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Adoptive γδ T cell therapy controls cytomegalovirus infection in preclinical transplantation models

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ABSTRACT

γδ T cells show promise for anti-tumoral therapies but have yet to be evaluated to treat infectious diseases. In this preclinical study, we assess a Vδ1+ γδ T cell-based adoptive cell therapy, named Delta One T cells, to treat cytomegalovirus (CMV) infection in high-risk transplant recipients. Even when expanded from CMV-naïve healthy donors, Delta One T cells efficiently control CMV dissemination in vitro. CMV recognition is independent of the γδTCR but requires LFA-1 co-stimulation. In an *in vivo* model, adoptive transfer of mouse γδ T cells recapitulating Delta One T cell features protects mice against lethal murine CMV infection. Importantly, CMV-reactive Delta One T cells can be successfully generated from kidney transplant recipients undergoing refractory CMV infections and maintain their functionality in the presence of immunosuppressive drugs. These findings broaden the scope of γδ T cell therapies to infectious diseases and uncover a universal adoptive T cell therapy to treat refractory CMV infections.

INTRODUCTION

Human cytomegalovirus (CMV) infection remains the most common opportunistic infection following transplantation. In solid organ transplantation (SOT), CMV disease is more frequent in CMV-naïve recipients with a CMV-seropositive donor (D+R-, 15-20%), than in CMV-seropositive recipients (R+, 5-10%)[1,2]. CMV disease is associated with an increased risk of graft loss in all solid organ transplant recipients (SOTR) and a higher mortality in lung transplant recipients[3,4]. In hematopoietic stem cell transplantation (HSCT), D-R+ patients have the highest risk of CMV disease and a decreased survival[5].

Besides, in patients with CMV disease recurrence (15-30%)[6,7] or refractory CMV infection (up to 10%)[5,8], second-line therapies are associated with significant adverse effects. Maribavir has recently been approved as a curative therapy for refractory or resistant CMV disease in both HSCT and SOT[9]. Nevertheless, its efficacy is limited since it is not able to clear CMV at 8 weeks in 45% of patients, and is associated with more than 30% of recurrence and 26% of emergent mutations associated with resistance[10]. There is thus an urgent need for strategies able to induce a robust and persistent CMV-specific immune response, which is essential for lifelong viral control[11].

Adoptive transfer of CMV-specific CD4+ and CD8+ αβ T cells has emerged as an alternative therapy in both hematopoietic stem cell transplant recipients (HSCTR) and SOTR, with the goal to restore a potent anti-CMV adaptive immunity[12,13]. Third-party CMV-specific αβ T cell adoptive transfer was recently shown to improve survival upon refractory CMV infection in HSCTR[14] and SOTR[15]. In SOTR, autologous CMV-specific αβ T cell therapy may also benefit patients with recurrent or resistant CMV infections[16,17]. However, αβ T cell therapies are human leukocyte antigen (HLA)-restricted and are therefore currently limited, in term of feasibility and efficacy, to only a fraction of transplant recipients, raising interest for complementary, HLA-

independent, cellular immunotherapies. It is notably challenging to obtain sufficient numbers of CMV-specific αβ T cells from CMV-naïve patients, who are those with the highest risk to develop refractory CMV infections.

The unique capability of γδ T cells to kill tumor cells independently of major histocompatibility complex (MHC) presentation and their natural trafficking to tissues recently put them at the forefront of immunotherapy strategies, with many ongoing clinical trials in cancer. However, clinical use of γδ T cells in infectious diseases has yet to be tested, despite their well-demonstrated protective functions in several infectious settings[18–20]. Human γδ T cells have traditionally been divided into two subsets according to their T cell receptors (TCR): Vγ9Vδ2 T cells (also called $V\delta2^{pos}$ T cells) and non-Vγ9Vδ2 T cells (also called $V\delta2^{neg}$ γδ T cells). $V\delta2^{neg}$ γδ T cells, mainly composed of Vδ1 and Vδ3 T cells, were shown to be involved in the anti-CMV immune response in HSCTR[21] and SOTR[22,23]. CMV-induced $V\delta2^{neg}$ γδ T cells are mainly composed of activated T effector memory CD45RA+ (TEMRA) cells[24], and $V\delta2^{neg}$ γδ T cell clones or cell lines were shown to inhibit CMV replication and kill CMV-infected cells in vitro[25,26]. In SOTR, $V\delta2^{neg}$ γδ T cell expansion was associated with recovery from CMV infection[27] and this protective role was confirmed by several studies in mice[28,29]. Importantly, while αβ T cells only recognize antigens presented by class I or class II MHC (HLA in humans), γδ T cells can recognize antigens via their T cell receptor (TCR) without MHC/HLA restriction, allowing allogeneic cell therapy strategies. Altogether, these data support the use of $V\delta2^{neg}$ γδ T cells as a potential adoptive immunotherapy for refractory CMV infections, especially in high-risk D-R+ HSCTR and D+R- SOTR.

Recently, a robust clinical-grade method for selective and large-scale expansion of $V\delta2^{neg}$ γδ T cells, termed Delta One T (DOT) cells due to predominance (>70%) of Vδ1+ cells in the final

product, was developed for adoptive immunotherapy in patients suffering from chronic lymphocytic leukemia[30] and acute myeloid leukemia[31,32,33]. Leveraging this expansion protocol, we aimed to evaluate DOT cells against CMV infection in an attempt to extend the applications of adoptive γδ T cell therapies. In this study, we expanded Vδ2^{neg} γδ T cells (hereafter called DOT cells) from both CMV-seropositive (CMVpos) and CMV-seronegative (CMVneg) healthy blood donors, as well as D+R- SOTR. Our objectives were to characterize and compare their anti-CMV reactivity in vitro and in vivo, to dissect the molecular mechanisms underlying CMV sensing by DOT cells, and to evaluate the feasibility of an autologous therapy in immunocompromised kidney transplant recipients (KTR), towards the use of DOT cells as an adoptive immunotherapy for post-transplant refractory CMV infection.

RESULTS

DOT cell expansion from CMVpos and CMVneg healthy donors

As previously described, Vδ2^{neg} γδ T cells from the peripheral blood of healthy donors can be selectively and robustly expanded in vitro to clinically relevant numbers using the DOT protocol[30,31]. This procedure leads to a highly-polyclonal expansion stemming from naïve Vδ2^{neg} γδ T cells[31]. However, CMV infection strongly drives these cells towards terminal differentiation, reducing the frequency of naïve Vδ2^{neg} γδ T cells from approximately 70% in CMVneg donors to around or below 30% in CMVpos donors[24]. We therefore first set out to uncover a potential negative impact of previous CMV exposure on the in vitro expansion capacity of DOT cells. For this, DOT cells were expanded from 10 CMVneg and 10 CMVpos healthy blood donors and analyzed by flow cytometry as depicted in **Fig. 1a**. After 21 days of culture, we obtained a mean ± standard deviation (SD) of 4.3 ± 1.8x10^7 Vδ2^{neg} γδ T cells for CMVneg donors, compared to 6.2 ± 4.7x10^7 cells from CMVpos donors (**Fig. 1b**), with corresponding Vδ2^{neg} γδ T cell fold increases in culture of 1.8 ± 1.5x10^3 and 1.2 ± 0.7x10^3 (**Fig. 1c**). A strongly enriched cellular product was obtained, as Vδ2^{neg} γδ T cells represented 85.8 ± 5.1% and 84.8 ± 7.6% of expanded cells for CMVneg and CMVpos donors respectively (**Fig. 1d**). All these parameters did not significantly differ between the two groups (two-tailed unpaired t-tests, absolute numbers P = 0.25, fold increases P = 0.34, and percentages P = 0.71). We also confirmed that Vδ1+ γδ T cells were the main population obtained after in vitro expansion, followed by other Vδ2^{neg} γδ T cell subtypes, and rare Vδ2+ γδ T cells and natural killer (NK) cells. This repartition is illustrated in **Fig. 1e** for 3 CMVneg and 3 CMVpos donors and was not significantly impacted by the CMV serotype (determined by two-tailed unpaired t-tests for each cell population: Vδ1+ γδ T cells P = 0.7463; Vδ2+ γδ T cells P = 0.3458; Vδ3+ γδ T cells P = 0.2220; Vδ1/2/3^{neg} γδ T cells P = 0.2732; CD3^{neg}

cells $P = 0.8933$; αβ T cells $P > 0.9999$). Altogether, these results show that previous CMV infection does not affect the clinical-grade expansion of DOT cells.

DOT cells display a CMV-independent effector profile

Donor CMV serostatus could potentially influence DOT cell phenotype, and eventually affect their suitability for cell therapy applications. To test this hypothesis, DOT cells expanded from CMVneg or CMVpos donors were analyzed by multiparametric flow cytometry. At day 0, Vδ2^{neg} γδ T cells from CMVneg donors were predominantly naïve (CD27+CD45RA+, 74.0 ± 18.8%) whereas CMVpos donors displayed a higher frequency of TEMRA Vδ2^{neg} γδ T cells (CD27^{neg}CD45RA+, 41.2 ± 19.8%). This previously described difference[24] was lost at the end of the expansion, with a vast majority of CD27+CD45RA^{neg} central memory cells obtained for both serotypes (88.1 ± 9.2% for CMVneg donors and 88.1 ± 9.6% for CMVpos donors) (**Fig. 1f** and **Fig. S1a**). Importantly, a central memory phenotype was previously shown to be favourable for the persistence and long-term efficacy of infused T cell therapies[34,35].

A more extensive phenotyping was then performed, comparing 3 CMVneg and 3 CMVpos donors **(Fig. 1g**). Gating strategies are presented in **Figure S1b-c**, with a representative analysis of activation markers, and in **Figure S2**, with a representative analysis of killer cell immunoglobulin-like receptors (KIRs). After expansion, Vδ2^{neg} γδ T cells fully expressed activation markers, but only small percentages of cells expressed the inhibitory checkpoints CTLA-4, LAG-3 and PD-1. Besides, TIGIT expression was not impacted by in vitro culture, while expanded cells became positive for TIM-3. Co-stimulatory receptors were absent (OX40, CD40L), scarcely expressed (CD28, CD30, 4-1BB, CD8αα), or homogeneously expressed (LFA-1) after expansion, while the NK activating receptors DNAM-1, NKG2D, NKp30 and NKp44 were

strongly up-regulated, as previously described[30]. Of note, although percentages of LFA-1+ cells were not modified by in vitro expansion (**Fig. 1g**), its expression was strongly upregulated on a per cell basis, as indicated by a significant increase in median fluorescence intensity (**Fig. 1h**). Importantly, percentages of cells positive for the cytotoxicity ligands TRAIL and FasL were markedly increased, similarly to previous observations on granzyme B and perforin[31], thus indicating an overt cytotoxic phenotype. It is noteworthy that neither CD16 nor KIRs were induced by the expansion protocol. Finally, we observed no significant impact of the CMV serotype in this phenotypic analysis, as illustrated for 3 CMVneg and 3 CMVpos donors (**Fig. 1g** and statistical comparisons in **Figure S3**). Altogether, we were able to manufacture a DOT product composed of strongly activated $V\delta2^{neg}$ γδ T cells, poised for effector (particularly cytotoxic) functions, regardless of the donor CMV serological status.

