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Specific Immunity to Cytomegalovirus in Pediatric Cardiac Transplantation

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Authorship

MCJ and MDIM participated in the performance of the research, data analysis, result interpretation, and in the writing of the paper. ESP and MF participated in patient consent and sample acquisition, result interpretation and in writing the paper. GLS participated in data analysis and writing the paper. NJK participated in research design, result interpretation, and writing the paper. MB participated in research design, patient consent and sample collection, result interpretation and writing the paper.

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Abbreviations

CMV, cytomegalovirus; D, donor; DNA, deoxyribonucleic acid; GAMM, generalised additive mixed model; GB, granzyme B; GOSH, Great Ormond Street Hospital; IFN-γ, interferon gamma; Ig, immunoglobulin; IL-2, interleukin 2; PBMC, peripheral blood mononuclear cells; PCR, polymerase chain reaction; R, recipient; TNF-α, tumor necrosis factor alpha, VL, viral load.

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ABSTRACT

Background: Cytomegalovirus (CMV) infection is implicated in endothelial dysfunction and graft damage after pediatric heart transplantation. CMV specific immune responses are thought to be necessary for CMV viral control but there is little data in pediatric heart transplantation.

Methods: We studied 28 consecutive pediatric heart transplant recipients for 1-year posttransplant. CMV-specific T cells expressing IFN-γ, TNF-α and IL-2 in response to *ex-vivo* stimulation with CMV lysates or peptides were measured. Circulating cytokines were measured in plasma. Generalised Additive Models were applied to the data to model changes of cell population dynamics over time.

Results: CMV-specific T cell mediated responses were impaired in the first 8 weeks posttransplant. During this period, 25% of patients had CMV viremia, of which those with viral loads ≥10,000 CMV DNA copies/mL were given ganciclovir. In this group, the frequency of CD4+ and CD8+ T cells producing IFN-γ and the CD8+CD57+GB+ T cell population increased at 12-24 weeks and remained elevated for the duration of the study.

Conclusions: We have shown that CMV viremia is associated with CMV specific immune responses and increased CD8+CD57+GB+ cells at 1-year posttransplant, however early responses were not predictive of impending CMV viremia. It remains to be seen if the early CMV immune response detected is associated with endothelial and allograft damage, in light of previous studies demonstrating increased vasculopathy in pediatric patients with CMV viremia.

Key words: Cytomegalovirus, inflammation, cytokines, endothelial dysfunction, coronary artery vasculopathy.

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Background

Human cytomegalovirus (CMV) is a ubiquitous double-stranded DNA virus. Although CMV infection is usually asymptomatic in the healthy population, it remains an important cause of morbidity and mortality among immune-compromised patients, including children undergoing cardiac transplants [1,2]. One peculiarity of CMV is its particular tropism for endothelial and epithelial cells [3] which may explain its postulated role in arterial hypertension and aortic atherosclerosis [4] and other cardiovascular diseases [5,6] including cardiac allograft vasculopathy (CAV) in adults [7–9]. CMV-specific T cell immune responses can be used to predict CMV tissue invasive diseases and complications in adult transplant recipients [10–15]. After heart transplantation in adults, an adequate immune response is crucial to control the viral burden, and appears to limit vascular damage [16]. We have previously linked CMV infection to pediatric allograft vasculopathy [17] and shown the association of CMV viremia in pediatric cardiac transplant recipients to endothelial dysfunction [18]. However, our studies were not prospective and we did not assess CMV cell mediated immune responses. Longitudinal immunological studies are difficult in pediatric heart transplantation because of the small populations and the difficulties in blood sampling, exemplified by a recent study highlighting only 3 heart transplant recipients with CMV as part of a wider cohort [13]. Understanding posttransplant immune reconstitution and CMV-specific immune response dynamics may provide insight to the risks of developing CMV related complications, such as cardiac allograft vasculopathy.

In the current prospective study, 28 children were followed up for the first year after heart transplant to explore the dynamics of the CMV-specific T cell population by evaluating the cellular response to total viral lysate and peptides by measuring intracellular cytokine production, specifically Interleukin-2 (IL-2), Tissue Necrosis Factor-alpha (TNF-α), and Interferon-gamma (IFN-γ). Reduced levels of IFN-γ have been associated with increased CMV

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VL [19]. Furthermore, reduced levels of polyfunctional T cells (those expressing more than 1 cytokine) have been associated with CMV viral replication [20], and that these polyfunctional T cells protect against the chronic long-term effects of viral infections. Levels of $CD8^+CD57^+$ T cells were also evaluated. These cells are known to replicate in response to CMV infection and produce Granzyme B [21,22], which can trigger apoptosis and graft damage. In addition, we examined soluble biomarkers of inflammation and vascular function. Data was analysed using a generalised additive mixed model (GAMM) to explore the dynamics of cell populations in different patient groups over time.

Methods

Study design and patient population

Children undergoing heart transplantation at Great Ormond Street Hospital (GOSH) from September 2009 to July 2011 were prospectively enrolled in the longitudinal cohort study. The study was conducted after obtaining the local ethical committee approval and in accordance with guidelines provided by the National Research Ethic Service. The immunosuppressive regimen for these patients was: induction immunosuppression with Basiliximab and high dose intravenous steroids. Maintenance was with tacrolimus and mycophenolate mofetil with oral steroids weaned by 6 months. Target tacrolimus levels were also reduced after 6 months from 9-12 to 7-10μg/L. Prophylactic treatment with nystatin, cotrimoxazole and aciclovir were given for the first 3 months after transplantation.

Patients were screened and monitored for CMV and Epstein-Barr Virus DNAemia, and tacrolimus levels, renal and liver function, and full blood count were checked at every clinical visit (weekly for the first month, every 2 weeks for 2 months, then monthly for the rest of the year). CMV DNAemia was detected by CMV polymerase chain reaction (PCR) at the GOSH

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diagnostic laboratory according to the method of Preiser [23]. The UK pediatric heart transplant units use pre-emptive treatment for CMV viremia, not prophylactic treatment. Antiviral therapy with ganciclovir was only given when CMV viral load ≥ 10 000 CMV DNA copies/mL.

