Characterization of CMV-specific CD4+ T-cell reconstitution following stem cell transplantation through the use of HLA Class II-peptide tetramers identifies patients at high risk of recurrent CMV reactivation

CMV reactivation remains a major complication following T-cell depleted (TCD) CMV seropositive allogeneic stem cell transplantation (HSCT), with nearly half of these patients developing recurrent episodes of viremia.[1] Although PCR monitoring for viremia can detect early reactivation, there is currently no established biomarker for predicting CMV recurrence.[2] Previous multiple regression analysis has identified low level CMV-specific CD8+ T-cells (CMV-CD8) as an independent predictor of CMV reactivation,[3] but the role of CMV-specific CD4+ T-cells (CMV-CD4) remains unclear due to the relative lack of direct assays.

In this study, we used HLA class I and novel class II tetramers to 1) analyse the kinetics and phenotype of CMV-CD4 reconstitution in relation to CMV-CD8,[4-7] and 2) to assess the clinical utility of monitoring using both tetramers[8] and a novel putative HLA-independent marker CD57 in 20 CMV-at-risk HLA-DRB1*0701 patients. CMV-CD4 and CMV-CD8 were detectable at low levels early after transplant, but expanded in parallel in response to viremia. Patients who failed to generate threshold levels of ≥0.7x10³/mL and ≥20x10³/mL CMV-CD4 and CMV-CD8 respectively, and ≥11x10³/mL CD4+CD57+ T-cells following the first episode of viremia, were found to be at high risk of recurrent viremia.

HLA-DRB1*0701 tetramers were custom made to incorporate a CMV-glycoprotein B-derived peptide DYS-

Table 1. Summary of CMV reactivation and magnitude of CMV specific T-cell responses in HSCT patients.

UPN	Conditioning/ donor	T-cell depletion	(CMV status) (D/R)	CMV episodes	Duration of viremia (days post-HSCT)	Maximum CMV-CD4* (% CD4)	Maximum CMV-CD8* (% CD8)	Log expansion of CMV-CD4	Log expansion of CMV-CD8**
01	RIC-Sib	C	+/+	1	63-78	50(15)	370(9.7)	3.07	1.36
02	RIC-MUD	C	+/+	2	21-28 100-120	1.5(5.2)	7(10)	1.2	2.40
03	RIC-Sib	C	+/+	1	28-70	17(7.5)	448(25)	2.03	3.04
04	MA-Sib	N	+/+	0	0	28.7(3.5)	ND	0.17	ND
05	MA-MUD	C	+/+	2	28-238 308-322	ND	ND	ND	ND
06	RIC-MUD	C	-/+	2	27-54 97-174	ND	1.9(0.5)	ND	3.5
7	RIC-Sib	C	+/+	1	24-50	15.5(10)	82(9.4)	1.49	1.79
08	RIC-MUD	C	+/+	2	34-41 126-147	1.2(1.4)	76(8)	3.07	1.94
09	RIC-MUD	C	+/+	1	35-56	6.4(4)	146(12)	1.34	1.61
10	RIC-Sib	C	+/+	1	41-62	0.5(0.23)	54(6.1)	0.17	0.77
11	RIC-MUD	C	+/+	3	63-91 144-164 203-224	ND	297(36)	ND	3.14
12	RIC-MUD	C	+/+	4	25-46 83-114 127-141 195-226	0.46 (0.35)	390(17)	1.60	2.57
13	MA-Sib	N	-/+	2	17-39 77-84	2.9(4.1)	15(2)	1.89	1.04
14	RIC-MUD	C	+/+	1	26-47	ND	25(1.2)	ND	2.77
15	RIC-MUD	C	+/+	1	47-68	ND	37(13)	ND	ND
16	RIC-MUD	ATG	-/+	3	43-78 100-120 280-295	30(25)	ND	3	ND
17	RIC-Sib	C	+/+	1	22-50	ND	8.7(1.3)	ND	0.53
18	RIC-MUD	C	-/+	1	22-43	ND	ND	ND	ND
19	RIC-MUD	C	+/+	1	28-56	ND	20(5)	ND	4.75
20	*RIC-MUD*	*ATG*	+/+	*2*	*27-62* *99-118*	*ND*	*ND*	*ND*	*ND*