DOT cells display anti-CMV effector functions

A primary objective of this study was to determine whether DOT cells can respond to CMV infection in vitro. The CMV clinical strain TB42/E was used to successfully infect three different target cell models, as evidenced by the presence of Immediate-Early (IE) CMV antigen in human foreskin fibroblasts, autologous monocyte-derived macrophages and glomerular endothelial cells (**Fig. S4a**). Building on their effector phenotype (**Fig. 1g**), we first explored the cytotoxic potential of DOT cells, by measuring the expression of the degranulation marker CD107a following monoculture or coculture with mock-infected or CMV-infected fibroblasts and macrophages (**Fig. S4b,c and Fig. 2a**). CMV infection significantly increased DOT cell degranulation against fibroblasts, and modestly against macrophages, with nevertheless a strong significance observed relative to DOT cells cultured in medium alone (**Fig. 2a**).

Next, we quantified the secretion of multiple cytokines, chemokines and growth factors (listed in the Methods section) in coculture supernatants to identify a potential CMV-induced secretome. The cytokines IFNγ and IL-10, as well as the chemokines GM-CSF, IP-10 and I-TAC, were induced by CMV infection in cocultures, with the strongest induction observed for IFNγ in cocultures with CMV-infected macrophages (**Fig. S4d**). We decided to further focus on IFNγ, which has been extensively characterized as a pleiotropic cytokine that modulates both innate and adaptive antiviral immune responses and can also inhibit directly CMV replication[36]. FNγ secretion in response to CMV-infected fibroblasts and macrophages was extensively confirmed in a larger cohort of DOT cell donors (**Fig. S4e**). Notably, as previously observed[37], IL-18 addition to cocultures potentiated IFNγ secretion in response to CMV. Indeed, in the presence of IL-18, IFNγ secretion was strongly induced by CMV-infection of fibroblasts, macrophages, but also endothelial cells (**Fig. 2b**).

Importantly, CMV-induced IFNγ secretion was independent of the donor's CMV serological status (means ± SD of 2.07 ± 2.05x10^3 pg/mL for CMVpos donors versus 2.18 ± 1.18x10^3 pg/mL for CMVneg donors, $P = 0.52$) (**Fig. 2c**). In addition, DOT cells secreted IFNγ in response to two distinct CMV strains (TB42/E and Merlin) but not in response to herpes simplex virus (HSV) or varicella zoster virus (VZV) infections (**Fig. 2d**). These results show a striking specificity of DOT cell reactivity to CMV infection, consistent with previous studies on Vδ2^{neg} γδ T cell clones or cell lines[22,24,25,38].

Finally, we examined whether degranulation, IFNγ secretion or other putative CMV-induced effector functions could be correlated with control of CMV spreading by DOT cells in vitro. Using a CMV dissemination assay (described in **Fig. 2e,** gating strategy shown in **Fig. S4f**), we found that DOT cells indeed inhibited CMV propagation in an effector-to-target (E:T) ratio-

dependent manner (**Fig. 2f**). Altogether, these results demonstrate that DOT cells are cytotoxic, secrete IFNγ in response to CMV infection, and are able to control CMV dissemination in vitro.

DOT cells do not need their TCR to recognize CMV-infected cells

The development of DOT cells as a therapeutic product requires a detailed understanding of the molecular mechanisms underlying CMV sensing by these cells. Using a Transwell system, we first observed that direct contact between DOT cells and fibroblasts was required for CMV-induced IFNγ secretion (**Fig. 3a**). We then tested which DOT cell receptors were involved in their reactivity against CMV-infected fibroblasts. We initially investigated the importance of the γδTCR, based on previous evidence that Vδ2^{neg} γδ T cells isolated from CMV-infected SOTR recognize CMV-infected fibroblasts in a TCR-dependent manner[25,38–40]. In contrast to these observations, adding a Vδ1 TCR blocking antibody to cocultures did not impact IFNγ secretion by DOT cells in response to CMV, as shown for eight independent donors in **Figure 3b**. However, as this result could have been explained by an incomplete Vδ1 TCR blocking (see **Fig. S5a**), we undertook to knock out the γδTCR of DOT cells obtained from three independent donors. Knockout (KO) yielded >90% γδTCR^{neg} cells in all three donors, as determined by TCR flow cytometry staining (**Fig. 3c**). KO efficiency was also verified by Sanger electropherogram analyses for donors 51 and 84, with 90% and 96% of editing respectively (given by the ICE analysis tool). We thus successfully generated viable γδ T cells lacking γδTCR expression, that we further sorted to obtain >99% of γδTCR^{neg} cells. γδTCR KO cells showed similar viability to control DOT cells in functional assays (**Fig. S5b and S5c**) and no longer secreted IFNγ in response to an agonist anti-γδTCR antibody, which validated the KO functionally (**Fig. S5d**). Nevertheless, γδTCR KO cell response to phorbol 12-myristate 13-acetate (PMA) and ionomycin stimulation was maintained

(**Fig. S5d**). This stimulation, commonly used as a positive control, bypasses TCR signaling by directly activating downstream signaling pathways.

Basal IFNγ secretion by DOT cells cultured alone or in the presence of mock-infected fibroblasts was completely abrogated in the absence of the γδTCR (**Fig. S5e**). In control cells (γδTCR+) basal IFNγ secretion is most likely explained by remaining TCR signaling following its stimulation by the anti-CD3 antibody used to expand DOT cells. In the presence of CMV-infected fibroblasts, IFNγ secretion by γδTCR KO cells was clearly reduced when compared to control γδ T cells (**Fig. S5e**), but was still quite substantial, and much higher than in the presence of mock-infected fibroblasts, as best illustrated with the fold differences shown in **Fig. 3d**. Altogether, these data show that DOT cells are still able to recognize CMV-induced signals on infected cells in the absence of γδTCR. This TCR-independent recognition is consistent with the highly polyclonal repertoire of DOT cells.

LFA-1 is required for CMV sensing by DOT cells

Because the NK activating receptors NKp30, NKp44, NKG2D and DNAM-1 were upregulated after expansion (**Fig. 1g and h**) and have previously been implicated in DOT cell response against tumor cells[30,31,33,41], we next tested whether they contribute to CMV recognition. Blocking these receptors in cocultures with CMV-infected fibroblasts had no significant impact on IFNγ secretion (**Fig. 3e**). This suggests that, in stark contrast to their key role in tumor cell recognition, these NK cell receptors are not involved in the anti-CMV reactivity of DOT cells.

Since DOT cells express high LFA-1 levels (**Fig. 1h**), and CMV-infection of fibroblasts strongly increases ICAM-1 surface expression[39], we investigated whether this axis plays a role in CMV sensing, as previously observed with $V\delta2^{neg}$ γδ T cell clones[39]. DOT cells could indeed

secrete IFNγ after stimulation with an anti-LFA-1 agonist antibody (**Fig. 3f**), or stimulation with its activating ligand ICAM-1 (**Fig. 3g**), in a dose dependent manner. Importantly, in coculture with CMV-infected fibroblasts, blockade of LFA-1 or ICAM-1 systematically reduced IFNγ secretion by DOT cells expanded from six independent donors, with the strongest effect observed when both were blocked simultaneously (**Fig. 3h**). We could not assess the functional impact of LFA-1 knockout, as repeated attempts failed to generate LFA-1^{neg} DOT cells, likely because of high mRNA or protein stability. Altogether, stimulation and blocking experiments suggest that LFA-1 contributes to DOT cell anti-CMV reactivity.

Expanded mouse γδ T cells control CMV infection in vivo

We next wanted to assess the efficacy of an adoptive γδ T cell therapy to treat CMV infection in an in vivo setting. However, CMV tropism being species-specific, mice can only be infected by murine CMV (MCMV), whereas human DOT cells can only recognize human CMV-infected cells. We therefore developed a method recapitulating γδ T cell expansion from mouse γδ T splenocytes in vitro, and used these expanded cells as an adoptive therapy to treat MCMV-infected mice. We adapted the human DOT protocol (see Methods section) to amplify γδ T cells from 9 MCMV-naïve (MCMVneg) and 9 MCMV-infected (MCMVpos) C57BL/6J wild type mice (**Fig. 4a-d).** After 7 days of culture, we obtained a mean ± SD of 2.8 ± 1.2 $x10^7$ γδ T cells from MCMVneg mice, compared to 2.5 ± 1.1 $x10^7$ cells from MCMVpos mice (**Fig. 4b**), with corresponding γδ T cell fold increases in culture of 1.15 ± 0.49 $x10^2$ and 1.16 ± 0.46 $x10^2$, respectively (**Fig. 4c**). A strongly enriched cellular product was obtained, as γδ T cells represented 84.4 ± 6.5% and 82.3 ± 8.9% of expanded cells for MCMVneg and MCMVpos mice, respectively (**Fig. 4d**). All these parameters did not significantly differ between the two groups (two-tailed

Mann-Whitney test, γδ T cell absolute numbers $P = 0.65$, fold increases $P = 0.80$, and percentages $P = 0.88$, respectively). In line with the human DOT product, a majority of central memory γδ T cells (CD62L+CD44+) was obtained after expansion: 57.2 ± 8.2% and 54.7 ± 4.9 for MCMVneg and MCMVpos mice respectively (**Fig. 4e,** representative dot plots shown in **Fig. S7a**). Phenotyping of murine γδ T cells before and after expansion showed an increase in activation (CD25), a lack of immune checkpoints such as PD-1 or TIM-3, and an upregulation of NK activating receptors (DNAM-1, NKG2D), cytotoxicity receptors (FasL and TRAIL), as well as LFA-1, independently of previous CMV priming (**Fig. 4f** and statistical analysis in **Fig. S6)**. Altogether, we were able to generate a cellular product of expanded murine γδ T cells similarly as and phenocopying human DOT cells, regardless of the MCMV status of the donor mouse.

We next evaluated whether in vitro-expanded murine γδ T cells could protect immunodeficient $Rag^{-/-}\gamma c^{-/-}$ mice from MCMV-induced mortality (**Fig. 4g**). Adoptive transfer of murine γδ T cells expanded from MCMVneg (n=22) and MCMVpos (n=13) mice significantly improved the survival of MCMV-infected $Rag^{-/-}\gamma c^{-/-}$ recipients, compared to RPMI-treated controls (n=18) ($P = 0.0001$ and $P < 0.0001$ respectively, log-rank Mantel-Cox test). This effect was independent of the MCMV status of donor mice ($P = 0.0837$) (**Fig. 4h**). In parallel, we were able to detect the presence of transferred γδ T cells in the spleens, livers and lungs of surviving mice at endpoint (n=11 analyzed from 5 MCMVneg and 6 MCMVpos donors, **Fig. 4i** and gating strategy illustrated in **Fig. S7b**), where they displayed a TEM phenotype as illustrated on **Fig. S7c**. In addition, this model gives us the opportunity to evaluate virus dissemination in different organs. Surviving mice that had received γδ T cells (n = 7 analyzed; from 4 MCMVneg and 3 MCMVpos donors) exhibited significantly lower viral loads in the spleen, liver and lung, than those that succumbed to infection ($P < 0.0001$, two-tailed Mann-Whitney test; **Fig. S7d**). To directly test

whether γδ T cell adoptive transfer can control MCMV viral load in organs, we transferred γδ T cells expanded from MCMVpos (n=5) or MCMVneg (n=5) C57BL/6J donor mice into MCMV-infected Rag-/-γc-/- mice and compared viral load to RPMI-treated controls (n=5) at day 14 post infection. Both γδ T cell treatment groups had significantly reduced viral loads in all three organs relative to controls (**Fig. 4j**). Together, these findings demonstrate that adoptively transferred γδ T cells can migrate to MCMV-infected organs, control viral replication, and protect immunodeficient mice from lethal MCMV infection.

