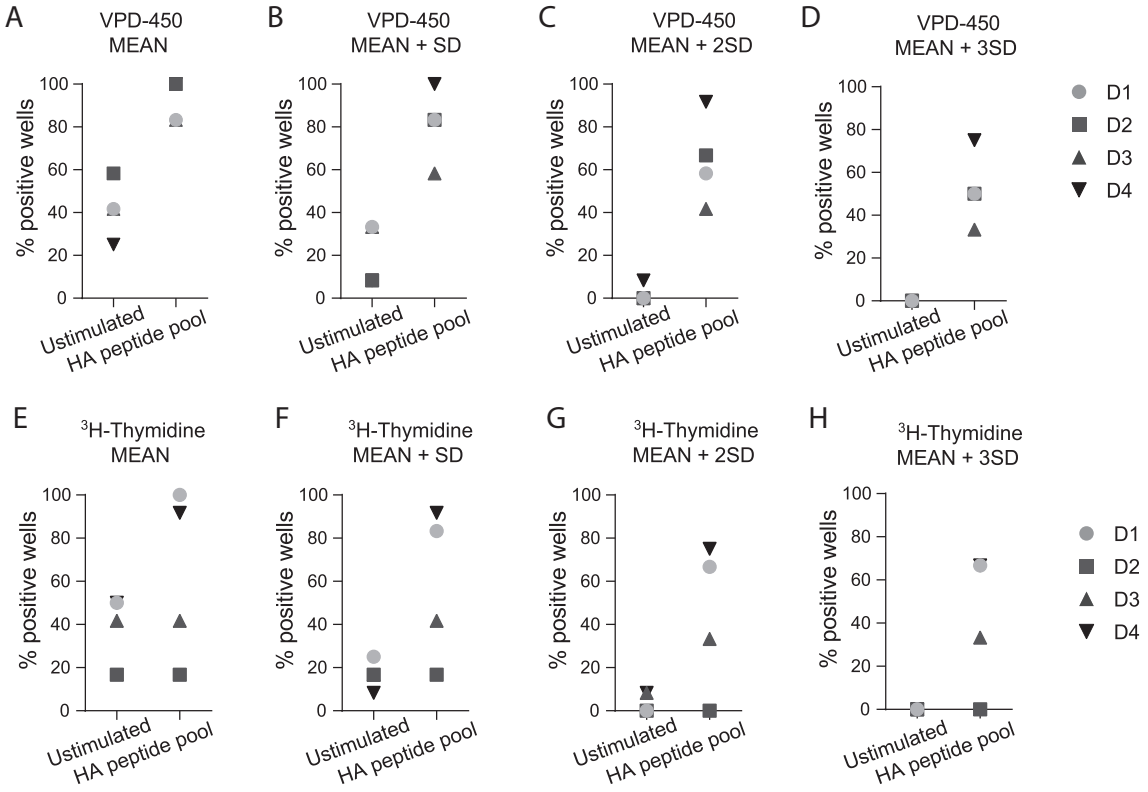

Fig. 3. Selection of cut-off value based on mean and SD of the unstimulated samples. Percentage of positive wells in unstimulated and HA peptide pool stimulated samples above the mean of proliferative response (% proliferating cells (A-D) or CPM (*E*-H), respectively) in the unstimulated samples (A, E), mean + standard deviation (SD) (B, F), mean + two times SD (2SD) (C, G), mean + three times SD (3SD) (D, H).

number of replicates for both stimulated and not stimulated samples to 36 due to the predicted low frequency T cell population. For defining a myelin-specific proliferative response, we calculated the percentage of wells with a proliferative response above the mean of CTRL +3SD. This resulted in the fact that in all three patients one well was considered positive in the unstimulated samples when mean of CTRL +3SD was used as cut-off (data not shown). Therefore, the mean of CTRL +3SD was not specific enough to select a positive Ag-specific proliferative response for this experimental setting. Based on our background values we then selected mean of CTRL +4SD as a cut-off to define a myelin-specific positive proliferative response (Fig. 6B). The use of this cut-off identifies patient A as a positive responder at each time point, while patients B and C were considered positive at two out of three times.

When SI > 2 is applied as cut-off for each individual well, in combination with $p < 0.05$ for the difference between the mean of unstimulated and stimulated samples, patient C would be considered as a negative responder for each time point (Fig. 6C).