The CMV serostatus of donors (D) and recipients (R) was established prior to transplantation by measuring specific immunoglobulin to CMV by enzyme-linked immunosorbent assay. For patients under 18 months old, IgM and IgG antibodies were measured, and CMV positive status given if the patient had IgM and IgG antibodies to CMV. At the time of transplant, patients were defined as being R+D+, R+D-, R-D+ and R-D-. Of the 6 patients that were less than 18 months, all except 1 patient were D+/R-. The 1 patient that was R+/D- was in the CMV+VL- group.

For the purposes of this study, children were divided into 3 groups: 1) patients, exposed to CMV prior to or at transplant, who had posttransplant viral replication (CMV+VL+); 2) patients exposed to CMV prior to or at time of transplant, who had no viral replication (CMV+VL-); and 3) patients who had neither previous CMV exposure (CMV-) nor viral replication (VL). Demographics of the patients included in the analysis are shown in Table 1.

Sample collection and processing

Blood samples were collected, when possible, before or on the day of the heart transplant (W0), at week 1, 2, and 4, and then 4-weekly, for up to a year. Plasma was separated and stored at -80°C, and peripheral blood mononuclear cells (PBMCs) were isolated from heparinised blood by density gradient centrifugation (lymphoprep - Axis-Shield PoC AS, Oslo, Norway) and processed immediately. CMV specific responses and ex vivo cell proportions were detected using flow cytometry. Soluble markers were detected using a MSD multi-array assay (Meso

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Scale Discovery; Rockville, MD, USA). Details of reagents and methods are described in Materials and Methods, SDC, http://links.lww.com/TP/B544.

Cell stimulation and staining:

Cells (2×10^6) were stimulated with 5 μg/mL of either viral peptides (IE-1 and pp65; JPT Peptide Technologies GmbH, Berlin, Germany) or purified viral lysate (Advanced Biotechnologies Inc., Columbia, MD, USA) or SEB (Sigma-Aldrich Company Ltd., Gillingham, UK) as positive control or left unstimulated (negative control) in the presence of 5 μg/mL costimulatory monoclonal antibody, anti-CD28 (BD Biosciences, Oxford, UK). After 2 h, complete medium containing Brefeldin A (Sigma-Aldrich; final concentration 10 μg/mL) was added and incubated for a further 14 h.

All antibodies were from BD biosciences (Oxford, UK) except for the anti-human Granzyme B antigen, which was from Life Technologies Ltd (Paisley, UK). Cells were_first costained with anti-CD3 (Pacific Blue™; clone MOPC-21) anti-CD4 (APC-Cy7;_clone SK3) and anti-CD8 (PerCP-Cy5.5; clone SK1), fixed with 4% paraformaldehyde (PFA), permeabilised with phosphate buffer saline containing 2% bovine serum albumin and 0.1% saponin, and then stained with anti-IL-2 (FITC; clone 3544.111), anti-TNF-α (PE; clone MAb11) and anti-IFN-γ (APC; clone B27). PBMCs were finally suspended in PFA and stored at 4°C until acquisition on an LSRII flow cytometer (BD biosciences), equipped with 4 lasers (350, 405, 488, and 633 nm). The instrument was calibrated on a weekly basis. At least, 100,000 events were collected. Acquired data were analysed using FlowJo v9.6.5 (TreeStar, Celeza GmbH, Olten, Switzerland).

Figure S1, SDC, http://links.lww.com/TP/B544 shows representative plots and gating strategy of a control sample, gated on IFN-γ expressing cells. CD3+CD4+ and CD3+CD8+ T cells were expressed as percent frequency of the total CD4 or CD8 T cell population. The CD8+

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or CD4+ T lymphocytes were considered to respond to stimulation when cytokines levels were equal to or above an arbitrary threshold, set on the unstimulated control at + 0.25%.

1×10^6 PBMCs were stained with anti-CD3 (Pacific Blue™), anti-CD4 (APC-Cy7), anti-CD8 (PerCP-Cy5.5) and anti-CD57 (FITC; clone HNK-1; Technologies Ltd (Paisley, UK). Cells were then fixed with 4% PFA and permeabilised as above before adding the anti-Granzyme B (GB; APC; clone GB11) antibody. After the staining, the cells were stored at 4°C until flow cytometry acquisition.

Statistical analysis

GAMMs were used to model the temporal pattern of the cell population dynamics. Where the response variable was expressed as 0/1 values (eg, the CD8-IFN-γ response to CMV lysate, pp65 or IE-1 peptide greater or equal to 0.25% above control) a binomial distribution with logit link function was used. The proportion of the CD57+GB+ population was modelled using a beta distribution with logit link function to handle the continuous and bounded (0-1) nature of the data. This model afforded considerably better fit to the observations and model diagnostics to the observations than a quasibinomial. We used GAMMs because we anticipated nonlinear functional responses on the scale of the linear predictor. GAMMs allow the estimated nonlinear relationships be determined from the data via the use of local spline basis functions, rather than be specified a priori by the analyst. Modern advances in GAM theory (eg, Wood et al, 2016 [24]; Wood 2017 [25]) have demonstrated the applicability and appropriateness of using GAMMs for applied statistical modelling where the true shape of the (partial) relationships between response and covariate are unknown or not representable using simple low-degree polynomials.

All models were fitted using the mgcv package [26–28] for the R statistical Software (version 3.2.2 [29]). Details of the GAMMs fitted are available in SDC, Materials and Methods.

