Received: 25 July 2020 | Revised: 1 December 2020 | Accepted: 2 December 2020

DOI: 10.1002/cyto.a.24286

ORIGINAL ARTICLE

MIFlowCyt

ISAC CYTOMETRY Journal of Quantitative Cell Science PART A

Flow cytometric measurement of STAT5 phosphorylation in cytomegalovirus-stimulated T cells

Michael Bitar[1] | Marcus Boettcher[1] | Andreas Boldt[1] | Fabian Hauck[2,3] | Ulrike Köhl[1,4,5] | Uwe G. Liebert[6] | Thomas Magg[2] | Marian S. Schulz[7] | Ulrich Sack[1]

[1]Institute of Clinical Immunology, Medical Faculty, University of Leipzig, Leipzig, Germany

[2]Department of Pediatrics, Dr. von Hauner Children's Hospital, University Hospital, Ludwig-Maximilians-University of Munich, Munich, Germany

[3]German Centre for Infection Research (DZIF), Munich, Germany

[4]Institute of Cellular Therapeutics, Hannover Medical School, Hannover, Germany

[5]Fraunhofer Institute for Immunology and Cell Therapy (IZI), Leipzig, Germany

[6]Institute of Virology, Medical Faculty, University of Leipzig, Leipzig, Germany

[7]Department of Women and Child Health, Hospital for Children and Adolescents, Hospitals University of Leipzig, Leipzig, Germany

Correspondence
Michael Bitar, Institute of Clinical Immunology, Medical Faculty, University of Leipzig, 04103 Leipzig, Germany.
Email: michael.bitar@medizin.uni-leipzig.de

Abstract

Cytomegalovirus (CMV)-specific T cells expand with CMV reactivation and are probably prerequisite for control and protection. Given the critical role STAT5A phosphorylation (pSTAT5A) in T cell proliferation, this study presents a simple and sensitive flow cytometric-based pSTAT5A assay to quickly identify CMV-specific T cell proliferation. We determined pSTAT5A in T cells treated with CMV-specific peptide mix (pp65 + IE1 peptides) from 20 healthy adult subjects and three immunodeficient patients with *CARMIL*-2 mutation. After stimulation, the percentage of pSTAT5A$^+$ T cells in CMV-seropositive (CMV$^+$) subjects significantly increased from 3.0% ± 1.9% (unstimulated) to 11.4% ± 5.9% (stimulated) for 24 h. After 7 days of stimulation, the percentage of expanded T cells amounted to 26% ± 17.2%. Conversely, the percentage of pSTAT5A$^+$ T cells and T cell proliferation from CMV-seronegative (CMV$^-$) subjects hardly changed (from 3.0% ± 1.3% to 3.7% ± 1.8% and from 4.3% ± 2.1% to 5.7% ± 1.7%, respectively). We analyzed the correlation between the percentage of pSTAT5A$^+$ T cells versus (1) CMV-IgG concentrations versus (2) the percentage of expanded T cells and versus (3) the percentage of initial CMV-specific T cells. In immunodeficient patients with *CARMIL-2* mutation, CMV-specific pSTAT5A and T cell proliferation were completely deficient. In conclusion, flow cytometric-based pSTAT5A assay represents an appropriate tool to quickly identify CMV-specific T cell proliferation and helps to understand dysfunctions in controlling other pathogens. Flow cytometric-based pSTAT5A assay may be a useful test in clinical practice and merits further validation in large studies.

KEYWORDS
CMV T cell proliferation, CMV-specific T cells, flow cytometry, STAT5 phosphorylation, T cell activation

[Correction added on 13 Jan, 2021, after first online publication: Projekt Deal funding statement has been added.]

Michael Bitar and Marcus Boettcher contributed equally to this study.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.
© 2020 The Authors. *Cytometry Part A* published by Wiley Periodicals LLC. on behalf of International Society for Advancement of Cytometry.

1 | INTRODUCTION

Human cytomegalovirus (CMV) is a common pathogen that often results in an asymptomatic infection [1]. In healthy CMV-infected individuals, pathogenesis is controlled by CMV-specific T cells involving both $CD4^+$ and $CD8^+$ T cells. Loss or suppression of adaptive immunity as accrues in primary and secondary immune deficiencies, transplant patients, or older people who suffer from age-associated changes in the immune system frequently leads to CMV reactivation [2–11].

CMV-specific T cells expand with CMV reactivation and are probably prerequisite for control and protection [12]. A variety of assays exist to measure CMV-specific cellular activation, including measurement of intracellular cytokine staining (ICS) by flow cytometry, enzyme-linked immunosorbent assay (ELISA), or enzyme-linked immunospot assay (ELISPOT) [13,14]. A different analysis consists of direct staining with CMV-peptide-loaded multimers and enumeration of CMV-specific $CD8^+$ T cells by flow cytometry [13].

Today, the most widely used method for detection of CMV-specific T cell proliferation is based on the measurement of cell proliferation by serial halving of the fluorescence intensity of the vital dye [4]. As initial CMV-specific T cell numbers vary from subject to subject, a prolonged culture time and an extensive number of cells are required to expand it, making the assay is labor- and resource-intensive and requiring several days (5–7 days) [2,3,5,15]. Different analysis has been used to measure specific T cell proliferation stimulated by antigens, is intracellular Ki67 expression. Ki67 is a nuclear protein that is detectable in every phase of the cell cycle of proliferating cells but is absent in G0 cells. Ki67 is expressed in T cells that had undergone in vitro proliferation after 6-day culture of human whole blood or PBMC with antigens [16,17].

These drawbacks might be impractical for routine diagnostic purposes specifically when an early diagnosis is required; therefore, new predictive biomarkers would have important clinical utility in efforts to prevent and treat CMV disease.

As previously described [18], upon T cell receptor (TCR) activation, T cell proliferation strongly depends on the phosphorylation of the signal transducer and activator of transcription 5A (pSTAT5A). The phosphorylation of STAT5A occurs within 24 h and can be used as an early marker to assess mitogen-driven T cell proliferation [18]. Based on this knowledge, the aim of the present study is to establish a rapid flow cytometric CMV-specific pSTAT5A assay to serve as a prediction marker for CMV-specific T cell proliferation. We analyze and compare pSTAT5A and T cell proliferation in T cells treated with a CMV-specific peptide mix (pp65 + IE1 peptides) from CMV^+ and CMV^- healthy subjects and show the correlation between the two events. Subsequently, we verify this assay by analyzing pSTAT5A in T cells from three *CARMIL*-2-deficient patients suffering from chronic CMV infection. Human *CARMIL*-2 deficiency is an autosomal recessive primary immunodeficiency disease associated with a deficiency of regulatory T cells and defective CD28 signaling, which leads to impaired T-cell stimulation, differentiation, and proliferation [19].

Given its simplicity and robustness, the flow cytometric-based pSTAT5 assay is especially appropriate for rapidly assessing CMV-specific T cell proliferation and understanding dysfunctions in controlling other pathogens.

2 | METHODS AND MATERIAL

2.1 | Collection of blood samples

After informed consent, blood was taken from 20 healthy adult subjects and three CMV-infected patients (P 1; P 2-1; P 2-2) with *CARMIL*-2 mutation for the serological and immunological analyses at the Institute of Clinical Immunology, University of Leipzig Medical Center. This study protocol was approved by the University of Leipzig Medical Center (092/2002 and 151/2006).

2.2 | Cytomegalovirus serology

Human serum was separated by centrifugation for 10 min at 600 x *g* and frozen at – 80°C. CMV-IgG levels were determined by ARCHITECT CMV IgG assay (Abbott Laboratories, Dublin, Ireland) at the Institute of Virology, University of Leipzig Medical Center. The cut-off of CMV-IgG levels was determined to be positive at concentration > 7.0 AU/ml.