4. Discussion

The reliable detection of Ag-specific T cell responses is important in translational medicine. In order to allow the immunological response to be correlated with the clinical results of different studies, strict attention must be paid to the setup and execution of the assay. (Ten Brinke et al., 2017) Differences in sample processing techniques for PBMC, including blood collection, cryopreservation and thawing, may have a significant effect on yield, viability, and downstream assay results, although of importance this is out of the scope of this paper. (Chen et al., 2020; Costantini et al., 2003).

Besides, an incorrect determination of a positive response may result in a misinterpretation of data. We observed a lack of information in literature about the methods used to critically interpret cell proliferation data and determine a cut-off value for a positive response to specific antigens, despite its importance in preventing false positive results. The intent of these paper is to provide researchers with practical and brief guidelines for execution and interpretation of results from low frequent Ag-specific proliferative responses of clinical samples.

In most diseases Ag-specific T cells have low frequency, therefore guidance for setting the cut-off to better determine the positive response with high sensitivity (i.e., highest number of positive responses in stimulated samples) and specificity (i.e., lowest number of positive responses in negative controls) might have a positive impact for the correct interpretation of data. Sensitivity and specificity are two mutually dependent parameters, as they are inversely related depending on the choice of a cut-off value. If the frequency of Ag-specific T cells is low, then only a small percentage of wells will respond to the Ag stimulation. When the Ag-specific response is measured, the outcomes are influenced by biological variability and random errors in the measurement process such that false-positive results can occur.

In this study we evaluated Ag-specific $CD4^+$ T cell responses to HA peptide pool stimulation. We tested two approaches to select the cut-off for a positive response, either using mean of proliferative response in the unstimulated samples + variation in the response or SI > 2. Both approaches have been used before for the interpretation of cellular proliferation data. (Bielekova et al., 2004; Seyfert-Margolis et al., 2006).

To determine an objective cut-off, the assay variation needs to be taken into consideration and the same number of replicates of unstimulated and stimulated samples should be analyzed. We defined a positive outcome by calculating the percentage of wells with a proliferative response above the mean of proliferative response in the unstimulated samples + x time SD (CTRL + SD). From our analysis on the HA response, it becomes clear that using mean + 2SD or a lower cut-off might lead to incorrect assignment of a positive response. Mean + 3SD was selected to evaluate Ag-specific $CD4^+$ T cell responses to HA peptide

