T-cell immune surveillance in allogenic stem cell transplant recipients: Are whole blood-based assays ready to challenge ELISPOT?

Chris D. Lauruschkat¹,‡, Lukas Page¹,‡, Sonja Etter¹, Philipp Weis³, Florian Gamon¹, Sabrina Kraus¹, Hermann Einsele¹, Sebastian Wurster²,#,‡, Juergen Loeffler¹,‡

¹) Department of Internal Medicine II, University Hospital Wuerzburg, Wuerzburg, Germany

²) Department of Infectious Diseases, Infection Control and Employee Health, The University of Texas MD Anderson Cancer Center, Houston, Texas, United States of America

‡ Contributed equally to this work.

© The Author(s) 2020. Published by Oxford University Press on behalf of Infectious Diseases Society of America.
This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs licence (http://creativecommons.org/licenses/by-nc-nd/4.0/), which permits non-commercial reproduction and distribution of the work, in any medium, provided the original work is not altered or transformed in any way, and that the work is properly cited. For commercial re-use, please contact journals.permissions@oup.com
* Corresponding author: Sebastian Wurster, MD

Department of Infectious Diseases, Infection Control and Employee Health

The University of Texas MD Anderson Cancer Center

6565 MD Anderson Boulevard, Z8.3002

Houston, TX 77030, United States of America

Email stwurster@mdanderson.org

Phone: +1-713-563-1753
Abstract

We compared the feasibility of four CMV- and Aspergillus-reactive T-cell immunoassay protocols in allogenic stem cell transplant recipients. While ELISPOT performed best overall, logistically advantageous whole blood-based assays performed comparably in patients with less severe lymphocytopenia. CMV-induced interferon-gamma responses correlated strongly across all protocols and showed high concordance with serology.

Keywords

Immunoassay, biomarker, aspergillosis, cytomegalovirus, T cells, cytokines, flow cytometry, ELISPOT
Introduction

Opportunistic infections including cytomegalovirus (CMV) disease and invasive aspergillosis cause significant morbidity and mortality in allogenic hematopoietic stem cell transplant (allo-HSCT) recipients [1,2]. A plethora of commercial and investigational T-cell immunoassays have been proposed to support the diagnosis of opportunistic infections in these highly vulnerable patients, with flow cytometry, enzyme-linked immunospot (ELISPOT), and enzyme-linked immunosorbent assays (ELISA) being the most common modalities [3-6]. Furthermore, several studies showed a prognostic value of T-cellular biomarkers, especially antigen-reactive interferon-gamma (IFN-γ) responses, which may facilitate individualized approaches for infection surveillance and prophylactic pharmacotherapy [5,6].

As systematic comparisons of immunoassay modalities are scarce, we sought to comparatively evaluate the feasibility of four CMV- and Aspergillus fumigatus-specific T-cell assay protocols (Suppl. Material 1) in allo-HSCT recipients. This cohort is notoriously challenging for immunoassays due to quantitative and qualitative aberrations of the leukocyte repertoire and immunosuppressive pharmacotherapy [7-10]. Specific end points of our study were the technical quality of the results, the concordance of pp65-specific IFN-γ responses with CMV serostatus and infection, and the correlation of results across the studied protocols.
Patients & Methods

Patient characteristics

Heparinized venous blood (30 ml) was obtained at three different time points after allo-HSCT (Suppl. Material 1). Thirteen patients (age: 35-69 years, 8 male, 5 female) were enrolled and 35 samples were available overall. For two patients, only the first sample could be collected before follow-up was lost. Detailed patient characteristics are provided in Suppl. Material 2.

