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Cytomegalovirus replication is associated with enrichment of distinct $\gamma\delta$ T cell subsets following lung transplantation: A novel therapeutic approach?

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BACKGROUND: Anti-viral treatments to control cytomegalovirus (CMV) after lung transplantation (LTx) are associated with toxicity and anti-viral resistance. Cellular immunotherapy with virus-specific cytotoxic T cells has yielded promising results but requires donor/recipient matching. $\gamma\delta$ T cells are involved in anti-viral immunity and can recognize antigens independently of major histocompatibility complex molecules and may not require the same level of matching. We assessed the phenotype of circulating $\gamma\delta$ T cells after LTx to identify the candidate populations for CMV immunotherapy.

METHODS: Peripheral blood mononuclear cells were isolated from lung transplant recipients before transplantation and at routine bronchoscopies after LTx. Patients were stratified by risk of CMV disease into moderate risk (recipient CMV seropositive, $n = 15$) or high risk (HR) (recipient CMV seronegative/donor CMV seropositive, $n = 10$). CMV replication was classified as polymerase chain reaction positive (>150 copies/ml) in blood and/or bronchoalveolar lavage within the first 18 months. The phenotype of $\gamma\delta$ T cells was assessed by multicolor flow cytometry, and T-cell receptor (TCR) sequences were determined by deep sequencing.

RESULTS: In HR lung transplant recipients with CMV replication, we observed striking phenotypic changes in $\gamma\delta$ T cells, marked by an increase in the proportion of effector V δ 1+ $\gamma\delta$ T cells expressing the activating natural killer cell receptor NKG2C. Moreover, we observed a remarkable increase in TCR diversity.

CONCLUSIONS: NKG2C+ V δ 1+ $\gamma\delta$ T cells were associated with CMV replication and may indicate their potential to control infection. As such, we propose that they could be a potential target for cellular therapy against CMV.

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Lung transplantation (LTx) is a life-saving procedure for end-stage lung disease. However, lung transplant recipients have lower long-term survival than recipients of other solid organ transplants, with a median survival of only 6.5 years.¹ Chronic lung allograft dysfunction is the major factor limiting LTx long-term survival and is the result of alloimmune- and infection-induced damage to the graft, resulting in allograft failure and death. In lung transplant recipients, cytomegalovirus (CMV) replication is common and impacts on survival directly through end-organ infection but is also associated with the development of chronic lung allograft dysfunction.² Whereas anti-viral drugs can effectively limit CMV replication, the dosing duration is largely empirical; side effects, including neutropenia are common; and indiscriminate dosing is associated with drug resistance.³ Furthermore, prolonged anti-viral use can result in inhibition of immune function, warranting an alternative approach to anti-viral treatment.⁴

Host control of CMV involves both the innate and adaptive immune systems,⁵ with a well-established contribution of $\alpha\beta$ T cells, B cells, and natural killer (NK) cells. An additional subset with a possible contribution in the immune control of CMV are $\gamma\delta$ T cells, which can provide protection from CMV in murine models,⁶ and human $\gamma\delta$ T cells, which can kill CMV-infected cells in vitro.⁷ After kidney and hematopoietic stem-cell transplantation, replication of CMV is associated with subset perturbations in the frequencies of circulating $\gamma\delta$ T cells. In particular, CMV replication has been linked to an expansion of the $\gamma\delta$ T cells lacking the *TRDV2* gene segment of the $\gamma\delta$ T cell receptor (TCR), called $V\delta 2^{\text{neg}}$ $\gamma\delta$ T cells.^{8,9} The increased frequency of $V\delta 2^{\text{neg}}$ $\gamma\delta$ T cells after CMV infection is substantial, often resulting in an expansion from 1% to more than 10% of the total circulating T cells,¹⁰ similar to that seen for CMV-specific CD8+ T cells.¹¹ However, there has been a minimal investigation of the contribution of these $\gamma\delta$ T cells in CMV immunity after LTx.

In addition to their TCR, $\gamma\delta$ T cells express several receptors that are typically associated with NK cells, including NKG2D, which engages stress-induced ligands such as the major histocompatibility complex (MHC) class I polypeptide-related sequence A and B.¹² $\gamma\delta$ T cells can also express the receptors from the CD94-NKG2 family, which recognize the non-classical MHC class I molecule human leukocyte antigen (HLA)-E.¹³ The upregulation of CD94-NKG2C (NKG2C) on NK cells has been associated with CMV seropositivity,¹⁴ and there are a number of reports describing the contribution of NKG2C+ NK cells in the control of CMV after solid organ and hematopoietic stem-cell transplantation.^{15,16} Our own studies¹⁷ and those of others¹⁸ have demonstrated the expansion of NKG2C+ NK cells after CMV replication after LTx, further implicating a role for this receptor in immunity to CMV. However, a role for NKG2C in the context of $\gamma\delta$ T cells remains largely unexplored.

In this study, we longitudinally assessed the phenotype of circulating $\gamma\delta$ T cells in lung transplant recipients at risk of CMV disease and temporally correlated this with CMV

replication within 18 months after LTx. The data suggest that there are changes in the composition of $\gamma\delta$ T cell subsets associated with CMV infection. Thus, clinical monitoring of this compartment might provide a guide for establishing the optimal duration of viral prophylaxis after LTx. Furthermore, the dramatic increases in the proportion of NKG2C+ $V\delta 1+$ $\gamma\delta$ T cells observed after infection raise the prospect that $\gamma\delta$ T cells could be a promising target for future cellular therapy.

Methods

Ethics

All patients gave written informed consent. The study was approved by the Alfred Hospital Ethics Committee (Project 401/13) and the University of Melbourne Human Research Ethics Committee (Project 1238243).

Participants

The clinical cohort consisted of 25 adult patients at risk of CMV (receiving a CMV seropositive donor and/or were CMV seropositive) who underwent a bilateral LTx between March 2014 and October 2016 at the Alfred Hospital, Melbourne, Australia. Peripheral blood was collected before LTx and at surveillance bronchoscopies (at 0.5, 1.5, 3, 6, 9, 12, and 18 months after LTx), separated into peripheral blood mononuclear cells (PBMCs) by Ficoll-Paque (GE Healthcare, Sydney, New South Wales, Australia), and then cryopreserved in 90% fetal calf serum/10% dimethyl sulfoxide until analysis. All patients were given the standard triple immunosuppressant regimen (prednisolone, tacrolimus, and azathioprine or mycophenolate).

CMV prophylaxis, monitoring, and treatment

The patient's risk of CMV replication was further grouped into moderate risk (MR) (recipient who was CMV seropositive, $n = 15$) or high risk (HR) (recipient who was CMV seronegative with a donor who was CMV seropositive, $n = 10$). Most patients at MR (12 of 15 recipients at MR) received standard anti-viral prophylaxis for 5.5 months, consisting of 2 weeks intravenous ganciclovir (5 g/kg body weight) followed by 450 mg twice daily oral valganciclovir. However, only 2 of 10 recipients at HR were on anti-viral prophylaxis for <6 months. In the presence of a negative Quantiferon-CMV assay (Qiagen, Valencia, CA), 3 of 15 lung transplant recipients at MR and 6 of 10 lung transplant recipients at HR received extended valganciclovir prophylaxis to 11 months after LTx. A total of 2 patients at HR received continuous valganciclovir throughout the entire monitoring period. As per protocol, recipients at HR also received CMV hyperimmune immunoglobulin (1.5 million U) on Days 1, 2, 3, 7, 14, 21, and 28 while also on valganciclovir. CMV replication was detected by COBAS Amplicor CMV monitor test (Roche Diagnostic Systems, New South Wales, Australia) in both the plasma and bronchoalveolar lavage (BAL), with a polymerase chain reaction (PCR) result >150 copies/ml (>137 international U) considered positive. Patients with high-level CMV replication in either blood or BAL (>10,000 copies/ml) were treated with intravenous ganciclovir for 2 weeks (5 mg/kg). Patients with low-level infection in the blood or BAL (600–10,000 copies/ml) were given oral valganciclovir for 2 weeks.