*RIC-Sib: reduced intensity conditioning; Sib: sibling; MA: myeloablative conditioning; MUD: matched unrelated donor transplant; T-cell depletion N: none; C: alemtuzumab; ATG: anti-thymocyte globulins. *Figures are cell numbers x10³/mL peripheral blood, figures in the brackets are percent of specific cells in whole CD4+ or CD8+ T-cells. **There was no statistically significant difference in expansion from baseline between CMV-specific CD4+ and CD8+ T-cells (median: 1.7 log and 2.2log, respectively. P=0.54). ND: not detected.*

NTHSTRYV (DYS),[9] that induces strong CMV-CD4 responses (*Online Supplementary Figure S1*). Together with HLA class I multimers, CMV-specific T-cells were monitored up to 36 weeks post-transplant (Figure 1). 18 patients received TCD reduced-intensity conditioning with either Alemtuzumab (n=16) or ATG (n=2) (Table 1) (*Online Supplementary Table S1* and *Online Supplementary Methods*) and 2 patients (patients 04 and 13) had myeloablative chemotherapy. CMV reactivated in 19 patients, of which 15 developed viremia within the first 6-7 weeks post-transplant. Ten out of 19 (53%) had a single episode of viral reactivation whilst the rest had multiple viremic relapses (Table 1) (*Online Supplementary Figure S2*). Within the latter group, patients 05 and 06 had prolonged episodes of viremia refractory to anti-viral treatment. Patients 11 and 16 had three episodes and patient 12 had four episodes of viral reactivation. Eight out of nine of this group had late recurrent reactivations beyond 100 days post-transplant (Table 1). In the group with multiple CMV reactivations, 4 out of 9 patients (patients 05, 11, 12, 13) were on systemic steroids for GVHD at the time of viremia whilst only 1 out of 10 (patient 14) with a single episode of CMV reactivation had steroids for GVHD.

Following the first CMV reactivation post-transplant, CMV-CD4 and CMV-CD8 proliferated rapidly and demonstrated parallel kinetics with an approximate 2 log expansion (Table 1) (Figure 1A,B). It was notable that the magnitude of the CMV-CD4 response was much smaller than that of CMV-CD8. In 11 out of 20 patients with detectable CMV-CD4, the median level at baseline before first reactivation was 0.11% of CD4$^+$ T-cells (range 0.02%