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Results

Patient recruitment and group demographics

Thirty-four children, aged between 5 months and 16 years were recruited for the study. Six children were excluded from the study. Three did not receive a transplant during the study period, 1 died, and insufficient sample was obtained from 2 to be included in the analysis. Patients were stratified according to their CMV status at transplant and whether or not they had viremia postoperatively. Patient demographics can be viewed on Table 1. Patients that were either R+ or D+ and had CMV viremia were CMV+VL+ (n = 7, median age 2.12 ± 6.45); patients that were R+ or D+, but did not have CMV viremia, were CMV+VL- (n = 10, median age 8.84 ± 5.65); patients that were D-/R- and had no CMV viremia were in the CMV-VL- group (n = 11, median age 7.58 ± 6.45). In the CMV+VL+ group, 2 patients were D+R+ and 5 D+R-. In the CMV+VL- cohort, 1 patient was D+R+, 3 patients were D+R- and 6 D-R+. All patients in the CMV-VL- group were D-R-.

Posttransplant CMV viremia

Seven of the 17 (41%) 'at risk' patients had CMV DNA detected in the blood within the first 12 weeks posttransplant (Figure 1). Among those children who had viremia, 2 out of 7 of them (22%; Figure 1; Patient number 1 and 2) were R+/D+ and had detectable CMV reactivation/reinfection (median 1045 copies/mL; range 0-2715 copies/mL) at week 9-11. However, the viral load was below the threshold at which anti-CMV treatment is administered, and these patients were able to control viral replication without medication. In the remaining 5 of the 7 viraemic children (78%; all R-/D+), CMV DNA was detected within the first 7 weeks after transplantation, with a maximal median CMV viral load of 20 924 copies/mL (range 0-182,316 copies/mL) at week 6-9. These patients required treatment with ganciclovir.

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Dynamics of CMV-specific T cell responses following peptides and viral lysate stimulation

To assess CMV-specific T cell response in the 3 cohorts, we measured the percentage of CD4+ and CD8+ T cell populations producing intracellular IFN-γ, TNF-α, and IL-2 (Figure S1, SDC, http://links.lww.com/TP/B544) following stimulation with whole CMV lysate, IE-1 and pp65 peptides. T cell responses to a nonantigenic stimulus were evaluated using bacterial superantigen SEB (Figure S2A and B, SDC, http://links.lww.com/TP/B544), and there was no statistically significant difference in response to SEB in CD4+ and CD8+ cells between the different patient groups.

In patients from the CMV+VL+ cohort, CD8+ and CD4+ T lymphocytes producing IFN-γ in response to 1 or more CMV stimuli were below the positive response threshold (>0.25% positive) for 8 and 12 weeks, respectively (Figure 2A). After 20-24 weeks, the frequency of IFN-γ producing T lymphocytes increased above the positive response threshold, peaking at 28 weeks (1.96% median CD4+IFN-γ+ cells; 3.53% median CD8+IFN-γ+ cells). Both CD4+ and CD8+ cells retained the capacity to produce IFN-γ above the response threshold until the end of the 52 week period. The frequency of TNF-α producing CD4+ and CD8+ T cells was low throughout the study period (data not shown). Lymphocytes from the CMV+VL+ cohort did not show any augmentation in IL-2 synthesis in response to stimulation (data not shown).

In the CMV+VL- cohort the frequency of detectable IFN-γ producing cells fluctuated around the response threshold. IFN-γ produced by CD8 cells was above the response threshold at 16 weeks (median 0.29%), and by week 52, both CD4 and CD8 T cells were producing detectable levels of IFN-γ (median 0.44% and 0.35% respectively (Figure 2B). As in the CMV+VL+ group, there was no significant increase in CD4+ or CD8+ cells producing TNF-α or IL-2 in the CMV+VL- patient group (data not shown).

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Lymphocytes from patients in the CMV-VL- cohort failed to produce IFN-γ from either CD4+ or CD8+ cells in response to any CMV stimulus (Figure 2C). TNF-α and IL-2 were rarely detected and always at low levels (data not shown).

Further investigation of any effects of CMV status and viral load on the production of IFN-γ by CD8+ T cells was conducted using the fitted GAMMs (Figure 3).

These models (Figure 3) demonstrated that in the CMV+VL+ cohort, levels of IFN-γ production by both CD4+ and CD8+ T cells increased significantly after 20 weeks (ie, after the reduction of CMV viremia to undetectable levels), and this increase continued for the duration of the study (χ^2 = 18.07, degrees of freedom (df) = 9, p<0.001 for CD4+ cells; χ^2 = 24.13, df = 9, p<0.001 for CD8+ cells respectively), whereas levels in the CMV+VL- group increased after 30-40 weeks posttransplant (p<0.001 for both CD4+ and CD8+ cells; Figure 3A and B). The model with patient-specific time curves provided a significantly better fit to the data than the common time-curve model (likelihood ratio test (LRT): χ^2 = 10.39, df = 1.96, p = 0.00528 for CD4 expressing cells and χ^2 = 33.51, df = 5.85, p<0.001 for CD8 expressing cells).

Granzyme B expression in CD57+ T cells

The patterns of CD57+ cells expressing GB were distinct for the 3 patient groups (Figures 2). In the CMV+VL+ patients, there was a decrease in levels of this population at 4-8 weeks, followed by a dramatic increase at week 12. Levels plateaued at 20 weeks, remaining elevated for the rest of the study (Figures 2A). In contrast, in the CMV+VL- group, these cells remained stable, only increasing at the end of the study period, yet never reaching the levels observed in the CMV+VL+ cohort (Figures 2B). Patients in the CMV-VL- group had low percentages of CD8+CD57+GB+ cells following transplant, rising at 12 weeks to levels that

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were maintained for the remainder of the study period (Figures 2C). Frequencies of these cells were always lower in the CMV-VL- group compared to levels in the other 2 groups.

The GAMM showed that in the CMV+VL+ patient cohort, there was an increase in CD57+GB+ CD8 cells around 8 weeks posttransplant, reaching a plateau after 30 weeks, suggesting that the increase in CD57+GB+ cells is after the peak in CMV viral load (Figure 2A and 3C). This increase in CD57+GB+ cell proportion in the CMV+VL+ patient cohort (Figure 2B and 3C) is significant (χ^2 = 367.97, df = 9, p<0.001). On the contrary, the CMV+VL- group has a shallower increase in CD57+GB+ cell proportion, and appears to reach a lower plateau after 40 weeks (χ^2 = 88.21, df = 9, p<0.001). Furthermore, there was a significant difference between the time curves of the 2 groups (LRT: χ^2 = 36.42, df = 2.13, p<0.001).