Flow cytometry

Longitudinal samples from a single recipient were analyzed on the same day. Cryopreserved PBMC were rapidly thawed and rinsed in phosphate-buffered saline containing 1% fetal calf serum and 5 mM ethylenediaminetetraacetic acid (fluorescence-activated cell sorting [FACS] buffer). PBMCs were then stained in 50 μ l FACS buffer containing a single antibody cocktail listed in [Supplementary Table S1](#) available online at www.jhltonline.org. After 30-minute incubation with the antibody cocktail at 4°C, PBMCs were rinsed in FACS buffer before fixing in 100 μ l Cytofix (BD Biosciences, Franklin Lakes, NJ). Samples were then centrifuged, supernatant removed, and the samples were resuspended in 100 μ l FACS buffer. Anti-mouse immunoglobulin compensation beads (BD Biosciences) were used for compensation controls for flow cytometry. Flow cytometry analysis was performed using an LSR Fortessa (Becton Dickinson, Franklin Lakes, NJ). Samples were gated on single, CD14⁻, and CD19⁻ live lymphocytes, followed by gating on CD3⁺ and $\gamma\delta$ TCR⁺, and analyzed using FlowJo software (Tree Star, San Carlos, CA). Data were excluded from the analysis where the proportion of $\gamma\delta$ T cells was 0% or where the number of events was <50 cells in $\gamma\delta$ T cell gate. Statistical significance was performed with GraphPad Prism. Comparisons between 2 groups (MR and HR) were tested using a 2-tailed Mann–Whitney test, and linear regression analysis was employed for HR, CMV-reactivated samples. Comparisons between 4 groups (recipients at MR and recipients at HR with and without CMV replication) was performed with Kruskal–Wallis test followed by Dunn’s multiple comparison tests.

$\gamma\delta$ TCR sequencing

We excluded $\gamma\delta$ T cells that were double positive for V γ 9 and V δ 2 from our analysis (see [Supplementary Figure S1a](#) online). V γ 9/V δ 2-negative $\gamma\delta$ T cells were sorted from 2 recipients at HR, 1 before LTx (15,000 cells) and at 9 months after LTx during CMV reactivation (15,000 cells) and for the other, V δ 1+ $\gamma\delta$ T cells at 6 months after LTx (before CMV detection, 1,000 cells) and 12 months after LTx (during CMV reactivation, 2,900 cells). Cells were sorted into RNeasy lysis buffer (Qiagen) according to the manufacturer’s instructions. Extracted RNA was then used for high-throughput deep sequencing of $\gamma\delta$ TCRs using amplicon-rescued multiplex PCR. After initial first-round reverse transcriptase-PCR using high concentrations of gene-specific primers, universal primers were used for the exponential phase of amplification (iRepertoire Inc., Huntsville, AL, patent: WO2009137255A2), allowing deep, quantitative amplification of TCR γ (TRG) and TCR δ (TRD) sequences. All complementary DNA synthesis, amplification, and next-generation sequencing library preparation were performed using TRG/TRD iRprofile kits (iRepertoire, Inc., Huntsville, AL), and subsequent libraries were pooled and deep sequenced using an Illumina MiSeq (Illumina, San Diego, CA) sequencer (Illumina, Melbourne, Australia).

TCR repertoire data analysis

Variable, diversity, and joining gene usage and complementarity-determining region (CDR) 3 sequences were identified and assigned, and tree maps were generated using iRweb tools (iRepertoire). Tree maps show each unique CDR3 as a colored rectangle; the size of each rectangle corresponds to each CDR3s abundance within the repertoire, and the positioning is determined

by the variable region usage. Analysis of TCR repertoire diversity was measured by D50 metric; this value indicates the percentage of the clonotypes required to occupy 50% of the total TCR repertoire from either TRD or TRG repertoires.

Results

CMV replication after LTx

CMV infection significantly impacts on LTx success. Therefore, a cohort of lung transplant recipients was assessed for the presence of actively replicating CMV at routine bronchoscopies after LTx. Before LTx, donor and recipient CMV serostatuses were assessed, and patients were stratified into 2 groups at risk of CMV reactivation: MR (recipient CMV-seropositive group, $n = 15$) and HR (recipient CMV-seronegative, donor CMV-seropositive group, $n = 10$). After LTx, evidence of CMV replication was assessed by quantitative PCR ([Table 1](#)). Overall, there was more CMV replication in the blood and/or BAL in the HR group, and 8 of 10 recipients (H3–H10, [Table 1](#)) showed evidence of viral replication, whereas CMV replication was observed in 7 of 15 recipients at MR (M9–M15, [Table 1](#)). Of the 2 recipients at HR in whom CMV replication was absent, 1 was on extended anti-viral prophylaxis for 338 days (H1), and the other was treated with valganciclovir for the entire monitoring period (H2). Viral replication was typically first detected at the cessation of anti-viral prophylaxis, at 6 months (4 recipients), 8 to 9 months (5 recipients), or 11 to 12 months (6 recipients) after LTx ([Table 1](#)). The virus was detected at multiple time points for 4 recipients at MR (M9, M11, M12, and M14) and 4 recipients at HR (H7, H8, H9, and H10). CMV replication was more frequently observed in the blood of individuals from the HR group (5 of 10 recipients at HR vs 3 of 15 recipients at MR, [Table 1](#)). Symptomatic CMV infection was observed in 4 individuals: 1 recipient at MR and 3 recipients at HR. In recipient M12 at MR, reduced lung function was observed coincidentally with high-level viral replication in the blood (>700,000 copies/ml). CMV pneumonitis was observed in recipient H7 at HR at 8 months after LTx, whereas CMV syndrome/gastritis was observed in recipients H3 and H4 at HR at 12 months after LTx ([Table 1](#)).

The proportion of V δ 1+ T cells correlates with pre-LTx CMV serology and increases on CMV replication

To determine whether there was an association between CMV infection and circulating $\gamma\delta$ T cells, we initially compared the proportion of $\gamma\delta$ T cells within PBMCs between recipients at MR and those at HR. The gating strategy employed to identify $\gamma\delta$ T cells is shown in [Figure 1a](#). Overall, the proportion of $\gamma\delta$ T cells did not differ between the different CMV risk groups at individual time points after LTx ([Figure 1b](#)). Stratifying results from recipients at HR in whom active CMV replication was detected by PCR, there was a small but significant increase in the proportion of $\gamma\delta$ T cells over the first 18 months after LTx ([Figure 1c](#)).

Table 1 CMV PCR Results

CMV prophylaxis and CMV PCR results	MR CMV (R+) (n = 15)	HR CMV (D+/R-) (n = 10) </TH1>
Extended prophylaxis	3 (M3, M4, M7)	8 (H1–H6, H8, H10)
BAL PCR results		
<150 copies/ml	M1–M8, M15	H1–H4, H8
150–600 copies/ml	M9 ^{12,18} , M12 ⁹ , M13 ⁹	H9 ¹²
600–10,000 copies/ml	M11 ¹⁸	H6 ¹² , H7 ¹² , H10 ¹²
>10,000 copies/ml	M9 ⁶ , M10 ⁹ , M11 ⁹ , M12 ⁶ , M14 ⁶	H5 ¹² , H9 ⁶
Blood PCR results		
<150 copies/ml	M1–M10, M13, M14	H1, H2, H6
150–600 copies/ml	M11 ⁹	H7 ¹² , H8 ¹⁸ , H10 ¹²
600–10,000 copies/ml	M12 ^{9,11} , M15 ⁸	H3 ^{12,a} , H4 ^{12,a} , H8 ¹² , H9 ¹²
>10,000 copies/ml	M12 ^{6,a}	H5 ¹² , H7 ^{8,a} , H9 ⁶

Abbreviations: BAL, bronchoalveolar lavage; CMV, cytomegalovirus; D, donor; H, recipient at HR; HR, high risk; M, recipient at MR; MR, moderate risk; PCR, polymerase chain reaction; R, recipient.

Superscript numbers indicate the timepoint after transplantation (in months) where the PCR positive result was obtained.

^aIndicates symptomatic CMV infection.