Immunoassays

Five hundred-microliter aliquots of heparinized blood were injected into ready-to-use stimulation tubes for whole blood (WB)-based flow cytometry and ELISA [8,11,12]. The remainder of the blood was used to isolate peripheral blood mononuclear cells (PBMCs) for flow cytometry and ELISPOT (Suppl. Material 1). Stimulation with an A. fumigatus mycelial lysate [13] or CMV pp65 (Lophius Biosciences) was performed according to previously optimized protocols [8,11,12,14]. All assays used dual α-CD28/α-CD49d co-stimulation except PBMC-based flow cytometry which only used α-CD28, as described before [11,14]. Unspecific background controls (“nil”) contained co-stimulatory factors but no antigens. Phytohemagglutinin (Sigma-Aldrich) served as a positive control. Flow cytometric samples were stained with α-CD4-FITC, α-IFN-γ-PE, and α-CD154-APC (Miltenyi Biotec), measured on a FACS Calibur flow cytometer (BD), and analyzed with FlowJo software. ELISPOT was performed using the T-Track CMV platform (Lophius Biosciences) and numbers of spot-forming cells (SFCs) were quantified with a Bioreader 5000a (BioSYS). For WB-ELISA, IFN-γ concentrations in plasma supernatants were quantified using ELISA-Max Deluxe Sets (Biolegend) and a NanoQuant Infinite 200M Pro microplate reader (Tecan). Detailed descriptions of the assays are provided in Suppl. Materials 3-5. Representative data for flow cytometry and ELISPOT are shown in Suppl. Materials 6 and 7. Technical acceptance criteria are summarized in Suppl. Material 8A.
Statistics

All immunoassay results presented in this manuscript are adjusted for unspecific background by subtraction of CD154 and/or IFN-γ response in “nil” controls. The binary classification efficacy depending on CMV serostatus and/or infection was determined with receiver-operating characteristics analysis. Correlation of results across different protocols was assessed by Spearman’s rank correlation. Applicable significance tests are specified in the figure legends. Statistical analyses and data visualization were performed using GraphPad Prism v8 and Microsoft Excel.

Results and Discussion

Considering all time points and antigens, CD154-based flow cytometry had 41% and 53% technical success rates for PBMCs and WB, respectively, with insufficient acquired CD4-cell numbers and elevated unspecific background being the predominant causes of non-evaluable measurements (Figure 1A). These results are consistent with an earlier report for Aspergillus-reactive T-cell quantification in high-risk patients [7]. Unlike in healthy donors [11], the WB-based assay produced more measurements with mildly increased background in allo-HSCT recipients. Adding IFN-γ as a second activation marker, technical performance improved to 63% (PBMCs) and 76% (WB), respectively (Figure 1A), largely due to mitigation of unspecific reactivity. The higher success rate of the WB assay is likely attributable to dual co-stimulation, which we recently recommended to attenuate the impact of immunosuppressive agents on CD154⁺ antigen-reactive T-cell quantification [8]. As these results were not available prior to the present study, dual co-stimulation was not used for PBMC-based flow cytometry; thus, our data may underestimate the actually achievable performance.

In line with prior reports [6,15], ELISPOT produced few non-evaluable results (7%, Figure 1A), mainly due to elevated unspecific background. WB-ELISA performed comparably to CD154⁺IFN-γ⁺ WB flow cytometry with 74% successful measurements. The feasibility of both WB-based assays improved
with increasing time after HSCT (Figure 1B, Suppl. Material 9) and increasing lymphocyte counts (Figure 1B). Of note, CD154⁺IFN-γ⁺ WB flow cytometry and WB-ELISA were non-inferior to ELISPOT in patients with >400 and >800 lymphocytes/µl, respectively (Figure 1B). The higher number of WB-ELISA than ELISPOT measurements with insufficient responses to the positive control in strongly lymphopenic patients is consistent with prior reports for commercial IFN-γ release assays [16,17].

Unlike our study, some commercial platforms for CMV immunoassays [18,19] do not apply specific cutoffs to the unstimulated control and/or use positive controls only to validate measurements that are non-reactive to the antigen of interest (Suppl. Material 8B). Applying stricter requirements, our data therefore provide a rather conservative estimation of the success rate of the investigational protocols, especially for WB-ELISA.

