mTOR Inhibitors Prevent CMV Infection through the Restoration of Functional $\alpha\beta$ and $\gamma\delta$ T cells in Kidney Transplantation.

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Abstract: Background: The reported association of mTOR-inhibitor (mTORi) treatment with a lower incidence of cytomegalovirus (CMV) infection in CMV-seropositive (R+) kidney transplant recipients (KTR) remains unexplained.
Methods: The incidence of CMV infection and T-cell profile was compared between mTORi-treated and mycophenolic acid (MPA)-treated KTR, and mTORi effects in vitro on T-cell phenotype and functions analyzed.
Results: In MPA-treated R+ KTR, both Î±Î² and Î³Î´ T-cells displayed a more dysfunctional phenotype (PD-1+, CD85j+) at day 0 of transplantation in the 16 KTR with severe CMV infection when compared to the 17 KTR without or with spontaneously resolving CMV infection. In mTORi-treated patients (n= 27), the proportion of PD-1+ and CD85j+ Î±Î² and Î³Î´ T cells decreased when compared to MPA-treated patients (n=44), as well as the frequency and severity of CMV infections. mTORi treatment also led to higher proportions of latedifferentiated and cytotoxic Î³Î´ T cells, and IFNÎ³-producing and cytotoxic Î±Î² T cells. In vitro, mTORi increased proliferation, viability, and CMV-induced IFNÎ³ production of T cells and (decreased PD-1 and CD85j expression in T cells that shifted to a more efficient EOMES^{low} Hobit^{high} profile. In Î³Î´ T cells the mTORi effect was related to increased TCR signaling.

Conclusion: Severe CMV replication is associated with a dysfunctional T-cell profile and mTORi improve T-cell fitness in association with better control of CMV. A dysfunctional Tcell phenotype could provide a new biomarker to predict post-transplantation infection and to stratify patients who should benefit from mTORi treatment.

Significance Statement

It has been reported that mTOR-inhibitors (mTORi) are associated with a reduction of the incidence of CMV infection in CMV-seropositive (R+) organ transplant patients but a mechanistic explanation has been lacking to date. This work showed that a dysfunctional T-cell phenotype (CD85j+ PD1+) was associated to a higher risk of uncontrolled CMV-infection after transplantation in R+ patients and that mTORi reduced CMV incidence and severity by reinvigorating αβ and γδ T-cell function. Dysfunctional T- cell phenotype could represent a new biomarker to predict post-transplantation infection in R+ patients and to stratify patients who should benefit from treatment with mTORi.

mTOR Inhibitors Prevent CMV Infection through the Restoration of Functional αβ and γδ T cells in Kidney Transplantation

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Abstract

Background: The reported association of mTOR-inhibitor (mTORi) treatment with a lower incidence of cytomegalovirus (CMV) infection in CMV-seropositive (R+) kidney transplant recipients (KTR) remains unexplained.

Methods: The incidence of CMV infection and T-cell profile was compared between mTORi- treated and mycophenolic acid (MPA)-treated KTR, and mTORi effects *in vitro* on T-cell phenotype and functions analyzed.

Results: In MPA-treated R+ KTR, both αβ and γδ T-cells displayed a more dysfunctional phenotype (PD-1+, CD85j+) at day 0 of transplantation in the 16 KTR with severe CMV infection when compared to the 17 KTR without or with spontaneously resolving CMV infection. In mTORi-treated patients (n= 27), the proportion of PD-1+ and CD85j+ αβ and γδ T cells decreased when compared to MPA-treated patients (n=44), as well as the frequency and severity of CMV infections. mTORi treatment also led to higher proportions of late-differentiated and cytotoxic γδ T cells, and IFNγ-producing and cytotoxic αβ T cells. *In vitro*, mTORi increased proliferation, viability, and CMV-induced IFNγ production of T cells and (decreased PD-1 and CD85j expression in T cells that shifted to a more efficient $EOMES^{low}$ $Hobit^{high}$ profile. In γδ T cells the mTORi effect was related to increased TCR signaling.

Conclusion: Severe CMV replication is associated with a dysfunctional T-cell profile and mTORi improve T-cell fitness in association with better control of CMV. A dysfunctional T-cell phenotype could provide a new biomarker to predict post-transplantation infection and to stratify patients who should benefit from mTORi treatment.

Introduction

CMV is by far the most common opportunistic infection in solid allograft recipients and induces direct and indirect morbidity (1). CMV-positive solid allograft recipient (R+) have an intermediate risk of CMV reactivation or superinfection but represent the vast majority (50-90 %) of transplant recipients over the world (2). A preformed cell-mediated immunity (3, 4) contributes to this reduced risk, but sometimes fails to control the virus, leading to clinical infections. A dysfunctional status of CMV-specific T-cells could be hypothesized to explain this emergence of CMV disease.

