# High-dimensional mass cytometry identified circulating natural killer T-cell subsets associated with protection from cytomegalovirus infection in kidney transplant recipients

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**Cytomegalovirus (CMV) infection is associated with poor kidney transplant outcomes. While innate and adaptive immune cells have been implicated in its prevention, an in-depth characterization of the *in vivo* kinetics of multiple cell subsets and their role in protecting against CMV infection has not been achieved. Here, we performed high-dimensional immune phenotyping by mass cytometry, and functional assays, on 112 serially collected samples from CMV seropositive kidney transplant recipients. Advanced unsupervised deep learning analysis was used to assess immune cell populations that significantly correlated with prevention against CMV infection and anti-viral immune function. Prior to infection, kidney transplant recipients who developed CMV infection showed significantly lower CMV-specific cell-mediated immune (CMI) frequencies than those that did not. A broad diversity of circulating cell subsets within innate and adaptive immune compartments were associated with CMV infection or protective CMV-specific CMI. While percentages of CMV (tetramer-stained)-specific T cells associated with high CMI responses and clinical protection, circulating $CD3^{+}CD8^{mid}CD56^{+}$ NK-T cells overall strongly associated with low CMI and subsequent infection. However, three NK-T cell subsets sharing the CD11b surface marker associated with CMV protection and correlated with strong anti-viral CMI frequencies *in vitro*. These data were validated in two external independent cohorts of kidney transplant recipients. Thus, we newly describe the kinetics of a novel NK-T cell subset that may have a protective role in post-transplantation CMV infection. Our findings pave the way to more mechanistic studies aimed at understanding the function of these cells in protection against CMV infection.**

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Cytomegalovirus (CMV) is a β herpes virus belonging to the *Herpesviridae* family, which primarily threatens immunocompromised patients, such as transplant recipients, in whom primary infection or viral reactivation after transplantation occurs frequently and associates with significant morbidity and poor graft outcomes.[1–4]

## Lay Summary

Cytomegalovirus (CMV) infection is a main opportunistic infection occurring after kidney transplantation and has a negative impact on both patient and graft outcomes. In the past years, numerous studies have highlighted the role of different components of the immune system in the protection against CMV. However, this diversity and how they behave over time prior and after transplantation has not been fully elucidated yet. Using advanced new technology, such as mass cytometry (with cytometry by time of flight), which enables the detection of cellular populations at an unprecedented precision level in peripheral blood as well as functional tests, we here show the broad diversity of circulating cell subsets of the innate and adaptive immune system that contribute to the protection against CMV infection in a cohort of CMV kidney transplant recipients (KTRs). Most interestingly, we describe a specific cell subset population within natural killer T cells, coexpressing the $CD11b^+$ surface marker, which are strongly associated with CMV protection. We further validated these data in 2 independent cohorts of KTRs.

Despite the fact that the presence of CMV-specific IgG antibodies are widely used for determining prior exposure to viral infection, they are not sufficient to prevent and control CMV infection after transplantation.[5,6] In the past years, an important body of evidence has highlighted the role of cell-mediated immunity (CMI), particularly driven by memory/effector $CD8^+$ T cells, promoting protection against CMV infection in kidney transplant patients, and its assessment is being proposed as a novel biomarker for immune-risk stratification in clinical transplantation.[7–10] Furthermore, recent works have shown an important role of other cell subsets, such as γδ-T and natural killer (NK) cells, both considered to display shared innate and adaptive immune features,[11,12] controlling CMV replication after transplantation.[13,14] Among these cellular compartments, $V\delta2^{neg}\gamma\delta$ and $NKG2C^+CD57^+$ NK T-cell subsets have been shown to be key in inhibiting and controlling viral spread in close collaboration with T cells.[15,16] These subsets share similar biological features with αβ T cells, as they may acquire effector memory phenotypes and anti-viral cytotoxic function.[17,18] However, although $V\delta2^{neg}\gamma\delta$ T cells do not recognize viral peptides presented by human leukocyte antigen molecules, but rather they recognize CMV-induced "stress self-antigens" at the infected cell surface, some NK-T cells harbor CMV-specific T-cell receptor signaling[19] and show a rapid and relatively early cytotoxic immune response, highlighting a memory-like signature enabling a quick anti-viral response.[20] Importantly, and unlike αβ T cells, these cells seem to have their main role in viremic patients and not before the viral onset.

Although most studies have investigated the contribution of these distinct cell subsets individually, a thorough deep phenotype assessment of the diversity, *in vivo* kinetics, as well as their contribution to protective anti-viral functional immune responses assessed *in vitro* has not been investigated yet. Herein, we performed deep phenotype analysis of peripheral blood mononuclear cells (PBMCs) using mass cytometry with cytometry by time of flight (CyTOF) and assessment of CMV-specific CMI *in vitro* at serial time points prior and after kidney transplantation to unravel the most prominent circulating immune cell subsets associated to both protection against CMV infection and to functional anti-viral cellular immune responses. We took advantage of kidney transplant recipients participating in a prospective, randomized clinical trial assessing the utility of measuring CMV-specific CMI for infection-risk stratification.[10] The advanced semiquantitative deep learning discriminant analysis used enabled us to show the wide diversity of cell subsets playing a distinct role in preventing CMV infection. Most interestingly, we identified specific cell subsets within the NK T-cell compartment that strongly associate with protective *in vitro* CMI and discriminate patients at higher risk of developing CMV infection, which was further validated in 2 external kidney transplant cohorts.

## METHODS

### Study design and patients of the study

The study included a training and 2 validation cohorts. The training cohort consisted of 28 seropositive kidney transplant patients receiving a seropositive graft ($D^+/R^+$) who participated in the multicenter, prospective randomized clinical trial (NCT02550639)[10] (Supplementary Figure S1). For these patients, 112 consecutive PBMC samples were available at 4 distinct time points: before transplantation, at 15 or 30 days and at 3 and 12 months after transplantation. Two additional independent cohorts of 56 $R^+$ kidney transplant patients were also analyzed. The first cohort of 28 consecutively transplanted patients between 2021 and 2022 was participating in a retrospective, observational study (PI18/01832) and PBMC samples were available before CMV infection, in which circulating cellular phenotypes are evaluated for their association with different opportunistic viral infections. Also, a second external validation cohort of 34 $R^+$ patients transplanted between 2017 and 2020, in whom PBMC samples were available at distinct time points before CMV infection, was included from the Horizon 2020 EU-TRAIN (The European Transplantation and Innovation Consortium for Risk Stratification in Kidney Transplant Patients) study (754995), in which distinct noninvasive peripheral blood biomarkers are assessed for predicting allograft rejection.[21]

All patients received an anti–interleukin-2 receptor monoclonal antibody induction and tacrolimus, mycophenolic mofetil, and corticosteroids as maintenance therapy. All patients were followed up for 12 months. Main clinical, demographic, and immunologic data from all donors and recipients from the 3 cohorts were collected for analyses in this study (Table 1; Supplementary Tables S1 and S2). The study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by our Institutional Review Board (AC148/13).