2.3 | Isolation of PBMCs and staining with violet proliferation dye 450

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh lithium-heparinized peripheral blood samples by density centrifugation over Ficoll–Hypaque (Pan Biotech, Germany), as described previously [18,20,21]. PBMCs ($1 * 10^7$ cells/ml) were diluted with phosphate-buffered saline (PBS, pH 7.2) (Gibco life Technologies, Carlsbad, USA) and stained with violet proliferation dye 450 (VPD450) (3 μM) (BD Biosciences, San Jose, USA) for 15 min at 37°C. Subsequently, PBMCs were washed and re-suspended in RPMI 1640 containing 10% fetal bovine serum, penicillin ($1 * 10^5$ mg/ml), and streptomycin ($1 * 10^5$ mg/ml) (Gibco life Technologies, Carlsbad, USA) and finally adjusted to $3 * 10^6$ cells/ml.

2.4 | PBMCs stimulation

PBMCs ($3 * 10^6$ cells/ml) were seeded into 48-well cell culture plates ($7.5 * 10^5$ cells/well) at 37°C. Afterwards, PBMCs were stimulated with the major target antigens of CMV-specific peptide mix (1 μg/ml) consisting of pool of Pp65 + IE1 peptides (ELSP5944, AID, Germany) as previously described [9,22,23], withCD3/CD28 (100 ng/ml) (eBioscience clones OKT3, CD28.2) as a positive control, or with IL-2 (100 ng/ml) (BD Biosciences, Heidelberg, Germany). Simultaneously, CMV- and CD3/CD28-stimulated cells were cultured for 24 h or 7 days in a humidified atmosphere of 5% CO_2 at 37°C to determine

pSTAT5A and T cell proliferation, respectively. IL-2-stimulated cells were cultured for 15 min at 37°C.

2.5 | Determination of CMV-stimulated pSTAT5A$^+$ T cells by flow cytometry

The cultivated PBMCs were harvested after 24 h, pelleted by centrifugation, lysed and fixed by using "lyse and fix" buffer (BD Biosciences), and incubated at 37°C in a water bath for 12 min. Subsequently, cells were centrifuged, the supernatant was discarded, and the pellet was washed with 4 ml PBS.

The samples were permeabilized by using cold perm buffer III (1 ml) (BD Biosciences) and were left on ice for 30 min. The pellet was then washed with a fetal bovine serum stain buffer (FBS) (2 mL) (BD Biosciences) and finally re-suspended in 200 µl FBS. For flow cytometric analysis, the T cells were stained with PerCP-Cy TM 5.5 mouse anti-human CD3 (2.5 µl, clone UCHT1, BD Biosciences) and Alexa Fluor 647 mouse anti-human STAT5A phosphorylation (10 µl, PY694, Clone 47/Stat5, BD Biosciences). Mouse IgG1-k-Alexa Fluor 647 was used as an isotype control for assessing the background staining of the cells. After 1 h of incubation in the dark at room temperature, cells were washed with 2 ml stain buffer, centrifuged and were suspended in 300 µl of stain buffer. FACSCantoII-based flow cytometry was conducted to measure the samples. Briefly, the system was set up with three lasers: a violet laser 405 nm, a blue laser 488 nm, and a red laser 647 nm [24,25]. Prior to running samples, the instrument was calibrated using calibration beads (BD Biosciences). BD FACSDiva software was used for acquisition of the events [25]. The analysis of the pSTAT5A is based on the following gating strategies: (1) forward scatter (FSC) versus side scatter (SSC), (2) FSC area (FSC-A) versus FSC height (FSC-H) (singlets gate), and (3) CD3 versus SSC. After the conduction of the previous gating strategies, the T cells (CD3$^+$) were separated. Now, after a clear separation of T cells from the non-T cells, 20,000 CD3$^+$ T cells per sample were acquired. The expression of pSTAT5A was calculated as a percentage of pSTAT5A$^+$ T cells (Figure S1).

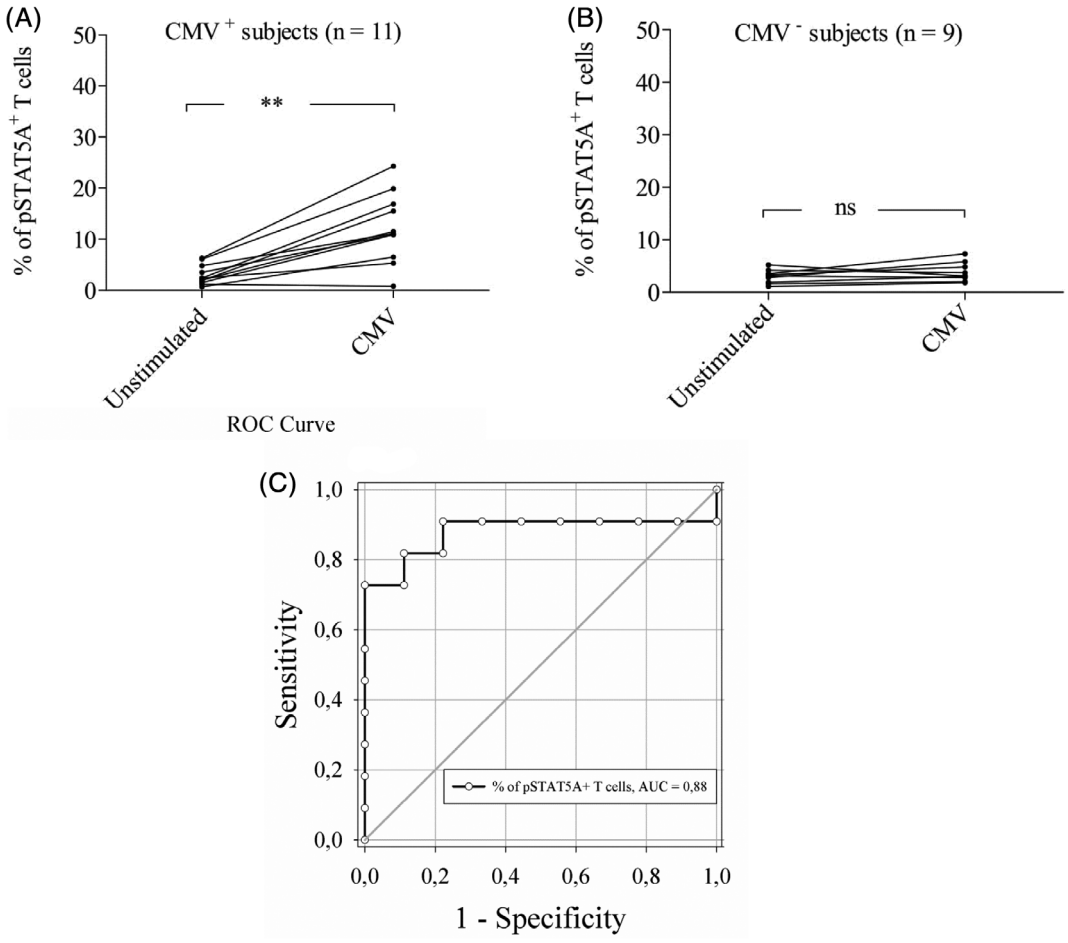

FIGURE 1 CMV-stimulated pSTAT5A profile and ROC analysis. PBMCs (3 * 10^6 cells/ml) were stimulated with CMV-specific peptide mix (1 µg/ml). After 24 h, the percentage of pSTAT5A in T cells was measured. (A) The percentage of CMV stimulated pSTAT5A$^+$ T cells in CMV$^+$ subjects; (B) the percentage of CMV-stimulated pSTAT5A$^+$ T cells in CMV$^-$ subjects; (C) the receiver operating characteristic (ROC) curve was plotted to evaluate the sensitivity, specificity, area under the curve (AUC) of the assay and to determine % pSTAT5A$^+$ T cells cut-off value. When % pSTAT5A$^+$ T cells cut-off value was set to 9.1, the ROC curve gave an AUC of 0.88 ($p < 0.004$) with a sensitivity (73%) and specificity (100%). Ns, non-significant; **, $p < 0.01$ (Wilcoxon's test)