CD8+ αβ T-cell response during CMV infection was described as inflationary i.e. leading to the lifelong accumulation of highly differentiated/functional T-cells (5, 6) that correlate to chronic but well-controlled low-level viral load (7). A "long-lived effector-type" phenotype has been distinguished for these T-cells expressing the transcription factor Hobit and strong capacities for both IFNγ and granzyme B production and for self-renewal (8-10).

Conversely, a dysfunctional effector T-cell profile has been deeply characterized in chronic lymphocytic choriomeningitis virus (LCMV) infection with clone C13 in mice (and also in humans during chronic hepatitis C, B and HIV) in which high virus load persists and is involved in the progressive hypo-responsiveness of antigen-specific effector T-cells (for review see (5)). Dysfunctional state is characterized by an increased expression of inhibitory receptors such as PD-1, a transcriptional program balance with high level of EOMES/low level of T-bet and by a decreased ability to produce cytokines and to proliferate (11). Conversely, "T-bet^{high}/EOMES^{low}/PD-1^{low}" effector T-cells are more functional (11, 12).

Our hypothesis is that multiple CMV reactivations or higher viral loads could occur in graft recipients that could lead to the emergence of such dysfunctional CMV-specific T-cells (9, 13-18). Interestingly, among immunosuppressive treatments, mTOR inhibitors (mTORi) have

been recently associated to a decreased number of CMV events after solid organ transplantation (19-21) but this association remains unexplained. Direct antiviral action of mTORi was reported but has only been observed *in vitro* and not in all type of CMV-infected cells (22). Alternatively, it could be due to an effect on CMV-specific immunity. Whereas mTORi have a strong anti-proliferative effect on naive cells (23), they also increase CD8 memory T-cells maintenance after LCMV infection in mice (24). However, data on mTORi effect on human T-cells remains elusive particularly on highly differentiated CMV-specific T-cells.

Since mTORi positive association with reduced CMV events has been observed in R+ patients (20) but not in D+R- patients (25), we hypothesized that mTORi could improve the functions of preformed CMV-specific effector T-cells.

T-cell response against CMV involves both CD8+ αβ and γδ T-cells (26). Only Vδ2^{neg} γδ T-cells are involved in the control of CMV, as demonstrated in many different studies (for review(27)), and their response is very similar to that of CMV-specific CD8+ αβ T-cells. They share similar kinetics, acquire the same late effector memory CD45RA+ (TEMRA) phenotype and antiviral functions (cytotoxicity, IFNγ production) (28, 29). However, Vδ2^{neg} γδ T-cells do not recognize viral peptides presented by HLA molecules, but CMV-induced "stress-self-antigens" at the surface of infected cells. Very few of these antigens have been described (30).

In the present study, our first aim was to assess if specific attributes of CMV-specific αβ and γδ T-cells in kidney recipients before transplantation could be associated with the risk of developing CMV reactivation after transplantation. Then, we investigated *in vitro* and *in vivo,* the effect of mTORi on the function and phenotype of CMV-specific effector αβ and γδ T-cells to understand why mTORi are associated with a better control of CMV infection in patients.

Methods

Sample and Patients

An ancillary study of a French multicenter phase IV clinical trial which compared the incidence of CMV DNAemia in CMV seropositive patients included at day 0 of transplantation and receiving everolimus with low doses of ciclosporin or mycophenolic acid with standard doses of ciclosporin (See supplemental methods for details) was performed among patients included at Bordeaux University Hospital (83 of 186 patients). Six patients discontinued the study prior to the first month and were therefore excluded (everolimus EVR, n=33 or mycophenolic acid MPA, n=44). Among EVR treated patients, 6 were switched to mycophenolic acid between month 1 and month 2, so were excluded of the study. Consequently, analyses of our ancillary study involved 71 patients, 44 MPA treated patients and 27 EVR treated patients (Flow chart in **Supplementary Figure 1)**. Among the 44 MPA treated patients, deep immunophenotyping of T cells has been performed on frozen PBMC at day 0 of transplantation to compare basal T cell profile of patients with "severe CMV" to patients with "well-controlled CMV" in absence of mTORi. CMV DNAemia requiring an antiviral treatment defined *severe CMV infections*, including CMV disease and CMV DNAemia for which antiviral drug has been introduced by physician. *Well-controlled CMV* is *defined* either by no post-transplant CMV DNAemias or CMV DNAemia with spontaneous negativation without any antiviral treatment. Flow chart of those 44 MPA-treated patients is described in **Supplementary Figure 2.** The protocol was approved by an independent ethics committee (CPP number: 2013/57) and registered in the ClinicalTrials.gov database (NCT02328963). All subjects signed a written informed consent before enrollment. CMV serostatus for each kidney transplant recipients had been determined the day of the graft and was positive for all patients. $V\delta2^{neg}$ γδ T cells immunophenotyping was performed at week 1, week 2 and week 24 post-transplantation. Peripheral blood mononuclear cells (PBMC) were isolated and frozen at day 0, month 1, month 3, month 6 and month 12 post-