Autologous CMV-reactive DOT cells can be obtained from SOTR

Both autologous and allogeneic virus-specific T cell therapies have been or are currently being tested in phase I trials in SOTR[15,17,42] (CTRN12621000323820) or in a phase III trial in HSCTR (TRACE study: NCT04832607). Among these two strategies, the autologous approach is particularly attractive in SOTR to avoid the risk of allosensitization deleterious to the transplant[43]. However, whether functional and CMV-reactive DOT cells can be expanded from immunosuppressed transplant recipients remains to be evaluated. In order to assess the feasibility of an autologous approach, peripheral blood mononuclear cells (PBMCs) were obtained from eight D+R- KTR (see **Table 1**) with recurrent and refractory CMV infections, among which five exhibited an antiviral drug resistance (UL97 mutation) requiring Foscavir or Maribavir treatment. Despite lymphopenia, DOT cells were expanded from all of these eight critical patients with, at day 21 of culture, a mean ± SD of 1.01 ± 1.04x10^8 Vδ2^{neg} γδ T cells, a fold increase of 0.72 ± 1.75x10^4 and a percentage in culture of 83.68 ± 8.17% (**Fig. 5a-c**). Importantly, these eight cellular products secreted IFNγ in response to CMV infection (**Fig. 5d)**. Altogether, these results show that CMV-reactive DOT cells can be obtained from SOTR to serve as autologous adoptive cell therapy.

DOT cells remain functional under immunosuppression

Another important requirement is that infused DOT cells persist and expand in SOTR despite immunosuppressive treatments. To test this, DOT cells expanded from healthy donors were subsequently cultured in the presence of IL-15 and different immunosuppressive drugs classically used in SOTR (two calcineurin inhibitors: tacrolimus and cyclosporin, the mTOR inhibitor everolimus and the antiproliferative drug mycophenolate mofetil). After seven days, their proliferation was, as expected, completely inhibited by mycophenolate mofetil (MMF), but was maintained in the presence of tacrolimus, cyclosporin and everolimus (**Fig. 5e and 5f**). These results were confirmed after longer treatments of 15 days, by analyzing DOT cell counts and viability (**Fig. 5g and 5h)**. To evaluate their functionality, DOT cells were cocultured during 24h with CMV-infected fibroblasts in the presence of the same immunosuppressive drugs. CMV-induced IFNγ secretion was entirely suppressed by MMF, but was maintained in the presence of tacrolimus, cyclosporin and everolimus alone (**Fig. 5i**). In addition to the immunosuppressive drugs tested above, glucocorticoids are a standard component of the immunosuppressive regimen after SOT. Importantly, treatment with the glucocorticoid methylprednisolone at the concentration typically used in the maintenance treatment of SOTR (around 10 ng/ml, 12 hours after ingestion[44]) did not inhibit DOT cell proliferation (**Fig. S8a-b**) or their IFNγ secretion in response to CMV-infected fibroblasts (**Fig. S8c**). In summary, these data show that DOT cells are maintained in vitro and respond to CMV despite the presence of immunosuppressive drugs, strongly supporting the feasibility of a DOT cell therapy in SOTR.

DISCUSSION

Effective αβ and γδ T cell responses are critical to prevent CMV infection and disease in HSCTR and SOTR. The indicence of CMV recurrence and disease after transplantation has indeed been associated with the amount of CMV-specific αβ T cells or Vδ2^{neg} γδ T cells present in the recipient before graft or during the first months post-transplantation[27,45]. Reconstituting these antiviral responses after transplantation is currently an unmet need, which could be reached through the use of adoptive cell therapies. αβ T cell-based therapies developed so far from CMVpos donors are promising and have reached the clinics. However, challenges arise in CMV-naïve HSCT donors and SOT recipients (reviewed in[18]). While the manufacturing of CMV-specific αβ T cells from CMV-naïve donors was recently achieved, it is a lengthy and complex procedure not yet ready for large-scale dissemination[46]. Encouragingly, a clinical trial successfully reported adoptive transfer of autologous αβ T cells expanded from SOTR, including patients with no prior detectable anti-CMV αβ T cell response[16,17]. Here, we focused on Vδ2^{neg} γδ T cells as a potential complementary or alternative cell therapy, based on strongly established evidence of their anti-CMV functions in vitro and in vivo. Vδ2^{neg} γδ T cells were for long poorly studied for clinical application in comparison with their Vδ2^{pos} counterparts. However, thanks to the development of good manufacturing practices (GMP)-compatible, clinical-grade protocols for their specific expansion, notably the DOT cell protocol[30,31] applied in this study, adoptive Vδ2^{neg} γδ T cell transfer has recently reached the clinical trial phase as a cancer treatment (NCT05886491).

Here, we demonstrate the reproducible and large-scale expansion of DOT cells responding to CMV in vitro, feasible from potential HSCT donors, independently of CMV previous exposure, and from D+R- KTR undergoing recurrent or resistant CMV infection, despite ongoing immunosuppressive treatments. Compared to the landmark clinical trial by Smith and colleagues using autologous αβ T cells[16,17], DOT cell yields obtained are relevant for the adoptive transfer of

several doses per patient. Because of their HLA independence, both autologous and allogeneic DOT cell transfers are possible. However, in SOTR, an autologous therapy could be prioritized to avoid HLA sensitization. In this context, the ability to generate CMV-reactive DOT cells from the naïve γδ T cell pool may offer an advantage in patients with no CMV-specific memory αβ T cells. In the allogeneic setting, being able to generate DOT cells from both CMV-seronegative and CMV-seropositive individuals is critical to ensure broad donor availability.

In an in vitro CMV dissemination assay with infected fibroblasts[47,48], DOT cells displayed a striking antiviral effect. Although we have shown that CMV infection induces the secretion of the antiviral cytokine IFNγ, a direct cytotoxic effect could also participate in this control, as suggested by degranulation assays. In addition to IFNγ, we observed other CMV-induced soluble effectors in coculture, which could possibly mediate additional DOT cell functions in vivo. For instance, IP-10, I-TAC and GM-CSF secretion could attract myeloid cells and lymphocytes to CMV infection sites and contribute to the establishment of anti-CMV adaptive immunity.

Although their physiology is distinct from Vδ2^{neg} γδ T cells, the molecular determinants of DOT cell reactivity against CMV must be thoroughly characterized to enable their development as a therapeutic product. CMV sensing by Vδ2^{neg} γδ T cells was previously shown to be TCR-dependent[18,38]. By contrast, we demonstrate here that DOT cells that already received TCR stimulation during their expansion no longer require TCR engagement to distinguish CMV-infected from uninfected cells. This does not imply that TCR signaling is irrelevant to DOT cell function, as these cells may carry a legacy of prior TCR activation, through altered downstream signalling pathways as well as epigenetic imprinting. A similar mechanism was previously proposed for DOT cell recognition of acute myeloid leukemia cells, which was TCR-independent but required the activating NK receptors NKp30 and DNAM-1[31]. On the other hand, previous

studies have shown the importance of LFA-1 co-stimulation in CD4+, CD8+ T cell[49,50], NK cell[51] and Vδ2^{neg} γδ T cell (Vγ4Vδ5 clone[39]) responses against CMV. Here, we extend these findings to polyclonal Vδ2^{neg} γδ T cells, and corroborate the importance of integrins in γδ T cell functions[52]. Altogether, our results show how multiple signals can tune DOT cell full activation in response to CMV: (i) stimulatory signals provided during the expansion step, namely TCR stimulation combined with IL-15, (ii) sensing of CMV-infected cells associated with co-stimulatory receptor engagement such as LFA-1, and (iii) an appropriate environment, for instance with the CMV-induced inflammatory mediator IL-18[37]. Notably, IL-18 is not only induced by CMV infection in vitro, but also elevated in vivo in transplant recipients with CMV disease[53,54]. This multiplicity illustrates the concept of CMV-induced multimolecular stress signature, previously shown to activate Vδ2^{neg} γδ T cell clones, which also appears to be critical for DOT cell activation.

The multifactorial activation of Vδ2^{neg} γδ T cells in response to CMV can indeed be seen as a safeguard, preventing excessive activation, and is of interest for safety. Regarding preclinical safety assessment, it was previously shown that DOT cells do not react against autologous PBMCs[30] and allogeneic lymphoid or myeloid populations[31]. Here, in complement to these observations, we did not see compelling IFNγ secretion in response to allogeneic mock-infected fibroblasts or glomerular endothelial cells. Although DOT cells secreted IFNγ in response to mock-infected macrophages, this result is in adequation with previous observations showing a cytotoxic activity of γδ T cells towards activated macrophages, as a regulatory mechanism for macrophage populations homeostasis[55,56]. In addition to these in vitro observations, results from the ongoing phase I clinical trial of DOT cell adoptive transfer in acute myeloid leukemia (NCT05886491) will provide important safety information. Besides, mouse γδ T cells were unable to induce graft versus host disease after HSCT[57] and, in human KTR, only the CD16+ γδ T cell subset was involved in

antibody-mediated rejection in HLA-sensitized patients[58]. As DOT cells do not express CD16, there is no rationale to think that this adoptive cell therapy can induce more rejection[59].

In the setting of anti-CMV adoptive therapies, preclinical murine models are difficult to implement, since HCMV does not infect mice and human DOT cells do not react against MCMV-infected cells. Although humanized mouse models exist, they are currently limited and far from replicating the features of CMV infection observed in humans. To evaluate in vivo efficacy, we therefore developed a protocol to expand murine γδ T cells recapitulating the phenotype of human DOT cells, and used these cells as adoptive therapy in immunodeficient mice infected with MCMV. The main advantage of this model is its similarity to human CMV pathogenesis, making it appropriate for testing new cell therapies. Using this model, we have previously shown that MCMV induces long-term expansion of murine γδ T cells that protect immunodeficient mice from CMV infection[28,60]. MCMV is thus an interesting model to make parallels between human and murine γδ T cells. After adoptive transfer, in vitro-expanded mouse γδ T cells were able to migrate and persist in infected organs, leading to a protection from MCMV-induced death. These findings strongly support the use of DOT cells as an adoptive therapy to improve the outcome of refractory or resistant CMV infection in HSCTR and SOTR.