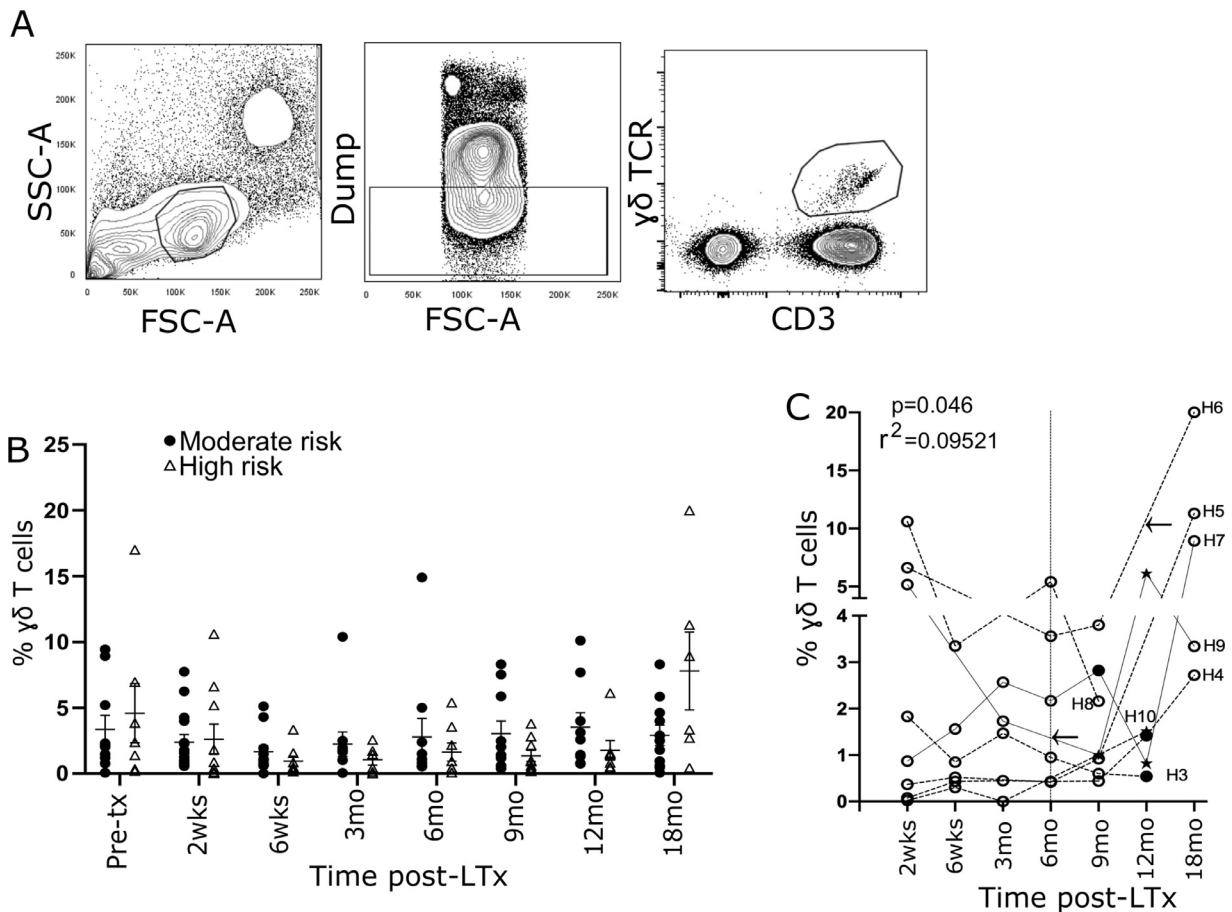


Figure 1 The proportion of circulating $\gamma\delta$ T cells is associated with pre-existing CMV immunity. (a) Flow cytometry plot of the gating strategy used to identify $\gamma\delta$ T cells in PBMCs samples. Dump = channels including CD14, CD19, and live/dead fixable to exclude monocytes, B cells, and dead cells, respectively. (b) $\gamma\delta$ T cell proportions segregated on timepoints of sample collection. Each symbol represents a sample within the assigned risk group (dark circles, MR; open triangles, HR). (c) Analysis of the proportion of $\gamma\delta$ T cells after LTx in recipients at HR who were CMV PCR positive. The joined solid lines indicate the changes in an individual recipient on standard anti-viral prophylaxis, whereas the dashed lines between points are changes for recipients at HR on extended anti-viral prophylaxis. The dashed vertical line indicates the cessation of standard anti-viral prophylaxis. The filled black circles indicate positive CMV PCR in blood. Stars indicate positive CMV PCR in both blood and BAL. Arrows indicate where CMV replication was observed for H6 and H9 at 12 mos and 6 mos, respectively, but where no sample was available for analysis. BAL, bronchoalveolar lavage; CMV, cytomegalovirus; FSC-A, forward scatter area; H, recipient at HR; HR, high risk; LTx, lung transplantation; mo, month; MR, moderate risk; PBMC, peripheral blood mononuclear cell; PCR, polymerase chain reaction; SSC-A, side scatter area; Tx, transplantation; wk, week.

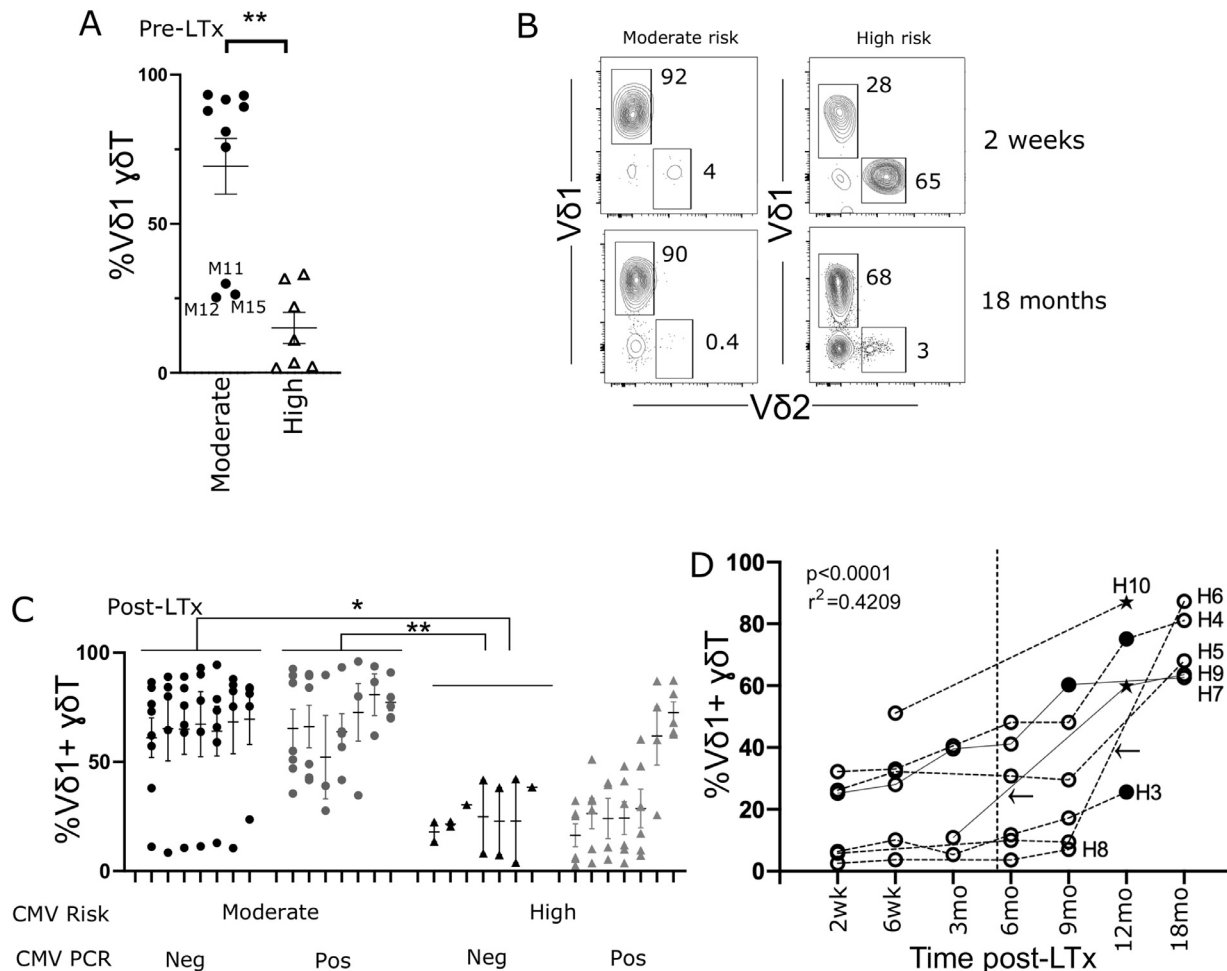


Figure 2 The predominance of Vδ1 chain expression by circulating γδ T cells is associated with CMV immunity. (a) The proportion of Vδ1+ cells within the γδ T-cell population in recipients at MR and recipients at HR before transplantation. A total of 3 recipients at MR with a lower proportion of Vδ1 γδ T cells are labeled (M11, M12, M15). ** $p=0.004$, Mann–Whitney test. (b) A flow cytometry plot of lung transplant recipients at MR and recipients at HR at 2 wks and 12 mos after LTx in whom CMV active replication was detected. Numbers show the proportion of cells within a given gate (gated on γδ T cells). (c) The analysis of post-transplantation Vδ1 chain usage within γδ T cells. Data shown are from all timepoints after LTx (the ticks on x-axis represent 2 wks, 6 wks, 3 mos, 6 mos, 9 mos, 12 mos, and 18 mos after LTx). Recipients were stratified by CMV risk group ($n=15$ and $n=10$ recipients at MR and recipients at HR, respectively) and by whether active CMV replication occurred (CMV PCR Neg or CMV PCR Pos). * $p=0.02$, ** $p=0.003$, Dunn’s multiple comparisons test. (d) Analysis of the proportion of Vδ1+ γδ T cells after LTx in recipients at HR with active CMV replication. The joined solid lines indicate changes in an individual recipient on standard anti-viral prophylaxis, whereas the dashed lines between points are changes for recipients at HR on extended anti-viral prophylaxis. The dashed vertical line indicates the cessation of standard anti-viral prophylaxis. The filled black circles indicate Pos CMV PCR in blood, and stars indicate the Pos CMV PCR in both blood and BAL. Arrows indicate where CMV replication was observed for H6 and H9 at 12 mos and 6 mos, respectively, but where no sample was available for analysis. BAL, bronchoalveolar lavage; CMV, cytomegalovirus; H, recipient at HR; HR, high risk; LTx, lung transplantation; M, recipient at MR; mo, month; MR, moderate risk; Neg, negative; PCR, polymerase chain reaction; Pos, positive; wk, week.

but not in the 2 recipients at HR where CMV replication was not observed (data not shown). Therefore, CMV replication in lung transplant recipients who were previously CMV seronegative was associated with relatively minor changes in the overall proportion of γδ T cells in the blood.