None of the enrolled patients developed possible or probable invasive mold infections during the study period (Suppl. Material 2). Consistently, the patients’ distributions of CD154⁺A. fumigatus-reactive T-cell frequencies and IFN-γ responses (Suppl. Material 10) were similar or slightly lower than those observed in healthy subjects [11,20,21].

Concordance of test results with serology is commonly used to validate CMV-specific T-cell assays [22,23]. In our study, all four IFN-γ assays showed excellent concordance with serology (Figure 2A). Only one false-positive measurement was observed for PBMC-based flow cytometry. Plausible CMV-induced IFN-γ kinetics, especially for ELISPOT and WB-ELISA, were seen in a patient with an asymptomatic primary CMV infection prior to the first T-cell measurement (Figure 2B). Importantly, poor IFN-γ response to CMV antigens in seropositive patients (with reactive positive controls) does not indicate technical failure, but is considered a prognostic indicator of an increased risk of CMV reactivation or disease in allo-HSCT recipients [6,15]. While data are limited, IFN-γ responses of two patients who experienced PCR-documented CMV reactivation clustered at the bottom of the range for seropositive patients in both WB-based assays but not ELISPOT (Figure 2A&C).
High correlation of IFN-γ response to CMV ($\rho = 0.74$-$0.88$, $p < 0.001$, Suppl. Material 11) and good concordance of test outcomes (80%-96%, Suppl. Material 12) were found across all assays. Of note, our retrospectively determined cutoffs for antigen reactivity closely resembled those of representative commercial protocols for both ELISPOT [19,24] and WB-ELISA [18] (Suppl. Material 8).

In summary, while ELISPOT had the highest success rate overall, our investigational WB-based IFN-γ assays performed comparably in patients with less severe lymphopenia. Additional considerations could tip the balance towards WB-based modalities. On one hand, as extensively discussed elsewhere [11], the logistical advantages, cost-effectiveness, and easier translatability to the bedside may favor WB-based protocols. Moreover, this study was performed in an investigational setting with short pre-analytic delays. Therefore, the relative performance in the clinical routine may shift towards the more robust WB-based protocols [11], especially when encountering long sample transportation times or when testing cytokines that require prolonged stimulation (e.g., IL-17) [25]. Although least robust, flow cytometry retains the advantage of an essentially infinite spectrum of assayable activation markers beyond cytokine induction. Similarly, the output of WB-ELISA can be maximized by using multiplex cytokine panels, whereas options for multiplexing in ELISPOT assays are limited. Consequently, larger PBMC quantities are needed for more comprehensive ELISPOT analyses to stimulate multiple plates containing different (combinations of) detection antibodies.

Several limitations of this small-scale study need to be considered. Although considered inferior to CD4-cell activation markers to predict clinically significant CMV events [26,27], inclusion of CD8-specific flow cytometry parameters (e.g., CD107a) may have been interesting. While not feasible due to limited blood volumes, inclusion of additional antigens (e.g., IE-1) would have allowed for a more comprehensive analysis of the anti-CMV T-cell response. Furthermore, we did not include an early measurement during the first six weeks after allo-HSCT, since considerable blood volumes would have been required to perform all four immunoassays simultaneously during a period of early T-cell
recovery. Lastly, our findings may not be transferrable to other centers using different regimens for GvHD prophylaxis and to other cohorts with hematological malignancies. Despite these limitations, our study provides important insights into the comparative technical performance of different platforms for (investigational) T-cell immunoassays in allo-HSCT recipients. The relatively high technical success rate, good concordance of CMV pp65-specific IFN-γ response and serostatus, and excellent correlation of test results across all protocols are encouraging for the continued investigation, and eventually, clinical translation of T-cellular immune surveillance of opportunistic infections in allo-HSCT recipients. Further in-depth comparison of ELISPOT and WB-based assays in larger studies evaluating additional antigens and cytokines could open new avenues for individualized immune monitoring approaches. In particular, our results support the selection of optimal immunoassay modalities based on blood count parameters, e.g. for future large-scale studies to evaluate protective T-cell responses as part of CMV surveillance algorithms [10].
Patient Consent Statement

The study was approved by the Ethics Committees of the University of Wuerzburg (protocol 178/16). Written informed consent was obtained from all patients.