Several key aspects regarding adoptive transfer potency will only be answered in a clinical trial. Efficacy will notably depend on the persistence and functionality of infused DOT cells. The immunosuppressive treatments currently used in SOTR are a combination of tacrolimus (or cyclosporin) with mycophenolate (or everolimus) with or without steroids. Patients with recurrent or refractory CMV disease often receive a weaker regimen, which may be tacrolimus monotherapy, cyclosporin monotherapy, or a combination of low-dose tacrolimus with everolimus. For now, in vitro cultures in the presence of these immunosuppressive drugs showed that DOT cell therapy would be compatible with regimens based on tacrolimus, cyclosporin, or

everolimus, since these drugs did not affect the persistence and functionality of DOT cells. Tacrolimus and cyclosporin are calcineurin inhibitors, which function by inhibiting IL-2 production in T cells and therefore impairing their proliferation. The independence of DOT cells on IL-2 for expansion and maintenance may explain their resistance to these two drugs. We have also previously shown that everolimus, at doses used in SOTR, has a positive effect on T cell proliferation and survival[61]. These observations are in line with αβ T cell adoptive transfer follow-up in SOTR, which showed persistence of infused cells despite immunosuppression in a recent study[17].

Research on DOT cells comprehensively established their potency against hematological malignancies[30–33], leading to an ongoing clinical trial in acute myeloid leukemia (NCT05886491). It exemplifies the rising interest in γδ T cell-based immunotherapies against cancer, with different strategies including adoptive cell therapy or agonist antibodies yielding promising results. Our findings now position DOT cells for immunotherapy of infectious diseases. They pave the way to solve the currently unmet need for strategies to treat recurrent, refractory or resistant CMV infections in transplant recipients. Based on this preclinical study, a DOT cell therapy appears ready to be tested in a dose-escalation phase I clinical trial.

METHODS

Study approval and design

Blood samples from healthy donors and kidney transplant recipients were obtained after written informed consent, in accordance with the Declaration of Helsinki. KTR were included in the SPARCKLING study (NCT03339661), approved by the Bordeaux University Hospital ethics committee CHUBX 2016/40, or the HORUS study (NCT05701228), approved by the Bordeaux University Hospital ethics committee CHUBX 2022/10. As CMV infection can affect all sexes and genders and sex is not a known risk factor, we did not perform sex- or gender- based analyses, and findings apply to individuals of all sexes and genders. Sex and gender were not considered in the design of the SPARCKLING and HORUS studies, and were self-reported. Besides, the in vivo protocols described in this study were approved by a local ethics committee (Bordeaux University, committee n°2016092917471799).

DOT cell expansion

Blood samples were obtained from healthy volunteers (50 mL), recruited at the local blood bank (Etablissement Français du Sang), or from KTR (20 mL). CMV serologies from healthy donors and KTR were determined by enzyme-linked immunosorbent assay (ELISA) upon request. When determined, the CMV serotype of healthy donors is provided as a source data file and indicated in the corresponding figures. PBMCs were isolated using lymphocyte separation medium (Eurobio Scientific), and stained with an antibody directed against the αβTCR, coupled to biotin (Miltenyi Biotec, clone REA652, diluted 1:50), followed by anti-biotin microbeads (Miltenyi Biotec). αβ T cells were removed from PBMCs by magnetic-activated cell sorting and remaining PBMCs were cultured for 21 days to expand $V\delta2^{neg}$ γδ T cells, following the DOT cell protocol[30,31]. Cells were

cultured in OpTmizer™ CTS™ serum-free medium (GIBCO, Life Technologies) supplemented with 10% human serum, 1% Penicillin-Streptomycin (PS), and 2mM L-glutamine (expansion medium), at a concentration of 160 000 cells/mL in the presence of the following recombinant human cytokines (all from Peprotech): 100 ng/mL interleukin 4 (IL-4), 70 ng/mL interferon γ (IFNγ), 7 ng/mL IL-21, 15 ng/mL IL-1β and an activating antibody directed against CD3 (1 µg/mL, clone OKT-3, Biolegend). At days 6, 11 and 16 of culture, expansion medium was renewed and supplemented with 70 ng/mL IL-15 (days 6, 11, 16), 13 ng/mL IL-21 (day 6), 70 ng/mL IFNγ (day 16), and 2 µg/mL anti-CD3 (days 6, 11, 16).

DOT cell culture in the presence of immunosuppressive drugs

After in vitro expansion, DOT cells were counted and resuspended at 1 x 10^6 cells/mL in the presence of 100 ng/mL IL-15 and the following immunosuppressive drugs: tacrolimus (7 ng/mL), cyclosporin (75 ng/mL), everolimus (0.5 nM), MMF (1 µg/mL), or methylprednisolone (1, 10 or 100 ng/mL). When indicated, DOT cells were labeled with 5µM carboxyfluorescein succinimidyl ester (CFSE) or CellTrace™ Violet (CTV), following the CellTrace™ Cell Proliferation Kit instructions (Thermofisher Scientific). After 7 days, the culture medium was renewed with fresh IL-15 and immunosuppressive drugs. After 7 to 15 days, DOT cells were harvested and viable cells were analyzed for viability by 4′,6-diamidino-2-phenylindole (DAPI) staining, or for proliferation based on CFSE or CTV dilution. Proliferation indexes were calculated using the proliferation tool in FlowJo V10.5 (TreeStar).

DOT cell phenotyping by flow cytometry

Cells were harvested at days 0, 11 and 21 of expansion, and stained for flow cytometry analysis[38]. Single cell suspensions were first washed twice in staining buffer: phosphate-buffered saline (PBS) supplemented with 0.5% bovine serum albumin (BSA) and 2mM ethylenediaminetetraacetic acid (EDTA), with centrifugations at 300 x g for 5 min. Cells were resuspended in staining buffer and stained with a viability dye (Fixable Viability Stain 780, BD Biosciences or Viobility 405/520 Fixable Dye, Miltenyi Biotec) for 10 min at room temperature in the dark, washed twice, and subsequently resuspended in staining buffer in the presence of the following antibodies, for 20 min at 4° C in the dark. For cell culture follow-up (at days 0, 11 and 21): antibodies directed against CD3, CD27, CD45RA, γδTCR, αβTCR, Vδ1 TCR and Vδ2 TCR were used. For characterization of cell populations among expanded cells, antibodies directed against CD3, γδTCR, Vδ1 TCR, Vδ2 TCR, Vδ3 TCR, CD14, CD16, CD19 and CD56 were used. For extensive phenotyping at days 0 and 21, cells were stained with antibodies directed against the population markers CD3, γδTCR, Vδ1 TCR, Vδ2 TCR; the activation markers CD25, CD69, CD71, HLA-DR; the immune checkpoints CTLA-4, LAG-3, PD-1, TIGIT and TIM-3; the co-stimulatory receptors CD8αα, CD11a-CD18 (LFA-1), CD28, CD30, CD134 (OX40), CD137 (4-1BB), CD150 (SLAMF1), CD154 (CD40L); the NK activating receptors CD16, CD226 (DNAM-1), NKG2D, NKp30, NKp44, NKp46; the KIRs KIR2DL1 (CD158a), KIR2DL1/S1 (CD158a/h), KIR2DL2/L3 (CD158b), KIR3DL1 (CD158e1), KIR3DL1/S1 (CD158e1/e2), KIR2DS4 (CD158i); and the cytotoxicity markers TRAIL and FasL. After antibody staining, cells were washed twice in staining buffer and analyzed on a Canto II cytometer (BD Biosciences) for cell culture follow up, or on a LSR II Fortessa cytometer (BD Biosciences) for other panels. Data were analyzed with FlowJo V10.5. Heatmap representation was created with the MeV (Multiple Experiment Viewer) software. Data regarding antibodies are available in **Supplementary Table 1**.

CRISPR-Cas9 knockout in DOT cells

Two guide RNAs (gRNAs) were designed using the CRISPOR algorythm[62,63], targeting the T cell receptor delta constant (TRDC) gene, coding for the constant region of the γδTCR δ chain: gRNA1 TGGAACAAATGTCGCTTGTC and gRNA2 AAGTACAATGCTGTCAAGCT. gRNAs were selected based on their high specificity scores, as calculated by CRISPOR using the cutting frequency determination (CFD) specificity metric[64,65], and by inspection of predicted off-target sites. Both gRNAs had CFD specificity scores above 90 (95 and 92, respectively), with no predicted off-target exonic sequences bearing fewer than four mismatches. Detailed information regarding gRNA characteristics is available in **Supplementary Table 2.**

After expansion, DOT cells were rested for 24h in expansion medium supplemented with 70 ng/mL IL-15, in order to allow complete surface expression of the TCR. CRISPR-Cas9 knockout was performed by Cas9/gRNA ribonucleoprotein transfection of DOT cells[66]. crRNA and tracrRNA oligonucleotides were first annealed at equimolar concentrations by heating at 95°C for 5min. Ribonucleoprotein complexes were formed by incubating duplexed gRNAs (100µM) with Cas9 (62 µM) at a 1:1 volume ratio for 10min at room temperature. The Alt-R™ S.p. HiFi Cas9 Nuclease V3 was used (engineered for reduced off-target activity). DOT cells were washed in PBS, resuspended in 20µL of supplemented Nucleofector solution (Lonza, V4XP-3032) and mixed with 5µL of ribonucleoprotein complex and 1µL of Alt-R™ CRISPR-Cas9 Electroporation Enhancer (100µM). Cells were electroporated using a 4D-Nucleofector system (Lonza) with the EH115-P3 program. Immediately after electroporation, cells were recovered in their pre-warmed expansion medium supplemented with 70 ng/mL IL-15. As a control, DOT cells were transfected with a ribonucleoprotein incorporating a non-targeting gRNA (Alt-R™ CRISPR-Cas9 Negative

Control crRNA). All CRISPR reagents were purchased from IDT. Knockout efficiency was assessed 96h after transfection by flow cytometry, using antibodies directed against CD3, γδTCR, Vδ1 TCR and Vδ2 TCR. Knockout efficiency was also verified by the analysis of Sanger electropherograms, using the ICE v2 CRISPR Analysis Tool (Synthego) which gives a percentage of editing. Remaining γδTCR+ cells were subsequently depleted using a FACS ARIA (BD Biosciences) in order to achieve >99% purity. After sorting, DOT cells were rested overnight and used for functional assays.

Target cell cultures

Human foreskin fibroblasts (HFF) were provided by Dr. Hamid-Reza Rezvani (INSERM, U1035, Bordeaux). They were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine and 1% PS (complete DMEM), and used up to passage 18. Conditionally immortalized glomerular endothelial cells (CiGEnC) were cultured in complete Endothelial Cell Growth Medium MV2 (Promocell) supplemented with 1% PS, maintained at 33°C and differentiated at 37°C for 5 days[67] prior to CMV infection. Autologous human monocytes were obtained from healthy donors' PBMCs. PBMCs were stained with human CD14 MicroBeads (Miltenyi Biotec) and positively sorted by magnetic-activated cell sorting. Sorted cells were stained with a viability dye (FVS780) and an antibody directed against CD14 (CD14-Vioblue, Miltenyi Biotec) to verify monocyte purity. Monocytes were seeded at an initial concentration of 500 000 cells/mL and differentiated into M1 macrophages for 6 days, in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FBS, 2mM L-glutamine, 1% PS, and 10 ng/mL recombinant human granulocyte-macrophage colony-stimulating

factor (GM-CSF, Peprotech). Additional 5 ng/mL GM-CSF was added to the culture after 3 days. All cell lines were regularly tested for mycoplasma contamination.