To determine whether there were gross changes in the γδ T cell repertoire in the LTx cohort, we initially enumerated the proportion of Vδ1+ and Vδ2+ γδ T cells and found a significantly higher proportion of Vδ1+ cells in the recipients who were CMV seropositive before transplantation, although there were 3 recipients at MR with a notably lower proportion than others (Figure 2a). Recipients at MR maintained higher proportions of Vδ1+ γδ T cells throughout the

post-transplantation period (Figure 2b and c) than recipients at HR who remained PCR negative (Figure 2c). Furthermore, recipients at MR with CMV replication and recipients at MR without CMV replication, both had a higher proportion of Vδ1+ γδ T cells than recipients at HR without CMV replication (the mean proportion of Vδ1+ γδ T cells in the recipients at MR with CMV was 68%, the mean proportion of Vδ1+ γδ T cells in the recipients at MR without CMV was 65%, and the mean proportion of Vδ1+ γδ T cells in the recipients at HR without CMV was 24%). Without considering timing over the post-LTx period, there were no significant differences between recipients at HR who experienced CMV replication and any other group (mean

proportion of $V\delta 1+$ $\gamma\delta$ T cells = 35%). However, in recipients at HR in whom CMV replication was confirmed, the proportion of circulating $V\delta 1+$ $\gamma\delta$ T cells significantly increased over time after LTx, which was not observed in the 2 recipients at HR on prolonged anti-viral treatment (Figure 2c and d). These changes were apparent after the cessation of anti-viral prophylaxis and occurred before or coincidentally with CMV replication, such that by 18 months after LTx, more than 60% of the circulating $\gamma\delta$ T cells were $V\delta 1+$ in the recipients at HR in whom CMV replication was detected (Figure 2d). Therefore, CMV infection results in skewing toward the $V\delta 1+$ subset in the $\gamma\delta$ T-cell repertoire.

$V\delta 1+$ $\gamma\delta$ T cells show high TCR diversity during CMV replication

To assess post-LTx changes in the $\gamma\delta$ TCR repertoire in more detail, we performed a pilot analysis of TCR sequences before and during active CMV replication. Given previous findings¹⁹ and our own observations that the archetypal blood $V\gamma 9/V\delta 2$ are not involved in CMV control,²⁰ we excluded the clones that were double positive for $V\gamma 9$ and $V\delta 2$ and only sequenced the $\gamma\delta$ T cells lacking this specific TRG and TRD combination (called $V\gamma 9/V\delta 2$ -negative cells, see Supplementary Figure S1a online). In an recipient at HR who had evidence of CMV replication (Recipient H7), we used deep sequencing to assess the pre- and post-transplant $\gamma\delta$ TCR repertoire. Analysis of the TCR repertoire of $V\gamma 9/V\delta 2$ -negative $\gamma\delta$ T cells by tree plot found a dramatic expansion of individual clonotypes during CMV replication (Figure 3a), which was not a result of the differences in the sequencing depth between each sample (see Supplementary Figure S1b online). Moreover, the total number of clonotypes and D50 diversity index sharply increased, and the accumulated frequency of the top 20 clonotypes decreased from the pre-transplant repertoire (see Supplementary Figure S1c online). The flow cytometry data for this recipient indicated that the proportion of $V\delta 1+$ $\gamma\delta$ T cells increased from ~20% before LTx to >50% of $\gamma\delta$ T cells during CMV replication, concurrent with a decrease in the proportion of $V\delta 2+$ $\gamma\delta$ T cells from ~40% to ~10% of the $\gamma\delta$ T cells over the same time frame (data not shown). Therefore, when filtering on the expanding $V\delta 1+$ fraction for this donor, we observed that the TCR repertoire was surprisingly diverse during the CMV replication (Figure 3b).

To exclude the possibility that the transplantation itself impacted on $V\delta 1+$ diversity rather than CMV replication, we performed deep sequencing on a second recipient at HR at 2 post-LTx timepoints: 1 before CMV replication (6 months after LTx) and the other during CMV replication (12 months after LTx). In this recipient, to focus on the $V\delta 1+$ fraction, $V\delta 1+$ $\gamma\delta$ T cells were sorted by flow cytometry, and the TRG repertoire was analyzed by deep sequencing. A similar reshaping of the repertoire occurred coincidentally with CMV replication compared with that of pre-CMV repertoire, showing a reduction of clonotypes utilizing $V\gamma 9$ and expansion of individual clonotypes utilizing $V\gamma 2$, $V\gamma 4$, and $V\gamma 5$

TCR gene segments, with $V\gamma 4$ showing the largest increase (Figure 3c). Within the $V\gamma 4$ repertoire, there was a significant expansion of diverse clonotypes during CMV replication (Figure 3c). Taken together, our pilot sequencing analysis of recipients at HR indicates that the pre-CMV clonal diversity of the $\gamma\delta$ T-cell compartment was similar to that reported for healthy individuals,²⁰ whereas in recipients at HR who had active CMV replication, clonal diversity was significantly increased (Figure 3 and see Supplementary Figure S1 online). In particular, our findings suggest that in the post-LTx period coincident with active CMV infection, there is a substantial remodeling of the $V\delta 1+$ $\gamma\delta$ TCR repertoire and the dynamics of this remodeling involved a dramatic expansion of individual $\gamma\delta$ TCR clonotypes into the immune repertoire.

The proportion of NKG2C+ effector $\gamma\delta$ T cells increases with CMV replication

Given the substantial diversity in TCR repertoires during the post-LTx period in recipients at HR, we investigated whether we could identify the expression of a common marker that was indicative of CMV exposure. Previous studies had shown that an increase in the proportions of NK cells expressing the NKG2C activating receptor was associated with active CMV replication.¹⁷ Therefore, the expression of NKG2C on $\gamma\delta$ T cells was compared between lung transplant recipients at MR and those at HR. Akin to NK cells, a higher proportion of NKG2C+ $\gamma\delta$ T cells before LTx was associated with recipients at MR, where the mean proportion of circulating $\gamma\delta$ T cells that expressed NKG2C was 40% in the recipients at MR, in contrast to <20% in the recipients at HR (Figure 4a). Interestingly, the same 3 recipients at MR with a lower proportion of $V\delta 1+$ cells also had a lower proportion of NKG2C+ $\gamma\delta$ T cells (M11, M12, and M15; Figure 4a), and flow cytometric analysis revealed that most NKG2C+ $\gamma\delta$ T cells were $V\delta 1+$ (Figure 4b). Most recipients at MR consistently maintained a high proportion of NKG2C+ $\gamma\delta$ T cells over the post-LTx period (Figure 4b and c), and similar to the results for $V\delta 1+$ analysis, recipients at HR without CMV replication had significantly lower proportions of NKG2C+ $\gamma\delta$ T cells than both MR groups (the mean percentages of NKG2C+ $\gamma\delta$ T cells in the recipients at MR without CMV replication and the recipients at MR with CMV were 32% and 35%, respectively, vs 8% in the recipients at HR without CMV; Figure 4c). Further analysis of the recipients at HR with active CMV replication indicated an associated increase in the proportion of NKG2C+ $\gamma\delta$ T cells over the post-LTx period ($p = 0.003$), with a mean proportion of NKG2C+ $\gamma\delta$ T cells of 9% at 2 weeks after LTx, increasing to 32% at 18 months after LTx (mean overall percentage of NKG2C+ $\gamma\delta$ T cells after LTx = 18%, Figure 4b and d). Importantly, this increase was observed after the cessation of anti-viral prophylaxis and occurred coincidentally with CMV replication. Moreover, this change was not evident in the 2 recipients at HR without CMV replication who were on prolonged anti-viral treatment (Figure 4c). Therefore, this suggests that the