Funding

This study was funded by the German Research Foundation (DFG) through the TR 124 FungiNet, “Pathogenic fungi and their human host: Networks of Interaction” (DFG project number 210879364, Project A2 to HE and JL) and by the Bavarian Ministry of Economics, Media, Energy and Technology (grant number BayBIO-1606-003, “T-cell based diagnostic monitoring of invasive aspergillosis in haematological patients” to JL).

Author Contribution Statement

CDL, PW, SW, and JL conceived the study and planned the experiments. CDL, LP, and SE performed the experiments. CDL and SW analyzed and visualized the data. FG and SK were responsible for patient acquisition and compilation of clinical data. CDL and SW wrote the paper. HE, SW, and JL supervised the study. HE and JL acquired funding. All authors provided revisions and have approved the final version of the manuscript.

Disclosure of potential conflicts of interests
The authors have no conflicts of interest related to this study. Lophius Biosciences kindly provided the ELISPOT plates for this study.

Acknowledgment

We would like to thank Lubov Darst for her effort and commitment in the acquisition of patient samples.
Figure legends

Figure 1: Comparative technical performance of T-cell immunoassays in allo-HSCT recipients.

(A) Summary of technical performance and quality control infringements (as defined in Suppl. Material 8A) for each assay considering all sampling time points and both stimuli (A. fumigatus mycelial lysate and CMV pp65).  (B) Technical success rates of each protocol depending on the sampling period and the patient’s lymphocyte count.

Figure 2: Concordance of CMV-induced IFN-γ responses with CMV serology and infection.

(A) CMV-induced IFN-γ response detectable by flow cytometry (CD154+IFN-γ+/CD4+ cells), ELISPOT (IFN-γ spot forming cells per million PBMCs), and WB-ELISA (IFN-γ pg/ml) depending on the patient’s CMV status (colored diamonds). Numbers in large green diamonds indicate the number of non-reactive measurements (no antigen-reactive response exceeding the “nil” control or < 0.001% specific T-cells detectable by flow cytometry). White horizontal bars and colored boxes represent medians and inter-quartile ranges, respectively. Dashed lines indicate the retrospectively determined cutoffs for test positivity. P-values above the panels were determined using the two-sided Mann-Whitney U-test. ROC AUC = area-under-the-curve of receiver operating characteristics analysis.  (B) Kinetics of CMV-induced IFN-γ responses in a CMV-seronegative patient (#7 in Suppl. Material 2) who had a positive CMV PCR blood test (red triangle) on day +56 (week 8) and subsequently received valganciclovir therapy. All other CMV PCR blood tests during the study period were negative (green triangles). The second WB-based flow cytometry test was non-evaluable due to elevated unspecific background (“B”). Results of all other assays peaked at the second measurement (43 days after the positive CMV PCR test).  (C) Kinetics of CMV-induced IFN-γ responses in two CMV-seropositive patients (#3 and #5 in Suppl. Material 2) who had asymptomatic
PCR-confirmed CMV reactivation. Golden and red triangles indicate positive CMV blood PCR results below the quantifiable threshold (< 300 copies/ml) and quantifiable results, respectively. The red hash sign (#) highlights negative IFN-γ assay results. “P” = T-cell assay not evaluable due to insufficient response to the positive control. “BC” = not evaluable due to low CD4-cell numbers acquired and elevated unspecific background.
References

1. Cho, S.Y.; Lee, D.G.; Kim, H.J. Cytomegalovirus Infections after Hematopoietic Stem Cell Transplantation: Current Status and Future Immunotherapy. *Int J Mol Sci* 2019, 20, doi:10.3390/ijms20112666.