Soluble markers in plasma of heart transplanted children exposed to CMV

There was no convincing evidence that the patterns of soluble markers differed between the 3 groups. The levels of cytokines were low in all groups throughout the year of follow-up (Table S1, SDC, http://links.lww.com/TP/B544).

Long term patient outcomes

In the years following this study, we have had 5 deaths in our patient population. Four deaths were due to antibody mediated rejection, of which 2 of those patients were in the CMV-VL- group, 1 was in the CMV+VL- group, and the last patient was in the CMV+VL+ group. This last patient was D+/R+ and the only patient that developed CAV. The 5th patient died due to unrelated circumstances. With our small patient sample size and only 1 patient with CAV, it will be difficult to assess the implication of the immunological markers on the development of CAV. Larger, multicenter studies will be required to truly discern the impact of CMV in a pediatric population unaffected by other confounding factors involved in adult CAV disease post heart transplant.

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Conclusions

This is the first prospective study of CMV-specific cell mediated immunity in pediatric heart transplantation. During the first 8 weeks following transplantation, CMV specific responses were rarely detectable. This reflects the impact of the early immunosuppression, which not only influenced CMV specific responses but also suppressed CD4+ and CD8+ IFN–γ expression in response to SEB stimulation. This period of immunosuppression coincided with detectable CMV viral load. CMV became undetectable just prior to emergence of specific CMV responses in both ganciclovir treated (5) and untreated (2) patient. The detection of CD4+ and CD8+ IFN–γ expression was significantly earlier in patients who had detectable CMV and coincided with reduction of CMV viremia to undetectable levels.

In both CMV+ cohorts, CD8+ and CD4+ cells producing either TNF-α or IL-2 were rarely detected and the plasma levels of these cytokines did not show any appreciable difference when compared to those of the CMV-VL- patients. These finding may at least be partially explained by a decreased capacity to produce CMV-specific IL-2 responses during primary infection [30] and by the ability of CMV to render the CD4+ cells unresponsive to immunological stimuli [31,32]. It may also reflect the impact of immunosuppression on the cytokine response.

In the UK all pediatric heart transplant patients have pre-emptive therapy for CMV infection. While reducing exposure to antiviral drugs effect [33], some patients will develop significant CMV viremia and this strategy can be logistically demanding. Universal prophylaxis reduces early CMV disease but exposes children to medication with potentially harmful side-effects [34]. Furthermore, recent work has shown no difference in patient outcome when comparing pre-emptive therapy and prophylaxis, however, pre-emptive therapy does reduce the burden of drug exposure in children with liver transplantation [35,36]. Biomarkers that predict which patients require prophylaxis would be an attractive means of allocating anti-viral therapy.

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In our study we did not find that CMV specific CD4 and CD8 responses were predictive of developing CMV viremia in CMV positive patients, largely because levels were below the positive threshold prior to CMV viremia. This indicates that other components of the immune system may be more important in controlling CMV replication and/or that the methods used for detecting CMV specific immune responses were too insensitive to detect patients at risk of viremia. Interestingly, the high detection levels of early CMV-specific T cell immune recovery by ELISpot in adult heart transplant recipients provides long-lasting protection to CMV disease [37], and this technique may be more sensitive for low level detection.

Adult allograft recipients have been shown to have increased numbers of CD8+CD57+ circulating lymphocytes [30], correlating with CMV carrier status [31]. Whilst all 3 groups showed an increase in CD8+CD57+GB+ cells following transplant, there was a substantially greater increase in these cells in the CMV+VL+ group at around 8 weeks posttransplant, reaching a plateau after 30 weeks, ie, after the peak in CMV viral load. This was significantly earlier than in patients in whom viral load was not detectable.

The significance of the increase in CD57+ cells is unclear. We found a strong association between CD57 positivity and GB expression. This population of cells has the potential to cause tissue injury [38], through the release of potent cytolytic enzymes. GB production has been linked to allograft rejection in renal transplantation [39], with inhibition of the cytolytic activity of GB leading to improved transplant survival. We and others have shown that CMV infection is related to cardiac allograft vasculopathy and posttransplant endothelial dysfunction [17,18]. We hypothesise that the high levels of CD57+ cells in patients with CMV may be involved in the vasculopathy observed in patients with CMV viremia posttransplant, in light of previous work from our group, where we associate CMV viremia with vasculopathy in a similar pediatric population of patients [18].

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In conclusion, we have shown that in the first year post pediatric heart transplant, patients with active CMV viral replication display both increased CMV specific immune responses and an increase in CD8+CD57+GB+ cells. While these responses were not predictive of impending CMV viremia, they may be involved in endothelial injury. Further studies are required to understand if these cells are responsible for CMV associated posttransplant vasculopathy, and if the virus and/or the potentially damaging immune responses should be targeted to reduce long-term mortality and morbidity.

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TABLE LEGEND

Table 1: **Patient demographics and clinical characteristics**

FIGURE LEGENDS

Figure 1: **Viral titers in CMV+VL+ group**

CMV Viral load of patients in the CMV+VL+ cohort up to 12 weeks posttransplant measured by real-time PCR. Patients denoted with black lines (■ and □) were D+/R+, who did not receive ganciclovir treatment. Patients with grey lines were D+/R-, and did receive ganciclovir treatment, as described in Materials and Methods.

Figure 2: **Frequency of CD8+ and CD4+ total T lymphocytes, the percentage of CD4+ and CD8+ T cell populations producing intracellular IFN-γ following the stimulation of total viral lysate or CMV peptides, and CD57+GB+ T cells**

Median lymphocyte populations in the 3 patient cohorts are shown. A) CMV+VL+ patient group (n=7); B) CMV+VL- patient group (n=10), C) CMV-VL- patient group (n=11). The CD4+ (●) and CD8+ (▲) populations stimulated by the total CMV viral lysate, and by the IE-1 and pp65 peptides were averaged per patient and per week, and plotted as a median. The grey line denotes the 0.25% positive response threshold for the CMV specific immune responses. The mean intracellular IFN-γ producing CD4+ (+) and CD8+ (□) T cells following stimulation with viral lysate or peptides were calculated and the median values drawn. The frequency (median) of the CD57+ T cells expressing Granzyme B (■) is also shown.