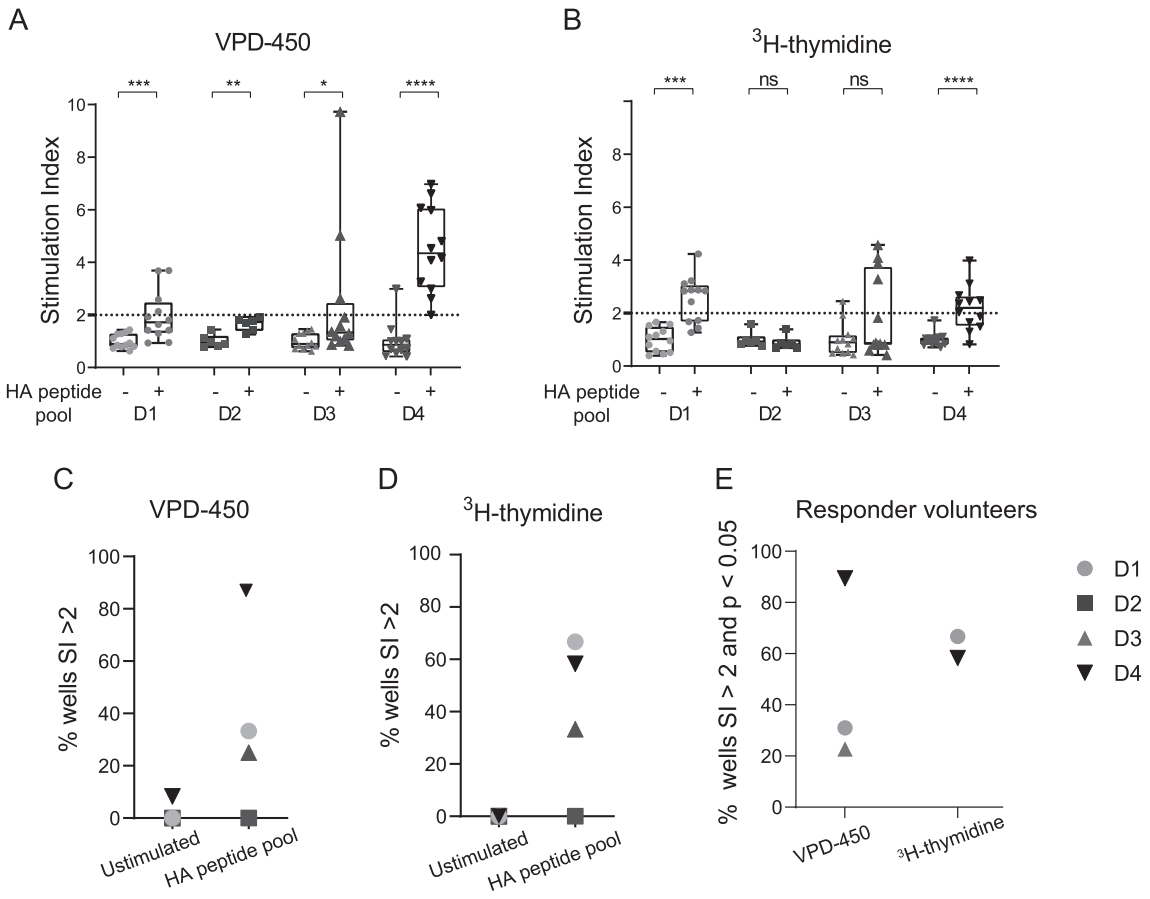

Fig. 4. SI > 2, as cut-off value for positive response. SI was calculated by dividing the percentage of proliferative response (A) or CPM ^{3}H thymidine incorporation (B) by the respective mean of the unstimulated cultures. Box plot show the data distribution and each dot represent one of the twelve replicates of PBMCs, either unstimulated or stimulated with HA peptide pool. Differences in means were evaluated with non-parametric Mann-Whitney test, which was performed to determine if the responses were significantly different. Cut-off for SI has been set >2. Percentage of wells with SI > 2 in unstimulated and HA peptide pool stimulated samples in VPD-450 and ^{3}H-thymidine (C and D, respectively).
Percentage of positive well (SI > 2 and $p < 0.05$) in HA peptide pool stimulated (E) samples from responder donor obtained using VPD-450 and ^{3}H thymidine assay. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$, ns: not significant.

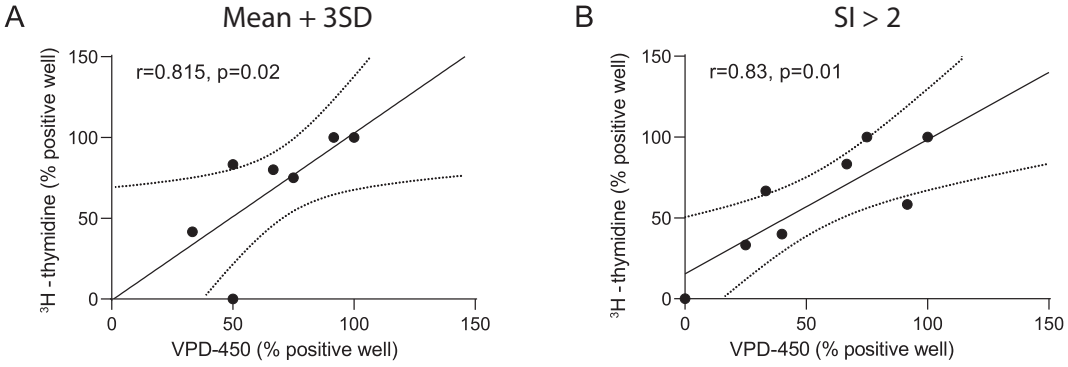

Fig. 5. Correlation between frequencies of Ag-specific T cell obtained using proliferation dye (VPD-450) assay and ^{3}H thymidine assay. Responses to HA peptide pool stimulation of PBMCs from vaccinated healthy volunteers are expressed as percentage of well with a positive proliferative response compared to the mean + 3SD of unstimulated samples (A) and SI > 2 (B).

pool stimulation since our data clearly showed that the mean of CTRL +2SD, or a lower cut-off, was not specific enough to select a positive Ag-specific proliferative response and the unstimulated conditions itself would otherwise also have been classified as positive.

Therefore, we suggest that using a cut-off lower than the mean of CTRL +3SD may result in the incorrect assignment of a positive response for this data-set.

It is important to take into account that T cell population frequencies vary for different antigens. (Pogorelyy et al., 2018).