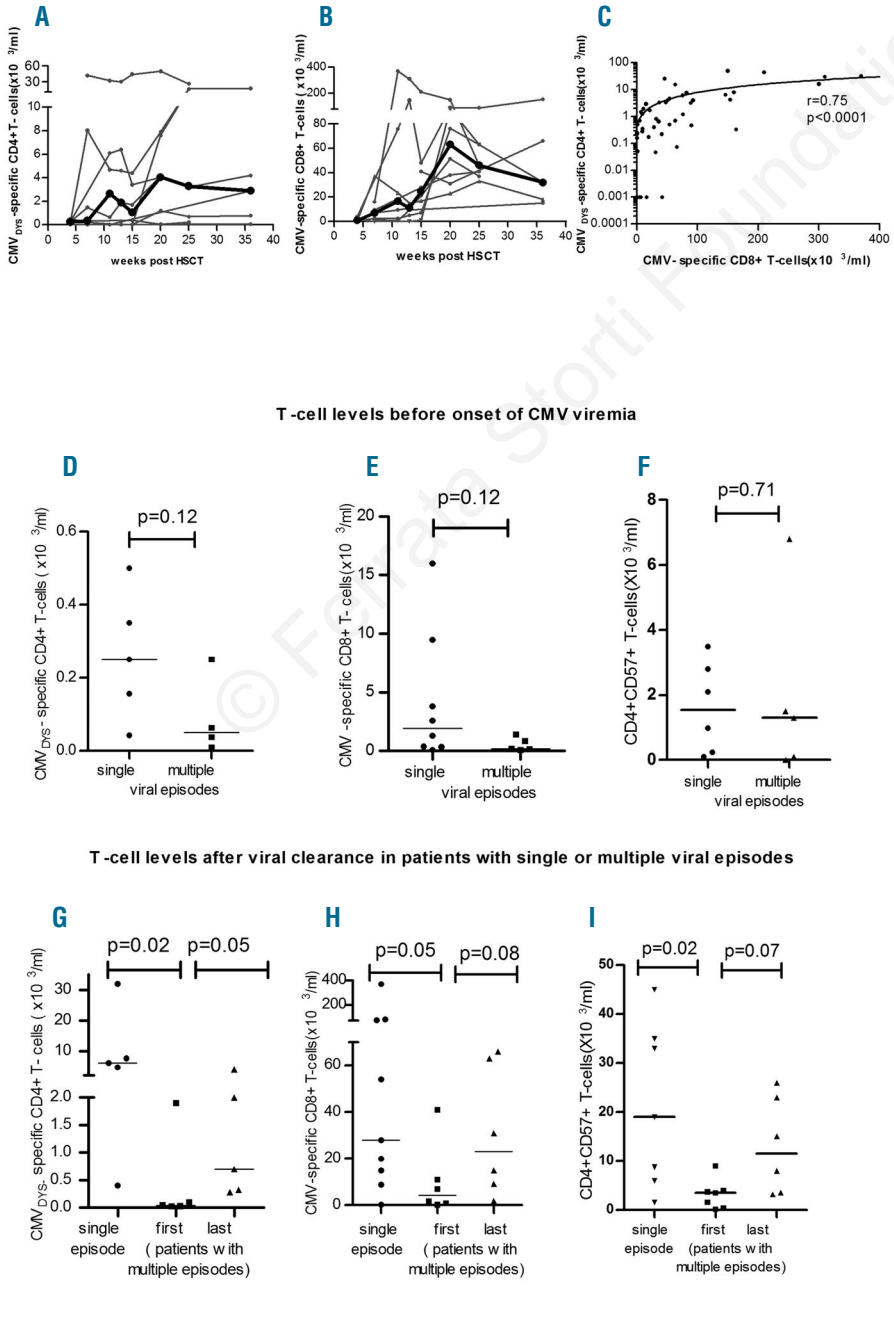

Figure 1. Reconstitution of CMV-specific T-cells post-HSCT before and after viremia in patients with single or multiple episodes of CMV reactivation. (A) Longitudinal monitoring of CMV-CD4 and (B) CMV-CD8 after HSCT using HLA-DRB1*0701-DYS and HLA-class I multimers, respectively. Thick black lines represent median cell number of CMV-CD4 and CMV-CD8 in peripheral blood of 9 patients. (C) Correlation between number of CMV-CD4 and CMV-CD8 during immune reconstitution after transplantation (r=0.75, *P*<0.0001). T-cell levels before onset of CMV reactivation: (D) number of CMV-CD4, (E) CMV-CD8 and (F) CD4$^+$CD57$^+$ T-cells before CMV viremia in patients who underwent single or multiple episodes of viral reactivation. T cell levels following viral clearance in patients with single or multiple episodes of viremia: (G) number of CMV-CD4, (H) CMV-CD8 and (I) CD4$^+$CD57$^+$ T-cells after clearance of viremia in patients with single reactivation, or after first and final episodes of reactivation in patients who suffered from multiple episodes of CMV reactivation. Patients with a single episode of CMV reactivation had higher numbers of all T-cell subsets after the first episode of viremia compared to patients with multiple reactivations (*P*=0.02).

to 4%). The corresponding absolute numbers ranged from 0.01 to 0.5x10³/mL (median: 0.15x10³/mL). This population expanded markedly after viral reactivation and reached a median peak of 4.8% of the CD4⁺ T-cell pool (range 0.23% to 25%) with corresponding absolute median count of 5.20 x10³ /mL (range 0.46 to 50x10³/mL). The magnitude of CMV-CD4 and CMV-CD8 following CMV reactivation within individual patients exhibited a strong correlation (r=0.75, *P*<0.0001) (Figure 1C).