Preparation of CMV, HSV and VZV stocks

Five virus strains were used for in vitro experiments: the CMV clinical strain TB42/E was a gift from Prof. Dr. Christian Sinzger (Ulm University, Germany), CMV clinical strains Merlin and Merlin UL32-GFP were kind gifts from Dr. Ceri Fielding (Cardiff University, UK). Herpes simplex virus-1 (HSV-1) KOS strain and Varicella Zoster Virus (VZV) OKA strain were kind gifts from Dr. Sonia Burrel (Virology Unit, Pitié Salpétrière University Hospital, France). CMV stocks were prepared by infecting HFF immortalized by human telomerase (HFF TERT) at a low multiplicity of infection (MOI) for 2-3 weeks. When 100% cytopathic effect was observed, supernatants were harvested, centrifuged at 300 x g for 10 min and stored at -80°C. HSV and VZV stocks were prepared by infecting MRC5 cells (obtained from Eurobio Scientific). When 50-100% cytopathic effect was observed, supernatants were harvested and directly frozen at -80°C, in 50% FBS for HSV and in 10% dimethyl sulfoxide (DMSO), 20% FBS for VZV. CMV, HSV and VZV stocks were titrated by plaque assays on HFF[68], using 2% Avicel (Sigma-Aldrich).

CMV, HSV and VZV infections

HFF were mock-infected or infected by CMV at a MOI of 0.01, by VZV at a MOI of 0.01, and by HSV at a MOI of 1 in complete DMEM. CiGEnC and macrophages were infected by CMV strain TB42/E at a MOI of 1 in complete DMEM. After overnight incubation, infection medium was replaced by fresh complete DMEM. HFF infection by CMV, HSV and VZV were monitored by evaluating cytopathic effects by microscopy. Additionally, percentages of CMV-infected HFF,

CiGEnC and macrophages were quantified by flow cytometry: mock-infected or infected cells were fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience), according to the manufacturer's instructions and stained with an antibody directed against the human CMV IE antigen (Merck Millipore). HFF were infected by CMV and VZV for 5 days, and by HSV for 2 days, in order to reach 50-100% of cells showing cytopathic effects at the start of functional assays. CiGEnC and macrophages were infected by CMV for 2 days, in order to maximize the % of CMV-IE cells, before functional assays.

Cocultures and reactivity assays

After 21 days of expansion, DOT cells were washed and rested for 2 days in expansion medium supplemented with 70 ng/mL IL-15 before functional assays. DOT cells were cocultured with CMV-infected or mock-infected target cells in 96-well plates, at an effector to target (E:T) ratio of 1:1, in complete RPMI 1640 medium. For experiments measuring IFNγ secretion, IL-18 (50 ng/mL) was added to cocultures when specified in the figure legends and panels.

To study the impact of immunosuppressive drugs, tacrolimus (7 ng/mL), cyclosporin (75 ng/mL), everolimus (0.5 nM), MMF (1 µg/mL) or methylprednisolone (1, 10 or 100 ng/mL) were added to the coculture medium.

For receptor blocking, DOT cells were preincubated for 30 min at room temperature with neutralizing antibodies directed against Vδ1 TCR, DNAM-1, NKG2D, NKp30, NKp44, LFA-1, or a control mouse IgG1k antibody. When indicated, target fibroblasts were preincubated with an antibody directed against ICAM-1 or a control mouse IgG1k antibody. Cells were subsequently washed before coculture. For antibody-mediated activation, DOT cells were incubated with coated antibodies directed against CD3 (clone OKT-3, Biolegend), LFA-1 (clone 25.3, Beckman Coulter)

or a control mouse IgG1k antibody. Information on antibodies used for blocking and stimulation is available in **Supplementary Table 3**. When indicated, DOT cells were activated with different concentrations of coated recombinant human ICAM-1 FC Chimera (R&D Systems, catalog number 720-IC-050), or with 25 ng/mL PMA (Sigma, catalog number P8139) and 1 µg/mL ionomycin (Sigma, catalog number A23187).

Transwell experiments were performed in 24-well plates. DOT cells were incubated in inserts (0.4µm pore polyester membrane from Corning) and target fibroblasts in the lower chambers. Cocultures were performed as described above.

After 24h, supernatants were harvested for ELISA and LEGENDplex assays. When indicated, cells were also harvested, stained with 1 µg/mL DAPI (Thermofisher Scientific, catalog number D1306) and percentages of viable cells ($DAPI^{neg}$) were quantified by flow cytometry on a Canto II cytometer (BD Biosciences).

Degranulation assays

DOT cells were labeled with 0.5µM CFSE, following the CellTrace™ CFSE Cell Proliferation Kit instructions (Thermofisher Scientific) and subsequently cocultured for 16h with target cells. An anti-CD107a antibody, 0.67 µL/mL monensin (BD GolgiStop, BD Biosciences) and 1 µg/mL brefeldin A (Sigma) were added to the coculture medium for the last 4h. Cells were then collected, stained with a viability dye (FVS780), antibodies directed against γδTCR and Vδ2 TCR, and analyzed on a LSR II Fortessa cytometer (BD Biosciences).

Control of CMV dissemination by DOT cells

To measure CMV dissemination in vitro, fibroblasts were seeded in 24-well plates and, when confluent, infected with the CMV strain Merlin UL32-GFP at a low MOI (0.005). 24h after infection, DOT cells were added to fibroblasts at different E:T ratios. Infected fibroblasts without DOT cells were used as a positive control and mock-infected fibroblasts as a negative control. After 6 days of coculture, fibroblasts were harvested using Accutase cell detachment solution (Sigma), washed, and the percentage of CMV-infected fibroblasts was measured by flow cytometry (% of GFP+ cells) on a Canto II cytometer (BD Biosciences). The control of CMV dissemination by DOT cells was measured by comparing percentages of GFP+ cells to the control condition without effector cells.

ELISA and LEGENDplex assays

Cell culture supernatants were analyzed for IFNγ concentrations by ELISA, using the human IFNγ ELISA development kit (Mabtech) and according to manufacturer's instructions. Additionally, cell culture supernatants were analyzed using LEGENDplex assays (Biolegend) for the concentration of the following cytokines, chemokines and growth factors: IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, CXCL10 (IP-10), CCL2 (MCP-1), CXCL8 (IL-8), IFNγ, TGF-β1, TNFα, (LEGENDplex Human Essential Immune Response Panel, Biolegend), CCL3 (MIP-1α) , CCL4 (MIP-1β), CCL5 (RANTES), CCL11 (Eotaxin), CCL17 (TARC), CCL20 (MIP-3α), CXCL1 (GROα), CXCL5 (ENA-78), CXCL9 (MIG), CXCL11 (I-TAC) (LEGENDplex Human Proinflammatory Chemokines Mix and Match Subpanel), Angiopoietin-2, EGF, FGF-basic, G-CSF, GM-CSF, M-CSF, PDGF-AA, PDGF-BB, VEGF (LEGENDplex Human Growth Factor Mix and Match Subpanel). LEGENDplex assays were performed according to manufacturer's instructions.

Mice

C57BL/6J and BALBcBy/J mice were purchased from Charles River Laboratories and $Rag^{-/-}\gamma c^{-/-}$ mice were obtained from the Centre de Distribution, Typage et Archivage animal (CDTA), Orleans, France. Mice were bred and maintained under specific pathogen-free conditions in an appropriate biosafety level 2 facility (Bordeaux University, approval n° B33-063-916). Mice were housed under controlled conditions (21°C, 55-65% humidity, 12h/12h light/dark cycle). Experiments were performed on 8-12 weeks-old mice, in accordance with governmental guidelines and european ethical regulations. The protocols described in this study were notably approved by an ethics committee (Bordeaux University, committee n°2016092917471799).

Mouse γδ T cell expansion and phenotyping

The MCMV Smith strain was obtained from the American Type Culture Collection (ATCC VR-194) and used to infect BALBcBy/J mice for virus propagation. 2-3 weeks after MCMV infection, salivary glands were collected from infected BALBcBy/J mice to generate the MCMV stock solution used for all mouse experiments. Virus titers were determined by standard plaque forming assay on monolayers of mouse embryonic fibroblasts. C57BL/6J wild-type mice were infected by intraperitoneal (i.p.) injection with 2.10^4 plaque-forming units (PFU) of MCMV, or mock-infected. Three weeks after infection, spleens were harvested from mock-infected (MCMV-negative) or MCMV-infected (MCMV-positive) mice. Splenocytes were isolated through cell strainers and magnetically-depleted of αβ T cells and NK cells. Depletion was performed by staining with biotin-coupled antibodies (anti-TCRβ, clone REA318, diluted 1:50 and anti-NKp46, clone 29A1.4.9, diluted 1:10, Miltenyi Biotec) followed by anti-biotin microbeads (Miltenyi Biotec) and magnetic sorting. Sorted cells were seeded at 200 000 cells/well in round-bottom 96-well plates,

in RPMI 1640 supplemented with 10% FBS, 1% PS, 2mM L-glutamine, 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 50uM β-Mercaptoethanol (mouse γδ T cell expansion medium). Expansion medium was supplemented with an ultra-LEAF™ purified anti-mouse CD3ε Antibody (1 µg/mL, clone 145-2C11, Biolegend) and the following cytokines: recombinant murine IL-1β (15 ng/mL), recombinant human IL-15 (20 ng/mL), recombinant murine IL-21 (7 ng/mL) and recombinant murine IFNγ (70 ng/mL), all from Peprotech. After 6 days of expansion, cells were harvested, washed, and rested overnight before flow cytometry analysis or adoptive transfer. Resting was performed in mouse γδ T cell expansion medium supplemented with 20 ng/mL IL-15. The phenotypes of αβ and NK-depleted splenocytes (day 0) as well as in vitro expanded γδ T cells (day 7) were analyzed by flow cytometry. Briefly, 2.10^6 cells were stained with a viability dye (FVS780) and antibodies directed against mouse CD3, γδTCR, CD11a (LFA-1), CD25, CD44, CD62L, CD178 (FasL), CD226 (DNAM-1), CD253 (TRAIL), CD279 (PD-1), CD314 (NKG2D) and CD366 (TIM-3) (**Supplementary Table 1**). Cells were analyzed on an Aurora cytometer (Cytek) and data analyzed with FlowJo v10.8.