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Figure 3: **Generalised Additive Models (GAM) of lymphocyte populations in the CMV+VL+ and CMV+VL- cohorts**

GAM models are presented for the CMV+VL+ group (dashed line) and CMV+VL- group (solid line) showing the IFN-γ producing CD8+ T cells (A), the IFN-γ producing CD4 T cells (B) in response to CMV lysate, pp65 or IE-1 peptide, and the CD8+ cells expressing CD57 and GB (C). The models were fitted as described in SDC, Materials and Methods.

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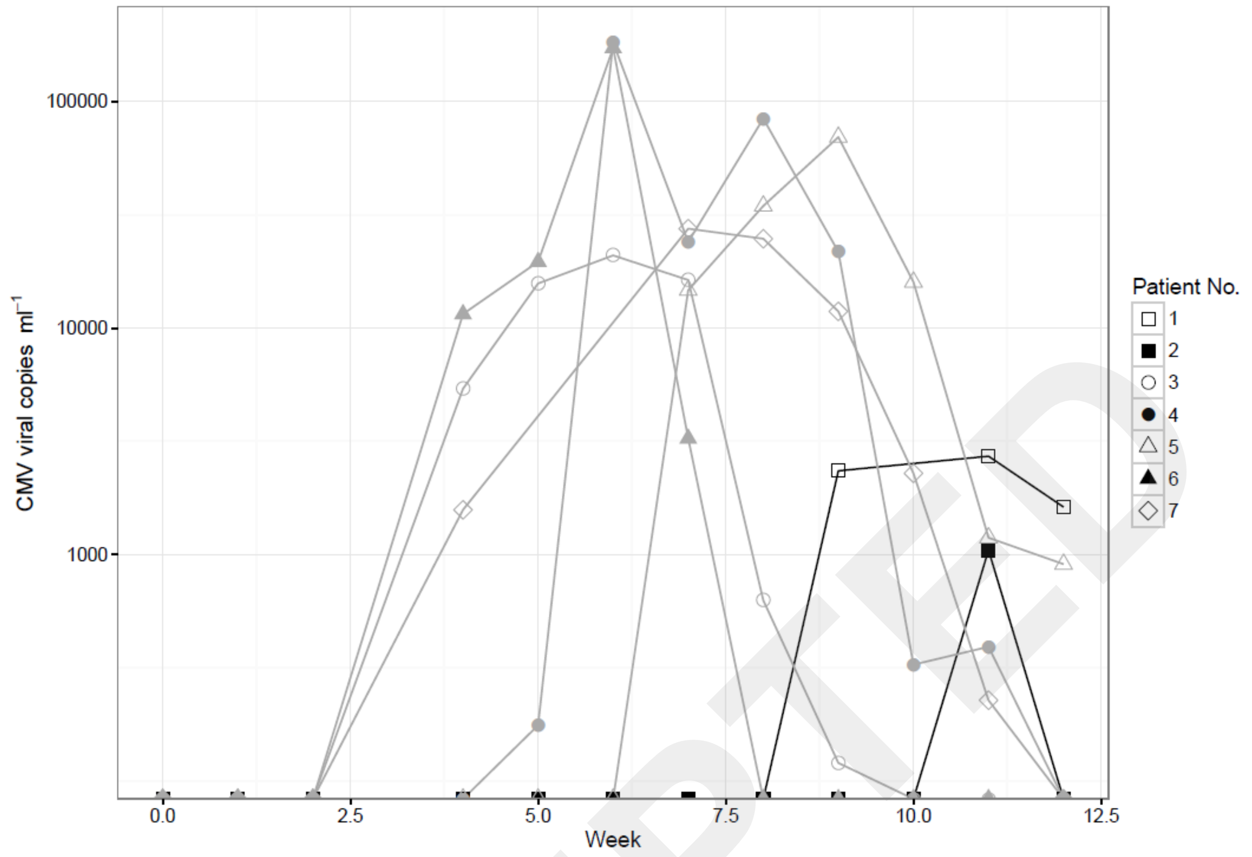