2. Ullmann, A.J.; Aguado, J.M.; Arikan-Akdagli, S.; et al. Diagnosis and management of Aspergillus diseases: executive summary of the 2017 ESCMID-ECMM-ERS guideline. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases* 2018, 24 Suppl 1, e1-e38, doi:10.1016/j.cmi.2018.01.002.

3. Bacher, P.; Steinbach, A.; Kniemeyer O; et al. Fungus-Specific CD4+ T Cells for Rapid Identification of Invasive Pulmonary Mold Infection. *Am J Respir Crit Care Med* 2015, 191(3):348-52.

4. Potenza, L.; Vallerini, D.; Barozzi, P.; et al. Characterization of specific immune responses to different Aspergillus antigens during the course of invasive Aspergillosis in hematologic patients. *PLoS One* 2013, 8, e74326, doi:10.1371/journal.pone.0074326.

5. Yong, M.K.; Cameron, P.U.; Slavin, M.; et al. Identifying Cytomegalovirus Complications Using the Quantiferon-CMV Assay After Allogeneic Hematopoietic Stem Cell Transplantation. *J Infect Dis* 2017, 215, 1684-1694, doi:10.1093/infdis/jix192.

6. Chemaly, R.F.; El Haddad, L.; Winston, D.J.; et al. Cytomegalovirus (CMV) Cell-Mediated Immunity and CMV Infection After Allogeneic Hematopoietic Cell Transplantation: The REACT Study. *Clin Infect Dis* 2020, 10.1093/cid/ciz1210, doi:10.1093/cid/ciz1210.

7. Steinbach, A.; Cornely, O.A.; Wisplinghoff, H.; et al. Mould-reactive T cells for the diagnosis of invasive mould infection-A prospective study. *Mycoses* 2019, 62, 562-569, doi:10.1111/myc.12919.

8. Page, L.; Lauruschkat, C.D.; Helm, J.; et al. Impact of immunosuppressive and antifungal drugs on PBMC- and whole blood-based flow cytometric CD154(+)Aspergillus fumigatus specific T-cell quantification. *Medical microbiology and immunology* 2020, 10.1007/s00430-020-00665-3, doi:10.1007/s00430-020-00665-3.

9. Potenza, L.; Vallerini, D.; Barozzi, P.; et al. Mucorales-Specific T Cells in Patients with Hematologic Malignancies. *PLoS One* 2016, 11, e0149108, doi:10.1371/journal.pone.0149108.

10. Nesher, L.; Shah, D.P.; Ariza-Heredia, E.J.; et al. Utility of the Enzyme-Linked Immunospot Interferon-gamma-Release Assay to Predict the Risk of Cytomegalovirus Infection in Hematopoietic Cell Transplant Recipients. *J Infect Dis* 2016, 213, 1701-1707, doi:10.1093/infdis/jiw064.
11. Weis, P.; Helm, J.; Page, L.; et al. Development and evaluation of a whole blood-based approach for flow cytometric quantification of CD154+ mould-reactive T cells. *Med Mycol* **2020**, *58*, 187-196, doi:10.1093/mmy/myz038.

12. Lauruschkat, C.D.; Page, L.; Etter, S.; et al. Establishment of a whole blood ELISA to quantify T-cellular cytokine release in response to Aspergillus fumigatus antigens. In Proceedings of Advances Against Aspergillosis and Mucormycosis, Lugano, Switzerland.

13. Bacher, P.; Hohnstein, T.; Beerbaum, E.; et al. Human Anti-fungal Th17 Immunity and Pathology Rely on Cross-Reactivity against Candida albicans. *Cell* **2019**, *176*, 1340-1355.e1315, doi:10.1016/j.cell.2019.01.041.