An important finding was that the magnitude of the CMV-specific T-cell response following the first viral clearance was predictive of protection from recurrent reactivation. CMV-CD4 and CMV-CD8 levels before the first reactivation were low, and although there is up to a 5-fold difference in the magnitude of the CMV-CD4 and CMV-CD8 between single reactivation and multiple reactivations, the difference was not significant (Figure 1D,E).

Instead, levels measured after the resolution of the first viremia showed that patients with a single viremic episode had a median peak CMV-CD4 level of 6.2x10³/mL, whereas those with multiple episodes of viremia had a median CMV-CD4 level of only 0.04x10³/mL (*P*=0.02) (Figure 1G). A similar pattern was seen with CMV-CD8, where the post-viremic count was 41.10x10³ /mL in patients who gained long term control of reactivation, compared to 4.30x10³/mL in those who had multiple reactivations (*P*=0.049) (Figure 1H). These data reveal that the peak CMV-specific T-cell count after the initial episode of viremia can determine the ability to control further reactivations. We estimated the minimum thresholds of CMV-CD4 and CMV-CD8 counts for long-term viral control by taking the median peak T-cell level following the last episode of viremia. A median level of ≥0.7x10³/mL CMV-CD4 and ≥20x10³/mL CMV-CD8 after initial clearance of CMV was associated with the