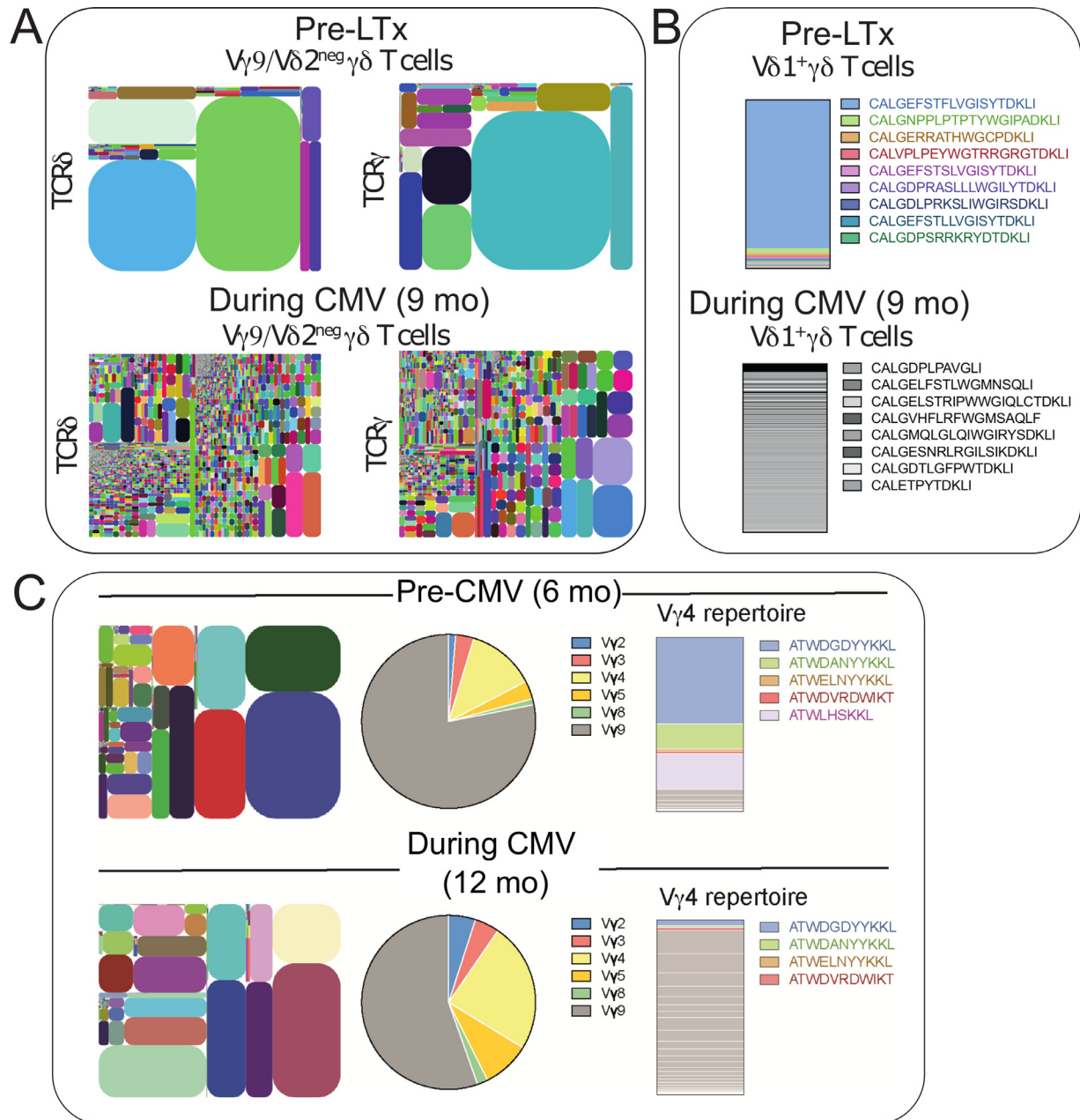


Figure 3 High TCR diversity in $\gamma\delta$ T cells observed after transplantation. (a) Tree maps showing TRG and TRD deep sequencing of $V\gamma 9/V\delta 2$ -negative $\gamma\delta$ T cells from a lung transplant recipient at HR before LTx and during CMV replication at 9 mos after transplantation. Each colored rectangle represents a unique TCR clonotype (colors do not match between plots). (b) Clonotype frequency showing the diversity within the filtered $V\delta 1^+$ T-cell fraction of the repertoire presented in (a). The top 9 clonotypes before LTx are colored. (c) TRG deep sequencing from a second lung transplant recipient at HR. Sequencing was from $V\delta 1^+$ $\gamma\delta$ T cells sorted from 6-mo and 12-mo post-LTx samples, with the 12-mo sample coincident with CMV replication. Left panels show the tree maps showing TRG clones within $V\delta 1^+$ $\gamma\delta$ T-cell repertoire at 6 and 12 mos after LTx, with each colored rectangle representing a unique TCR clonotype (colors do not match between plots). Middle panels show the pie charts showing the V-region usage within the $V\delta 1^+$ $\gamma\delta$ T-cell repertoire at 6 and 12 mo after LTx. Right panels show the $V\gamma 4$ TCR repertoire at 6 and 12 mos after LTx. Individual clonotypes at 6 mos are colored, and frequencies of the same clonotypes are shown in the same color at 12 mos (if present). CMV, cytomegalovirus; HR, high risk; LTx, lung transplantation; mo, month; TCR, T-cell receptor; TRD, TCR δ ; TRG, TCR γ .

induction of NKG2C on $\gamma\delta$ T cells after CMV infection is at least partially dependent on active viral replication.

To better understand whether the remodeling of the $\gamma\delta$ T-cell compartment was accompanied by changes in the phenotypic marker expression after LTx, $\gamma\delta$ T cells were assessed for the expression of CD27 and CD45RA, 2 receptors used to define naive (CD27+/CD45RA+) and effector

(CD27^{low} CD45RA⁺) $\gamma\delta$ T cells.^{20,21} Before LTx, recipients at MR had more effector $\gamma\delta$ T cells than recipients at HR, although the same 3 recipients at MR with lower $V\delta 1^+$ $\gamma\delta$ T cells and NKG2C+ $\gamma\delta$ T cells also had a lower proportion of effector $\gamma\delta$ T cells (Figure 5a). Consistent with previous data, individuals from the MR group maintained higher proportions of effector $\gamma\delta$ T cells after LTx