### CMV preventive strategies

In the training cohort, 2 patients received prophylaxis (7.14%) and were treated with valganciclovir, 900 mg/d (and adjusted to the glomerular filtrate rate of the patients), during

**Table 1 | Clinical and demographic characteristics of the training cohort**

| Demographics | Training cohort (n = 28) | | |
|---|---|---|---|
| | Infected (n = 12 [43%]) | Noninfected (n = 16 [57%]) | *P* value |
| Recipient age, yr | 65.18 [47–76.25] | 63.96 [53.16–71.68] | 0.529 |
| Recipient sex (F) | 4 (33.33) | 6 (37.50) | 0.820 |
| Caucasian ethnicity (yes) | 12 (100) | 15 (93.75) | 0.378 |
| CMV serostatus MM $D^+/R^+$ | 12 (100) | 16 (100) | 1.000 |
| Type of donor (deceased) | 11 (91.67) | 14 (87.50) | 0.724 |
| First transplant (yes) | 12 (100) | 16 (100) | 1.000 |
| Immunosupression (yes) | | | |
| Calcineurin inhibitors (TAC/CsA) | 12 (100) | 16 (100) | 1.000 |
| MMF/MPA | 12 (100) | 16 (100) | 1.000 |
| Corticosteroids | 9 (75) | 15 (93.75) | 0.161 |
| No. of HLA mismatches | | | |
| Class I mismatch | 3 [2–4] | 3 [2–3] | 0.473 |
| Class II mismatch | 1 [1–2] | 1 [1–1] | 0.130 |
| DGF (yes) | 2 (16.67) | 4 (25) | 0.595 |
| BPAR | | | |
| TCMR (yes) | 1 (3.6) | 0 (0) | 0.238 |
| ABMR (yes) | 1 (3.6) | 0 (0) | 0.238 |
| CMV screening strategy | | | |
| Preemptive (yes) | 10 (93.30) | 16 (100) | 0.090 |
| Antiviral prophylaxis (yes) | 2 (16.70) | 0 (0) | NA |
| CMV events | | | |
| Infection (yes) | 12 (100) | 0 (0) | NA |
| Disease (yes) | 2 (16.67) | 0 (0) | NA |
| Peak CMV viral load, IU/mL | 9396 [1745–20,614] | NA | NA |
| Mean time of CMV infection, mo | 2.38 [1.25–3.50] | NA | NA |
| Mean time of CMV disease, mo | 2.63 [1.19–3.75] | NA | NA |
| Antiviral treatment (yes) | 8 (66.70) | 0 (0) | <0.001 |
| Graft loss (yes) | 0 (0) | 0 (0) | 1.000 |
| Patient death (yes) | 0 (0) | 0 (0) | 1.000 |

ABMR, antibody-mediated rejection; BPAR, biopsy-proven acute rejection; CMV, cytomegalovirus; CsA, cyclosporine; $D^+$, donor with CMV-IgG+ serology; DGF, delayed graft function; F, female; HLA, human leukocyte antigen; MM, mismatch; MMF, mycophenolate mofetil; MPA, mycophenolic acid; NA, not available; $R^+$, recipient with CMV-IgG+ serology; TAC, tacrolimus; TCMR, T cell–mediated rejection.
Data are given as median [interquartile range] or n (%).

the first 100 days after transplantation. After stopping prophylaxis, patients were followed preemptively with CMV quantitative nucleic acid testing every 15 days until month 4 and at months 6 and 12 after transplantation. On the other hand, 26 patients (92.86%) followed the preemptive strategy and were tested for CMV quantitative nucleic acid testing during the first month every 2 weeks until month 3, monthly until month 6, and every 2 months until month 12 after transplantation.

### Clinical definitions of CMV infection

The definition of CMV infection and disease was based on the criteria recommended by the CMV Drug Development Forum.[10] CMV infection was defined as the detection of CMV DNA replication in plasma, whereas CMV disease was defined as evidence of CMV DNA replication with compatible symptoms, including both viral syndrome and invasive tissue disease.

### CMV-specific CMI

All samples from the first transplant cohort were assessed at all time points of the study for CMV-specific CMI against the 2 main CMV immunogenic antigens (IE-1 and pp65) using an interferon (IFN)-γ Enzyme-linked ImmunoSpot assay (T-Spot.CMV), as previously described.[10]

### CyTOF sample preparation and data acquisition and analysis

CyTOF data were acquired at Icahn School of Medicine at Mount Sinai, as previously reported.[22–26] CyTOF sample preparation (Supplementary Table S3) and data acquisition and data analysis (Supplementary Figure S2) are thoroughly described in the Supplementary Methods (Figure 1).

### Flow cytometry analysis

PBMC samples from 62 kidney transplant recipients of the 2 validation cohorts (N = 28 validation cohort A and N = 34 validation cohort B), harvested at the time and post-transplantation (before CMV infection), were evaluated with flow cytometry (LSRFortessa; BD Biosciences) for the presence and frequencies of most determinant cell effectors discriminating patients from CMV infection observed by CyTOF analyses. PBMCs were stained with anti–CC chemokine receptor 7 (BV786, clone 2-L1-A, BD Bioscience), CD38 (B650, clone HB-7, BioLegend), CD3 (BV605, clone OKT3, BioLegend), programmed death-1 (PD1; BV421, clone EH12.1, BD Bioscience), CD45 (BB700, clone HI30, BD Bioscience), CD57 (AF488, TB01, FisherScientific), CD56 (PECy7, clone NCAM-1, BD Bioscience), CD11b (PECy55, clone M1/70, BioLegend), CD45RA (ECD, clone 2H4, Beckman Coulter), CD95 (PE, clone UB2, Beckman Coulter),