14. Lauruschkat, C.D.; Wurster, S.; Page, L.; et al. Susceptibility of A. fumigatus-specific T-cell assays to pre-analytic blood storage and PBMC cryopreservation greatly depends on readout platform and analytes. *Mycoses* **2018**, *61*, 549-560, doi:10.1111/myc.12780.

15. Wagner-Drouet, E.; Teschner, D.; Wolschke, C.; et al. Standardized monitoring of cytomegalovirus-specific immunity can improve risk stratification of recurrent cytomegalovirus reactivation after hematopoietic stem cell transplantation. *Haematologica* **2019**, 10.3324/haematol.2019.229252, doi:10.3324/haematol.2019.229252.

16. Giulieri, S.; Manuel, O. QuantiFERON®-CMV assay for the assessment of cytomegalovirus cell-mediated immunity. *Expert review of molecular diagnostics* **2011**, *11*, 17-25, doi:10.1586/erm.10.109.

17. Kumar, D.; Chernenko, S.; Moussa, G.; et al. Cell-mediated immunity to predict cytomegalovirus disease in high-risk solid organ transplant recipients. *Am J Transplant* **2009**, *9*, 1214-1222, doi:10.1111/j.1600-6143.2009.02618.x.

18. QuantiFeron. QuantiFeron-CMV Package Insert [https://www.quantiferon.com/wp-content/uploads/2018/10/L1075110-R05-QF-CMV-ELISA-IFU-CE.pdf](https://www.quantiferon.com/wp-content/uploads/2018/10/L1075110-R05-QF-CMV-ELISA-IFU-CE.pdf) (visited on 08.26.2020, 5.20 pm)

19. OxfordImmunotec. T-Spot.CMV Package Insert [http://www.oxfordimmunotec.com/international/wp-content/uploads/sites/3/PI-CMV-IVD-UK-V2.pdf](http://www.oxfordimmunotec.com/international/wp-content/uploads/sites/3/PI-CMV-IVD-UK-V2.pdf) (visited on 08.26.2020, 5.22 pm).

20. Wurster, S.; Weis, P.; Page, L.; et al. Intra- and inter-individual variability of Aspergillus fumigatus reactive T-cell frequencies in healthy volunteers in dependency of mould exposure in residential and working environment. *Mycoses* **2017**, *60*, 668-675, doi:10.1111/myc.12643.

21. Page, L.; Weis, P.; Müller, T.; et al. Evaluation of Aspergillus and Mucorales specific T-cells and peripheral blood mononuclear cell cytokine signatures as biomarkers of environmental mold exposure. *International journal of medical microbiology : IJMM* **2018**, *308*, 1018-1026, doi:10.1016/j.ijmm.2018.09.002.
22. Abate, D.; Saldan, A.; Mengoli, C.; et al. Comparison of cytomegalovirus (CMV) enzyme-linked immunosorbent spot and CMV quantiferon gamma interferon-releasing assays in assessing risk of CMV infection in kidney transplant recipients. *J Clin Microbiol* 2013, 51, 2501-2507, doi:10.1128/jcm.00563-13.

23. Banas, B.; Böger, C.A.; Lückhoff, G.; et al. Validation of T-Track® CMV to assess the functionality of cytomegalovirus-reactive cell-mediated immunity in hemodialysis patients. *BMC Immunology* 2017, 18, 15, doi:10.1186/s12865-017-0194-z.

24. LophiusBiosciences. T-Track CMV Instructions for Use. [https://www.lophius.com/fileadmin/user_upload/Downloads/IFUs/IFU_UREA_CMV_LI-P90003-EN-10-20191213.pdf](https://www.lophius.com/fileadmin/user_upload/Downloads/IFUs/IFU_UREA_CMV_LI-P90003-EN-10-20191213.pdf) (visited on 08.26.2020, 5.38 pm).

25. Duechting, A.; Przybyla, A.; Kuerten, S.; Lehmann, P.V. Delayed Activation Kinetics of Th2- and Th17 Cells Compared to Th1 Cells. *Cells* 2017, 6, doi:10.3390/cells6030029.