2.6 | Determination of CMV-stimulated T cell proliferation by flow cytometry

Cultivated PBMCs were harvested after 7 days and washed with 2 mM EDTA PBS. The pellet was re-suspended in 200 µl PBS and stained with FITC mouse anti-human CD3 (5 µl, clone SK7, BD Biosciences) at 37°C. After 15 min, cells were washed with 2 ml 2 mM EDTA PBS, centrifuged, and suspended in 300 µl PBS. 7-Amino-Actinomycin D staining (7-AAD) (10 µl, BD Biosciences) was added to pre-stained T cells for 10 min before the measurement was conducted. 7-AAD was used to determine cell viability. The analysis of the T cell proliferation is based on two lymphocyte gates (1) FSC versus SSC and (2) FSC-A versus FSC-H (singlets gate) where $CD3^+$ live cells (T cells) were separated in a third dot plot (CD3 vs. 7-AAD). Now, the decrease of VPD450 dye intensity in expanded $CD3^+$ cells was measured and 50,000 $CD3^+$ cells per sample were acquired (Figure S2). Data analysis was conducted using FlowJo.7.6.5 software (Ashland, OR, USA) [26]. FlowJo presents graphical display as well as information about each generation in the subset. The proliferation platform also provides information about the fraction of cells from the original population that have divided (Initial CMV-specific T cells) and the number of times these cells have divided.

2.7 | Determination of IFN-γ and TNF-α production in CMV-stimulated T cells

PBMCs (3 * 10^6 cells/ml) from three CMV seropositive healthy volunteers enrolled in the study were cultured with CMV-specific peptide mix (1 µg/ml) (ELSP5944, AID, Germany) or with CD3/CD28 (100 ng/ml) (eBioscience clones OKT3, CD28.2) for 24 h. The supernatants were then collected and assayed for IFN-γ and TNF-α by enzyme immunoassay (EI A; R&D system, Minneapolis, USA).

2.8 | Adaptation of methods to DIN EN ISO 15189 requirements

The international standard DIN EN ISO 15189 suggested proceedings (performing intra-assay and inter-assay precision) to fulfill highest requirements for the quality and competency of medical laboratories to exclude or diminish false positive and false negative results [25,27]. Please note that in case of using two or more different flow cytometers, differences in technical adjustments among different devices leading to various results in mean

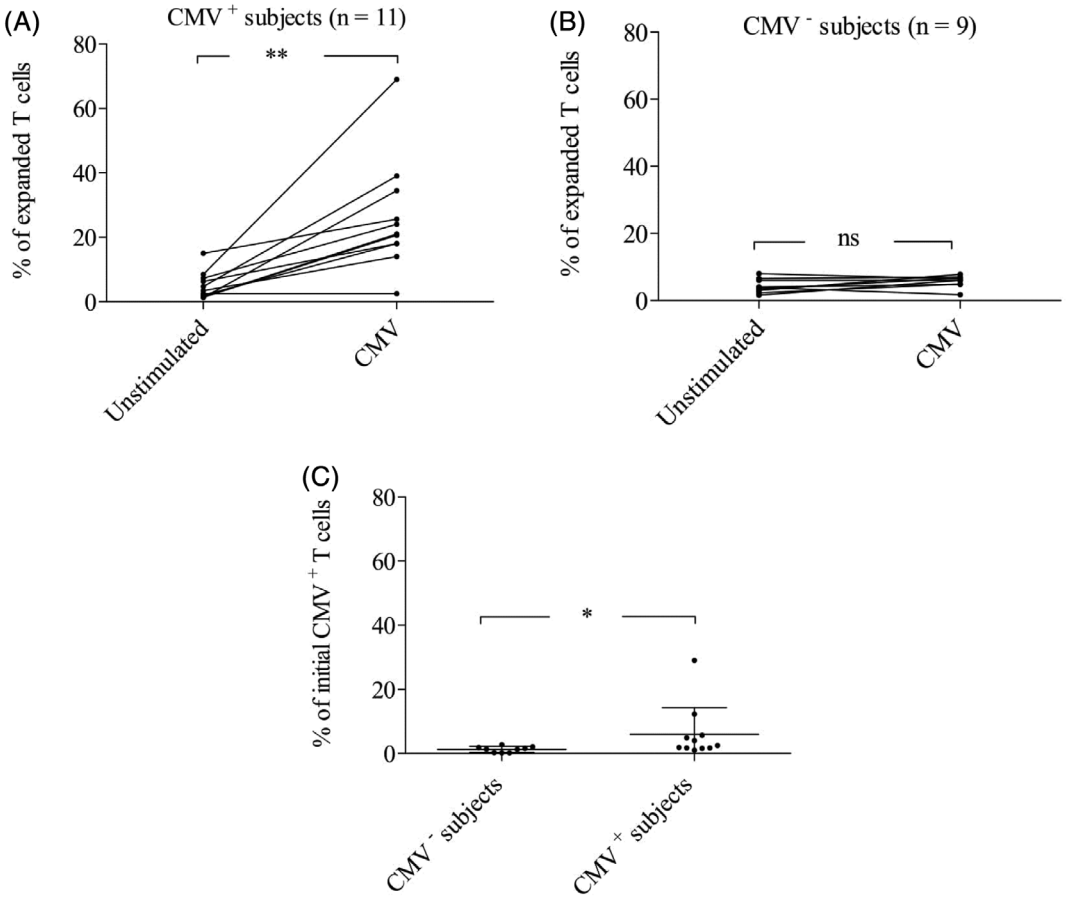

FIGURE 2 Analysis of CMV-specific T cell proliferation and initial CMV-specific T cells from CMV^+ and CMV^- subjects. PBMCs (3 * 10^6 cells/ml) were stimulated with CMV specific peptide mix (1 µg/ml). After 7 days, the percentage of expanded T cells was measured. (A) The percentage of expanded T cells from 11 CMV^+ subjects. (B) The percentage of expanded T cells from 9 CMV^- subjects; (C) comparison of the percentage of initial CMV specific T cells in CMV^- and CMV^+ subjects. Ns, non-significant; **, $p < 0.01$ (Wilcoxon's test)

fluorescence measurement should be considered. Therefore, a transfer of the instrument setting among the devices has to be performed.

2.9 | Statistical analysis

Statistical analysis was performed using the Graph Pad Prism 5 software (graph Pad Prism software, Inc., San Diego, CA, USA) and Sigmaplot.14.0 software (Systat Software Inc, San Jose, CA). The adjusted p-values were estimated by Wilcoxon's test (Ns not significant, *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001). Correlation analysis was used to identify Spearman's correlation coefficient (*r*). The receiver operating characteristic (ROC) curve was plotted to evaluate the sensitivity, specificity, and area under the curve (AUC) value of the system.

3 | RESULTS

3.1 | Cytomegalovirus serology

CMV-IgG concentrations in subjects enrolled in the study were determined. Eleven of the 20 healthy subjects tested positive for CMV-IgG. The samples were considered seropositive if the CMV-IgG level is higher than 7.0 AU/ml.