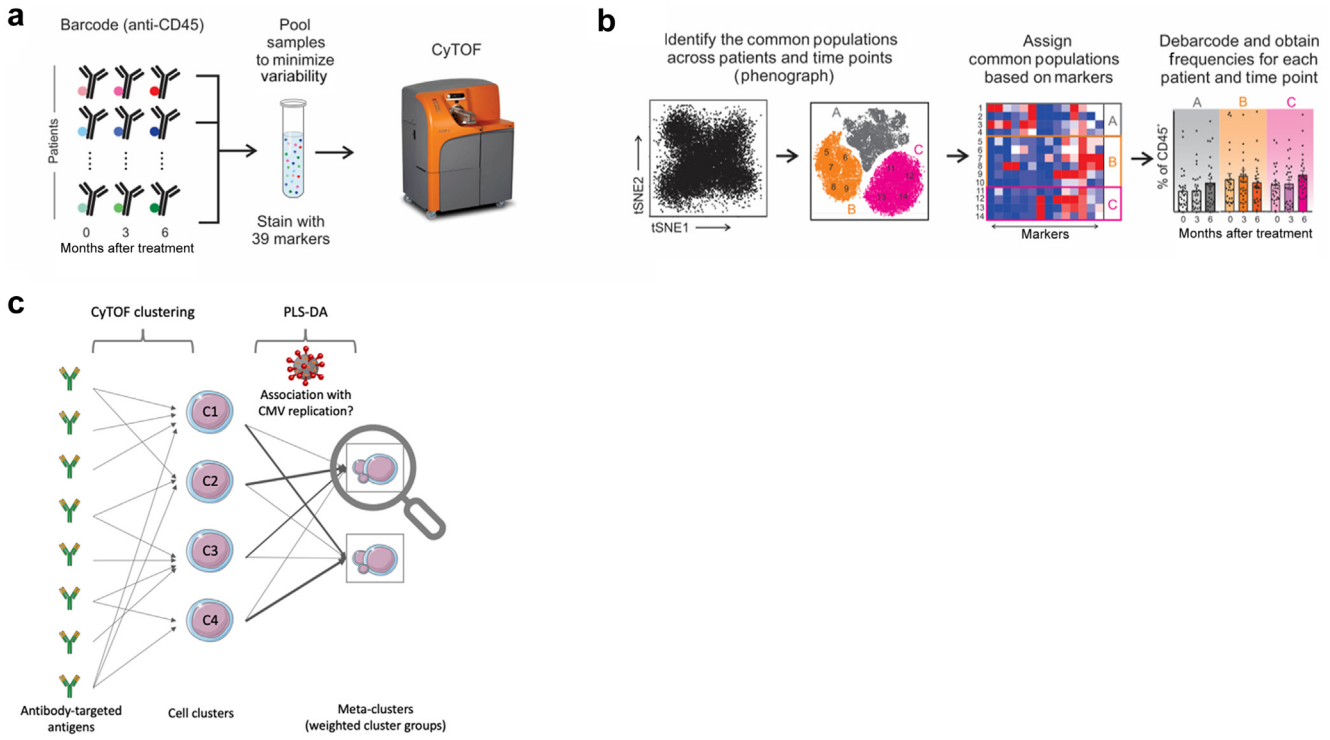


**Figure 1 | Overall procedure of the experimental work and analysis performed in the study.** (**a**) Cytometry by time of flight (CyTOF) sample preparation. Samples were barcoded with anti-CD45 antibodies conjugated to unique metal isotopes before pooling them together. All peripheral blood mononuclear cells were stained with a panel of 40 antibodies and processed in 3 separate batches using 5 barcoding antibodies. (**b**) CyTOF data acquisition. Samples were acquired on a CyTOF2 (Fluidigm) at a concentration of 1 million cells/ml at an event rate of <500 events/second. (**c**) CyTOF data analysis. Unsupervised clustering on cells was performed by Phenograph algorithm and the analysis strategy was structured in 3 layers: (i) major immune cell types, (ii) immune subtype of CD4 and CD8 T cells, and (iii) further investigation of cell subtypes of γδ-T cells, natural killer T cells, and cytomegalovirus (CMV)–specific T cells. Projection to latent structures–discriminant analysis (PLS-DA) was used to evaluate the association between cell phenotypes and CMV infection events. tSNE, t-distributed stochastic neighbor embedding.

CD8 (AF700, clone B9.11, Beckman Coulter), CD127 (APC, clone A019D5, BioLegend), and LIVE/DEAD Fixable Aqua (ThermoFisher Scientific). The CD11b-positive NK-T cells were assessed with a gating of $CD3^+$ $CD8^{mid}$ $CD56^+CD11b^+$ (Supplementary Figure S3).

### Statistical analyses

We analyzed the frequencies of major immune compartments observed by CyTOF in peripheral blood using unbiased clustering (Phenograph). Correlation between CyTOF clusters and CMV infection events was assessed using the clusters of cells defined by the CyTOF analysis. We used a projection to latent structures–discriminant analysis (also referred to as partial least squares–discriminant analysis)[22,27] to evaluate the association between cell phenotypes and CMV infection events, as recently shown.[28] In brief, numbers of cells within each of the original cell populations (e.g., $CD4^+$ T cells, or the various NK-T clusters) were used as independent covariates for the projection to latent structures–discriminant analysis algorithm, predicting the clinical end point "CMV infection at any point post-transplantation" (binary outcome, either the patient underwent a CMV infection or not). This enabled us to define meta-clusters (i.e., linear combination of predictors [cell populations, or cell clusters] that were associated with CMV infection). The main aim of the assessment was to detect cell populations, or clusters, associated with a subsequent CMV infection. We therefore restricted our analysis to samples analyzed at any time point before any CMV event. Each cluster has a weight within the meta-cluster. A positive result translates into an increased number of cells from this cluster within the corresponding meta-cluster, whereas a negative result translates into a decreased population within this meta-cluster. This analysis allows us to weight different variables toward a specific direction and therefore, it allows us to investigate protective or detrimental associations between cell clusters and CMV infection events.

Also, the correlation between cell populations and peripheral CMV-specific CMI at each time point was investigated by Spearman rank correlation test.

Continuous variables were expressed as median (interquartile range) and categorical variables as number of total (n) and percentage. A comparison between groups was performed using Pearson $\chi^2$ test for categorical data. Continuous measurements were compared among groups using analysis of variance and *t* tests. $P < 0.05$ was considered statistically

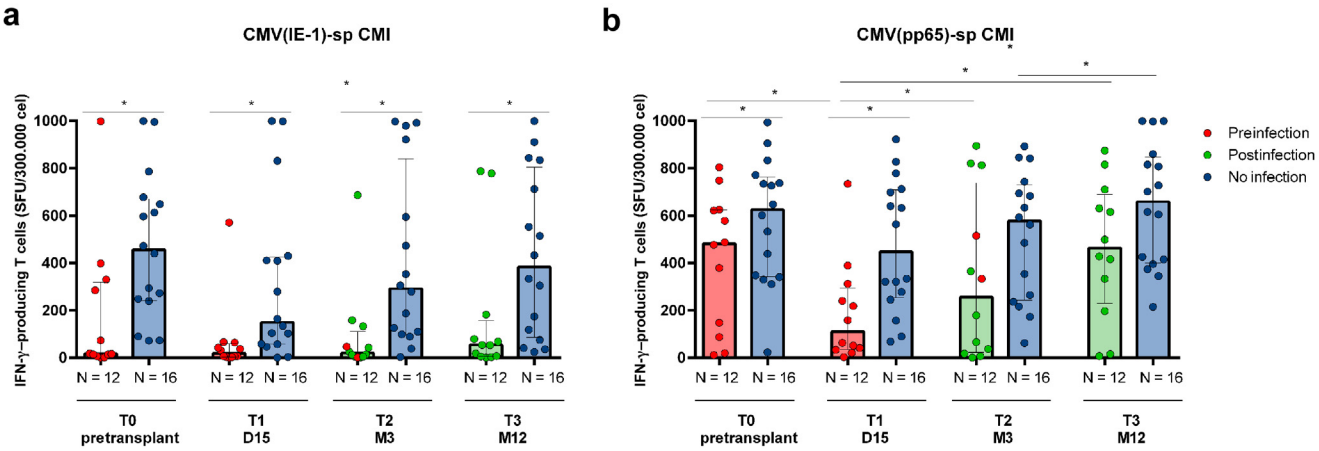


**Figure 2 | Functional cytomegalovirus (CMV)–specific (sp) cell-mediated immunity (CMI) and CMV infection.** Before CMV infection, CMI against (**a**) IE-1 and (**b**) pp65. D15, 15 or 30 days after transplantation; IFN-γ, interferon-γ; M3, 3 months after transplantation; M12, 12 months after transplantation; SFU, spot forming unit.

significant. Univariate comparison of cell populations frequencies, at different time points, were performed by a Wilcoxon test. The Benjamini and Hochberg correction was used to account for multiple tests and to limit the false discovery rate to 5%.

## RESULTS

### Study cohort and main clinical outcomes

All patients in the training cohort received the same immunosuppressive regimen (Table 1). Twelve (43%) developed CMV infection and 2 of them CMV disease with gastrointestinal involvement, whereas 16 (57%) did not develop CMV infection during the 12-month study follow-up period. The peak of viremia was 9396 copies (range, 1745–20,614 copies). No major demographic, clinical, and immunologic differences were observed between patients with viremia and patients without viremia.