26. Rogers, R.; Saharia, K.; Chandorkar, A.; et al. Clinical experience with a novel assay measuring cytomegalovirus (CMV)-specific CD4+ and CD8+ T-cell immunity by flow cytometry and intracellular cytokine staining to predict clinically significant CMV events. *BMC Infect Dis* 2020, 20, 58, doi:10.1186/s12879-020-4787-4.

27. Sester, M.; Sester, U.; Gärtner, B.; et al. Levels of virus-specific CD4 T cells correlate with cytomegalovirus control and predict virus-induced disease after renal transplantation. *Transplantation* 2001, 71, 1287-1294, doi:10.1097/00007890-200105150-00018.
Figure 1

A

Evaluation of technical performance

- 100%
- 90%
- 80%
- 70%
- 60%
- 50%
- 40%
- 30%
- 20%
- 10%
- 0%

Flow cytometry PBMC CD154
Flow cytometry WB CD154
Flow cytometry PBMC CD154 IFN-γ
Flow cytometry WB CD154 IFN-γ
ELISPOT IFN-γ
WB-ELISA IFN-γ

Evaluatable measurements
- No quality control concerns
- Mildly elevated unspecific background

Non-evaluatable measurements
- Insufficient cell number (C)
- Strongly elevated unspecific background (B)
- Insufficient response to positive control (P)
- QC infringements (B) and (C)
- QC infringements (B) and (P)

B

| Week post-transplant (Visit) | Lymphocyte count / μl |
|-----------------------------|-----------------------|
| 7-11 (V1)                  | ≤ 400                 |
| 11-15 (V2)                 | 401-800               |
| 18-25 (V3)                 | > 800                 |

Flow cytometry PBMC CD154:
- 39% ≤ 400
- 45% 401-800
- 41% > 800

Flow cytometry WB CD154:
- 50% ≤ 400
- 64% 401-800
- 67% > 800

Flow cytometry PBMC CD154 IFN-γ:
- 58% ≤ 400
- 77% 401-800
- 55% > 800

Flow cytometry WB CD154 IFN-γ:
- 73% ≤ 400
- 73% 401-800
- 82% > 800

ELISPOT IFN-γ:
- 96% ≤ 400
- 100% 401-800
- 82% > 800

WB-ELISA IFN-γ:
- 54% ≤ 400
- 91% 401-800
- 82% > 800
Figure 2

A

MW Test

| Test | Flow cyt. PBMC | Flow cyt. WB |
|------|----------------|--------------|
| ROC AUC | 0.97 | 1.00 |
| P-value of AUC | < 0.001 *** | < 0.001 *** |

- p < 0.001 ***

Donor & recipient CMV seronegative
Donor & recipient CMV seropositive, no reactivation
Donor & recipient CMV seropositive, reactivation
Patient with primary CMV infection

B

Week after allo-HSCT

| Week | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 |
|------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| CMV PCR | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ |
| Flow cytometry PBMC CD154 IFN-γ | 0.147% | 0.296% | 0.241% |
| Flow cytometry WB CD154 IFN-γ | 0.247% | 0.131% |
| ELISPOT IFN-γ (SFCs / ml. PBMCs) | 235.0 | 1678.8 | 1346.3 |
| WB-ELISA IFN-γ (pg/ml) | 329.1 | 1691.0 | 1307.2 |

C

Week after allo-HSCT

| Week | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 |
|------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| CMV PCR | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ |
| Flow cytometry PBMC CD154 IFN-γ | 0.188% | 0.229% | 0.095% |
| Flow cytometry WB CD154 IFN-γ | 8C | 0.248% | 0.122% |
| ELISPOT IFN-γ (SFCs / ml. PBMCs) | 3.8 # | 65.4 | 678.8 |
| WB-ELISA IFN-γ (pg/ml) | 2.6 # | 4.4 # | 30.2 |

- p < 0.001 ***