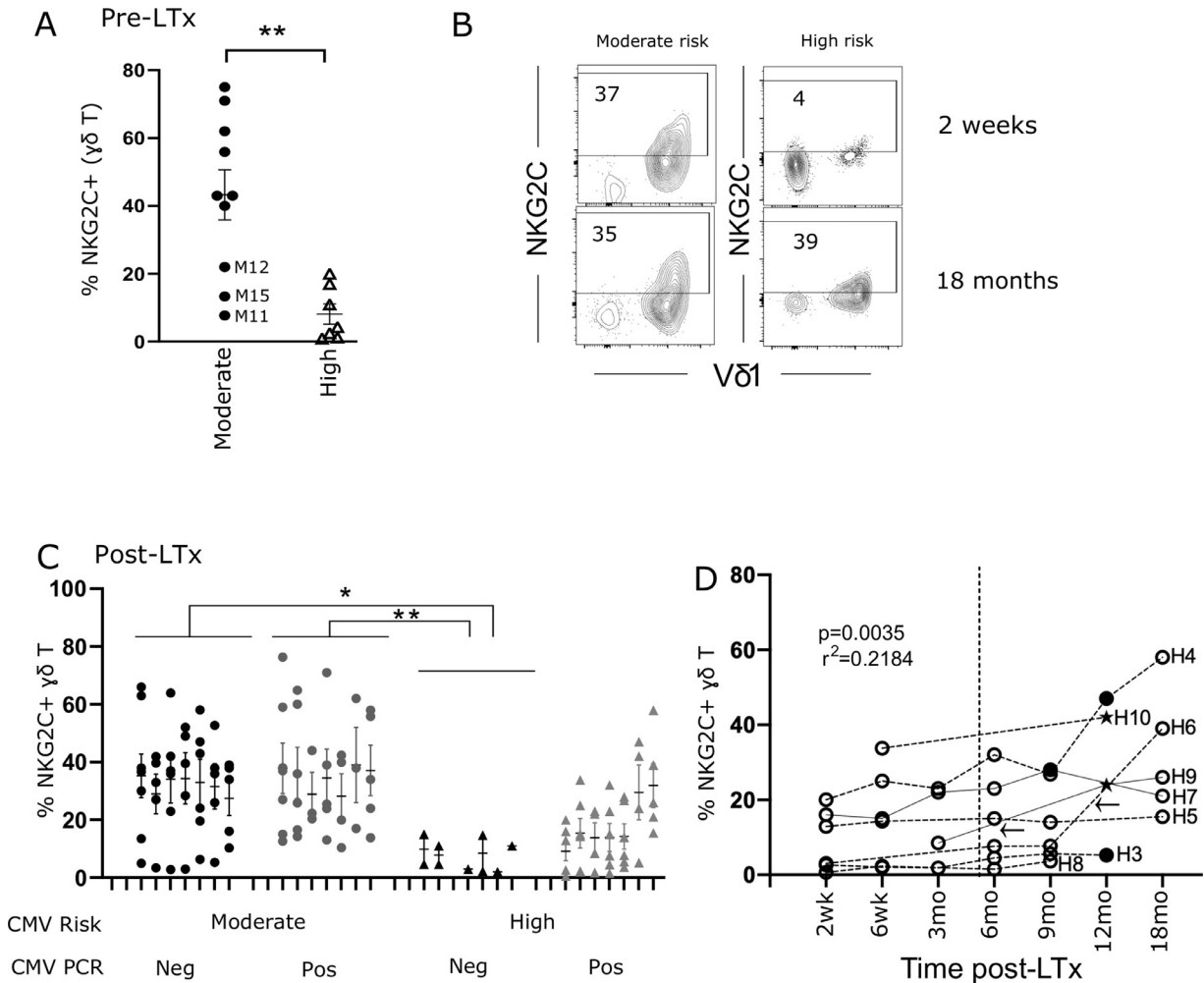


Figure 4 The proportion of NKG2C+ $\gamma\delta$ T cells is associated with CMV immunity. (a) The proportion of NKG2C+ $\gamma\delta$ T cells of total blood $\gamma\delta$ T cells is shown between transplant recipients who were at MR and those who were at HR before transplantation. Each symbol represents 1 recipient with mean \pm SEM shown. A total of 3 recipients at MR with a lower proportion of NKG2C+ $\gamma\delta$ T cells are labeled (M11, M12, and M15). ** $p=0.0018$, Mann–Whitney test. (b) A flow cytometry plot of lung transplant recipients at MR and at HR at 2 wks and 12 mos after LTx in whom CMV active replication was detected. Numbers show the proportion of cells within a given gate (gated on $\gamma\delta$ T cells). (c) Pooled values for the proportion of NKG2C+ $\gamma\delta$ T cells from recipients at MR and recipients at HR stratified by the absence or presence of CMV replication (CMV PCR Neg or CMV PCR Pos), with individual timepoints designated by discrete groups with mean \pm SEM shown for each (the ticks on the x-axis represent 2 wks, 6 wks, 3 mos, 6 mos, 9 mos, 12 mos, and 18 mos after LTx). * $p=0.007$, ** $p=0.0004$, Dunn’s multiple comparisons test. (d) Analysis of NKG2C+ $\gamma\delta$ T cells over time after transplantation in recipients at HR who experienced active viral replication. The joined solid lines indicate the changes in an individual recipient on standard anti-viral prophylaxis, whereas the dashed lines between points are changes for recipients at HR on extended anti-viral prophylaxis. The dashed vertical line indicates the cessation of standard anti-viral prophylaxis. The filled black circles indicate the Pos CMV PCR in blood, and the stars indicate the Pos CMV PCR in both blood and BAL. Arrows indicate where CMV replication was observed for H6 and H9 at 12 and 6 mos, respectively, but where no sample was available for analysis. BAL, bronchoalveolar lavage; CMV, cytomegalovirus; H, recipient at HR; HR, high risk; LTx, lung transplantation; M, recipient at MR; mo, month; MR, moderate risk; Neg, negative; PCR, polymerase chain reaction; Pos, positive; wk, week.

(Figure 5b and c), and both MR groups had significantly higher proportions of effector $\gamma\delta$ T cells than patients at HR without CMV replication (recipients at MR without CMV replication = 70%, recipients at MR with CMV = 67% vs recipients at HR without CMV; Figure 5c). In contrast, there was a dramatic proportional increase in CD27low CD45RA+ effector $\gamma\delta$ T cells in the HR group who went on to experience an episode of CMV replication (Figure 5b–d). Overall, the proportion of CD27low CD45RA+ $\gamma\delta$ T cells in the recipients at HR with CMV replication was 48%; however, at 2 weeks, this proportion was 33%,

increasing to 90% at 18 months after LTx. Indeed, the higher proportions of effector $\gamma\delta$ T cells were not seen in the recipients at HR without CMV replication on extended prophylaxis (Figure 5c).

Our data suggested that CMV replication resulted in an expansion of effector $\gamma\delta$ T cells that coexpressed V δ 1 and NKG2C. Indeed, a detailed analysis of the 2 recipients at HR (H6 and H9) showed similar trends before and after CMV replication (Figure 6). After LTx but before CMV replication, the proportions of $\gamma\delta$ T cells in the blood were similar in both recipients (~2% of lymphocytes), which