3.2 | Time-dependent STAT5A phosphorylation

To determine the optimal time to typically analyze the percentage of pSTAT5A$^+$ T cells, a series of kinetics was performed. CMV (1 µg/ml)-stimulated pSTAT5A was reliably detected after 6 h of stimulation and reached a peak value after 12 h. It declined thereafter (Figure S3(A)). Clearly, the measurement of IFN-γ and TNF-α by EIA in supernatants from three CMV$^+$ subjects showed increased concentrations of the two cytokines after 24 h (Figure S3(B),(C), respectively). Because of difficulties in maintaining the working routine of the laboratory, we analyzed pSTAT5A after 24 h.

3.3 | Determination of the percentage of CMV-stimulated pSTAT5A$^+$ T cells

CMV-stimulated T cells from CMV$^+$ subjects showed a significant increase in the percentage of pSTAT5A$^+$ T cells (from 3.0% ± 1.9% (unstimulated) to 11.4% ± 5.9% (CMV)) at 24 h (Figure 1(A)). Conversely, the percentage of pSTAT5A$^+$ T cells from CMV$^-$ subjects

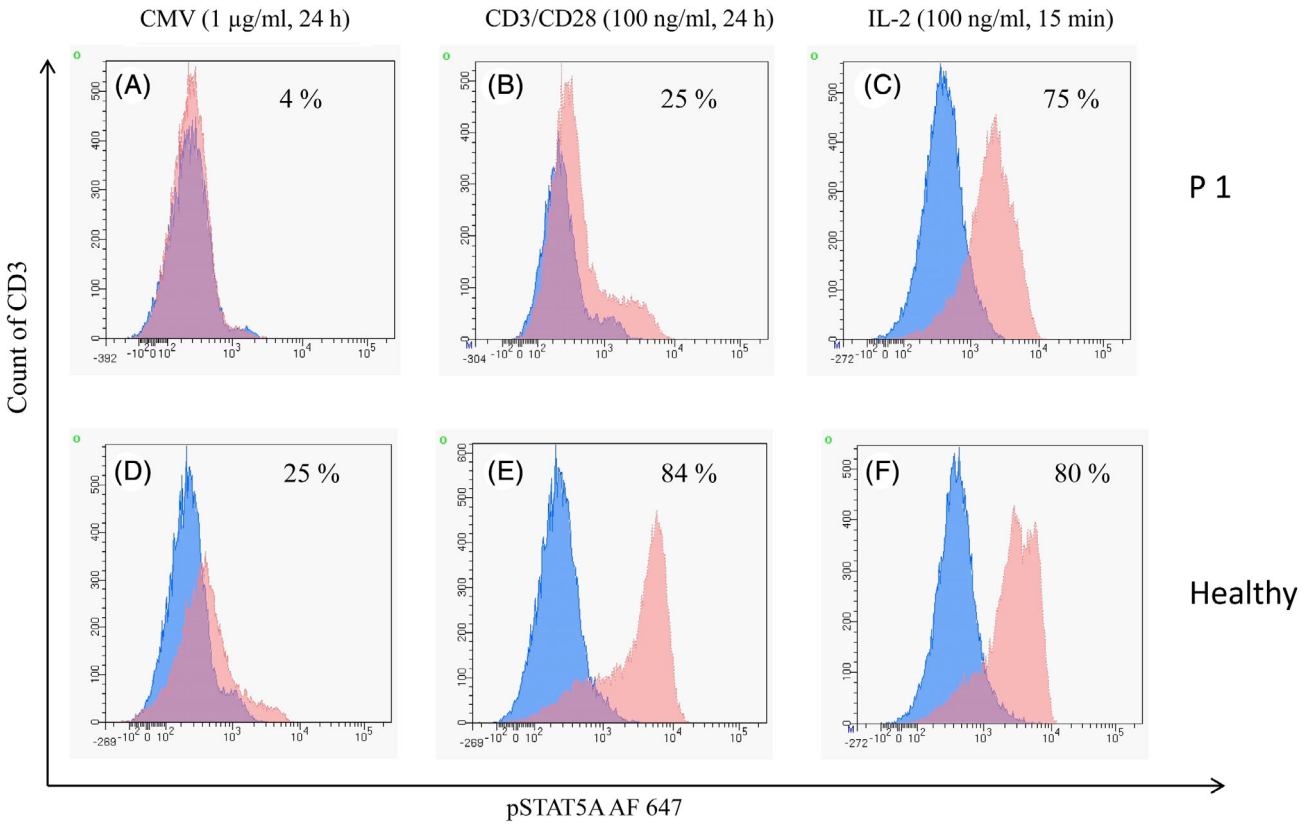

FIGURE 3 Flow cytometric analysis of pSTAT5A in T cells from a patient (P 1) with CARMIL-2 mutation compared to a healthy control sample. PBMCs (3 * 10^6 cells/ml) were stimulated (red histogram) with (A, D) CMV specific peptide mix (1 µg/ml, 24 h); (B, E) CD3/CD28 (100 ng/ml, 24 h); (C, F) IL-2 (100 ng/ml, 15 min) or unstimulated (blue histogram). Percentages of pSTAT5A$^+$ T cells are detailed within the histogram boxes

hardly changed (from 3.0% ± 1.3% (unstimulated) to 3.7% ± 1.8% (CMV)) at 24 h (Figure 1(B)).

The ROC curve was plotted to determine the specificity, sensitivity, and AUC value of the assay. As shown in Figure 1(C), when the percentage of pSTAT5A$^+$ T cells cut-off value was set to 9.1 (pre-test probability 0.5, Cost ratio 1.0), the ROC curve gave an AUC of 0.88 ($p < 0.004$) with a specificity and sensitivity of 100% and 73%, respectively.

A strong correlation was observed between the percentage of pSTAT5A$^+$ T cells with CMV-IgG concentrations ($r = 0.6$, $p = 0.04$) (Figure S4(A)).

3.4 | Determination of the percentage of expanded T cells and the initial CMV-specific T cells

Furthermore, after antigen stimulation for 7 days, CMV-specific T cells from CMV$^+$ subjects expanded from 4.9% ± 4% to 26% ± 17.2% as determined by VPD 450 staining (Figure 2(A)). In contrast to CMV$^+$ subjects, the percentage of expanded T cells from CMV$^-$ subjects hardly changed (from 4.3% ± 2.1% (unstimulated) to 5.7% ± 1.7% (CMV)) (Figure 2(B)). The percentage of initial CMV-specific T cells was 6% ± 8.2% in the original population from CMV$^+$ subjects, as calculated by FlowJo.7.6.5 software (Figure 2(C)). A significant difference between CMV$^+$ and CMV$^-$ subjects in relation to the percentage of initial CMV-specific T cells was observed (Figure 2(C)).

We found no significant correlation between the percentage of pSTAT5A$^+$ T cells versus (1) the percentage of expanded T cells ($r = 0.16$, $p > 0.05$) and versus (2) the percentage of initial CMV-specific T cells ($r = 0.13$, $p > 0.05$) (Figure S4(B),(C), respectively).

3.5 | Validation of CMV-stimulated STAT5A phosphorylation in 20 healthy adult subjects to DIN EN ISO 15189 requirements and for use in diagnostic application

To ensure that our analysis is accurate, reproducible, and precise, validation of our data included the definition of intra- and inter-assay precision values [27]. For intra-assay precision, whole blood of healthy donor was used to determine all data fivefold during 1 day (Table S1). Inter-assay precision was assessed by measuring all samples in single evaluations on five consecutive days (Table S2). The standard deviation and coefficient of variation were calculated. A coefficient of variation of up to 20% was considered tolerable and fulfilled the criteria of the International Standard EN ISO 15189.