transplantation. The course of CMV infection was preemptively monitored by longitudinal whole blood quantitative nucleic acid testing (QNAT) for CMV viral load every week from day 0 to month 3, then at month 4, 5, 6, 9 and 12, as previously described (31). The results were all given in IU/ml, calibrated with the International Standard WHO (32). The lower limit of quantification was 1000 IU/ml and of detection 250IU/ml; consequently, when the result was "weak positive", we put 999IU/ml. CMV infection was defined as a positive QNAT (33). Antiviral treatment with ganciclovir or valganciclovir was started at the time of the first >2000 IU/mL positive CMV QNAT.

PBMC cultures for T-cell expansions.

For γδ T-cells, PBMC were cultured in complete medium (RPMI medium supplemented with 2mM glutamine and 10% human serum) with 1000 IU/ml recombinant human IL-2 (rIL2, n°200-02), (all from Peprotech, France) for 28 days with 0; 0.5 or 10nM of the mTORi everolimus (HY-102018, Medchemexpress EU). In some experiments 10 ng/ml recombinant human IL-15 (rIL15, n°200-15) was added.

For CMV-specific αβ T-cell cultures, PBMC were cultured in complete medium with 10 IU/ml recombinant human IL-2 for 16 days with 0 or 0.5nM of everolimus. 0.6 nmol/ml of peptivator CMV pp65 (130-093-438, Miltenyi) was added at day 0 and day 9.

Additional experiments were performed as previously described for γδ T-cells and CMV-specific αβ T-cell cultures with the addition of 0/25/75/200ng.mL^{-1} ciclosporin (Novartis Pharma, Basel, Switzerland) with or without either 0.5nM everolimus or 1000ng.mL^{-1} mycophenolate sodium (Cellcept, Roche, batch B8003).

Proliferation and viability assay for γδ and CMV-specific αβ T-cells

Proliferation within TEMRA cells was first assessed by CFSE assay. PBMC were labelled with 5 μM 5(6)-Carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE, n°V12883, eBioscience, France) and cultured with or without 1000 U/ml rIL-2 and 10 ng/ml rIL-15. At day 7 of culture, $2x10^5$ cells were incubated with monoclonal antibodies anti-pan TCRδ (PE), TCRVδ2-PC-7 and anti-CD3 (BV510), anti-CD45RA (BV786), CD27 (BV650), washed and processed on the BD LSRFortessa. Proliferation was also assessed by counting on a Neubauer cell counting chamber and phenotyping with monoclonal antibodies anti-CD3 (V450), CD27 (APC),CD45RA (FITC), panδ (PE),Vδ2 (PC7) and processed with a BD Canto II (BD biosciences).

For CMV-specific αβ T-cells, PBMC were incubated in 96 well plates with 150μL of the previously described media. At day 9, 0.6 nmol/mL of peptivator CMV pp65 was added with a protein transport inhibitor (n°554724, BD biosciences) overnight at 37°C, cells were stained with anti-CD3 (V450), CD27 (BV650), CD45RA (FITC), pan-αβ (APC), CD69-PE monoclonal antibodies. Fixation-permeabilization was performed for staining with the anti-IFNγ (BV786) antibody. The assessment of CMV-specific CD8+ T cells was performed using flow cytometry with a gating of double positive CD69+IFNγ+ αβ CD8+ cells as previously described (34).

Absolute numbers are the percentages of CMV-specific αβ and $V\delta2^{neg}$ T-cells among PBMC multiplied by the total PBMC count.

Cell viability was measured by incubating $2x10^5$ cultured PBMC with the viability marker FVS575.

Expression of co-receptors and transcription factors for γδ and CMV-specific αβ T-cells

For $V\delta2^{neg}$ γδ T-cells, $5x10^5$ PBMC at day 21 of culture were stained with either the viability marker FVS575, then with antibodies anti-CD3 (BV510), PD-1 (BV650), TIM3 (BV711),

DNAM-1 (BV786), panδ (PE), Vδ2 (PC7) and KLRG1 (FITC); or the viability marker FVS780, then with antibodies anti-CD3 (BV510), Vδ2 (pacific blue) and either panδ (APC) with Blimp1 (PE), T-bet (PC5.5), or EOMES (Pe-Cy7) alone or panδ (PE) with Hobit (alex-fluor 647) (BD bioscience). PBMC were permeabilized with the FOXP3 transcription factor staining buffer (Fisher scientific). For CMV-specific αβ T-cells, $5x10^5$ PBMC cultured as described above in 96-wells plates, were stained with FVS575, then either with antibodies anti-PD-1 (BV650), KLRG1 (FITC), pan-αβ (APC), CD3 (V450), CD69 (PE) and intracellular staining with anti-IFNγ (BV786) or anti-pan-αβ (PE), CD3 (V450), CD69 (Alexa Fluor 700) antibodies , then anti-EOMES (PC7), Hobit (alexa-fluor 647) and IFNγ (BV786) antibodies (processed on the BD LSRFortessa)

S6 and Akt phosphorylation.