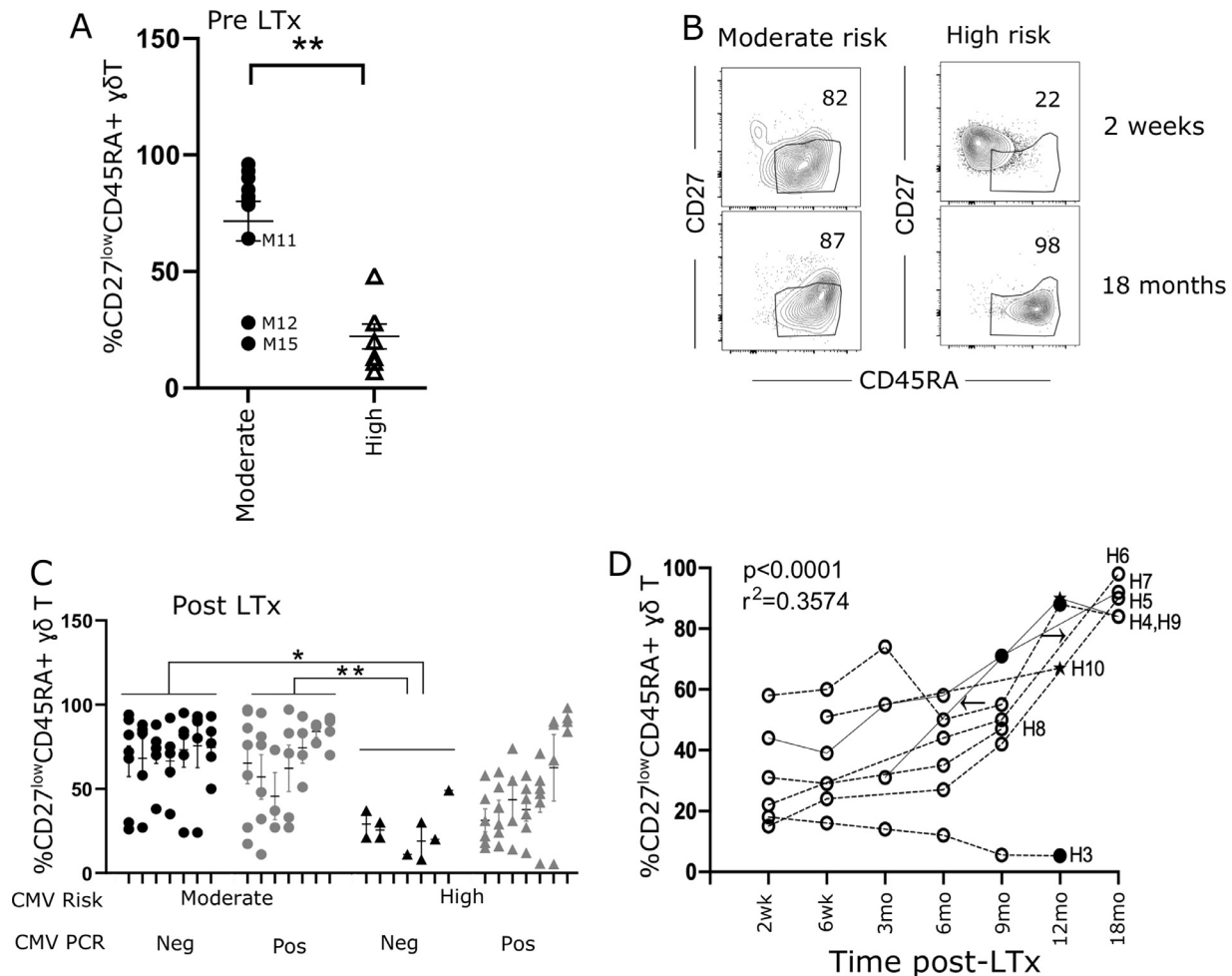


Figure 5 An increase in $\gamma\delta$ T cells with an effector phenotype is associated with CMV replication in individuals at HR. (a) The proportion of effector (CD27^{low} CD45RA⁺) $\gamma\delta$ T cells of total blood $\gamma\delta$ T cells between recipients at MR and recipients at HR with CMV before transplantation. Each symbol represents 1 recipient with mean \pm SEM shown. A total of 3 recipients at MR with a lower proportion of effector $\gamma\delta$ T cells are labeled (M11, M12, and M15). ** $p = 0.0018$, Mann–Whitney test. (b) A flow cytometry plot of lung transplant recipients at MR and lung transplant recipients at HR at 2 wks and 12 mos after LTx in whom CMV active replication was detected. Numbers show the proportion of cells within a given gate (gated on $\gamma\delta$ T cells). (c) Pooled values for the proportion of effector (CD27^{low} CD45RA⁺) $\gamma\delta$ T cells from recipients at MR and recipients at HR stratified by the absence or presence of CMV replication (CMV PCR Neg or CMV PCR Pos), with individual timepoints designated by discrete groups with mean \pm SEM shown for each (the ticks on x-axis represent 2 wks, 6 wks, 3 mos, 6 mos, 9 mos, 12 mos, and 18 mos after LTx). * $p = 0.002$, ** $p = 0.01$, Dunn’s multiple comparisons test. (d) Analysis of effector (CD27^{low} CD45RA⁺) $\gamma\delta$ T cells over time after transplantation in recipients at HR who were CMV PCR Pos. The joined solid lines indicate changes in an individual recipient on standard anti-viral prophylaxis, whereas the dashed lines between points are changes for recipients at HR on extended anti-viral prophylaxis. The dashed vertical line indicates the cessation of standard anti-viral prophylaxis. The filled black circles indicate the Pos CMV PCR in blood, and the stars indicate Pos CMV PCR in both blood and BAL. Arrows indicate where CMV replication was observed for H6 and H9 at 12 and 6 mos, respectively, but where no sample was available for analysis. BAL, bronchoalveolar lavage; CMV, cytomegalovirus; H, recipient at HR; HR, high risk; LTx, lung transplantation; M, recipient at MR; mo, month; MR, moderate risk; Neg, negative; PCR, polymerase chain reaction; Pos, positive; wk, week.

increased after CMV replication to 22% and 7% in H6 and H9, respectively (Figure 6a). In both recipients at HR before CMV replication, the vast majority of the $\gamma\delta$ T cells expressed V δ 2 (~80%), whereas the proportion that expressed V δ 1 in the circulation was only 14% (Figure 6b). Strikingly, after CMV replication, this ratio changed markedly as the proportion of V δ 1⁺ $\gamma\delta$ T cell increased to 87% and 65% of $\gamma\delta$ T cells in H6 and H9, respectively, which was accompanied by a concomitant decrease in the proportion of V δ 2⁺ $\gamma\delta$ T cells to the point where they were almost absent in recipient H6 (Figure 6b). Notably, it was the expanded V δ 1⁺ $\gamma\delta$ T-cell population that expressed

NKG2C at much higher levels than V δ 2⁺ $\gamma\delta$ T cells and CD3⁺ non- $\gamma\delta$ T cells (Figure 6c). Moreover, the proportion of V δ 1⁺ $\gamma\delta$ T cells expressing an effector phenotype (CD27^{low} CD45RA⁺) increased dramatically after CMV replication, namely, from 30% to 100% in H6 and from 15% to 85% in H9 (Figure 6d). Although there was also an increase in the proportion of effector V δ 2⁺ $\gamma\delta$ T cells in recipient H9 (19%–54%, Figure 6d), this was not to the same extent as V δ 1⁺ $\gamma\delta$ T cells. Moreover, as mentioned earlier, the overall proportion of V δ 2⁺ $\gamma\delta$ T cells had dramatically decreased after CMV replication in recipient H9. Notably, V δ 2⁺ $\gamma\delta$ T cells were virtually absent in the

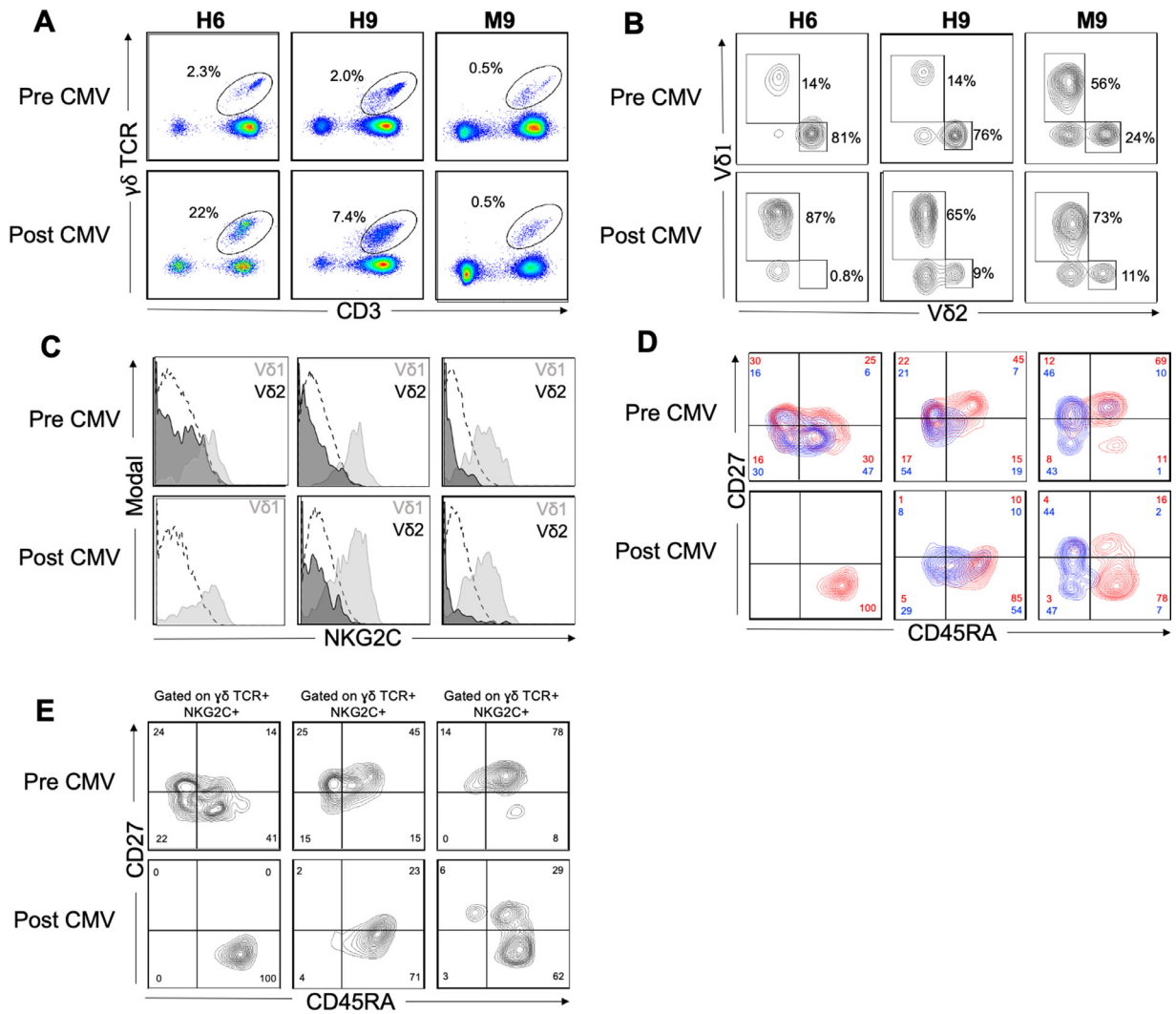


Figure 6 NKG2C+ V δ 1+ effector $\gamma\delta$ T cells are associated with CMV replication. Flow cytometry plots of recipients at HR (H6 and H9) and a recipient at MR (M9) before the detection of replicating CMV (before CMV: 6 months, 3 months, and 6 weeks after LTx for H6, H9, and M9, respectively) and after the detection of CMV (after CMV: 18 months, 12 months, and 18 months after LTx for H6, H9, and M9, respectively). (a) The proportion of $\gamma\delta$ T cells in the blood pre-CMV and post-CMV detection. Numbers represent $\gamma\delta$ T cells as a percentage of total lymphocytes. (b) The proportion of V δ 1 and V δ 2 $\gamma\delta$ T cells before and after the detection of CMV replication. Numbers represent the proportion as a percentage of total $\gamma\delta$ T cells. (c) The expression of NKG2C on V δ 1 (light gray histogram) and V δ 2 (dark gray histogram) $\gamma\delta$ T cells, compared with that of CD3-positive $\gamma\delta$ TCR-negative T cells (dashed histogram). (d) The proportion of V δ 1 (red) and V δ 2 (blue) $\gamma\delta$ T cells expressing CD27 and CD45RA before and after CMV replication. Numbers represent proportions of cells in each quadrant, with the proportions of V δ 1 showing in red and V δ 2 in blue. (e) The proportion of NKG2C+ $\gamma\delta$ T cells expressing CD27 and CD45RA before and after CMV replication. Plots are gated on NKG2C+ $\gamma\delta$ TCR+ cells. Numbers represent the proportions of cells in each quadrant. CMV, cytomegalovirus; H, recipient at HR; HR, high risk; LTx, lung transplantation; M, recipient at MR; MR, moderate risk; TCR, T-cell receptor.

circulation of recipient H6 after CMV replication (Figure 6b). A higher proportion of NKG2C+ $\gamma\delta$ T cells also expressed an effector phenotype after CMV replication in both recipients H6 and H9 (Figure 6e), likely as a result of the coexpression of NKG2C on V δ 1+ $\gamma\delta$ T cells (Figure 6c).