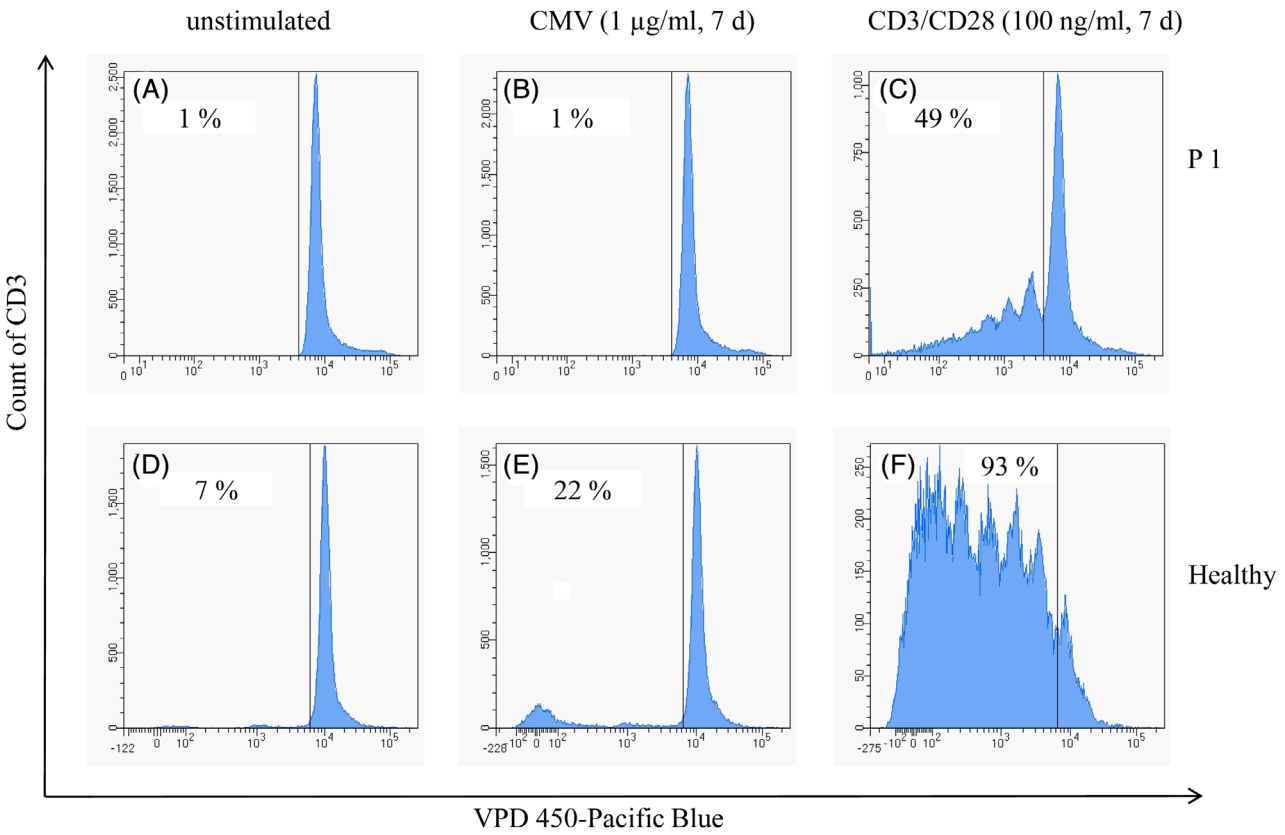

FIGURE 4 Flow cytometric analysis of T cell proliferation in PBMCs from a patient with CARMIL-2 mutation compared to a healthy control sample. PBMCs (3 * 10^6 cell/ml) were stimulated with (B, E) CMV-specific peptide mix (1 µg/ml, 7 days); (C, F) CD3/CD28 (100 ng/ml, 7 days) or (A, D) unstimulated. Percentages of expanded T cells are detailed within the histogram boxes

3.6 | Evaluation of pSTAT5A profile at three patients with *CARMIL2* mutations suffering from chronic CMV infection

Subsequently, we verified the established measurement by CMV-specific T cells stimulation in three selected patients diagnosed with *CARMIL2* mutation and suffering from chronic CMV infection.

Patient 1 (P 1, male) has consanguine parents. PCR analysis showed high CMV viral load. The profile of flow cytometric analysis demonstrated that most T cells were naïve or effector T cells, whereas memory T cells could not be detected. Furthermore, after 24 h of stimulation, pSTAT5A was completely or partially deficient in CMV or CD3/CD28 stimulation, respectively (Figure 3(A), (B), (D), (E)). The complete and the partial deficiency of CMV- and CD3/CD28-stimulated pSTAT5A correlated with the complete and the partial deficiency of CMV- and CD3/CD28-stimulated T cells proliferation, respectively (Figure 4). IL-2-stimulated T cells for 15 min showed a regular phosphorylation of STAT5A (Figure 3(C), (F)). Trio-exome sequencing revealed to *CARMIL*-2 mutation, which is characterized by CD28 signaling deficiency.

Patients P 2-1 and P 2-2 were diagnosed with homozygous loss-of-function *CARMIL-2* mutation. Our results demonstrated that after 24 h of stimulation, pSTAT5A was completely or partially deficient in CMV- and CD3/CD28-stimulated T cells, respectively (Figure 5). The complete and the partial deficiency of CMV- and CD3/CD28-stimulated pSTAT5A correlated with the complete and the partial deficiency of CMV- and CD3/CD28-stimulated T cells proliferation, respectively (Figure 6).

4 | DISCUSSION

It is known that measurement of cell proliferation presents the standard procedure to detect cellular immune responses to antigens; therefore, we attempted to introduce a rapid and straightforward