Mouse γδ T cell adoptive transfer in a MCMV infection model

$Rag^{-/-}\gamma c^{-/-}$ mice received either 10^6 γδ T cells (expanded from MCMVneg or MCMVpos C57/BL6J mice) or RPMI 1640 medium, by retro-orbital intravenous injection at day 0 and day 7. One day post adoptive transfer (day 1), mice were infected with MCMV (Smith strain, 2.10^2 PFU, i.p.). Infected mice were monitored twice per week for clinical signs of infection (weight loss, round back, tip-toe walking and/or absence of hair care) and sacrificed upon reaching humane endpoints, as defined in accordance with the ethics committee. At the end of the experiments, spleen, liver and lungs were sampled for viral load measurement and processed to analyze γδ T

cell infiltration. For the latter analysis, spleens were dissociated on a 70 µm cell strainer followed by red blood cell lysis in ACK lysis buffer (A1049201, Thermofisher Scientific) and washes before splenocyte staining. Lungs were digested at 37°C during 40min in RPMI 1640 supplemented with 1 mg/mL collagenase I and 50 µg/mL DNAse I (Sigma), then passed through 70 µm cell strainers. Livers were directly dissociated on 70 µm cell strainers. After red blood cell lysis, lung and liver cell suspensions were separated by a discontinuous 40/80% Percoll gradient (according to manufacturer's instructions, Sigma catalog number P4937). Immune cells were then harvested and stained with a viability dye (FVS780) and antibodies directed against mouse CD3, γδTCR, CD44 and CD62L. Cells were analyzed on a Canto II (BD) and data was analyzed on FlowJo v10.8.

Viral load quantifications

Viral DNA was extracted from organs using the QIAwave DNA Blood & Tissues kit (Qiagen). Lysis was performed with TissueLyser II (Qiagen). DNA was quantified using DeNovix Nanodrop (Thermofisher Scientific) and 300 ng to 500 ng DNA was used for PCR amplification. Reactions were prepared using GoTaq qPCR MasterMix (Promega) and MCMV-specific primers targeting glycoprotein B (gB) (forward primer: GGTAAGGCGTGGACTAGCGAT; reverse primer: CTAGCTGTTTTAACGCGCGG). Viral loads were quantified using standard curves prepared with 10^1 to 10^7 copies of plasmids comprising a 517 bp fragment of DNA encoding MCMV gB[28].

Statistics & reproducibility

Analyses were performed using conventional statistical methods. Wilcoxon matched-pairs signed rank test, two-tailed Mann-Whitney test, paired or unpaired t-tests, Welch's t-tests, ordinary or repeated-measures one-way ANOVA with Dunnett's or Tukey's multiple comparisons were used

when appropriate. Kaplan-Meier survival curves were analyzed with log-rank (Mantel-Cox) tests. $P < 0.05$ was considered statistically significant.

To address experimental variations, all in vitro experiments were performed in duplicates or triplicates of culture. This information is indicated in the corresponding figure legends. To address biological variations, all in vitro experiments were repeated on independent healthy donors or patients. This information is indicated in the corresponding figure legends. Each analysis presented in this study is based on a minimum of three independent experiments performed on independent donors or patients.

Sample sizes for experiments involving healthy human donors were not determined using statistical power calculations, but were instead based on prior experience and experimental observations to ensure data robustness and reproducibility. For in vivo experiments, sample sizes were determined upfront based on prior experimental data to detect meaningful differences while complying with ethical guidelines. For experiments involving patient samples, sample sizes were determined by the number of eligible participants that could be recruited during the study period. Sample sizes for each experiment are provided in the corresponding figure legends. No data were excluded from the analysis. Mice used for in vivo experiments were randomly assigned to the different groups. The follow-up of MCMV-infected mice after γδ T cell adoptive transfer was performed blinded to the conditions of the experiment. Blinding was not applicable to in vitro experiments, in which well-controlled quantitative measurement methods were used to exclude bias.

List of antibodies and reagents

Data regarding primary antibodies (used for flow cytometry, blocking and activation experiments), isotype controls and flow cytometry reagents are recapitulated in **Supplementary Tables 1** and **3**.

DATA AVAILABILITY

All datasets generated and analyzed during this study are available in the Source Data file provided.

REFERENCES

1. Humar, A. *et al.* The efficacy and safety of 200 days valganciclovir cytomegalovirus prophylaxis in high-risk kidney transplant recipients. *Am. J. Transplant.* **10**, 1228–1237 (2010).
2. Witzke, O. *et al.* Valganciclovir Prophylaxis Versus Preemptive Therapy in Cytomegalovirus-Positive Renal Allograft Recipients: 1-Year Results of a Randomized Clinical Trial. *Transplantation* **93**, 61–68 (2012).
3. Kliem, V. *et al.* Improvement in long-term renal graft survival due to CMV prophylaxis with oral ganciclovir: Results of a randomized clinical trial. *Am. J. Transplant.* **8**, 975–983 (2008).
4. Kotton, C. N. *et al. The Third International Consensus Guidelines on the Management of Cytomegalovirus in Solid-Organ Transplantation*. *Transplantation* vol. 102 (2018).
5. Ljungman, P. *et al.* Guidelines for the management of cytomegalovirus infection in patients with haematological malignancies and after stem cell transplantation from the 2017 European Conference on Infections in Leukaemia (ECIL 7). *Lancet Infect. Dis.* **19**, e260–e272 (2019).
6. Natori, Y. *et al.* Recurrence of CMV infection & the effect of prolonged antivirals in organ transplant recipients. *Transplantation* **101**, 1449–1454 (2017).
7. Ljungman, P., Hakki, M. & Boeckh, M. Cytomegalovirus in Hematopoietic Stem Cell Transplant Recipients. *Hematol. Oncol. Clin. North Am.* **25**, 151–169 (2011).
8. Couzi, L. *et al.* High incidence of anticytomegalovirus drug resistance among D+R-kidney transplant recipients receiving preemptive therapy. *Am. J. Transplant.* **12**, 202–209 (2012).
9. Avery, R. K. *et al.* Maribavir for Refractory Cytomegalovirus Infections With or Without Resistance Post-Transplant: Results From a Phase 3 Randomized Clinical Trial. *Clin. Infect. Dis.* **75**, 690–701 (2022).
10. Chou, S. *et al.* Drug Resistance Assessed in a Phase 3 Clinical Trial of Maribavir Therapy for Refractory or Resistant Cytomegalovirus Infection in Transplant Recipients. *J. Infect. Dis.* **229**, 413–421 (2024).
11. Chemaly, R. F. *et al.* Cytomegalovirus (CMV) Cell-Mediated Immunity and CMV Infection after Allogeneic Hematopoietic Cell Transplantation: The REACT Study. *Clin. Infect. Dis.* **71**, 2365–2374 (2020).

12. Nicholson, E. & Peggs, K. S. Cytomegalovirus-specific T-cell therapies: Current status and future prospects. *Immunotherapy* **7**, 135–146 (2015).
13. Houghtelin, A. & Bollard, C. M. Virus-specific T cells for the immunocompromised patient. *Front. Immunol.* **8**, (2017).
14. Prockop, S. E. *et al.* Third-party cytomegalovirus-specific T cells improved survival in refractory cytomegalovirus viremia after hematopoietic transplant. *J. Clin. Invest.* **133**, e165476 (2023).
15. Khoury, R. *et al.* Third-party virus specific T cells for the treatment of double stranded DNA viral reactivation and PTLD after solid organ transplant. *Am. J. Transplant.* S1600613524002806 (2024) doi:10.1016/j.ajt.2024.04.009.
16. Smith, C. *et al.* Autologous Adoptive T-cell Therapy for Recurrent or Drug-resistant Cytomegalovirus Complications in Solid Organ Transplant Recipients: A Single-arm Open-label Phase I Clinical Trial. *Clin. Infect. Dis.* 1–9 (2018) doi:10.1093/cid/ciy549.
17. Smith, C. *et al.* T cell repertoire remodeling following post-transplant T cell therapy coincides with clinical response. *J. Clin. Invest.* **129**, 5020–5032 (2019).
18. Kaminski, H. *et al.* Understanding human γδ T cell biology toward a better management of cytomegalovirus infection. *Immunol. Rev.* **298**, 264–288 (2020).
19. Shen, L. *et al.* Fast-acting γδ T-cell subpopulation and protective immunity against infections. *Immunol. Rev.* **298**, 254–263 (2020).
20. Pamplona, A. & Silva-Santos, B. γδ T cells in malaria: a double-edged sword. *FEBS J.* **288**, 1118–1129 (2021).
21. Prinz, I. *et al.* Donor Vδ1+ γδ T cells expand after allogeneic hematopoietic stem cell transplantation and show reactivity against CMV-infected cells but not against progressing B-CLL. *Exp. Hematol. Oncol.* **2**, 2–5 (2013).
22. Déchanet, J. *et al.* Implication of γδ T cells in the human immune response to cytomegalovirus. *J. Clin. Invest.* **103**, 1437–1449 (1999).
23. Couzi, L. *et al.* Gamma-delta T cell expansion is closely associated with cytomegalovirus infection in all solid organ transplant recipients. *Transpl. Int. Off. J. Eur. Soc. Organ Transplant.* **24**, e40-42 (2011).
24. Pitard, V. *et al.* Long-term expansion of effector/memory Vδ2− γδ T cells is a specific blood signature of CMV infection. *Blood* **112**, 1317–1324 (2008).
25. Halary, F. *et al.* Shared reactivity of Vδ2 neg γδ T cells against cytomegalovirus-infected cells and tumor intestinal epithelial cells. *J. Exp. Med.* **201**, 1567–1578 (2005).
26. Couzi, L. *et al.* Antibody-dependent anti-cytomegalovirus activity of human γδ T cells expressing CD16 (FcγRIIIa). *Blood* **119**, 1418–1427 (2012).
27. Kaminski, H. *et al.* Surveillance of γδ T Cells Predicts Cytomegalovirus Infection Resolution in Kidney Transplants . *J. Am. Soc. Nephrol.* **27**, 637–645 (2016).
28. Khairallah, C. *et al.* γδ T Cells Confer Protection against Murine Cytomegalovirus (MCMV). *PLoS Pathog.* **11**, 1–22 (2015).