### Functional CMV-specific CMI and CMV infection

Patients developing CMV infection displayed significantly lower CMV-specific CMI against both IE-1 and pp65 CMV antigens than those who did not at all time points before infection (17 [8–318.75] vs. 456 [241.25–669.75], $P = 0.004$ for IE-1 pre-transplant; 482 [102.25–624] vs. 624 [342–762.50], $P = 0.189$ for pp65 pre-transplant; 18.50 [3.75–58.50] vs. 149 [57.25–425.25], $P = 0.006$ for IE-1 at 15 or 30 days after transplantation; 110.50 [35–294] vs. 448 [253.25–708.50], $P = 0.004$ for pp65 at 15 or 30 days after transplantation; Figure 2). CMV-specific CMI, especially those reactive to pp65, significantly recovered after infection (19.50 [4–111.50] vs. 292 [101.75–839.25], $P = 0.003$ for IE-1 at 3 months after transplantation; 256 [22–737.75] vs. 577.50 [243.25–730.75], $P = 0.133$ for pp65 at 3 months after transplantation; 53.50 [5.50–156.25] vs. 383 [85.75–803.50], $P = 0.013$ for IE-1 at 12 months after transplantation; and 463.50 [231–690] vs. 658 [400.75–848], $P = 0.189$ for pp65 at 12 months after transplantation).

### Major immune cell subset populations and CMV infection

We next analyzed the frequencies of distinct cell subsets within major immune cell compartments at all study time points (Figure 3; Supplementary Table S4), and we compared them between patients who developed CMV infection and patients who did not. As illustrated in Supplementary Figures S4A–C, statistically significant differences in major immune cell compartments were only present in NK and dendritic cells before CMV infection between the 2 cohorts of transplant recipients. Differences in NK-T cells were observed between the 2 groups after CMV viremia (Supplementary Table S5). No differences over time or between severity of CMV viremia were observed for any of the cellular populations (data not shown).

### Role of parental cellular meta-clusters on CMV infection and CMI

Although *in vivo* protection and *in vitro* CMI have been predominantly attributed to peripheral $CD4^+$ and especially $CD8^+$ T cells, other cell subsets may produce IFN-γ in response to CMV antigens.

We aimed to merge all major immune cell compartments together to decipher their global effect driving CMV immune protection. As shown in Figure 4a, although higher percentages of NK-T and $CD4^+$ T cells associated with higher risk of CMV infection, high percentages of CMV-specific $CD8^+$, γδ T cells, and $CD8^+$ T cells were associated with protection against CMV infection. Indeed, the temporal evolution of this meta-cluster discriminated patients who subsequently developed CMV infection from those who did not (Figure 4b). The association between these global cellular compartments with CMV-specific CMI did not reveal any significant correlations, but surprisingly only a modest negative correlation with $CD4^+$ T cells ($R = -0.205$, $P = 0.003$ for IE-1 and $R = -0.208$, $P = 0.038$ for pp65; Figure 4c).

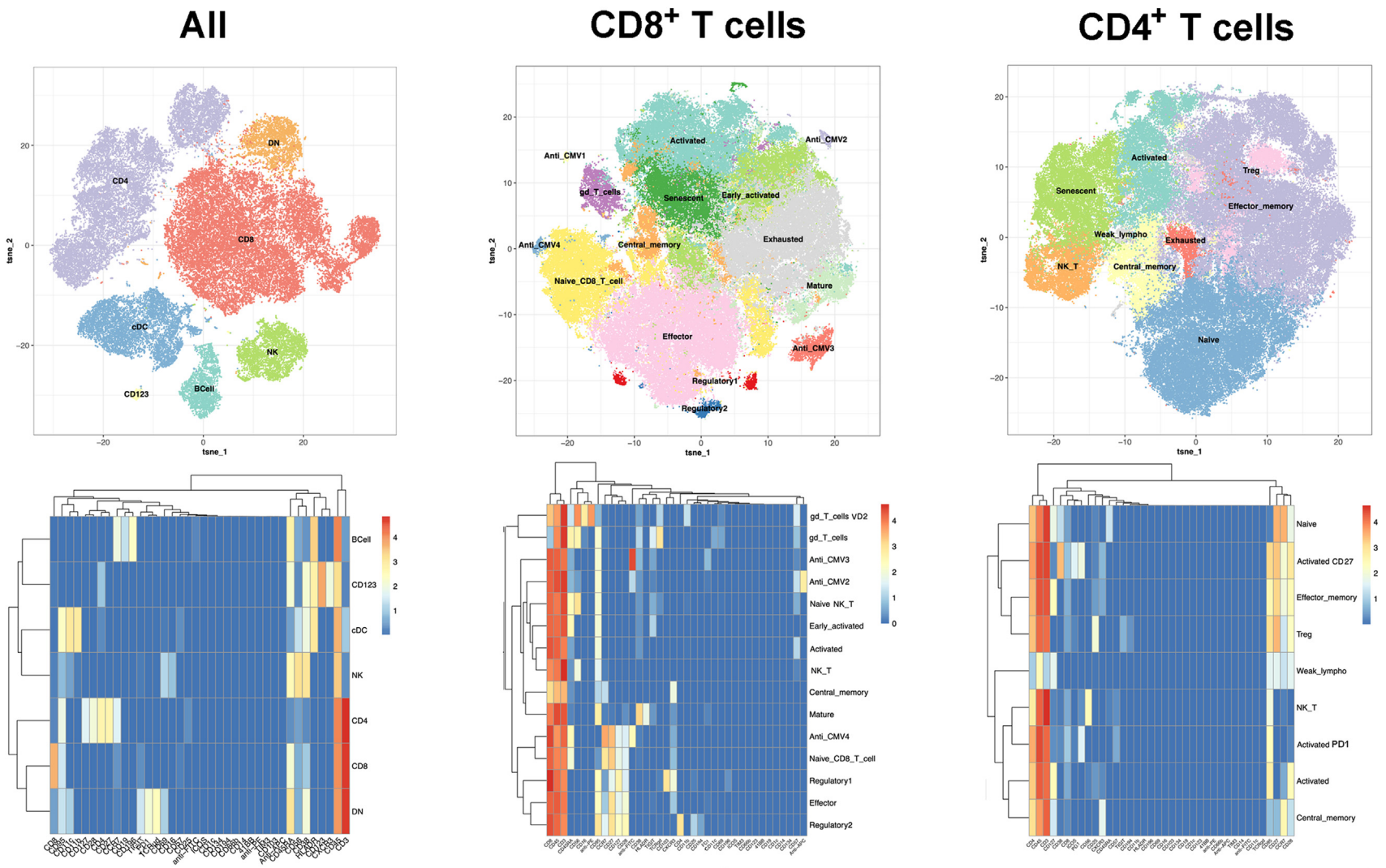


**Figure 3| Unbiased immune cell clustering analysis of major immune cell compartments in peripheral blood.** Two-dimensional projection graphs were created with visual stochastic neighbor embedding (viSNE). Heat maps represent all clusters and cellular populations in the *y* axes (right) and extracellular markers in the *x* axes. CMV, cytomegalovirus; NK, natural killer; sp, specific. (Continued)