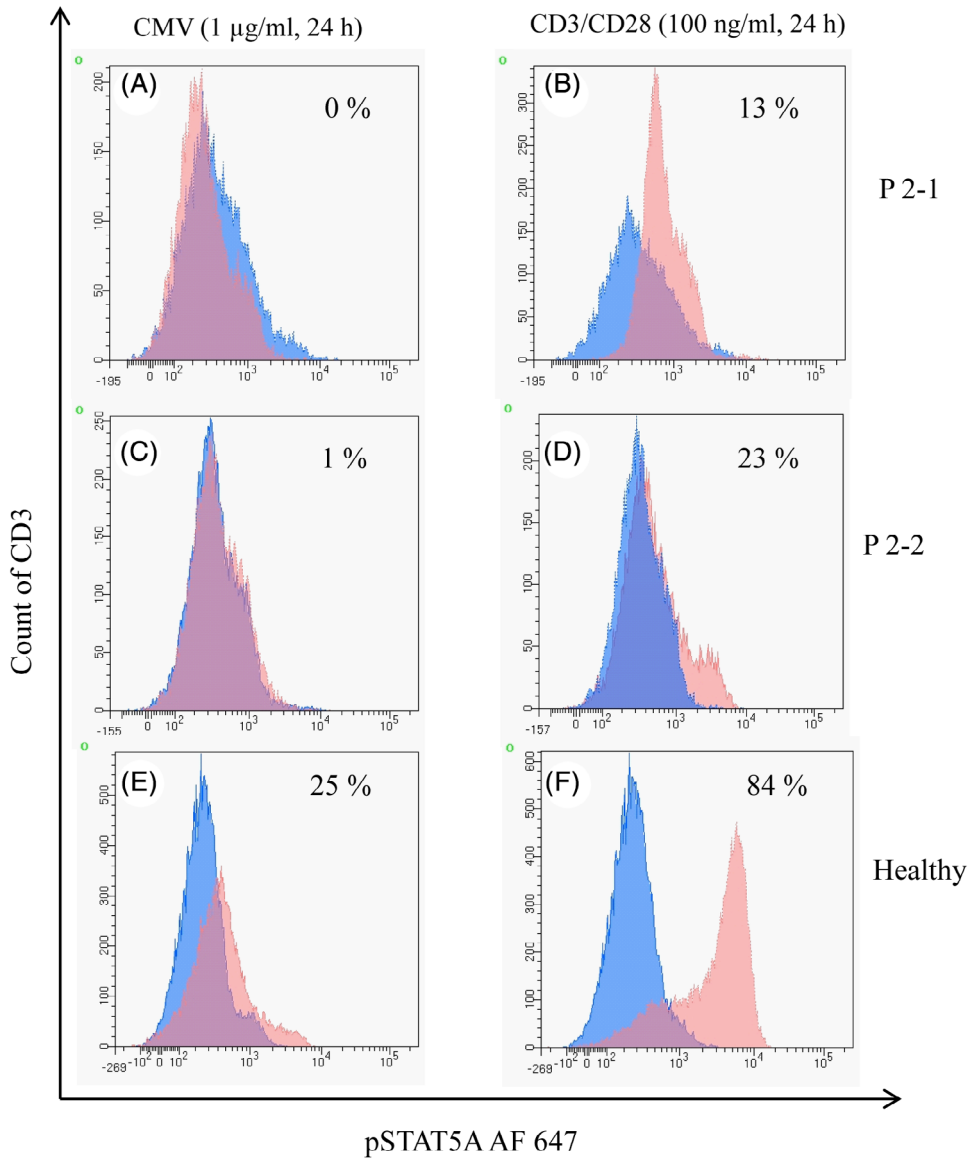

FIGURE 5 Flow cytometric analysis of pSTAT5A in PBMCs from two patients (P 2-1, P 2-2) diagnosed with CARMIL-2 mutation compared to a healthy control sample. PBMCs ($3 * 10^6$ cells/ml) were stimulated (red histogram) with (A, C, E) CMV-specific peptide mix (1 µg/ml, 24 h); (B, D, F) CD3/CD28 (100 ng/ml, 24 h) or unstimulated (blue histogram). Percentages of $pSTAT5A^+$ T cells are detailed within the histogram boxes

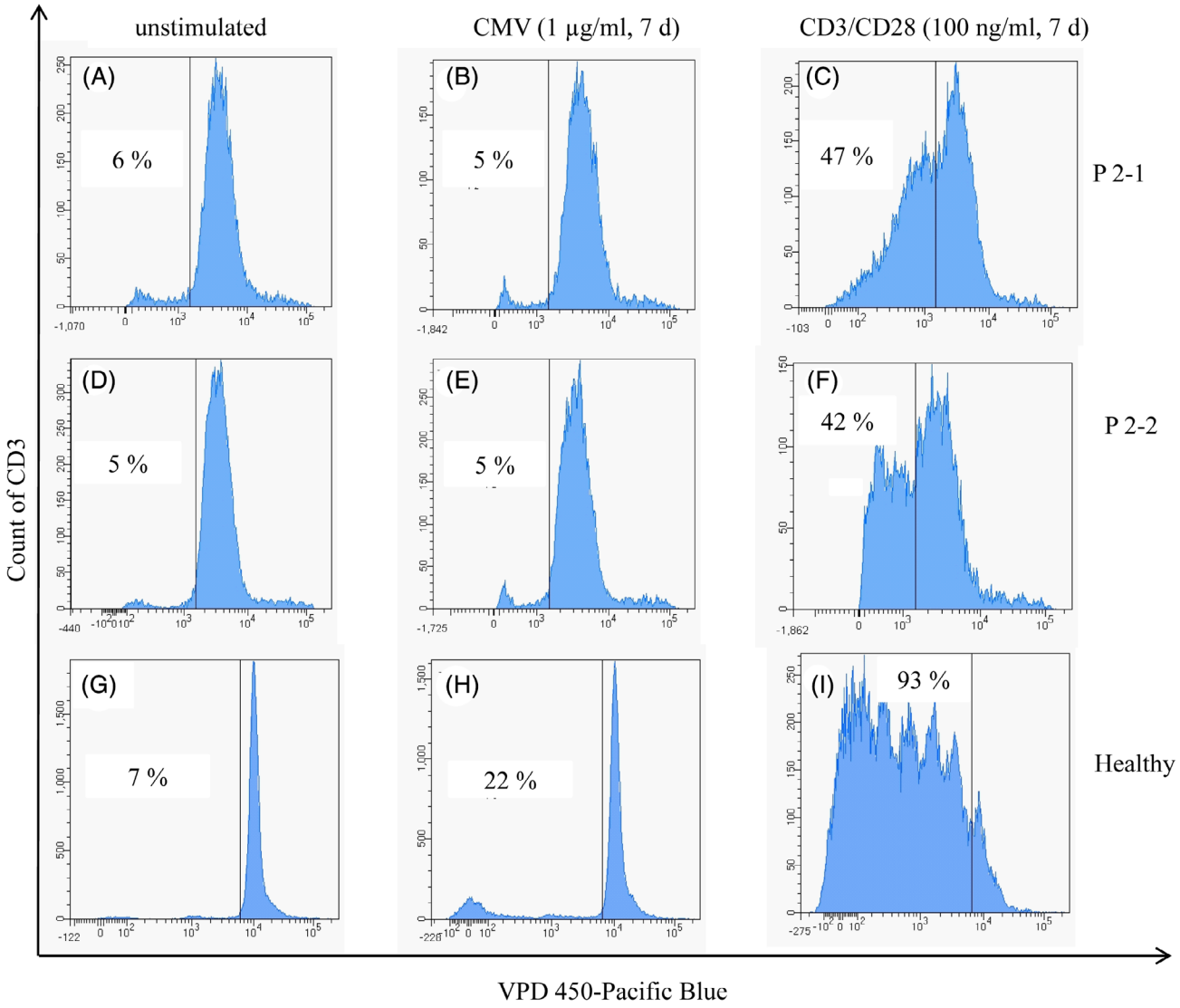

FIGURE 6 Flow cytometric analysis of T cell proliferation in PBMCs from two patients (P 2-1, P 2-2) diagnosed with CARMIL-2 mutation compared to healthy control sample. PBMCs (3 * 10^6 cells/ml) were stimulated with (B, E, H) CMV-specific peptide mix (1 µg/ml, 7 days); (C, F, I) CD3/CD28 (100 ng/ml, 7 days) or (A, D, G) left unstimulated. Percentages of expanded T cells are detailed within the histogram boxes

flow-cytometric method for the assessment of CMV-specific T cell proliferation [28,29]. In the present study, we showed that STAT5A phosphorylation could be a functional surrogate marker for the identification of CMV-T cell proliferation.

We demonstrated that a moderate expression of pSTAT5A starts after 6 h of stimulation by CMV-specific peptide mix in CMV^+ subjects and reaches a peak value after 12 h, which leads to CMV-specific T cell proliferation after 7 days. Conversely, T cells treated with CMV-specific peptide mix from CMV^- subjects did not respond. These observations confirm that STAT5A phosphorylation mediates and plays an important role in CMV-specific T cell proliferation.

We evaluated the diagnostic value of the pSTAT5 assay and determined the percentage of $pSTAT5A^+$ T cells cut-off value at which the pSTAT5 assay has the greatest diagnostic potency. Our data showed that a cut-off value of 9.1% could be used to assess CMV-specific T cell proliferation with a specificity and sensitivity of 100% and 73%, respectively.

Using pSTAT5-simplified protocol, we can predict CMV-specific cell proliferation earlier (within 24 h) and introduce important information on T cell biology, which in current CMV-specific assays such as ELISA-and ICS-based assays cannot be yet achieved.

ELISA-based assays require low blood volumes and are easy to perform [30], but they are restricted to the detection of IFN-g producing $CD8^+$ T cells and do not allow single-cell-level analysis.