29. Sell, S. *et al.* Control of Murine Cytomegalovirus Infection by γδ T Cells. *PLoS Pathog.* **11**, 1–21 (2015).
30. Almeida, A. R. *et al.* Delta One T Cells for Immunotherapy of Chronic Lymphocytic Leukemia: Clinical-Grade Expansion/Differentiation and Preclinical Proof of Concept. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **22**, 5795–5804 (2016).
31. Di Lorenzo, B. *et al.* Broad cytotoxic targeting of acute myeloid leukemia by polyclonal delta one T cells. *Cancer Immunol. Res.* **7**, 552–558 (2019).
32. Sánchez Martínez, D. *et al.* Generation and proof-of-concept for allogeneic CD123 CAR-Delta One T (DOT) cells in acute myeloid leukemia. *J. Immunother. Cancer* **10**, e005400 (2022).
33. Mensurado, S. *et al.* CD155/PVR determines Acute Myeloid Leukemia targeting by Delta One T cells. *Blood J.* blood.2023022992 (2024) doi:10.1182/blood.2023022992.
34. Gattinoni, L. Acquisition of full effector function in vitro paradoxically impairs the in vivo antitumor efficacy of adoptively transferred CD8+ T cells. *J. Clin. Invest.* **115**, 1616–1626 (2005).
35. Berger, C. *et al.* Adoptive transfer of effector CD8+ T cells derived from central memory cells establishes persistent T cell memory in primates. *J. Clin. Invest.* **118**, 294–305 (2008).
36. Kang, S., Brown, H. M. & Hwang, S. Direct Antiviral Mechanisms of Interferon-Gamma. *Immune Netw.* **18**, e33 (2018).
37. Guerville, F. *et al.* TCR-dependent sensitization of human γδ T cells to non-myeloid IL-18 in cytomegalovirus and tumor stress surveillance. *OncoImmunology* **4**, 1–13 (2015).
38. Kaminski, H. *et al.* Characterization of a Unique γδ T-Cell Subset as a Specific Marker of Cytomegalovirus Infection Severity. *J. Infect. Dis.* (2020) doi:10.1093/infdis/jiaa400.
39. Willcox, C. R. *et al.* Cytomegalovirus and tumor stress surveillance by binding of a human γδ T cell antigen receptor to endothelial protein C receptor. *Nat. Immunol.* **13**, 872–879 (2012).
40. Marlin, R. *et al.* Sensing of cell stress by human γδ TCR-dependent recognition of annexin A2. *Proc. Natl. Acad. Sci.* **114**, 3163–3168 (2017).
41. Simões, A. E., Di Lorenzo, B. & Silva-Santos, B. Molecular determinants of target cell recognition by human γδ T cells. *Front. Immunol.* **9**, 1–7 (2018).
42. Amini, L. *et al.* Comprehensive Characterization of a Next-Generation Antiviral T-Cell Product and Feasibility for Application in Immunosuppressed Transplant Patients. *Front. Immunol.* **10**, 1148 (2019).
43. Ferrandiz, I. *et al.* Impact of Early Blood Transfusion After Kidney Transplantation on the Incidence of Donor-Specific Anti-LA Antibodies. *Am. J. Transplant.* **16**, 2661–2669 (2016).
44. Varis, T., Kaukonen, K.-M., Kivistö, K. T. & Neuvonen, P. J. Plasma concentrations and effects of oral methylprednisolone are considerably increased by itraconazole*. *Clin. Pharmacol. Ther.* **64**, 363–368 (1998).
45. Jarque, M. *et al.* Cellular Immunity to Predict the Risk of Cytomegalovirus Infection in Kidney Transplantation: A Prospective, Interventional, Multicenter Clinical Trial. *Clin. Infect. Dis.* **1**, 1–11 (2020).

46. Hanley, P. J. *et al.* Erratum for the Research Article: "CMV-specific T cells generated from naïve T cells recognize atypical epitopes and may be protective in vivo" by P. J. Hanley, J. J. Melenhorst, S. Nikiforow, P. Scheinberg, J. W. Blaney, G. Demmler-Harrison, C. R. Cruz, . *Sci. Transl. Med.* **8**, 321er1-321er1 (2016).
47. Jackson, S. E., Mason, G. M., Okecha, G., Sissons, J. G. P. & Wills, M. R. Diverse Specificities, Phenotypes, and Antiviral Activities of Cytomegalovirus-Specific CD8+ T Cells. *J. Virol.* **88**, 10894–10908 (2014).
48. Houldcroft, C. J. *et al.* Assessing Anti-HCMV Cell Mediated Immune Responses in Transplant Recipients and Healthy Controls Using a Novel Functional Assay. **10**, 1–21 (2020).
49. Watanabe, M., Ito, M., Kamiya, H. & Sakurai, M. Adherence of peripheral blood leukocytes to cytomegalovirus-infected fibroblasts. *Microbiol. Immunol.* **40**, 519–523 (1996).
50. Waldman, W. J., Knight, D. A. & Huang, E. H. An in vitro model of T cell activation by autologous cytomegalovirus (CMV)-infected human adult endothelial cells: Contribution of CMV-enhanced endothelial ICAM-1. *J. Immunol.* **160**, 3143–3151 (1998).
51. Leong, C. C. *et al.* Modulation of natural killer cell cytotoxicity in human cytomegalovirus infection: The role of endogenous class I major histocompatibility complex and a viral class I homolog. *J. Exp. Med.* **187**, 1681–1687 (1998).
52. Siegers, G. M. Integral roles for integrins in γδ T. *Front. Immunol.* **9**, (2018).
53. van de Berg, P. J. *et al.* Human Cytomegalovirus Induces Systemic Immune Activation Characterized by a Type 1 Cytokine Signature. *J. Infect. Dis.* **202**, 690–699 (2010).
54. Karaba, A. H. *et al.* Interleukin-18 and tumor necrosis factor-α are elevated in solid organ transplant recipients with possible cytomegalovirus end-organ disease. *Transpl. Infect. Dis. Off. J. Transplant. Soc.* **23**, e13682 (2021).
55. Dalton, J. E., Pearson, J., Scott, P. & Carding, S. R. The Interaction of γδ T Cells with Activated Macrophages Is a Property of the Vγ1 Subset. *J. Immunol.* **171**, 6488–6494 (2003).
56. Hirsh, M. I. & Junger, W. G. Roles of heat shock proteins and γδT cells in inflammation. *Am. J. Respir. Cell Mol. Biol.* **39**, 509–513 (2008).
57. Drobyski, W. R., Majewski, D. & Hanson, G. Graft-facilitating doses of ex vivo activated γδ T cells do not cause lethal murine graft-vs.-host disease. *Biol. Blood Marrow Transplant.* **5**, 222–230 (1999).
58. Bachelet, T. *et al.* Cytomegalovirus-Responsive γδ T Cells: Novel Effector Cells in Antibody-Mediated Kidney Allograft Microcirculation Lesions. *J. Am. Soc. Nephrol.* **25**, 2471–2482 (2014).
59. Charmetant, X. *et al.* γδ T Cells' Role in Donor-Specific Antibody Generation: Insights From Transplant Recipients and Experimental Models. *Transpl. Int. Off. J. Eur. Soc. Organ Transplant.* **38**, 12859 (2025).
60. Yared, N. *et al.* Long-lived central memory γδ T cells confer protection against murine cytomegalovirus reinfection. *PLOS Pathog.* **20**, e1010785 (2024).

61. Kaminski, H. *et al.* mTOR Inhibitors Prevent CMV Infection through the Restoration of Functional ab and gd T cells in Kidney Transplantation. *J. Am. Soc. Nephrol.* **33**, 121–137 (2022).
62. Concordet, J. P. & Haeussler, M. CRISPOR: Intuitive guide selection for CRISPR/Cas9 genome editing experiments and screens. *Nucleic Acids Res.* **46**, W242–W245 (2018).
63. Haeussler, M. *et al.* Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR. *Genome Biol.* **17**, 148 (2016).
64. Doench, J. G. *et al.* Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. *Nat. Biotechnol.* **34**, 184–191 (2016).
65. Tycko, J. *et al.* Mitigation of off-target toxicity in CRISPR-Cas9 screens for essential non-coding elements. *Nat. Commun.* **10**, 4063 (2019).
66. Oh, S. A., Seki, A. & Rutz, S. Ribonucleoprotein Transfection for CRISPR/Cas9-Mediated Gene Knockout in Primary T Cells. *Curr. Protoc. Immunol.* **124**, (2019).
67. Satchell, S. C. *et al.* Conditionally immortalized human glomerular endothelial cells expressing fenestrations in response to VEGF. *Kidney Int.* **69**, 1633–1640 (2006).
68. Baer, A. & Kehn-Hall, K. Viral Concentration Determination Through Plaque Assays: Using Traditional and Novel Overlay Systems. *J. Vis. Exp.* 1–10 (2014) doi:10.3791/52065.

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AUTHOR CONTRIBUTIONS

Conceptualization, G.M., J.D.-M. and L.C.; Methodology, G.M., C.G., V.P., V.P.-M., B.T., B.S.-S, H.K., M.Ca., J.D.-M. and L.C.; Investigation, G.M., C.G., M.C., C.T., A.C., S.C.-C., V.B., J.I., V.P., A.Z., I.G., M.Ca. and V.P.-M.; Writing: Original Draft, G.M., C.G., J.D.-M. and L.C.; Writing: Review & Editing, G.M., C.G., J.D.-M., L.C., H.K., V.P.-M., B.T., E.F., P.M., B.S.-S. and M.Ca.; Funding Acquisition, M.C., J.D-M. and L.C.; Resources, D.A.; Supervision, J.D-M. and L.C.

COMPETING INTERESTS

B.S.-S. was a co-inventor of the DOT cell technology now owned by Takeda, which supports his work via a sponsored research agreement. The other authors declare no competing interests.

TABLES

Patient	P1	P2	P3	P4	P5	P6	P7	P8
Sex	F	M	M	M	M	M	M	M
Time post-transplantation (months)	16	10	11	18	10	6	6	3
Number of CMV episodes	4	3	2	4	2	2	3	2
Refractory CMV infection	Yes	No	Yes	Yes	No	Yes	Yes	Yes
Antiviral drug resistance	UL97	No	UL97	UL97	No	No	UL97	UL97
Antiviral treatment	VGCV, FOS	VGCV	VGCV, FOS	VGCV, FOS	VGCV	VGCV, MBV	VGCV, MBV	VGCV, MBV
Intravenous Immunoglobulin	Yes	No	Yes	No	No	No	No	No
Immunosuppression	Everolimus	Cyclosporin - steroid	Steroid	Tacrolimus - everolimus	Tacrolimus - mycophenolic acid	Tacrolimus - everolimus	Everolimus	Tacrolimus - everolimus
Lymphocyte count (/mm3))	390	1140	860	410	500	1000	830	300
CMV serostatus	D+R-	D+R-	D+R-	D+R-	D+R-	D+R-	D+R-	D+R-

Table 1. Patient clinical data

D, donor; F, female; FOS, foscavir; M, male; MBV, maribavir; P, patient; R, recipient; VGCV, valganciclovir

FIGURE LEGENDS

Figure 1: Large-scale expansion and characterization of DOT cells from CMV-seronegative and positive healthy donors.

(a) Gating strategy to monitor Vδ2^{neg} γδ T cells during *in vitro* expansion. Percentages of γδ T cell sub-populations are shown on day 0 (after αβ T cell depletion) and on day 21 (one representative donor). **(b-d)** DOT cells were expanded independently from 10 CMV-seronegative (CMVneg) and 10 CMV-seropositive (CMVpos) donors. Absolute numbers **(b)**, fold increases **(c)** and percentages **(d)** of Vδ2^{neg} γδ T cell are shown at days 0, 11 and 21. Shown are means ± SD, compared between CMVneg and CMVpos donors using unpaired two-tailed t-tests (*P* values are indicated). **(e)** Cell population percentages at day 21 are shown as means ± SD for CMVneg (n=3) and CMVpos (n=3) donors. P>0.05 determined by unpaired two-tailed t-tests for each cell population. **(f)** Differentiation states of Vδ2^{neg} γδ T cells expanded from 10 CMVneg and 10 CMVpos donors at days 0, 11 and 21 of culture, assessed using CD27 and CD45RA, and presented as means ± SD. Populations were compared in between CMVneg and CMVpos donors using two-tailed unpaired t-tests. Labels correspond to naïve: CD27+ CD45RA+; TEMRA: CD27- CD45RA+; TCM: CD27+ CD45RA-; TEM: CD27- CD45RA-. **(g)** Vδ2^{neg} γδ T cells expanded independently from 3 CMVneg and 3 CMVpos healthy donors were phenotyped before (left) and after (right) expansion. Heatmaps show percentages of positive Vδ2^{neg} γδ T cells per marker. For CD8, LFA-1 and KIR expression (separated heatmaps), analysis was performed on distinct groups of CMV-seronegative and positive donors. Individual donor numbers are indicated. **(h)** MdFI of DNAM-1, NKG2D and LFA-1 before and after DOT cell expansion from six independent donors: 3 CMVneg (grey) and 3 CMVpos (red). Results are presented as means ± SD, compared between day 0 and day 21 using a two-tailed Wilcoxon matched-pairs signed rank test. The source data underlying this figure are

provided in the Source Data file. A: area, CMV: cytomegalovirus, FSC: forward scatter, KIRs: killer cell immunoglobulin-like receptors, MdFI: median fluorescence intensity, neg: negative, PBMCs: peripheral blood mononuclear cells, pos: positive, SSC: side scatter, TCM: T central memory, TCR: T cell receptor, TEM: T effector memory, TEMRA: T effector memory reexpressing CD45RA, W: width.