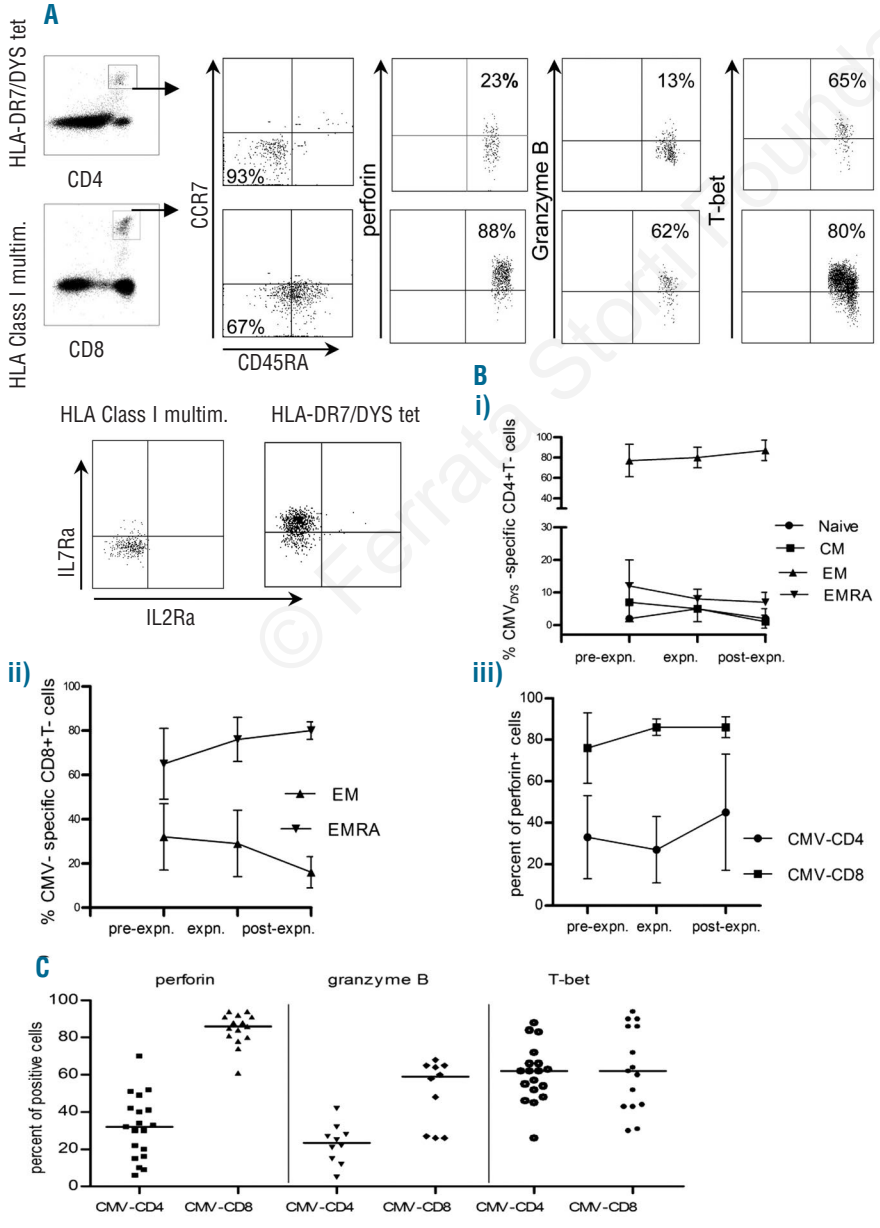

Figure 2. Phenotypic and functional analysis of CMV-specific CD4⁺ and CD8⁺ T-cells during immune reconstitution after transplantation. (A) Representative flow cytometric dot plot analysis of expression of CD45RA, CCR7, perforin, granzyme B, T-bet, IL2R and IL7R on tetramer-staining CMV-CD4 and CMV-CD8. (B) Differentiation phenotype of reconstituting CMV-specific T-cells in response to viremia at pre-expansion (pre-expn), peak (expn) and post-expansion (post-expn). (i) Percentage of CMV-CD4 which express a naïve, central memory, effector memory and 'revertant' memory phenotype in relation to reactivation; (ii) percentage of effector memory and effector memory (revertant) CMV-CD8 in relation to reactivation; (iii) mean frequency of perforin-positive CMV-CD4 and CMV-CD8 in relation to reactivation. Results reveal a reduction in the perforin level at the time of peak expansion of CMV-CD4 but not of CMV-CD8. (C) Percentage intracellular expression of perforin, granzyme B and T-bet within CMV-CD4 and CMV-CD8 during immune reconstitution. The expression of perforin and granzyme B by CMV-CD8 is increased compared to CMV-CD4 (*P*<0.0001, *P*<0.002, respectively).

absence of further reactivations. Interestingly, this magnitude of CMV-CD8 T-cells is consistent with previously reported protective levels of 10 to 20x10³/mL.[8]

As the detection of CMV-CD4 was restricted to a single epitope, we explored a surrogate marker of CMV-CD4. CD4⁺CD57⁺ T-cells are present almost exclusively in CMV-seropositive patients and expressed by CMV-CD4.[10] The number of CD4⁺CD57⁺ T-cells did indeed correlate strongly with DYS-specific CD4 T-cells (r=0.93, *P*<0.0001) (*Online Supplementary Figure S3A*). A higher level of CD4⁺CD57⁺ T-cells was observed after first CMV reactivation in patients with only a single reactivation episode, than in those with multiple reactivations (median 19x10³/mL *vs.* 4x10³/mL) (p=0.02) (Figure 1I). Of interest, the median protective number of CD4⁺CD57⁺ T-cells after final viral clearance in multiply reactivated patients was 11.5x10³/mL. Indeed, in cases where no CMV-CD4 were detected with the HLADR7 tetramer, the level of CD4⁺CD57⁺ T-cells was able to discriminate between those developing single or multiple CMV episodes.

Notwithstanding the strong correlation between CMV-CD8, CMV-CD4 and CD4⁺CD57⁺ T-cells, in one case (patient 12) where there was a high CMV-CD8 and a CMV-CD4 below the protective threshold, the patient developed recurrent reactivation. This may have been an example of the failure of CD4 in maintaining CD8 functionality.

A key feature of HLA-multimers is their ability to allow detailed single cell analysis. Longitudinal monitoring demonstrated largely stable CMV-CD4 and CMV-CD8 phenotypes throughout viremia (Figure 2A,Bi, ii). The majority of CMV-CD4 maintained a CCR7-CD45RA- effector memory phenotype (EM) (median 82%), whereas CMV-CD8 demonstrated a CCR7⁻CD45RA⁺ EMRA phenotype.