To examine whether subtle changes were also present in the recipients with CMV reactivation at MR, we performed a similar analysis of the coexpression of V δ 1, NKG2C, and effector $\gamma\delta$ T-cell subsets in a recipient at MR (M9). Indeed, although the proportion of $\gamma\delta$ T cells in the blood did not change before and after CMV replication in this recipient (Figure 6a), there was a proportional increase in the V δ 1+

population and decrease in V δ 2+ populations after CMV replication (Figure 6b). Notably, it was the V δ 1+ subset that expressed NKG2C (Figure 6c), and both the V δ 1+ (Figure 6d) and the NKG2C+ populations (Figure 6e) transitioned to an effector phenotype, whereas the V δ 2+ population lacked NKG2C (Figure 6c) and did not possess an effector phenotype (Figure 6d). Indeed, an analysis of the recipients with CMV replication at MR indicated changes in the composition of the $\gamma\delta$ T-cell compartment. Of particular note were recipients M11 and M15 who had low proportions of pre-LTx V δ 1+, NKG2C+, and effector $\gamma\delta$ T cells (Figures 2a, 4a, and 5a) but showed dramatic changes after CMV replication (see Supplementary Figure S2

online). Thus, taken together, the data show that CMV replication was associated with the development of effector V δ 1+ NKG2C+ $\gamma\delta$ T-cell population after LTx.

Discussion

Our study has found marked changes in circulating $\gamma\delta$ T cells after LTx, with a profound expansion of V δ 1+ NKG2C+ $\gamma\delta$ T cells strongly correlating with CMV replication after LTx. $\gamma\delta$ T cells have previously been shown to expand in response to CMV infection in patients who have undergone kidney transplantation and change the proportion of $\gamma\delta$ TCR sub-types²² from V δ 2+ to V δ 2-, with V δ 1+ cells specifically correlated with CMV immunity.²⁰ Our results suggest this to also be the case in lung transplant recipients at HR after CMV exposure, in keeping with what has been observed in other solid organ transplantations.⁹ Interestingly, most recipients who were CMV seropositive had stable, elevated frequencies of effector V δ 1+ cells that were similar in the pre-and post-LTx period, irrespective of subsequent episodes of CMV replication. In contrast, in lung transplant recipients at HR, there was a marked change in the composition of the $\gamma\delta$ T-cell population with CMV replication toward a cytotoxic effector phenotype, marked by the loss of CD27.²⁰ However, 3 recipients who were CMV seropositive (M11, M12, and M15) had lower frequencies of effector $\gamma\delta$ cells, and all the 3 of them experienced CMV replication. Notably, 2 of these recipients (M11 and M15) showed subsequent changes in the composition of $\gamma\delta$ T cells after CMV replication, similar to those seen in recipients at HR. Interestingly, M11 showed the highest CMV replication in the BAL, whereas for M15, it was in the blood (Table 1), indicating that the site of CMV replication did not impact on whether this subset was observed in the circulation. In contrast, recipient M12 failed to show such alterations in the $\gamma\delta$ T cell compartment and experienced multiple recurrences of CMV replication at 6, 9, and 11 months after LTx. It is tempting to speculate that CMV replication not only initiates the expansion of effector $\gamma\delta$ cells but that high levels of this subset also protect from further replication. A larger cohort from several transplant centers will verify these findings.

In contrast to previous studies, we did not observe an expansion of selected clones in the post-LTx period,^{8,20,23} rather we found a substantial increase in TCR diversity with CMV replication after LTx. Our observations may reflect expanded minor clones or thymic emigrants recruited into the immune repertoire in response to CMV. Indeed, a polyclonal CD8+ T cell response to CMV has been observed after umbilical cord blood transplantation.²⁴ It is also possible that this diversity is due to immunologic space created by immunosuppression after transplantation or inflammatory cytokines allowing for the non-specific expansion of unique clones. This, however, is unlikely because it does not explain the preferentially increased diversity in V δ 1+ cells, and moreover, V δ 1+ cells are reportedly refractory to expansion by key inflammatory cytokines (interleukin 12 or interleukin 18).²⁰ However, we acknowledge that our sequencing analyzes only 2 recipients

at HR and requires more recipients HR and MR to draw solid conclusions, which will be a focus of future studies.

Although the V δ 1+ $\gamma\delta$ T cell population after CMV replication in LTx had incredibly diverse TCRs, we found the coexpression of NKG2C. Intriguingly, and similar to that observed for NK cells,¹⁶ the proportion of NKG2C+ V δ 1+ $\gamma\delta$ T cells appeared to remain high and stable after the clearance of actively replicating virus. NKG2C expression on V δ 1+ $\gamma\delta$ T cells has previously been associated with cytotoxicity against human immunodeficiency virus-infected CD4+ $\alpha\beta$ T cells.¹³ Here, we associated the expression of NKG2C on $\gamma\delta$ T cells with CMV replication, suggesting an additional role for this subset in anti-viral immunity. NKG2C recognizes a monomorphic non-classical MHC class I molecule HLA-E. Moreover, NKG2C binds HLA-E, presenting the CMV-derived UL40 peptide, with a high affinity.²⁵ NKG2C expression on lymphocytes has been directly associated with CMV seropositivity²⁶ and a higher incidence of CMV replication in the LTx cohort in whom NKG2C gene deletion exists.²⁷ Although TCR ligand(s) for V δ 1+ $\gamma\delta$ T cells remain poorly defined, they have not been explicitly linked to CMV infection but include molecules induced by cellular stress. Although cells can be activated through NKG2C without the engagement of TCR,²⁸ their action is likely to be classical HLA-independent, favoring them as good candidates for cellular therapy sourced clinically from a third party without being HLA matched to the recipient.²⁹ Moreover, although NKG2C has been largely associated with CMV immunity, it is possible that V δ 1+ $\gamma\delta$ T cells are effective against other diseases where HLA-E is overexpressed, such as *Epstein-Barr virus*-associated cancers.³⁰ Intriguingly, a recent report found an association between the genetic lack of NKG2C and more severe coronavirus disease 2019 (Vietzen et al, unpublished data, 2020). Future research will be required to investigate the functional potential of NKG2C+ $\gamma\delta$ T cells in these settings. Moreover, investigations of this subset in the lung allograft itself will be of great benefit in pinpointing their contribution to local CMV immunity.

One drawback of our study was that all the recipients were on valganciclovir after LTx and that the withdrawal of anti-viral prophylaxis could have been responsible for initiating the expansion of this subset rather than active CMV replication. However, most recipients at MR and 2 recipients at HR ceased anti-viral prophylaxis before 6 months after LTx; yet, the enrichment of NKG2C+ V δ 1+ $\gamma\delta$ T cells only occurred coincidentally with CMV replication later in the post-LTx period. For example, recipient M6 who did not experience CMV reactivation exhibited consistently lower proportions of this subset up to 18 months post-LTx period, despite ceasing anti-viral prophylaxis at 160 days after transplantation. However, clearly, the importance of this $\gamma\delta$ T cell subset in preventing CMV reactivation in this particular individual is not apparent. Notably, this recipient had a very strong positive result from the QuantiFERON-CMV test, indicating a robust CMV-specific CD8+ T-cell response, and highlighting the likelihood that CMV control requires an orchestrated response from several immune compartments.

$\gamma\delta$ T cells represent an under-researched population of immune cells with the potential to contribute to both CMV immunity as a therapy or diagnostic tool. Monitoring transplant recipients for the phenotype of $\gamma\delta$ T cells could be highly relevant to developing new therapies against CMV after transplantation. Moreover, this cell subset may have utility in therapies aimed at reducing other viral infections.

Disclosure statement

The authors have no conflicts of interest to declare.

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Supplementary materials

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Supplementary data

Supplementary data associated with this article can be found in the online version at www.jhltonline.org/.

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