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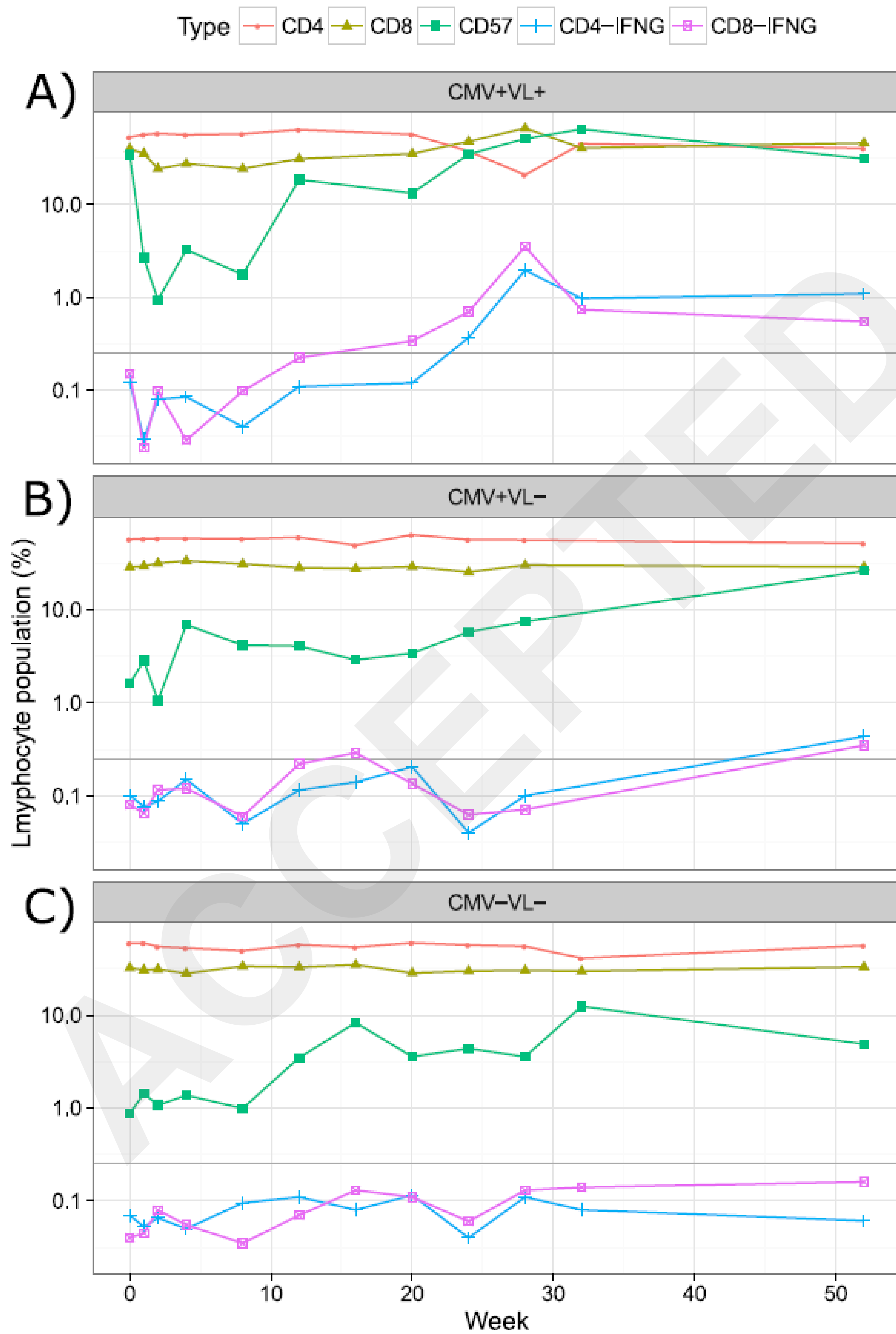

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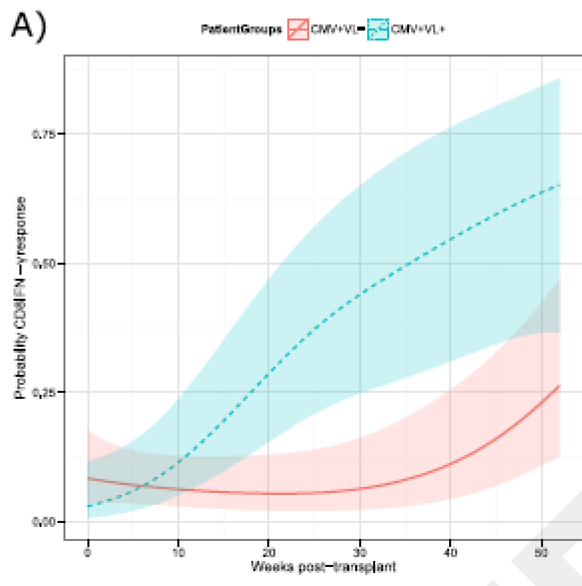