Since cytokines play a major role in immune reconstitution post HSCT, tetramer-positive cells were co-stained with anti-CD25 (IL2R) and anti-CD127 (IL7R). Neither of these receptors was expressed on CMV-CD8 but a mean of 46% of CMV-CD4 retained stable expression of IL7R but not IL2R (Figure 2A), indicating their capacity to proliferate and to survive long-term.

Studies have shown that a considerable percentage of CMV-CD4 from healthy individuals display cytotoxic activity.[10,11] Indeed, perforin and granzyme B expression was observed in 32% and 22% of CMV-CD4 respectively, confirming cytotoxic function within a substantial minority of the population (Figure 2). Intracellular analysis of perforin expression suggests that CD4⁺CD57⁺ T-cells represent cytotoxic cells. Both within the CD4⁺CD57⁺ and DYS-specific CD4⁺ T-cell pools, the median perforin expression was around 50%, in contrast to the whole CD4 population where only a median of 25% of cells expressed perforin (*Online Supplementary Figure S4*).

The expression levels of T-bet, a T-box transcription factor with an important role in the development of Th1 cells, was similar in CMV-CD4 and CMV-CD8 at a median of 62% of the antigen-specific population (Figure 2). The CMV-CD4 had higher T-bet expression compared to the global CD4⁺ T-cell population (*P*<0.03), supporting the predominant Th1 profile of CMV-CD4.[12] T-bet expression by CMV-CD8 after primary infection has been reported to predict control of recurrent pulmonary CMV viremia in lung transplant patients.[13] However, due to the limited number of frozen cells it was not possible to investigate the relation between CMV-CD4 and CMV-CD8 function.

Nevertheless, as CD4⁺ T-cells are known to support antibody production,[14] we assessed whether CMV-CD4 reconstitution correlated with the magnitude of the anti-CMV humoral response. A longitudinal analysis did indeed reveal that CMV-specific antibody titres correlated strongly with reconstituting CMV-CD4 (r=0.45, *P*=0.01) (*Online Supplementary Figure S3B*). This likely reflects the helper function of CMV-CD4, and forms part of the immune response against CMV. In addition to the neutralising effect of anti-CMV IgG, they may be involved in ADCC of infected cells mediated by NK cells.[15]

In conclusion, the study using novel HLA class II tetramers led to three major findings in the setting of reconstitution, and immune following HSCT. Firstly, CMV-CD4 were shown to reconstitute in parallel with CMV-CD8 following CMV reactivation but at a much lower magnitude. Secondly, the phenotype of the CMV-CD4 population is that of an effector memory subset and contains a considerable proportion of cytotoxic cells. Lastly, the enumeration of CMV-CD4 and CMV-CD8 responses after first episode of viral reactivation is predictive of the subsequent risk of recurrence.

Currently available CMV-specific tetramers cover around 80% of Caucasian tissue types. The addition of the HLA-DRB1*0701 tetramer expands the coverage but may also improve the prediction of reactivation. Larger prospective trials are needed to confirm the utility of these assays as biomarkers for risk-stratifying patients into a low risk group with high CMV-T-cell levels that do not suffer from further reactivation, and into a high risk group with low CMV-T-cell levels at high risk of recurrent CMV reactivation. The assay could help guide appropriate introduction of prophylactic T-cell immunotherapy or second line anti-viral drug therapy.

Mohammad Raeiszadeh,[1,2] Annette Pachnio,[2] Jusnara Begum,[2] Charles Craddock,[2,3] Paul Moss,[2,3] and Frederick E. Chen[1,2,3]

[1]NHS Blood and Transplant, Birmingham; [2]School of Cancer Sciences, University of Birmingham; and [3]Centre for Clinical Haematology, Queen Elizabeth Hospital, University Hospitals Birmingham NHS Foundation Trust, Birmingham, UK

Correspondence: frederick.chen@nhsbt.nhs.uk
doi:10.3324/haematol.2015.123687

Funding: The study was supported by the Lymphoma Leukaemia Research Grant (LLR0571) awarded to F.C., C.C., P.M.: National Institute of Health Research grant (RP-PG-0310-10003) to F.C.; Medical Research Council grant (G0901755) to P.M.