2.10^5 cells from the $V\delta2^{neg}$ γδ cultures at day 21, were washed and incubated in 400µL of RPMI (2mM glutamine, 8% FCS alone or with 0.5nM everolimus) and activated with an anti-Vδ1 mAb (10µg/ml) for the indicated period of time. As previously described for phosphorylated proteins staining (35) , cells were stained in the phosphoflow buffer with monoclonal antibodies anti-CD3 (V450), Vδ2 (FITC) and panδ (APC) and with either anti-pS6 (PeCy7), S6 (PE), pAkt T308 (PE), pAkt S473 (FITC), Akt (PE) (processed on the Canto II).

pSLP-76, p-ERK and p38 expression T-cells

At day 21 of culture with rIL2, $V\delta2^{neg}$ γδ T-cells were purified after magnetic negative sorting with pan T-cell isolation kit; αβ–biotin and anti-biotin (Miltenyi Biotec), Vδ2 FITC (Beckman) and anti-FITC (Miltenyi Biotec). Purity was controlled by staining sorted cells with anti-CD3 (V450), panδ (PE), and Vδ2 (PC7) resulting in a 85-96% purity. Cells were activated with

10µg/ml of purified UCHT1 (Beckman). Intracellular staining was performed as previously described (35) (processed on Canto II).

***Ex vivo* phenotyping of γδ T cells and CMV-specific CD8+ T cells.**

For complete reference of antibodies, see supplemental Methods.

Flow cytometry phenotyping of patients' Vδ2^{neg} γδ T cells was performed on whole blood as previously described (36) using the following monoclonal antibodies: anti-CD45-APC, anti-panδ-PE, anti-Vδ2-FITC, anti-CD27 Pe-Cy7 and anti-CD45RA-ECD and with the Lysing Solution IOTest®3 10X Concentrate (Beckman). The samples were processed on a NAVIOS flow cytometer (Beckman coulter).

One million frozen PBMC were used for each multi-color flow cytometry analysis, with the same viability marker FVS575, the same monoclonal antibodies against CD3 (PerCP), Vδ2 (PC-7), pan-delta (PE or APC), CD8 (either BV510 or PE-texas-red) and IFN-γ (BV786) and CD69 (AF-700) for the staining of CMV-specific CD8+ T cells after overnight pp65 stimulation with a protein transport inhibitor. Then, the staining included either CD45RA (FITC), and CD27 (BV786); or CD85j (FITC), CD161 (BV650), CD16 (BV786), and KLRG1 (PE-Vio 615); or granulysin (FITC), perforin (APC) and granzyme (BV421); or PD-1 (BV650), TIM-3 (BV711), LAG3 (BV421), and DNAM-1 (BV786). CMV-specific CD8+ T cells were gated as double positive cells for IFNγ and CD69 as previously described (34).

PBMC stained for intracellular markers were permeabilized,fixed using Fixation/Permeabilization Solution (BD biosciences) , and processed on the **BD LSRFortessa cytometer (BD biosciences).**

IFNγ production in co-cultures with CMV-infected cells

After 21 days of culture with rIL-2 and rIL-15, $V\delta2^{neg}$ γδ T-cells were negatively purified as described above. 50.10^4 cells were incubated *per* 96 wells-plate with RPMI 8% FCS, 2mM glutamine and 50ng/ml rIL18 (MBL international, B003-5, Massachusetts-US) (see (37)) with or without everolimus alone, or with either non-infected or CMV-infected fibroblasts during 24 hours at 37°C. For CMV-specific αβ T-cells, PBMC were cultured as described above but without pp-65 stimulation and after 7 days, cells were incubated alone or with peptivator CMV-pp65 0.6nmol/mL or PMA 25ng/ml/ionomycine 1µg/ml for 37°C during 24 hours. An extra well was added to perform an IFNγ and CD69 staining after overnight pp65 stimulation with a protein transport inhibitor.

Then, IFNγ was measured in supernatants using the Human IFN-γ ELISA development kit (Mabtech, n°3420-1H-6).

Preparation of CMV-infected fibroblasts

Human foreskin fibroblasts (HFF, kindly provided by Dr H. Rezvani, INSERM, U1035, Bordeaux), grown in Dulbecco's Modified Eagle Medium (DMEM) containing 8% FCS and 2mM glutamine, were infected with the TB42/E strain of human CMV at a multiplicity of infection (MOI) of 0.1. After virus adsorption overnight at 37°C, cells were washed and covered with fresh growth medium. Co-cultures were performed when cytopathic effects were ≥90%, 4 days after infection. Non infected (NI) cells grown in parallel were mock-infected using medium alone.