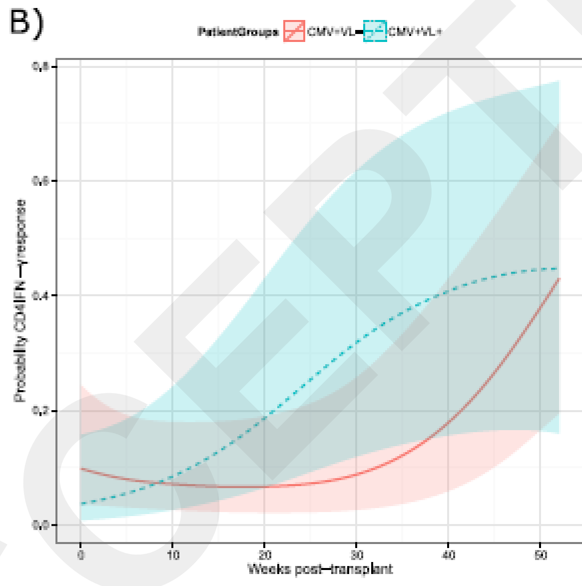

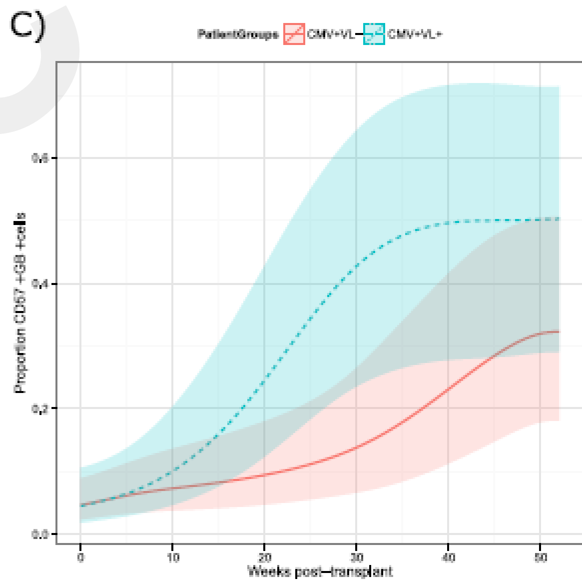

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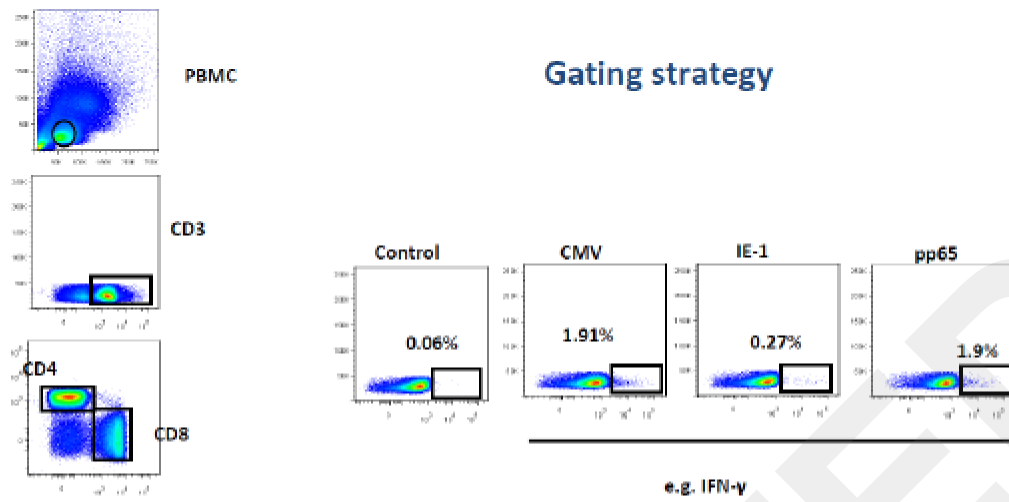
Figure S1:



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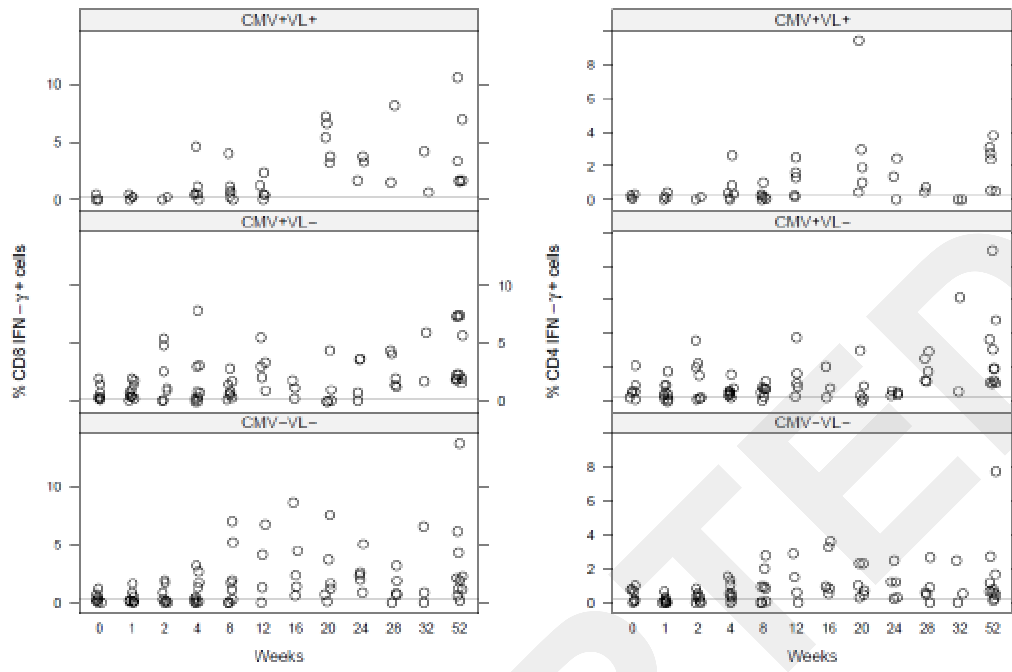

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Table S1

Week	CMV+VL+							CMV+VL-							CMV-VL-						
	IFN-γ (pg/mL)	TNF-α (pg/mL)	IL-2 (pg/mL)	IL-15 (pg/mL)	TM (pg/mL)	VEGF (pg/mL)	FKN (pg/mL)	IFN-γ (pg/mL)	TNF-α (pg/mL)	IL-2 (pg/mL)	IL-15 (pg/mL)	TM (pg/mL)	VEGF (pg/mL)	FKN (pg/mL)	IFN-γ (pg/mL)	TNF-α (pg/mL)	IL-2 (pg/mL)	IL-15 (pg/mL)	TM (pg/mL)	VEGF (pg/mL)	FKN (pg/mL)
0	0.04	0.44	7.83	0.92	1 611.2	24.2	1 055.2	0.17	0.69	5.06	0.87	1 131.7	100.5	1 020.9	0.10	0.65	25.55	0.76	1 422.1	41.5	724.6
1	0.00	0.19	5.37	0.77	1 381.0	66.7	977.5	0.11	0.63	3.92	1.98	1 053.5	131.1	833.6	0.04	0.52	8.77	1.74	1 523.7	78.4	711.0
2	0.05	0.33	0.64	0.47	845.2	88.8	659.7	0.08	0.48	1.38	0.81	935.8	150.0	620.6	0.13	0.66	3.70	1.31	1 240.7	85.4	922.1
4	0.18	0.57	1.91	0.50	1 308.2	97.0	587.5	0.15	0.65	1.38	0.73	1 154.4	107.1	691.1	0.07	0.73	3.01	0.76	1 322.6	98.0	778.1
8	1.49	0.80	2.60	0.55	1 774.2	49.4	776.3	0.25	0.67	0.82	0.82	1 262.7	78.3	879.7	0.43	0.69	1.97	0.80	1 272.4	81.5	626.3
12	0.47	0.59	0.20	0.29	1 620.5	45.6	760.8	0.38	0.68	0.31	0.67	1 183.0	76.8	617.7	0.18	0.57	0.88	0.79	1 827.5	43.0	n.d.
16	0.29	0.54	4.75	0.30	1 982.6	45.7	883.0	0.37	0.59	0.00	0.89	1 239.6	38.7	603.8	0.11	0.36	0.26	0.39	1 773.9	24.4	585.9
20	0.33	0.78	0.60	0.07	1 806.7	37.7	893.8	0.20	0.66	0.10	0.72	1 101.3	64.4	577.2	0.37	0.54	0.63	0.59	1 943.7	46.7	770.3
24	2.06	1.29	0.63	0.10	3 541.6	258.7	727.8	0.16	0.55	0.12	0.53	1 444.6	48.8	694.6	0.18	0.54	0.45	0.69	1 950.5	95.7	590.6
28	0.43	0.93	2.81	0.63	1 345.4	50.6	1 600.6	0.27	0.57	0.10	0.49	1 326.8	61.3	596.1	0.28	0.70	0.61	0.60	1 992.9	28.5	958.7
32	0.35	0.98	10.40	0.16	1 319.6	110.8	750.8	0.39	0.78	0.21	0.72	1 387.6	47.8	707.9	0.45	0.78	2.12	0.73	1 876.3	41.9	n.d.
36	0.55	1.36	0.74	0.05	3 292.5	93.2	730.2	0.17	0.66	0.10	0.67	963.2	46.9	670.1	0.04	0.44	0.41	0.54	2 405.1	42.1	n.d.
40	0.05	0.21	0.17	0.07	1 299.1	6.1	894.3	0.32	0.88	0.40	0.61	1 156.9	235.9	651.6	0.48	0.74	0.85	0.73	2 106.4	78.4	1 446.2
44	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.42	0.64	0.00	0.78	1 147.8	28.6	690.7	0.31	0.87	1.01	0.66	1 952.5	44.4	1 471.6
48	0.01	0.93	0.53	0.12	3 006.5	64.8	825.0	0.21	0.79	0.41	0.76	1 573.8	69.3	762.5	0.03	0.62	0.51	0.63	1 928.9	37.8	n.d.
52	0.60	0.98	1.42	0.29	971.3	64.8	664.5	0.14	1.00	0.53	0.56	1 094.7	72.3	848.7	0.40	0.78	0.59	0.69	1 862.4	81.8	688.1