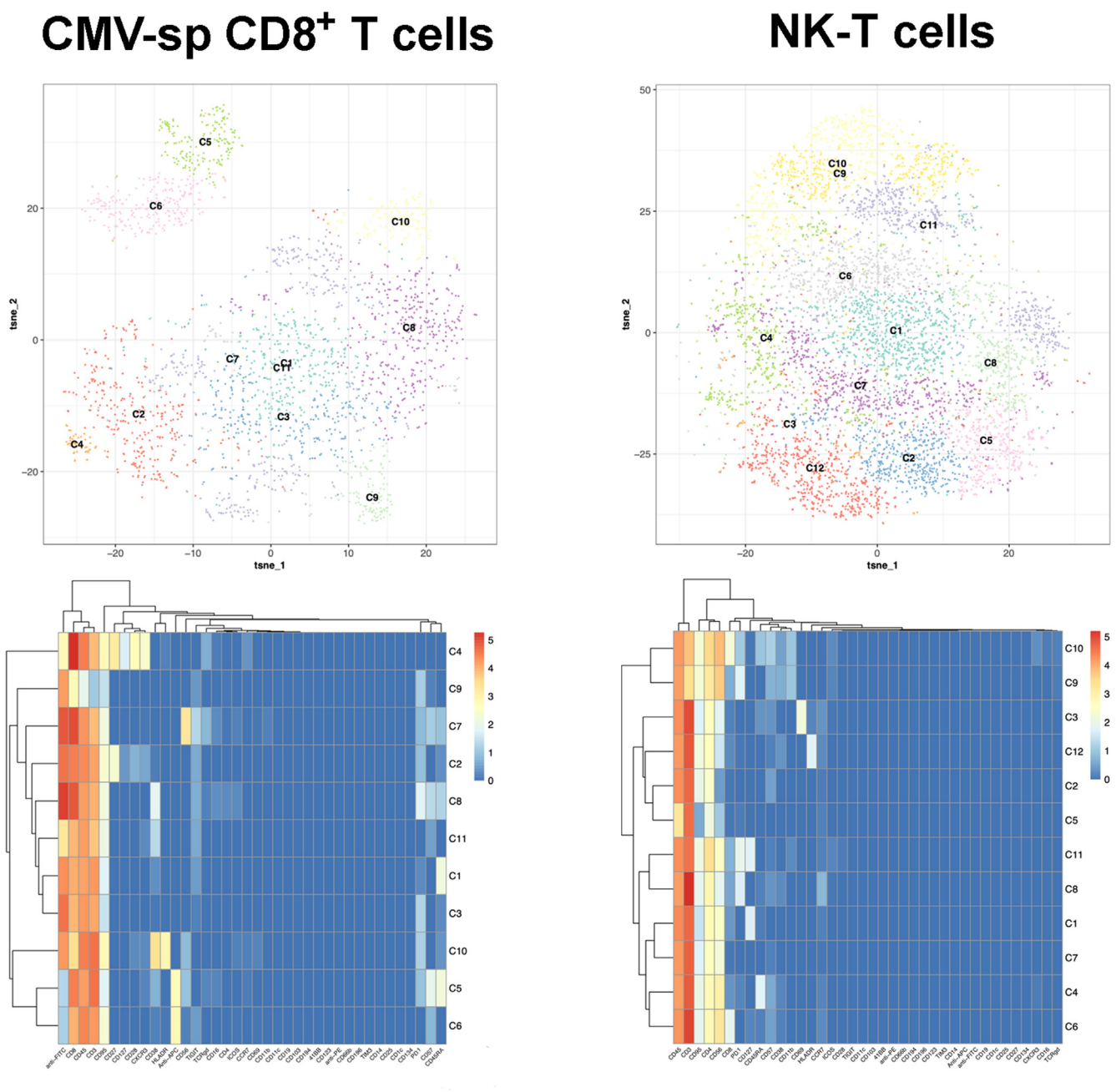


**Figure 3 |** (Continued)

The barely detectable frequencies of circulating γδ T cells did not enable us to perform any cluster analysis.

### Association between circulating T-cell subsets and CMV infection

We next aimed to break down all parental cell compartments into the different cell subsets and combine all the different emerging cell clusters into significant meta-clusters discriminating transplant patients subsequently developing CMV infection using the projection to latent structures–discriminant analysis statistical method (Figure 1). As for the $CD8^+$ T-cell subsets, although higher percentages of NK-T, naive NK-T, and naive $CD8^+$ T cells associated with higher risk of CMV infection, high percentages of activated and central/memory $CD8^+$ T cells correlated with CMV protection (Figure 5a). The temporal progression of this $CD8^+$ T-cell meta-cluster discriminated patients with subsequent development of CMV infection from those who did not (Figure 5b).

The analysis of the distinct $CD4^+$ T-cell clusters revealed that higher percentages of naive, effector/memory, and activated $CD4^+$ T cells associated with higher risk of CMV

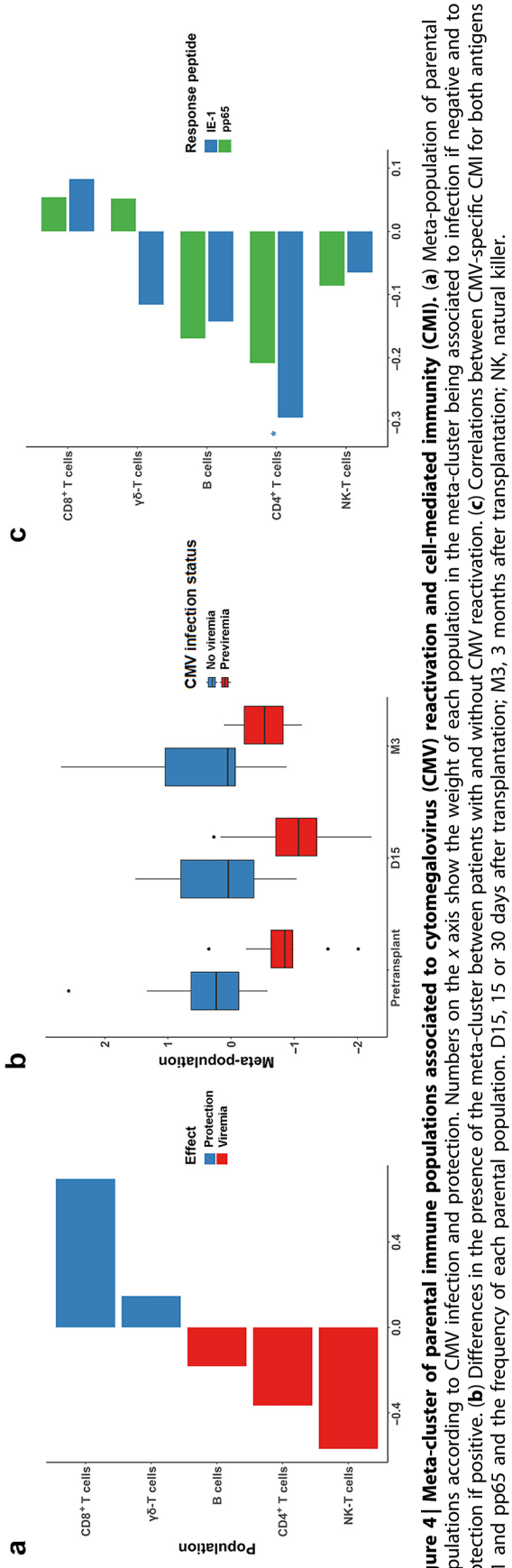


**Figure 4| Meta-cluster of parental immune populations associated to cytomegalovirus (CMV) reactivation and cell-mediated immunity (CMI).** (**a**) Meta-population of parental populations according to CMV infection and protection. Numbers on the *x* axis show the weight of each population in the meta-cluster being associated to infection if negative and to protection if positive. (**b**) Differences in the presence of the meta-cluster between patients with and without CMV reactivation. (**c**) Correlations between CMV-specific CMI for both antigens IE-1 and pp65 and the frequency of each parental population. D15, 15 or 30 days after transplantation; M3, 3 months after transplantation; NK, natural killer.