Moreover, the variation within the unstimulated samples might be different because of the diverse nature of the samples. This implies that using different samples, including those used for the analysis of myelin

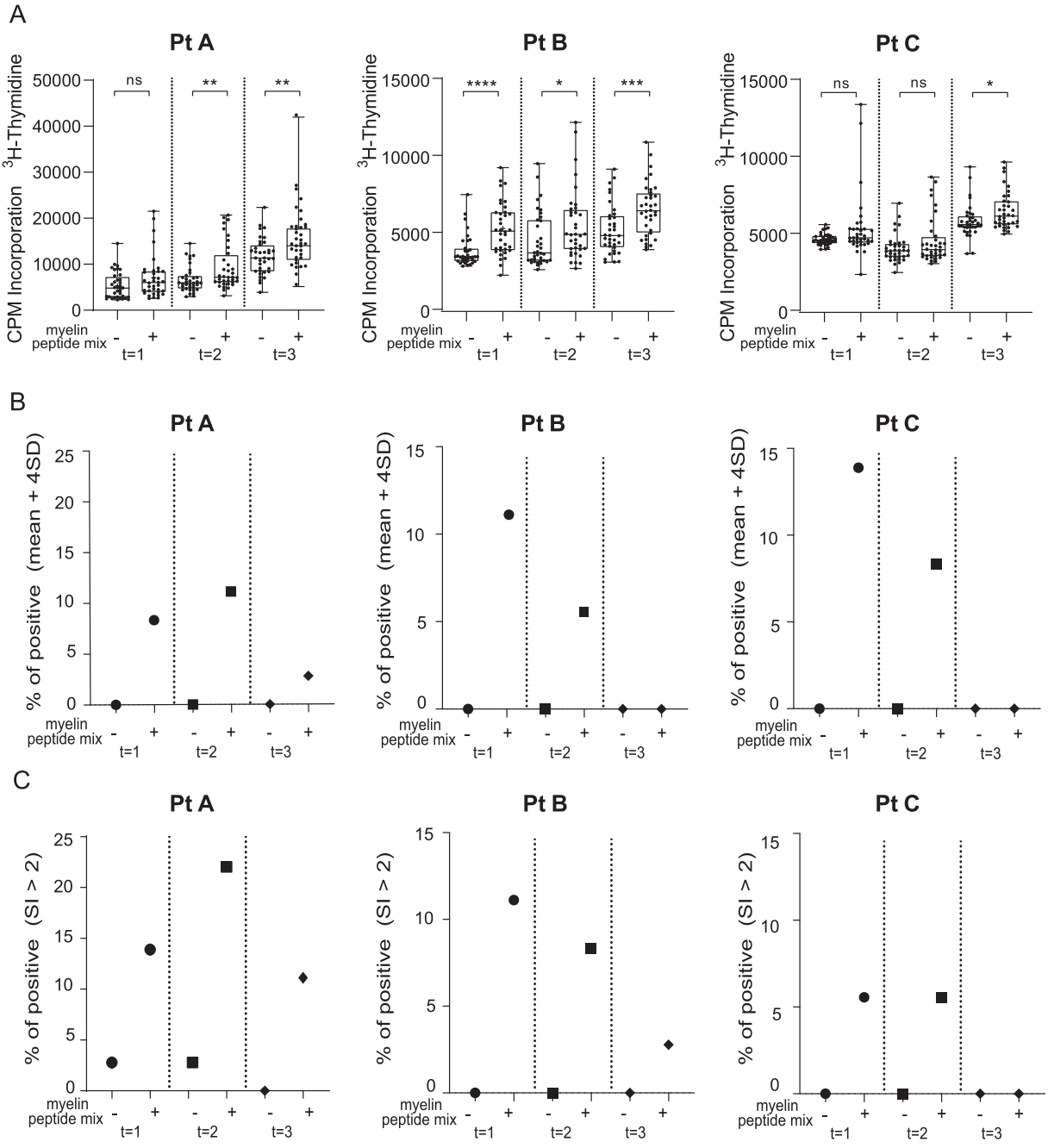

Fig. 6. Detection of very low-frequency human antigen-specific CD4$^+$T cell response in MS. The proliferative response to the in vitro stimulation with myelin peptide mix was evaluated by ^{3}H thymidine assay. PBMC from MS patients undergoing natalizumab treatment were tested at three time points ($t = 1$, 2 and 3). Box plot show the data distribution and each dot represents one of the thirty-six replicates of PBMCs, either unstimulated or stimulated with myelin peptide mix (A). A positive response has been defined as percentage of positive wells in the myelin peptide stimulated samples above the mean + 4SD of proliferative response in the unstimulated samples (B) or as percentage of positive well with a SI > 2 (C). Differences in means were evaluated with non-parametric Mann-Whitney test, which was performed to determine if the responses were significantly different. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$, ns: not significant.

specific response, mean of CTRL +3SD may not be enough to exclude unstimulated conditions as providing a positive response. Therefore, based on our data to distinguish a positive from a false positive response we showed that for the myelin specific dataset, the cut-off to be considered is mean of CTRL +4SD.