ICS analysis can allow detailed analysis of the percentage of T cells, T-cell subsets requisite to confer protection from CMV [31–33], but it cannot always be assumed that all T cells expressing IFN-g will undergo proliferation [13]. As thresholds for a positive test may exist depending on the immune assay used, patient group studied, and the

clinical setting, the comparison between the current method and ICS analysis is difficult due to lack of standardization.

Subsequently, we verify this assay by analyzing pSTAT5A in T cells from three *CARMIL*-2-deficient patients suffering from chronic CMV infection. The results showed that the complete and partial deficiency of CMV- and CD3/CD28-stimulated pSTAT5A in T cells from the three patients correlated with the complete and the partial deficiency of CMV- and CD3/CD28-stimulated T cell proliferation, respectively. Importantly, the positive IL-2-stimulated pSTAT5A in T cells from patient 1 excluded defects in JAK3/STAT5 signaling [18]. These results correlated with Trio-exome sequencing revealing to *CARMIL*-2 mutation, characterized by CD28 signaling deficiency, impaired naïve T cell activation, proliferation, and deficient gain of T cell memory [19]. In agreement with other studies, Goldeck et al. (2013) [34] showed that lower phosphorylation signal is identified due to lower CD28 expression impacting on TCR/CD28 signaling crosstalk, which is essential for T cell activation.

This implied that CMV-specific pSTAT5A detection could be used as a fast tool to detect CMV-specific T cells' regeneration and proliferation without requiring several days of culture. Because CMV-specific T cell proliferation critically influences viral replication, monitoring of CMV-specific T cells may be useful for identifying patients at risk of viral replication specifically in post-transplant periods. Our outcomes were variable and showed subject dependency. These variables would likely result in differences in human leukocytes antigen, epitope dominance, and variability in the relative number of $CD3^+$ expressions and memory T cells [35]. The limitations of our study are small sample size and no staining for sub-population such $CD4^+$ and $CD8^+$ T cells was done.

In conclusion, disorders in the TCR – IL-2 –JAK3/STAT5 signal pathway in T cells may result in an insufficient response to pathogens. As this method is rapid, robust, and adaptable, FCM-based pSTAT5A profiling is an effective tool for clinical laboratory diagnostics [1] to understand the susceptibility to recurrent opportunistic infections, [2] to quickly identify patients who are at risk of viral replication, and to further improve clinical management of CMV infection, for example, after allogeneic stem cell transplantation [3], and to identify and characterize well-known PIDs such as *CARMIL*-2 mutations.

ACKNOWLEDGMENTS

The authors thank Heike Knaack, Katrin Bauer, and Bettina Glatte for their excellent technical assistance, and Anja Grahnert, Ronald Weiß, Erik Schilling for support and advice, and Dr. Danny Issa and Sarah Semrau for reviewing the manuscript. Open Access funding enabled and organized by ProjektDEAL.

AUTHOR CONTRIBUTIONS

Thomas Magg: Investigation. **Marian Schulz:** Investigation. **Ulrich Sack:** Project administration; supervision. **Marcus Boettcher:** Methodology; software. **Uwe Gerd Liebert:** Validation; visualization; writing-original draft. **Andreas Boldt:** Validation; visualization. **Ulrike Koehl:** Supervision; validation. **Fabian Hauck:** Investigation. **Michael Bitar:** Data curation; formal analysis; validation; writing-original draft.

CONFLICT OF INTEREST

Authors declared no conflict of interest.

REFERENCES

1. Cannon, M. J., Schmid, D. S., & Hyde, T. B. (2010). Review of cytomegalovirus seroprevalence and demographic characteristics associated with infection. Rev Med Virol, 20, 202–213. https://doi.org/10.1002/rmv.655
2. Walker, J. D., Maier, C. L., & Pober, J. S. (2009). Cytomegalovirus-infected human endothelial cells can stimulate allogeneic CD4+ memory T cells by releasing antigenic exosomes. J Immunol, 182, 1548–1559. https://doi.org/10.4049/jimmunol.182.3.1548
3. Moro-García, M. A., Alonso-Arias, R., López-Vázquez, A., Suárez-García, F. M., Solano-Jaurrieta, J. J., Baltar, J., & López-Larrea, C. (2012). Relationship between functional ability in older people, immune system status, and intensity of response to CMV. Age (Dordr), 34, 479–495. https://doi.org/10.1007/s11357-011-9240-6
4. Hobeika, A., Osada, T., Serra, D., Peplinski, S., Hanson, K., Tanaka, Y., et al. (2008). Detailed analysis of cytomegalovirus (CMV)-specific T cells expanded for adoptive immunotherapy of CMV infection following allogeneic stem cell transplantation for malignant disease. Cytotherapy, 10, 289–302. https://doi.org/10.1080/14653240801927040
5. Albiero, E., Amati, E., Baumeister, E., Einsele, H., Grigoleit, G. U., & Rodeghiero, F. (2016). Heterogeneity of specific CD4+ and CD8+ T cells stimulated by CMV pp65 and IE1 antigens. J Immunother, 39, 329–342. https://doi.org/10.1097/CJI.0000000000000140
6. Hebart, H., Daginik, S., Stevanovic, S., Grigoleit, U., Dobler, A., Baur, M., … Einsele, H. (2002). Sensitive detection of human cytomegalovirus peptide-specific cytotoxic T-lymphocyte responses by interferon-gamma-enzyme-linked immunospot assay and flow cytometry in healthy individuals and in patients after allogeneic stem cell transplantation. Blood, 99, 3830–3837. https://doi.org/10.1182/blood.v99.10.3830
7. Boeckh, M., Leisenring, W., Riddell, S. R., Bowden, R. A., Huang, M.-L., Myerson, D., … Corey, L. (2003). Late cytomegalovirus disease and mortality in recipients of allogeneic hematopoietic stem cell transplants: importance of viral load and T-cell immunity. Blood, 101, 407–414. https://doi.org/10.1182/blood-2002-03-0993
8. Ljungman, P., Brand, R., Einsele, H., Frassoni, F., Niederwieser, D., & Cordonnier, C. (2003). Donor CMV serologic status and outcome of CMV-seropositive recipients after unrelated donor stem cell transplantation: an EBMT megafile analysis. Blood, 102, 4255–4260. https://doi.org/10.1182/blood-2002-10-3263
9. Widmann, T., Sester, U., Schmidt, T., Gärtner, B. C., Schubert, J., Pfreundschuh, M., & Sester, M. (2018). Rapid reconstitution of CMV-specific T-cells after stem-cell transplantation. Eur J Haematol, 101, 38–47. https://doi.org/10.1111/ejh.13077
10. Hebart, H., & Einsele, H. (2004). Clinical aspects of CMV infection after stem cell transplantation. Hum Immunol, 65, 432–436. https://doi.org/10.1016/j.humimm.2004.02.022
11. Marek, A., Stern, M., Chalandon, Y., Ansari, M., Ozsahin, H., Güngör, T., et al. (2014). The impact of T-cell depletion techniques on the outcome after haploidentical hematopoietic SCT. Bone Marrow Transplant, 49, 55–61. https://doi.org/10.1038/bmt.2013.132
12. Stern, L., Withers, B., Avdic, S., Gottlieb, D., Abendroth, A., Blyth, E., & Slobedman, B. (2019). Human cytomegalovirus latency and reactivation in allogeneic hematopoietic stem cell transplant recipients. Front Microbiol, 10, 1186. https://doi.org/10.3389/fmicb.2019.01186