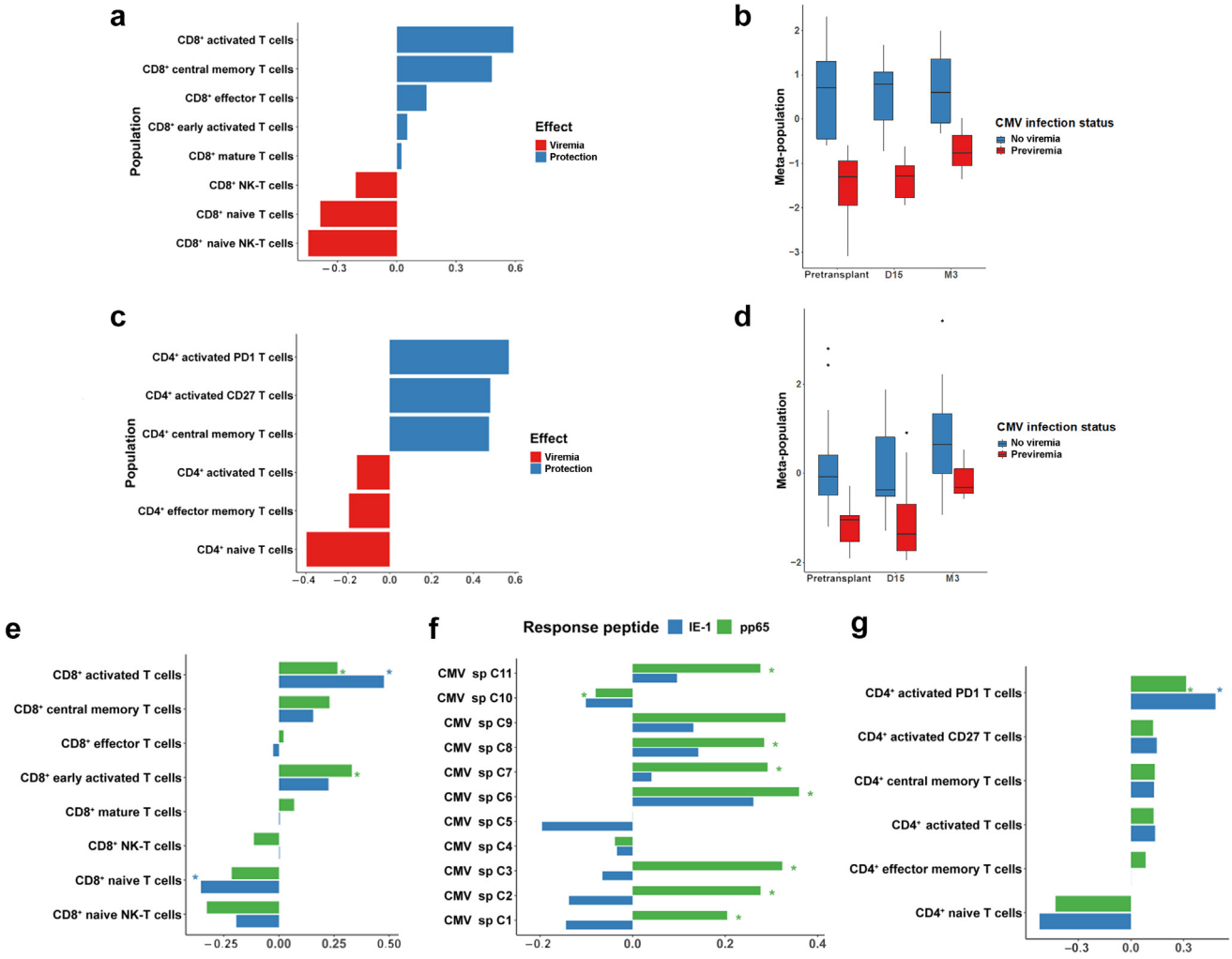


**Figure 5 | CD8$^+$ and CD4$^+$ T-cell meta-clusters associated to cytomegalovirus (CMV) reactivation and CMV-specific (sp) cell-mediated immunity (CMI).** (**a**) Meta-population of all CD8$^+$ T-cell populations according to CMV infection and protection. Numbers on the *x* axis show the weight of each population in the meta-cluster being associated to infection if negative and to protection if positive. (**b**) Differences in the presence of the meta-cluster between patients with and without CMV reactivation. (**c**) Meta-population of all CD4$^+$ T-cell populations according to CMV infection and protection. Numbers on the *x* axis show the weight of each population in the meta-cluster being associated to infection if negative and to protection if positive. (**d**) Differences in the presence of the meta-cluster between patients with and without CMV reactivation. (**e**) Correlations between CMV-specific CMI and CD8$^+$ T-cell subpopulations. (**f**) Correlations between CMV-specific CMI for IE-1 and pp65 antigens and the frequency of CMV-specific CD8$^+$ T-cell subpopulations. (**g**) Correlations between CMV-specific CMI and CD4$^+$ T-cell subpopulations. D15, 15 or 30 days after transplantation; M3, 3 months after transplantation; NK, natural killer.

infection, whereas high percentages of PD1$^+$ and CD27$^+$ activated CD4$^+$ T cells and central/memory CD4$^+$ T cells positively associated with CMV protection (Figure 5c). The temporal progression of this CD4$^+$ T-cell meta-cluster discriminated patients subsequently developing CMV infection from those who did not (Figure 5d).

### Association between circulating T-cell subsets and CMI

The correlation of the CD8$^+$ T cells cell meta-cluster with CMV-specific CMI showed a positive correlation between activated and early/activated CD8$^+$ T cells with CMV-specific CMI (CD8 activated: $R = 0.476$, $P < 0.001$ for IE-1; and CD8 early activated: $R = 0.331$, $P < 0.001$ for pp65), whereas naive CD8$^+$ T cells inversely correlated with CMI responses ($R = –0.353$, $P = 0.03$ for IE-1; Figure 5e). We then specifically analyzed the correlation between different CMV (tetramer-stained)–specific CD8$^+$ T-cell subsets with CMV-specific CMI. Although both IE-1– and pp65-specific CD8$^+$ T cells were detected in peripheral blood, a positive correlation was predominantly observed between CMV-specific CMI against pp65 antigen and CD8$^+$(pp65) CMV-specific T cells at different time points (T0: $R = 0.651$, $P = 0.003$; T1: $R = 0.542$, $P = 0.017$; T2: $R = 0.418$, $P = 0.075$; T3: $R = 0.514$, $P = 0.024$), as well as with the different CD8$^+$(pp65) CMV-specific T-cell sub-clusters before viral replication assessed by CyTOF (C1: $R = 0.304$, $P = 0.002$; C2: $R = 0.297$, $P = 0.047$; C3: $R = 0.323$, $P = 0.002$; C7: $R = 0.292$, $P = 0.024$; C8: $R = 0.498$, $P = 0.032$; and C11: $R = 0.276$, $P = 0.002$; Figure 5f; Supplementary Table S6).