Figure 2: DOT cells control CMV dissemination in vitro.

(a) Analysis of DOT cell degranulation in response to CMV infection. Mean percentages of CD107a+ cells are shown, from cocultures with fibroblasts (n=6 donors) and macrophages (n=7 donors) conducted in technical duplicates. **(b)** DOT cells were cultured alone (medium) or cocultured with mock- or CMV-infected fibroblasts (n=27 donors), macrophages (n=12 donors) or endothelial cells (n=10 donors), in the presence of IL-18 and in technical triplicates. Means of IFNγ secretion are shown. **(a, b)** Lines connect conditions from the same donor. Statistical analysis was performed using a repeated-measures one-way ANOVA in **(a)** or a mixed-effects model in **(b)**, both with Geisser-Greenhouse correction followed by Tukey's multiple comparisons test. **(c)** Comparison of IFNγ secretion by DOT cells expanded from CMVneg (n=13) versus CMVpos (n=14) donors in fibroblast cultures. Shown are means ± SD, compared using two-tailed Mann-Whitney tests. **(d)** Fibroblasts were mock-infected or infected with CMV TB42/E, CMV Merlin, HSV or VZV, then cultured alone (white) or with DOT cells (grey) in the presence of IL-18. IFNγ secretion is represented as a fold change compared to a DOT cell-alone control. Box plots show the median (centre line), 25th-75th percentile (box) and minimum-maximum values (whiskers) of six independent donors (technical triplicates). Statistical analysis was performed using two-tailed Wilcoxon matched-pairs signed rank tests. **(e)** Schematic of the CMV dissemination assay. **(f)**

Fibroblasts infected with CMV strain Merlin UL32-GFP were cultured alone or with DOT cells at various E:T ratios. Shown are percentages of GFP-positive fibroblasts, normalized to the infected monoculture control (set to 100%). Each bar represents the mean ± SD of 5 (mock and E:T 0.05:1 conditions) or 7 (other conditions) independent experiments using different DOT donors, each performed in technical duplicates. Statistical comparisons used a mixed-effects model with Geisser-Greenhouse correction and Dunnett's two-tailed multiple comparisons test, comparing each condition to the infected monoculture control. Source data is provided as a Source Data file. CMV: cytomegalovirus, DOT: Delta One T, E:T: effector to target, GFP: green fluorescent protein, HFF: human foreskin fibroblasts, HSV: herpes simplex virus, IFN: interferon, IL: interleukin, Mϕ: macrophages, neg: negative, pos: positive, VZV: varicella-zoster virus.

Figure 3: DOT cell recognition of CMV-infected targets is γδTCR-independent and involves LFA-1.

(a) DOT cells were cultured alone or in the presence of mock- or CMV-infected fibroblasts, in direct contact or separated by a Transwell insert. IFNγ secretion (fold change relative to DOT cells alone) is shown as means ± SD from three independent donors analyzed in technical triplicates. **(b)** IFNγ secretion by DOT cells cocultured with CMV-infected fibroblasts, in the presence of an anti-Vδ1 TCR blocking antibody (αVδ1) or an isotype control. Shown are means from n=8 donors. Lines connect conditions from the same donor. **(a, b)** Groups were compared using two-tailed paired t-tests. **(c)** DOT cells from three independent donors: 51 (CMVpos), 84 (CMV serology unknown), and R6 (CMVneg) were knocked out for their γδTCR. γδTCR staining is shown for control (orange), γδTCR knockout (green) and FMO (grey) conditions, pre-gated on live cells. **(d)** γδTCR KO and control DOT cells were cultured alone or with mock- or CMV-infected fibroblasts. IFNγ secretion levels (means ± SD of technical triplicates) are represented as fold change relative

to DOT cells alone and were compared using two-tailed Welch's t-tests. **(e)** IFNγ secretion (fold change relative to isotype control condition) in cocultures with CMV-infected fibroblasts (n=6 donors), in the presence of an isotype control or blocking antibodies targeting NK activating receptors. Means ± SD are shown and were compared using repeated-measures one-way ANOVA with Geisser-Greenhouse correction, followed by Dunnett's two-tailed multiple comparisons test. **(f-g)** DOT cells from n=3 donors were treated with an isotype control or with increasing concentrations of **(f)** coated anti-LFA-1 antibody or **(g)** recombinant human ICAM-1 Fc chimera to activate LFA-1. Shown are means ± SD of IFNγ secretion. **(h)** IFNγ secretion (means ± SD) by DOT cells (n=6 independent donors, technical triplicates) cultured alone or with mock-infected or CMV-infected fibroblasts ± blocking antibodies targeting ICAM-1 or LFA-1, alone or in combination, and compared by paired two-tailed t-tests. **(a-h)** IL-18 was added in all culture conditions. Source data are provided as a Source Data file. Ab: antibody, CMV: cytomegalovirus, DOT: Delta One T, FMO: fluorescence minus one, IFN: interferon, IL: interleukin, KO: knockout, neg: negative, pos: positive, TCR: T cell receptor.

Figure 4: In vitro expanded mouse γδ T cells are protective against MCMV infection in vivo. **(a)** Gating strategy to monitor mouse γδ T cells during in vitro expansion (one representative culture). At days 0 and 7, γδ T cells were analyzed for purity and differentiation using CD44 and CD62L to define naïve, TCM and TEM populations. **(b-d)** γδ T cells were expanded from 9 MCMV-negative (MCMVneg) and 9 MCMV-positive (MCMVpos) C57BL/6J mice in 5 independent experiments. γδ T cell absolute numbers **(b)**, fold increases **(c)** and percentages **(d)** are shown as means ± SD. MCMVneg and MCMVpos groups were compared using two-tailed Mann-Whitney tests. **(e)** Differentiation states of mouse γδ T cells expanded from 5 MCMVneg

and 5 MCMVpos C57BL/6J mice, at day 0 and day 7, shown as mean percentages ± SD. MCMVneg and MCMVpos groups were compared using two-tailed unpaired t-tests. **(f)** γδ T cells from 4 MCMVneg and 4 MCMVpos C57BL/6J mice were phenotyped before and after expansion. The heatmap shows % of positive γδ T cells per marker. **(g)** Experimental workflow for panels **(h-j)**. **(h)** Kaplan-Meier survival curves of MCMV-infected $Rag^{-/-}\gamma c^{-/-}$ mice treated twice with RPMI (n=18) or γδ T cells from MCMVneg (n=22) or MCMVpos (n=13) mice. Mice were monitored until humane endpoints. Survival differences were analyzed by log-rank (Mantel-Cox) test. Data are pooled from three independent experiments. **(i)** Spleen, liver and lung infiltration of γδ T cells in surviving mice (n=5 MCMVneg, n=6 MCMVpos), shown as mean percentages ± SD of live cells. **(j)** Viral loads in spleen, liver and lungs at day 14 post-MCMV infection in $Rag^{-/-}\gamma c^{-/-}$ mice treated with RPMI (n=5) or γδ T cells from MCMVneg (n=5) or MCMVpos (n=5) mice. Shown are means ± SD of MCMV DNA copy numbers per 300 ng genomic DNA. Viral loads were compared by ordinary one-way ANOVA followed by Dunnett's two-tailed multiple comparisons test. The source data underlying this figure are provided in the Source Data file. A: area, FACS: fluorescence-activated cell sorting, FSC: forward scatter, H: height, MCMV: mouse cytomegalovirus, neg: negative, IL: interleukin, i.p: intraperitoneal, i.v: intravenous, NK: natural killer, pos: positive, SSC: side scatter, TCM: central memory T cell, TEM: effector memory T cell.

Figure 5: Feasibility of a DOT cell therapy in kidney transplant recipients.

(a-c) DOT cells were expanded from eight D+R- kidney transplant recipients. **(a)** Absolute numbers **(b)**, fold increases and **(c)** percentages of $V\delta2^{neg}$ γδ T cells are shown at days 0, 11 and 21 of culture. **(d)** Following expansion, DOT cells (n=8) were cultured without target cells (medium) or with mock- or CMV-infected fibroblasts in the presence of IL-18. IFNγ secretion was

measured. Data represent means, with lines connecting conditions from the same patient. Each datapoint represents the mean of technical triplicates. Statistical analysis: repeated measures one-way ANOVA with Geisser-Greenhouse correction, followed by Tukey's multiple comparisons test. **(e-h)** Proliferation of DOT cells in the presence of immunosuppressive drugs. DOT cells from four **(e-f)** or three **(g-h)** independent healthy donors were stained with CFSE **(e-f)** and cultured in expansion medium supplemented with IL-15 and the following immunosuppressive drugs: tacrolimus, cyclosporin, everolimus or MMF. **(e-f)** After 7 days, proliferation was assessed. **(e)** Histograms from a representative donor out of four. **(f)** Proliferation index of $V\delta2^{neg}$ γδ T cells, shown as means ± SD. **(g)** Number of cells per well were counted at days 3, 7 and 15 of culture and are displayed as means ± SD from three independent healthy donors, each analyzed in culture triplicates. **(h)** After 15 days, cell viability was analyzed by DAPI staining. Percentages of viable cells are shown as means ± SD. **(i)** DOT cells from five independent healthy donors were cocultured with mock-infected or CMV-infected fibroblasts during 24 hours in the presence of IL-18 and analyzed for IFNγ secretion. When indicated, immunosuppressive drugs were added to cocultures. Means ± SD of five independent donors are shown, each analyzed in culture triplicates. **(f-i)** Statistical analysis was performed using repeated-measures one-way ANOVA with Geisser-Greenhouse correction, followed by Dunnett's two-tailed multiple comparisons test. The source data underlying this figure are provided in the Source Data file. CFSE: carboxyfluorescein diacetate succinimidyl ester, CMV: cytomegalovirus, Cyclo: cyclosporin, DAPI: 4′,6-diamidino-2-phenylindole, DOT: Delta One T, D+R-: CMV-seronegative recipient of a CMV-seropositive graft, Evero: everolimus, IL: interleukin, IFN: interferon, MMF: mycophenolate mofetil, neg: negative, pos: positive, Tacro: tacrolimus.

Editorial Summary:
Gamma delta T cells as a therapeutic strategy have shown great promise in a range of contexts. Here the authors explore the use of a gamma delta T cell therapy in experimental models of cytomegalovirus infection.

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