IFN-γ = interferon gamma, TNF-α = tumor necrosis factor alpha, IL-2 = Interleukin 2, IL-15 = Interleukin 15, TM = Thrombomodulin, VEGF= vascular endothelial growth factor, FKN = CX3CL1/fractalkine, n.d.= not determined.

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SDC, Materials and Methods

Detection of soluble biomarkers

Plasma samples from were defrosted and used to measure VEGF, Thrombomodulin, IL-2, TNF-α, IFN-γ, IL-15 and the atypical chemokine CX3CL1 (Fractalkine). Soluble biomarkers were detected by electrochemiluminescence using a MSD multi-array assays (Meso Scale Discovery; Rockville, MD, USA). For each biomarker, the plasma samples were diluted in the appropriate medium and the tests were performed according to manufacturer's instructions.

Statistical model:

GAMMs were used to model the temporal pattern in biomarker responses. Where the response variable was expressed as 0/1 values (eg, the CD8-IFNγ response to CMV lysate, pp65 or IE1 peptide greater or equal to 0.25% above control) a binomial distribution with logit link function was used. The proportion of the CD57+GB+ population was modelled using a beta distribution with logit link function to handle the continuous and bounded (0-1) nature of the data. This model afforded considerably better fit to the observations and model diagnostics to the observations than a quasibinomial.

Smooth functions of the temporal pattern were fitted to the 3 patient groups via a smooth-factor interaction GAMM

$$\mu_{ij} = g\left(\beta_j PatientGroup_i + s_j(Week_i)\right)^{-1}$$

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$$Y_i = f_{\theta_i}(y_i)$$

where μ_{ij} is the expectation of the response variable for the ith patient in the jth patient group, $g(\ )^{-1}$ is the inverse of the link function $g(\ )$, β_j represents the mean response for the jth group and $s_j(\ )$ is a separate smooth function of the number of weeks posttransplant representing the temporal trend for each patient group. f_{θ_i} indicates an extended exponential family distribution (binomial or beta) with parameters θ determined by μ_{ij}. y_i is an observation of the random variable Y_i.

To account for the repeated measurements on multiple patients, a random intercept term for Patient was introduced to the model above via a random effect smooth, which equates to an assumption of i.i.d. Gaussian random effects [22].

Smoothness estimation for $s_j(\ )$ was performed via the restricted maximum likelihood (REML) method with an additional penalty on the null space of each smooth. The additional penalty applies a form of shrinkage which allows for smooths to be penalized towards zero degrees of freedom and hence selected out of the model during fitting [33].

To determine whether there were different responses in the 3 patient groups, models that included a single smooth for number of weeks posttransplant were also fitted. A likelihood ratio test was used to compare these nested models (refitted using maximum likelihood) to evaluate if patient group-specific time curves resulted in a better fit to the data than a single time curve for all 3 patient groups.

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All models were fitted using the mgcv package [21–23] for the R statistical Software (version 3.2.2; [24]).

Table S1: **Plasma levels of biomarkers**

Figure S1: **Analysis of PBMC populations and gating strategy**

The density plot profiles of PBMCs are shown as examples of the selected cell populations of interest for the identification of IFN-γ positive CD8+ and CD4+ T cells. 100 000 PBMC events were collected and selected. Cells were gated on $CD3^+$ positive cells, and then on CD4+ and CD8+ positive cells. A region was set using the unstimulated control sample, and this area was then used to identify cell populations expressing surface markers CD4+ and CD8+ cells and within those cells producing IFN-γ on samples stimulated with SEB, CMV lysate, IE-1 or pp65. For the analysis of cytokine production, a threshold of 0.25% above un-stimulated control was considered as positive.

Figure S2: **Detection of IFN-γ produced by CD8+ and CD4+ T cells of the 3 groups of patients stimulated with SEB**

IFN-γ responses by CD8+ (left panel) and CD4+ (right panel) cells in response to *Staphylococcus aureus* enterotoxin B at the week of the transplant (W0), week 1, week 2, week 4, then at 4-weekly intervals until week 32, and a final sample at week 52. Upper panels show patients in the CMV+VL+ group, middle panels are the CMV+VL- patient group, and the lower panel are patients in the CMV-VL- group. Each circle represents 1 patient. A line has been drawn at 0.25% to illustrate where the positive response threshold was set.

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