Preparation of free CMV

To produce free CMV (TB42/E strain), human foreskin fibroblasts were infected at a MOI of

0.1 and incubated at 37°C in culture medium DMEM, 8% bovine serum and glutamine for 10 days or until cytopathic effects were ≥90%. The supernatant was stored at -80°C. The

preparation had a titer of 2,5 10^6 PFU (plaque-forming unit)/ml, the titration was performed as previously described [1]. All virus stocks and cells were tested negative for the presence of *mycoplasma*.

Viability of Vδ2^{neg} γδ T cells during co-culture with and without blocking anti-CD3 antibody analyzed with DAPI

Vδ2^{neg} γδ T cells after 21 days of culture with 0 and 0.5 nM everolimus were negatively sorted with magnetic beads and were cultured in medium alone (either with 0 or 0.5 nM everolimus), non-infected (NI), CMV-infected fibroblasts with or without blocking anti-CD3 mAb (10µg/ml) during 24 hours and cells were stained with 1µM DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) and after 15 minutes of incubation at room temperature, cells were analyzed by flow cytometry Canto II.

***Ex vivo* quanti-FERON-CMV.**

QuantiFERON-CMV V (n°0350-0201, Qiagen) was performed as previously described (38) from frozen plasma and read with the QUANTA-Lyser® 2 Inova Diagnostics; at day 14 (32 mTORi-treated and 43 MPA-treated patients) and month 6 post transplantation (24 mTORi-treated and 39 MPA-treated patients). Results were analyzed with the CMV v3.03 software.

Statistics

The Mann-Whitney U test, the χ^2 test or the Fisher-test, the unpaired t test were used when appropriate. Alternatively, paired-t test was used for paired data. Paired tests were used when two sets of data from the same patients were compared whereas unpaired tests were used to

compare data from two different group of patients. $p<0.05$ was considered statistically significant. Analyses were performed with conventional statistical methods using GraphPad Prism. Figures were obtained with FlowJo software (V.10) and GraphPad Prism.

Results

Increased percentages of T-cells expressing inhibitory receptors at baseline in MPA-treated patients with severe CMV infections.

Thirty-two of 44 MPA-treated patients undergo CMV infection: 14 with a peak viral load<999IU/ml spontaneously cleared and thus belong to the "well-controlled CMV" group, 18 with CMV DNAemia requiring treatment called "CMV-severe" among which 6 with CMV disease. Among those 44 MPA-treated patients of the ancillary study, 33 had available frozen PBMC at day 0 of transplantation and we consequently characterized T-cell phenotype at day 0 of transplantation (baseline phenotype) in a first subgroup of 21 MPA-treated patients. Nine had "severe CMV", either CMV disease (n=3) or CMV DNAemia for which antiviral drug has been introduced by physician (n=6; peak viral load: mean=6916 IU/ml, standard deviation=3762IU/ml) and 12 had a well-controlled CMV, either no CMV DNAemia (n=6) or CMV DNAemia spontaneously cleared without any antiviral treatment (n=6, all with a peak viral load< 999IU/ml). Late-differentiated CD45RA+ T effector cells (TEMRA) were highly represented in γδ T-cells and CD8+ T-cells (**Figure 1A**) as previously observed (29). Both T-cell compartments presented high percentages of cells expressing activation receptors such as DNAM-1 (CD226) (also CD8αα and CD16 for γδ T-cells), as well as KLRG1, a receptor expressed on highly differentiated CD8+ T-cells with high cytotoxic but low proliferative capacities in CMV infection (39). A significant proportion of T-cells also expressed inhibitory receptors, such as CD85j, PD-1 and TIM3 (**Figure 1B**). These phenotypes were then compared between patients with post-transplant *severe CMV infections* (n=9) and patients with *well-controlled CMV* (n=12). No difference in group differentiation status (**Supplemental Figure 3A**) or KLRG1, DNAM-1, CD8αα and CD16 expression **(Figure 1C)** were observed. However, patients who will present severe CMV infection had a higher percentage of T-cells expressing inhibitory receptors, such as PD-1 and CD85j, when compared to patients with well-