Key words: CMV, CD4, HLA-Class II tetramers, CD57, stem cell transplantation.

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

References

1. Chakrabarti S, Mackinnon S, Chopra R, et al. High incidence of cytomegalovirus infection after nonmyeloablative stem cell transplantation: potential role of Campath-1H in delaying immune reconstitution. Blood. 2002;99(12):4357-4363.
2. Ljungman P, Hakki M, Boeckh M. Cytomegalovirus in hematopoietic stem cell transplant recipients. Hematol Oncol Clin North Am. 2011;25(1):151-169.
3. Gratama GW, Boeckh M, Nakomura R, et al. Immune monitoring with iTAg MHC Tetramers for prediction of recurrent or persistent cytomegalovirus infection or disease in allogeneic hematopoietic stem cell transplant recipients: a prospective multicenter study. Blood. 2010;116(10):1655-1662.
4. Widmann T, Sester U, Gartner BC, et al. Levels of CMV specific CD4 T cells are dynamic and correlate with CMV viremia after allogeneic

stem cell transplantation. PloS one. 2008;3(11):e3634.
5. Eid AJ, Brown RA, Hogan WJ, Lahr BD, Eckel-Passow JE, Litzow MR. Kinetics of interferon-gamma producing cytomegalovirus (CMV)-specific CD4+ and CD8+ T lymphocytes and the risk of subsequent CMV viremia after allogeneic hematopoietic stem cell transplantation. Transpl Infect Dis. 2009;11(6):519-528.
6. Quinnan GV, Kirmani N, Rook AH, et al. Cytotoxic T Cells in Cytomegalovirus Infection. New Engl J Med. 1982;307(1):7-13.
7. Gamadia LE, Remmerswaal EBM, Weel JF, Bemelman F, van Lier RAW, Ten Berge IJM. Primary immune responses to human CMV: a critical role for IFN-γ–producing CD4+ T cells in protection against CMV disease. Blood. 2003;101(7):2686-2692.
8. Aubert G, Hassan-Walker AF, Madrigal JA, et al. Cytomegalovirus-Specific Cellular Immune Responses and Viremia in Recipients of Allogeneic Stem Cell Transplants. J Infect Dis. 2001;184(8):955-963.
9. Crompton L, Khan N, Khanna R, Nayak L, Moss PAH. CD4+ T cells specific for glycoprotein B from cytomegalovirus exhibit extreme conservation of T-cell receptor usage between different individuals. Blood. 2008;111(4):2053-2061.
10. van Leeuwen EMM, Remmerswaal EBM, Vossen MTM, et al. Emergence of a CD4+CD28– Granzyme B+, Cytomegalovirus-Specific T Cell Subset after Recovery of Primary Cytomegalovirus Infection. J Immunol. 2004;173(3):1834-1841.
11. Casazza JP, Betts MR, Price DA, Precopio ML, Ruff LE, Brenchley JM. Acquisition of direct antiviral effector functions by CMV-specific CD4+ T lymphocytes with cellular maturation. J Exp Med. 2006;203(13):2865-2877.
12. Edwards ESJ, Smith C, Khanna R. Phenotypic and transcriptional profile correlates with functional plasticity of antigen-specific CD4+ T cells. Immunol Cell Biol. 2014;92(2):181-190.
13. Pipeling MR, John ER, Orens JB, Lechtzin N, McDyer JF. Primary cytomegalovirus phosphoprotein 65-specific CD8+ T-cell responses and T-bet levels predict immune control during early chronic infection in lung transplant recipients. J Infect Dis. 2011;204(11):1663-1671.
14. Ludwig B, Kraus FB, Kipp M, et al. Cytomegalovirus-specific CD4 T-cell and glycoprotein B specific antibody response in recipients of allogenic stem cell transplantation. J Clin Virol. 2006;35(2):160-166.
15. Aicheler RJ,Wang ECY, Tomasec P, Wilkinson GW ,Stanton RJ. Potential for natural killer cell-mediated antibody-dependent cellular cytotoxicity for control of human Cytomegalovirus. Antibodies. 2013;2(4):617-635.