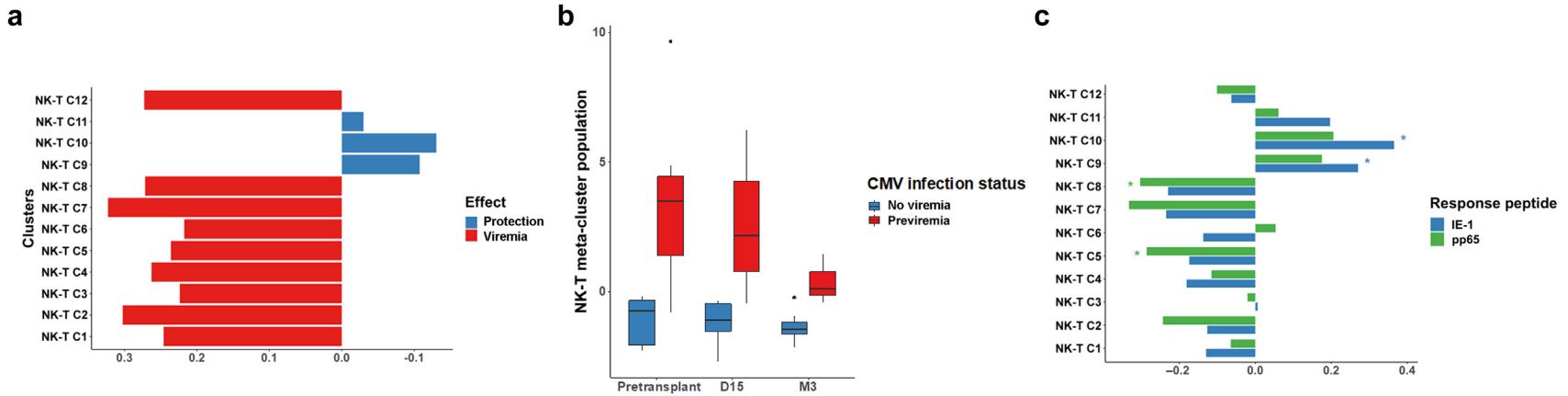


**Figure 6| Natural killer (NK) T-cell meta-cluster associated to cytomegalovirus (CMV) reactivation and cell-mediated immunity (CMI).** (**a**) Meta-population of all NK T-cell populations according to CMV infection and protection. Numbers on the *x* axis show the weight of each population in the meta-cluster being associated to infection if negative and to protection if positive. (**b**) Differences in the presence of the meta-cluster between patients with and without CMV reactivation. (**c**) Correlations between CMV-specific CMI for both antigens IE-1 and pp65 and the frequency of each NK T-cell population. D15, 15 or 30 days after transplantation; M3, 3 months after transplantation.

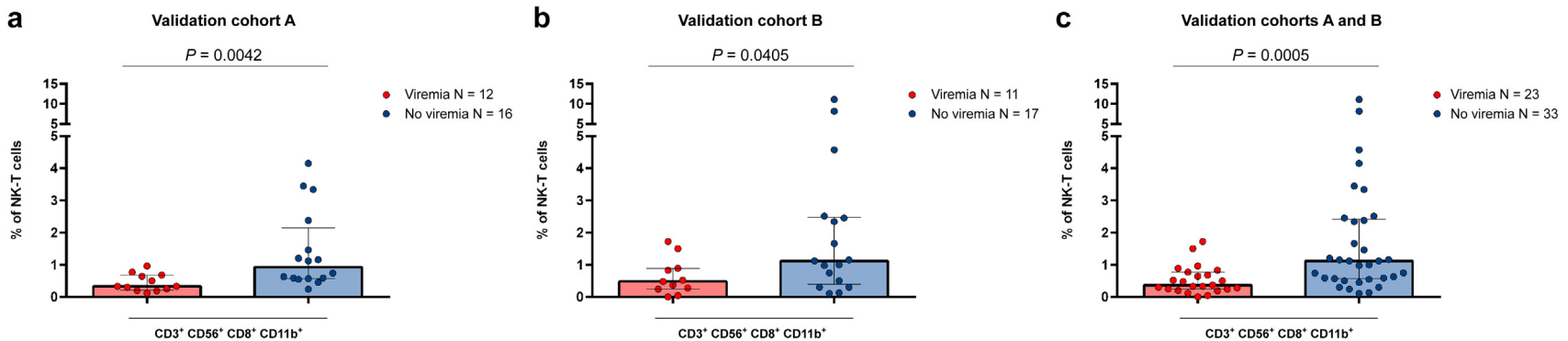


**Figure 7| Frequencies of CD11b$^{+}$ natural killer (NK) T cells in patients with cytomegalovirus viremia and without viremia.** (**a**) Validation cohort A. (**b**) Validation cohort B. (**c**) All patients from validation cohorts A and B.

The correlation between the $CD4^+$ T-cell meta-cluster with CMV-specific CMI showed a positive correlation of $PD1^+$ activated $CD4^+$ T cells ($R = 0.482$, $P < 0.001$ and $R = 0.315$, $P < 0.001$, for IE-1 and pp65, respectively), whereas naive $CD4^+$ T cells inversely correlated with CMI responses ($R = –0.521$, $P < 0.001$ for IE-1 and $R = –0.430$, $P < 0.001$ for pp65; Figure 5g). Although not statistically significant, patients not developing CMV replication showed a progressive increase of these $CD8^+$(pp65) CMV-specific T cells during the first months after transplantation (T1: 0.012 [0.003–0.049] vs. T2: 0.040 [0.004–0.087], $P =$ not significant) and were also numerically higher than in those patients who subsequently developed CMV replication (T1: 0.004 [0.001–0.010] vs. T2: 0.004 [0.001–0.036], $P =$ not significant). Interestingly, after CMV replication, these cells significantly expanded over time (T3: 0.009 [0.035–0.061]).

### Specific circulating NK T-cell subsets associate with immune protection against CMV infection

In view of the strikingly strong association between NK T-cell subsets with CMV infection, we further assessed the role of the distinct NK T-cell clusters by building new meta-clusters related to CMV infection and *in vitro* CMI. As illustrated in Figure 6a, most of the 12 cluster components of the NK T-cell meta-cluster favored the subsequent development of CMV infection and negatively correlated with CMV-specific CMI, similar to the global NK T-cell meta-cluster. Conversely, 3 $CD3^+CD8^{mid}CD56^+CD11b^+$, NK T-cell subsets (clusters 9, 10, and 11) associated with CMV protection and peripheral CMV-specific CMI against the 2 main immunogenic CMV antigens ($R = 0.365$, $P = 0.003$ and $R = 0.206$, $P < 0.001$ for IE-1 and pp65, respectively; Figure 6a–c; Supplementary Table S7).

To validate the association between this specific NK T-cell subset population with lower incidence of subsequent CMV infection, we measured these cells in 2 independent kidney transplant cohorts (Supplementary Tables S1 and S2). As shown in Figure 7, patients developing CMV infection showed statistically significant lower percentages of $CD3^+CD8^{mid}CD56^+CD11b^+$ NK-T cells before infection than patients not developing CMV infection (0.33 [0.21–0.67] vs. 0.93 [0.58–2.15], $P = 0.0042$ and 0.48 [0.24–0.89] vs. 1.12 [0.4–2.48], $P = 0.0405$) in both validation cohorts A (Figure 7a) and B (Figure 7b), respectively. The same significant difference persisted when we combined the 2 validation cohorts (0.37 [0.24–0.77] vs. 1.12 [0.56–2.42], $P = 0.0005$ between patients with viremia and without viremia, respectively; Figure 7c).

## DISCUSSION

Herein, we unraveled the kinetics and diversity of circulating immune cell subsets associated to either protection or risk of developing CMV reactivation after kidney transplantation, and we identified an NK T-cell subset whose concentration in peripheral blood can discriminate patients at higher risk of developing CMV reactivation. Such subsets were not previously identified using conventional flow-cytometry technologies. Furthermore, the prospective assessment of CMI specific to CMV performed in all patients allowed us to identify those circulating cell subsets associated with such protective anti-viral immune response.