controlled CMV (**Figure 1C**). We validated and extended these results by analyzing concomitantly γδ T-cells, total CD8+ T-cells (**Supplemental Figure 3B**) and also CMV-specific CD8+ T-cells in an internal validation subgroup of 12 additional patients (belonging to the 44 MPA-treated patients of the ancillary study): 7 with "severe CMV infection" of which 3 with CMV disease and 4 with CMV DNAemia for which antiviral drug has been introduced by physician (peak viral load: mean=7930 IU/ml, standard deviation=3976 IU/ml); 5 with well-controlled CMV (one patient without CMV DNAemia, 4 with CMV DNAemia spontaneously cleared, peak viral load <999 IU/ml). We confirmed that patients with severe CMV infection also displayed significantly higher percentages of PD-1+, and CD85j+ cells in CMV-specific CD8+ T-cells (**Figure 1D)** and validated those markers in total CD8+ and γδ T-cells (**Supplemental Figure 3B**). At baseline, T-cells from CMV seropositive patients could thus display a pre-existing dysfunctional profile, characterized by expression of inhibitory receptors, correlating to severe CMV infection after transplantation. Finally PD1 and CD85j appeared to be the more convincing markers differently expressed by both γδ T cells and CMV-specific CD8+ T cells. From a clinical perspective, only using two markers would be easier to implement as a routine assay than a multicolor staining.

The proportion of functional T-cells is enhanced by mTORi treatment and correlates to a subsequent lower incidence of CMV infection

To address the issue of mTORi effect on dysfunctional T-cells, 44 KTR treated with MPA were compared to 27 KTR treated with mTORi (everolimus). As shown in **Table 1**, no major difference was observed regarding age, sex, CMV serostatus of donor, rank of transplantation, living donor, expanded criteria donor and acute rejection. We confirmed that mTORi treatment

protects from CMV-infection since 26 % (7/27) of mTORi-treated patients displayed CMV infection contrasting with 70 % (32/44) in MPA-treated patients ($p<0.001$) **(Figure 2A**). Between day 7 and day 14, total and TEMRA γδ T-cell percentages increased significantly in mTORi-treated patients whereas did not change in MPA-treated patients (prior to any CMV replication) (**Figure 2B and 2C**). For CMV-specific αβ T-cell immunity assessed by QuantiFERON-CMV (**Figure 2D**), a significant difference between mTORi- and MPA-treated patients already exists at day 7 but CMV-quantiFERON significantly increased between day 7 and day 14 in mTORi- but did not in MPA-treated patients.

Then, we compared the effect of MPA (n=7 patients) and mTORi (n=8 patients) on the evolution of T cell phenotype before any CMV replication". mTORi treatment was associated with a decreased percentage of PD-1+ and CD85j+ T-cells (in γδ T-cells, CD85j was unchanged) together with an increase of perforin+ T-cells while MPA was associated to increased or stable PD-1+ and CD85j+ cell percentages (**Figure 2E**). Importantly, there was a direct association between the decrease of CD85j- or PD1-expressing T-cells percentage during the first month of transplantation and an absence or a low (<1000 IU/ml) CMV DNAemia (**Figure 2F**).

mTORi treatment increases the percentages of T-cells expressing a functional profile which is associated to a low incidence of CMV infection post-transplantation.

mTORi treatment is associated with spontaneously cleared CMV replication that correlate to an enhanced specific T-cell response

We then focused on the patients who developed post-transplantation CMV infection. The mean of CMV infection occurrence was 51 days (standard deviation 37 days) post transplantation.

Viral load was much lower (never exceeding 999 IU/ml) in patients with mTORi (n=7) than with MPA (n=32; mean 7482 IU/ml) (**Figure 3A**). Moreover, mTORi-treated patients did not require any anti-viral treatment since they all spontaneously cleared the virus by contrast to MPA-treated patients in which 56.25% (18/32) required anti-viral treatment (p=0.009) (**Figure 3B**). Six MPA-treated patients underwent CMV disease whereas none of everolimus-treated patients did (data not shown).

During the course of CMV infections (all occurring between month 1 and month 3 post-transplantation), γδ T-cell expansion (**Figure 3C**) and increase of TEMRA proportion (**Figure 3D**) were higher in mTORi- than MPA-treated patients. Moreover, CMV-specific αβ T-cell response was also improved since IFNγ measured by QuantiFERON-CMV increased significantly during the course of CMV infection only in mTORi-treated patients (p=0.015) (**Figure 3E**).

The time points chosen to perform the immunophenotyping were day 0 and at the closest time-point of CMV replication (month 1 or month 3 depending on when CMV replication happened for each patient) in order to analyze the effect of both immunosuppressive drug and CMV replication.

The proportion of γδ T-cells and total CD8+ T-cells expressing perforin increased more significantly in mTORi-treated patients, when compared to MPA-treated patients (**Figure 3F**). KLRG1 was expressed on significantly less T-cells in mTORi-treated patients (**Figure 3F**). Finally, the decrease of KLRG1+ cells and the increase of perforin+ cells among γδ T-cells was directly associated to low viral loads (<999 IU/ml) independently of the immunosuppressive treatment (**Figure 3G**).

Thus a better control of CMV infection in mTORi-treated patients compared to MPA-treated patients, was associated with a better expansion and a functional reinforcement of T-cells.