13. Barabas, S., Spindler, T., Kiener, R., Tonar, C., Lugner, T., Batzilla, J., … Deml, L. (2017). An optimized IFN-γ ELISpot assay for the sensitive and standardized monitoring of CMV protein-reactive effector cells of cell-mediated immunity. BMC Immunol, 18, 14. https://doi.org/10.1186/s12865-017-0195-y
14. Clari, M. Á., Muñoz-Cobo, B., Solano, C., Benet, I., Costa, E., Remigia, M. J., … Navarro, D. (2012). Performance of the QuantiFERON-cytomegalovirus (CMV) assay for detection and estimation of the magnitude and functionality of the CMV-specific gamma interferon-producing CD8(+) T-cell response in allogeneic stem cell transplant recipients. Clin Vaccine Immunol, 19, 791–796. https://doi.org/10.1128/CVI.05633-11
15. Lilleri, D., Gerna, G., Zelini, P., Chiesa, A., Rognoni, V., Mastronuzzi, A., … Locatelli, F. (2012). Monitoring of human cytomegalovirus and virus-specific T-cell response in young patients receiving allogeneic hematopoietic stem cell transplantation. PLoS One, 7, e41648. https://doi.org/10.1371/journal.pone.0041648
16. Soares, A., Govender, L., Hughes, J., Mavakla, W., de Kock, M., Barnard, C., et al. (2010). Novel application of Ki67 to quantify antigen-specific in vitro lymphoproliferation. J Immunol Methods, 362, 43–50. https://doi.org/10.1016/j.jim.2010.08.007
17. Ghanim, B., Klikovits, T., Hoda, M. A., Lang, G., Szirtes, I., Setinek, U., et al. (2015). Ki67 index is an independent prognostic factor in epithelioid but not in non-epithelioid malignant pleural mesothelioma: A multicenter study. Br J Cancer, 112, 783–792. https://doi.org/10.1038/bjc.2015.9
18. Bitar, M., Boldt, A., Freitag, M.-T., Gruhn, B., Köhl, U., & Sack, U. (2019). Evaluating STAT5 phosphorylation as a mean to assess T cell proliferation. Front Immunol, 10, 722. https://doi.org/10.3389/fimmu.2019.00722
19. Schober, T., Magg, T., Laschinger, M., Rohlfs, M., Linhares, N. D., Puchalka, J., … Hauck, F. (2017). A human immunodeficiency syndrome caused by mutations in CARMIL2. Nat Commun, 8, 14209. https://doi.org/10.1038/ncomms14209
20. Kuo, Y. C., Yang, N. S., Chou, C. J., Lin, L. C., & Tsai, W. J. (2000). Regulation of cell proliferation, gene expression, production of cytokines, and cell cycle progression in primary human T lymphocytes by piperlactam S isolated from Piper kadsura. Mol Pharmacol, 58, 1057–1066. https://doi.org/10.1124/mol.58.5.1057
21. Tsai, W.-J., Yang, S.-C., Huang, Y.-L., Chen, C.-C., Chuang, K.-A., & Kuo, Y.-C. (2013). 4-Hydroxy-17-methylincisterol from Agaricus blazei decreased cytokine production and cell proliferation in human peripheral blood mononuclear cells via inhibition of NF-AT and NF-κB activation. Evid Based Complement Alternat Med, 2013, 435916–435914. https://doi.org/10.1155/2013/435916
22. McLaughlin-Taylor, E., Pande, H., Forman, S. J., Tanamachi, B., Li, C. R., Zaia, J. A., … Riddell, S. R. (1994). Identification of the major late human cytomegalovirus matrix protein pp65 as a target antigen for CD8+ virus-specific cytotoxic T lymphocytes. J Med Virol, 43, 103–110. https://doi.org/10.1002/jmv.1890430119
23. Diamond, D. J., York, J., Sun, J. Y., Wright, C. L., & Forman, S. J. (1997). Development of a candidate HLA A*0201 restricted peptide-based vaccine against human cytomegalovirus infection. Blood, 90, 1751–1767.
24. Bitar, M., Boldt, A., Binder, S., Borte, M., Kentouche, K., Borte, S., & Sack, U. (2017). Flow cytometric measurement of STAT1 and STAT3 phosphorylation in CD4+ and CD8+ T cells-clinical applications in primary immunodeficiency diagnostics. J Allergy Clin Immunol, 140, 1439–1441.e9. https://doi.org/10.1016/j.jaci.2017.05.017
25. Boldt, A., Borte, S., Fricke, S., Kentouche, K., Emmrich, F., Borte, M., … Sack, U. (2014). Eight-color immunophenotyping of T-, B-, and NK-cell subpopulations for characterization of chronic immunodeficiencies. Cytometry B Clin Cytom, 86, 191–206. https://doi.org/10.1002/cyto.b.21162
26. Roederer, M. (2011). Interpretation of cellular proliferation data: avoid the panglossian. Cytometry A, 79, 95–101. https://doi.org/10.1002/cyto.a.21010
27. Sack, U., Barnett, D., Demirel, G. Y., Fossat, C., Fricke, S., Kafassi, N., … Lambert, C. (2013). Accreditation of flow cytometry in Europe. Cytometry B Clin Cytom, 84, 135–142. https://doi.org/10.1002/cyto.b.21079
28. Caruso, A., Licenziati, S., Corulli, M., Canaris, A. D., de Francesco, M. A., Fiorentini, S., … Turano, A. (1997). Flow cytometric analysis of activation markers on stimulated T cells and their correlation with cell proliferation. Cytometry, 27, 71–76 10.1002/(sici)1097-0320(19970101)27:1<71:aid-cyto9>3.0.co;2-o.
29. Cretel, E., Touchard, D., Bongrand, P., & Pierres, A. (2011). A new method for rapid detection of T lymphocyte decision to proliferate after encountering activating surfaces. J Immunol Methods, 364, 33–39. https://doi.org/10.1016/j.jim.2010.10.007
30. Walker, S., Fazou, C., Crough, T., Holdsworth, R., Kiely, P., Veale, M., et al. (2007). Ex vivo monitoring of human cytomegalovirus-specific CD8+ T-cell responses using QuantiFERON-CMV. Transpl Infect Dis, 9, 165–170. https://doi.org/10.1111/j.1399-3062.2006.00199.x
31. Sester, U., Gärtner, B. C., Wilkens, H., Schwaab, B., Wössner, R., Kindermann, I., et al. (2005). Differences in CMV-specific T-cell levels and long-term susceptibility to CMV infection after kidney, heart and lung transplantation. Am J Transplant, 5, 1483–1489. https://doi.org/10.1111/j.1600-6143.2005.00871.x
32. Egli, A., Humar, A., & Kumar, D. (2012). State-of-the-art monitoring of cytomegalovirus-specific cell-mediated immunity after organ transplant: A primer for the clinician. Clin Infect Dis, 55, 1678–1689. https://doi.org/10.1093/cid/cis818
33. Egli, A., Binet, I., Binggeli, S., Jäger, C., Dumoulin, A., Schaub, S., … Hirsch, H. H. (2008). Cytomegalovirus-specific T-cell responses and viral replication in kidney transplant recipients. J Transl Med, 6, 29. https://doi.org/10.1186/1479-5876-6-29
34. Goldeck, D., Low, I., Shadan, N. B., Mustafah, S., Pawelec, G., & Larbi, A. (2013). Multi-parametric phospho-flow cytometry: a crucial tool for T lymphocyte signaling studies. Cytometry A, 83, 265–272. https://doi.org/10.1002/cyto.a.22252
35. Ameres, S., Mautner, J., Schlott, F., Neuenhahn, M., Busch, D. H., Plachter, B., & Moosmann, A. (2013). Presentation of an immunodominant immediate-early CD8+ T cell epitope resists human cytomegalovirus immunoevasion. PLoS Pathogens, 9, e1003383. https://doi.org/10.1371/journal.ppat.1003383

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Bitar M, Boettcher M, Boldt A, et al. Flow cytometric measurement of STAT5 phosphorylation in cytomegalovirus-stimulated T cells. *Cytometry*. 2021;1–10. https://doi.org/10.1002/cyto.a.24286