By using CyTOF and advanced unsupervised deep learning analysis, we deciphered the association between each cell subset taken singularly and as meta-clusters and the subsequent development of CMV infection. We found that, although activated and central/memory $CD4^+$ and $CD8^+$ T-cell numbers directly correlated with CMV immune protection and CMV-specific ELISPOT, dendritic cells, $CD8^+$ and $CD4^+$ naive T cells, as well as NK-T cells seem to counterbalance such immune-infection risk and associated with higher infection risk and inversely with CMV-specific CMI. Most interestingly, we identified up to 11 different CMV-specific $CD8^+$ T-cell subsets, stained with major histocompatibility complex class-I peptide (IE-1/pp65) tetramers that were overexpressed in patients not developing CMV infection and correlated with *in vitro* functional immunity, highlighting the contribution of these distinct CMV-specific cells in promoting protection against CMV infection in seropositive kidney transplant recipients (KTRs). Interestingly, although most CMV(tetramer)-specific cells were conventional CD8 T cells, a small cell subset population did also express CD56, thus suggesting that a small proportion of these cells do also express NK-cell markers. Although the association between dendritic cells and increased incidence of CMV reactivation may seem counterintuitive, it has been postulated that because CMV may also infect these cells, it may lead to functional perturbations and suboptimal recognition of antigens preventing robust effector adaptive immune responses.[29]

Although *in vitro* CMI has been predominantly attributed to peripheral $CD8^+$ and, to a lower extent, $CD4^+$ T cells, we tested the contribution of other immune populations to these protective immune responses. Therefore, we further clustered NK cells into their different subsets, including NK-T cells, which express an αβ T-cell receptor and NK-like markers (CD56), and are considered a unique population at the interface between the innate and adaptive immune system with a strong potential to regulate immune responses through different mechanisms.[30,31] Through T- or NK cell–like effector mechanisms, NK-T cells may rapidly respond and produce a variety of cytokines, following T-cell receptor activation, including CMV immunogenic proteins.[32,33] Although bulk NK-T cell did not associate with CMV infection or peripheral CMI function, 3 $CD11b^+$ NK-T cell subsets displayed a strong association with CMV protection and antiviral immune responses assessed by IFN-γ ELISPOT. These data strongly suggest that these cells may contribute to anti-CMV IFN-γ production by either reinforcing or supporting CMV-specific effector T cells or by directly releasing IFN-γ or other cytokines that prevent CMV replication. Because we could not find any cellular cluster expressing CD11b within the $CD8^+$ CMV-specific T-cell compartment, we postulate

that these cells are likely to be invariant rather than antigen specific. Indeed, invariant NK-T cells have been shown to have a direct role in both anti-tumour and anti-viral immunity on recognition of glycolipid antigens, which may lead to either rapidly secreting immunomodulatory cytokines or influencing downstream immune activity by reinforcing antigen-specific T cells through the recruitment and activation of cytotoxic innate and adaptive immune cells.[31,33–38]

The presence of $CD11b^+$ NK and T cells has also been previously reported in the setting of different viral infections, including CMV, as a marker of immune activation and of response to viral infection.[39–46] To the best of our knowledge, however, this is the first report describing the role of $CD11b^+$ NK-T in the context of immune protection against CMV infection in kidney transplantation. Although we do not have a specific explanation on why the other NK T-cell clusters were not associated with protection and/or CMV-specific CMI, it is tempting to speculate that these cells exert a protective role at other stages during the CMV infection course, as shown with other cellular subsets, such as γδ T cells and other specific NK cells.[14,18,30]

The protective effect of these NK-T cells is further supported by the inverse association between $CD11b^+$ NK T-cell numbers and CMV replication also in 2 additional independent kidney transplant cohorts. Although the number of these cells seems to be rather low compared with other cellular counterparts, our data set the basis for further investigating the potential of these cells for their anti-viral role and thus, also as anti-viral immunotherapy. Indeed, there are attempts in developing chimeric antigen receptor–invariant NK-T cells, which have shown to be safe and effective in the context of certain malignancies.[36] Other recent studies using CyTOF to immune phenotype retrospective kidney transplant patient cohorts[18] underscored the role of a new cytotoxic $NKG2C^+CD8^+$ T-cell subset ($NKG2C^+CD57^+Fc\varepsilon RI\gamma^{low\text{-}dim}$) together with γδ T cells facilitating early CMV infection control during viral replication in $R^+$ kidney transplant patients. Furthermore, Pickering *et al.*[30] assessed the dynamics of NK and T cells in viremic and nonviremic KTRs and found a different NK cell subset playing a role during the different stages of CMV reactivation with memory NK cells and NK-like $CD28^-CD8^+$ T cells associated with viral control. Although all these studies underscore the complexity of the immune response specific to CMV driving immune protection, none of them assessed NK-T cells nor CMV(tetramer)-specific T cells in their cellular phenotype studies and thus, they could not analyze the association between our newly described $CD11^+$ NK-T cells as well as CMV-specific T cells with CMV infection outcomes after kidney transplantation.

There are caveats that should be considered when interpreting our findings. We only analyzed CMV-seropositive KTRs; therefore, our data may not be applicable in serologically negative transplant patients, in whom CMV infection does also have severe clinical consequences. The number of patients investigated in this study is relatively low; therefore, the association with clinical outcomes should be taken with caution. However, all the patients included in the study were carefully characterized and followed up as participants of a randomized clinical trial in which CMV infection was strongly associated to the CMV-specific functional immune responses, and we validated our data in 2 independent kidney transplant cohorts to further strengthen our findings. Finally, most of our patients followed a preemptive strategy against CMV; thus, the findings may not directly apply to patients receiving universal prophylaxis.

In summary, our study newly shows the distinct contribution of multiple immune cell subsets in the protection against CMV infection after kidney transplantation. The use of high-dimensional analysis using mass cytometry and an unsupervised deep learning algorithms has enabled us to gain further insight into the complex network and kinetics of the immune response against CMV in KTRs with viral infection. This approach also allowed us to identify new key immune cell components contributing to the prevention of CMV infection. Our data pave the way to further studies testing the role of our newly identified cell subsets, such as $CD11b^+$ NK-T cells, in the immunopathology of CMV infection and as a biomarker of infection-risk stratification in KTRs.

## DISCLOSURE

All the authors declared no competing interests.

## DATA STATEMENT

The data collected for the study, including individual patient data and a data dictionary defining each field in the data set, will be made available as deidentified participant data to researchers who propose to use the data for individual patient data meta-analysis. Data will be shared following approval of the proposal by the corresponding author and a signed data access agreement.

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## SUPPLEMENTARY MATERIAL

Supplementary File (Word)

**Supplementary Methods.**

**Supplementary Figure S1.** Flowchart of the study.

**Supplementary Figure S2.** Expression density viSNE graphs of $CD11b^+$ NK-T cell markers.

**Supplementary Figure S3.** Representative gating strategy to identify lymphocyte subsets.

**Supplementary Figure S4.** Percentages of cellular populations between patients with CMV-viremia and without viremia over time.

**Supplementary Table S1.** Clinical and demographic characteristics of validation cohort A.
**Supplementary Table S2.** Clinical and demographic characteristics of validation cohort B.
**Supplementary Table S3.** Mass cytometry panel.
**Supplementary Table S4.** Cellular populations identified by mass cytometry and the signature of surface markers representative of each population shown in Figure 3.
**Supplementary Table S5.** Differences in cellular populations frequencies between CMV-infected and noninfected patients at different time points. T0: prior to transplantation, T1: at 15/30 days post-transplant, T2: at 3 months after transplantation, T3: at 12 months after transplantation.
**Supplementary Table S6.** The signature of surface markers representative of each CMV-specific $CD8^+$ T cell subset.
**Supplementary Table S7.** The signature of surface markers representative of each natural NK-T cell subset.

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