Long-term *in vitro* treatment of T-cells with therapeutic doses of mTORi increase their proliferation and viability

Proliferation and viability were assessed *in vitro* on R+ KTR PBMC comprising predominantly TEMRA cells among both γδ T-cells (more than 95%) and CMV-specific αβ T-cells (up to 85%) (**Supplemental Figure 4**) at day 0.

TEMRA γδ T-cell can proliferate despite their late-differentiated phenotype, as shown through CFSE experiments at day 7 of culture (**Supplemental Figure 4B**), and through the strong increase of both percentages (**Supplemental Figure 4C)** and numbers (**Supplemental Figure 4D**).

Then mTORi was added either at dose mimicking anti-proliferative effect (10 nM), or at dose used in immunosuppressive treatment of solid organ transplantation (0.5 nM) for γδ T-cells, and only at the lowest dose for αβ T-cells.

As expected and shown in **Figure 4A**, high dose of mTORi inhibited γδ T-cell proliferation. By contrast, culture performed with low dose mTORi (0.5 nM) resulted in a better proliferation than in the absence of mTORi (**Figure 4A and C**). Yet, the dose of 0.5 nM was as potent as that of 10 nM to inhibit S6 phosphorylation in TEMRA γδ T-cells while not affecting the expression of total S6 protein level (**Figure 4B**). Interestingly, low and high doses of mTORi were associated with an increased viability of the cells during the late period of the culture (day 28) (**Figure 4D**). Like γδ T-cells, low dose mTORi-treated CMV-specific αβ T-cells show a better proliferation (**Figure 4E**) and viability (**Figure 4F**). Hence, low dose mTORi improves the proliferation and the viability of TEMRA T-cells consistently with our previous observations in mTORi-treated patients (**Figure 2**).

Low dose of mTORi promotes T-cells functional profile

When cultured in the presence of low or high dose of mTORi, a decrease in the proportion of PD-1+ and KLRG1+ γδ T-cells was observed while DNAM-1 and TIM-3 receptors were expressed at the same levels (**Figure 5A**). These results suggested that mTORi were able to modify *in vitro* the dysfunctional profile of γδ T-cells observed earlier *in vivo* at baseline (**Figure 1**), recapitulating the effect of mTORi observed in patients after transplantation.

Second, we tested whether mTORi were acting on transcription factors regulating IFNγ, *i.e.* Tbet, EOMES, Hobit and Blimp-1 Low and high mTORi doses resulted in an increase of Hobit and a decrease of EOMES, which may favor high IFNγ production (40). Low dose mTORi maintained, whereas high dose decreased, Tbet expression (**Figure 5B**). Like γδ T-cells, CMV-specific αβ T-cells treated with a low mTORi dose showed a lower expression of EOMES concomitantly to an increased expression of Hobit (**Figure 5D**), while PD-1+ cells were decreased (**Figure 5C**). Low mTORi dose also reduced the percentage of CD85j+ cells in both T-cell subsets (**Supplemental Figure 5**).

Third, we wondered if this new phenotype induced by mTORi correlated with a better antiviral function. γδ T-cells treated with low dose mTORi produced higher amount of IFNγ against CMV-infected cells, which was remarkably not the case when high mTORi dose was used (**Figure 6A**) in accordance with its inhibitory action on Tbet expression (**Figure 5B**). Regarding CMV-specific αβ T-cells, PBMC activated with CMV peptides after 7 days of mTORi culture comprised a similar quantity of CMV-specific αβ T-cells (IFNγ+CD69+ αβ T-cells) (**Supplemental Figure 6**) but an increased IFNγ production (**Figure 6B**) compared to PBMC cultured without mTORi.

In summary, a low mTORi dose improves functional fitness of T-cells with the reinforcement of CMV-induced IFNγ production. mTORi drives an increased percentage of "Hobit^{high}/ EOMES^{low}/PD-1^{low}" cells, previously associated to an efficient antiviral potential (10, 12).

mTORi improvement of the T cell dysfunctional profile is maintained when combined with Ciclosporin.

To mimic the patients immunosuppressive regimen, we also performed γδ and CMV-specific

αβ T-cells T cells culture combining either 0/25/75/200ng.mL^{-1} ciclosporin alone or with 0.5nM everolimus or with 1000ng.mL^{-1} mycophenolate sodium. Mycophenolate sodium was actually strongly inhibiting both γδ and CMV-specific αβ T cells proliferation. Ciclosporin inhibits in a dose-dependent manner γδ and CMV-specific αβ T cells proliferation (**Supplemental Figure 7A**). However, for the same concentration of ciclosporin, everolimus kept a significant effect on increasing proliferation (**Supplemental Figure 7A**) and on decreasing PD1 and CD85j expression (**Supplemental Figure 7B**). Consequently, our *in vitro* observations about everolimus effects were confirmed together with ciclosporin and led us to extrapolate what we observed *ex vivo* in patients.