SI is one of the most commonly reported methods to assess the positive response to in vitro stimulation. The choice of the cut-off for the SI is arbitrary, in practice it is often set empirically between 1.5 and 3. (Schultz et al., 2017; Mascarenhas et al., 2006; Karle et al., 2016).

A drawback of using SI as cut-off value is that the variation in the unstimulated control wells is not taken into account, because only the mean of the unstimulated control wells is used in the analysis.

Our data showed a variation in basal activation state of CD4$^+$ T cells in the absence of peptide-specific stimulation. We propose that the variation in the negative control should be used as a measure of the specific response of the Ag-specific T cell proliferation. Given that SI is an empirically chosen threshold, we propose that this should be combined with a statistical analysis that determines statistically significant differences between non-stimulated and stimulated conditions, so that fluctuations in all measurements are also considered. For low-frequency

T-cell population a small number of wells can results to be positive. In such circumstances, the choice of SI > 2 and $p < 0.05$ as cut-offs might lead to misinterpretation of the data and lead to a false exclusion of a positive response, as the low frequency/percentage of positive wells results in a p value >0.05.

In conclusion of the study of antigen-specific T cell response, we reason that mean of CTRL + x times SD, would be the optimal cut-off for detecting a positive response without misinterpretation of the results. The value of x depends on the experimental setting in order to not classify the unstimulated conditions itself as positive.

Another important aspect which should be considered is the number and the treatment of unstimulated wells. Since the definition of a positive response is derived from the comparison between the two groups (stimulated and non-stimulated cells), an equal number of replicate wells (unstimulated and Ag stimulated) have to be analyzed. Only by taking into account the variability in the responses of the unstimulated samples and an equal number of control and stimulated wells, the most appropriate cut-off value for detecting the positive response of low frequency Ag-specific T cells can be determined. Moreover, the negative control wells should be treated with the same solutions in which the peptides/antigens are solvated, and at the same final concentrations that have been used in the stimulated wells.

A secondary objective of this paper is to compare the applicability of ^{3}H-thymidine incorporation and dye-based proliferation assay to detect low frequency Ag-specific responses under the same experimental conditions. ^{3}H-thymidine and VPD-450 dilution assays using protocols that are performed regularly in our laboratories were applied to the same clinical sample and measured the proliferative response to HA peptide pool stimulation of PBMCs from vaccinated healthy volunteers. The obtained results highlight the suitability of using either method to detect Ag-specific T cell responses to influenza vaccine peptides. To evaluate the correlation between the results obtained with the ^{3}H-thymidine and VPD-450 dilution assays, we analyzed the distribution of positive responses among vaccinated healthy donors. The results obtained in our study show a strong correlation between the ^{3}H-thymidine and VPD-450 dilution assays.

Each of the ^{3}H-thymidine and VPD-450 dilution assays have advantages and disadvantages. The VPD-450 dilution assay is a good choice for most studies, allowing both phenotypic and functional analysis of individual proliferating cells. Our results indicate that VPD-450 dilution assay is sensitive enough to detect an Ag-specific T cell response. However, when measuring high number of replicates, the ^{3}H-thymidine may be a better choice due to its fast detection method. Some of the drawbacks of this assay include the fact that the assay uses radioactive reagent for which special facilities are needed, which are not available at every institute. In addition, and more importantly, ^{3}H-thymidine assay does not allow the assessment of which specific cell subpopulation is proliferating in response to Ag-specific stimulation.

In summary the results described in this study show the equal suitability of ^{3}H-thymidine and proliferation dye-based assays for the detection of low frequency antigen-specific T cell responses. Neither method seems to be more reliable or sensitive than the other, thus both are applicable for immunomonitoring analyses. Moreover, we provide a better understanding of the impact of the cut-off value for defining a positive response in these type of proliferation assays. Even more importantly we offer guidelines how to select a proper cut-off for monitoring of Ag-specific CD4^{+} T cell responses to avoid misinterpretation of the results, in which the variation of control wells has to be taken into account and needs to be analyzed in equal number of replicates compared to stimulated conditions.

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