Effect of mTORi on TCR engagement and signaling of γδ T-cells

Since it has been previously demonstrated that dysfunctional T-cells are hypo-responsive to TCR signaling (41, 42), we took the opportunity that high quantities of γδ T-cells were obtainable (compared to CMV-specific αβ T-cells) to investigate their TCR-signaling efficiency with or without mTORi. TCR neutralization significantly inhibited the production of IFNγ by γδ T-cells against CMV-infected cells (**Figure 7A**), and even more when γδ T-cells

were pre-incubated with mTORi, without viability issue (**Supplemental Figure 8**). mTORi may amplify the intrinsic ability of γδ T-cells to respond to the TCR engagement. In agreement with this hypothesis, in mTORi-treated γδ T-cells, IFNγ production T-cells after anti-TCR Vδ1 activation was increased (**Figure 7B**) together with a higher internalization of the TCR(**Figure 7C**). TCR signaling-induced phosphorylation of the kinase ERK was enhanced in mTORi-treated γδ T-cells (**Figure 7D**) while no difference was observed for MAPK-38 or of SLP-76 suggesting that mTORi was acting downstream of this very proximal event of TCR signaling (**Supplemental Figure 9)**.

Moreover, while S6 phosphorylation was decreased at baseline in mTORi-treated γδ T-cells (**Figure 7E, left**), the induction of S6 phosphorylation upon TCR triggering was much more important in mTORi-treated than in untreated cells, which was not associated to a feedback activation of Akt (**Supplemental figure 10A and 10B**).

Overall, inhibition of the mTOR pathway conditions γδ T-cells to respond more efficiently to an antigenic challenge, as occurs during CMV infection.

Discussion

It has been well established that mTORi treatment is associated with a reduction of the incidence of CMV infection in CMV-seropositive organ transplant patients (19, 20), but a mechanistic explanation for this effect has been lacking to date. In the present study, we suggest that a multimodal functional action on both αβ and γδ effector-T-cells is involved. In contrast with mTORi immunosuppressive functions on naive allogenic T-cells, our overall results indicate that mTORi treatment of specific-effector T-cells improves their response to CMV through improving expansion, viability, and restoring their functional phenotype characterized by increased cytotoxic potential, increased IFNγ production and decreased expression of inhibitory check-points.

Our first original observation was to describe at baseline a higher proportion of T-cells with a dysfunctional phenotype in CMV seropositive patients who were found unable to control CMV infection without antiviral treatment. We characterized this dysfunctional phenotype through the expression of several inhibitory receptors (PD-1, LAG3, TIM3, CD161, CD85j). As usually described, effector memory T-cells maintaining after CMV infection have a specific profile with CD45RA reexpression, low PD-1, and high KLRG1, Tbet and Hobit expression without loss of function (6). Here, we described that in cells from CMV seropositive KTR cultured *in vitro*, among effector memory T-cells, a percentage of dysfunctional cells expressed PD-1, high level of EOMES, low level of Hobit for the same level of Tbet. This profile of dysfunctional T-cells is usually described in a viral context of a persistent uncontrolled viral load during chronic phases of infection (e.g.LCMV) (43, 44), whereas the effector T-cell response described during CMV infection was called inflationary (5, 6) and correlated to well-controlled low viral load. However, CMV seropositive patients awaiting for kidney graft could be in a relative immunosuppressive state due to the end-stage renal disease (45), to previous

immunosuppressive treatment of their renal disease or to previous kidney transplantation. This context could lead to a chronic higher level of viral load favoring the emergence of those dysfunctional CMV-effector T-cells. Interestingly, documenting the presence of these dysfunctional T-cells prior to the introduction of immunosuppressive drug regimen may be used to predict the risk of CMV reactivation after transplantation in CMV seropositive patients to avoid useless treatment. Here, PD-1 and CD85j, expressed on both CMV-specific CD8+ and γδ T-cells were the most discriminating markers in R+ patients for predicting severe CMV infection. Especially, CD85j is a well-known inhibitory receptor induced on both αβ (46) and γδ T-cells (47) during CMV infection, and binding with high affinity the CMV encoded UL18 protein (48).

Secondly, we observed that mTORi improved the functional profile (decreased inhibitory receptors, increased functionality) of effector T-cells together with a lower incidence and severity of CMV infection after transplantation. In addition, during the course of CMV infection, decreased proportion of T-cells expressing the inhibitory receptor KLRG1 and an increased proportion of perforin-producing T-cells were directly associated to a better control of CMV, independently of the immunosuppressive treatments and then these changes were shown to be mostly induced by mTORi.

In vitro, low dose of mTORi induced a long-lived cell profile (PD1 low, Tbet high and EOMES low) which was associated with increased proliferation, viability and IFNγ production both on γδ and αβ T-cells. Those characteristics were previously associated with improved effector T-cells functionality (11, 12). Moreover, mTORi also increased the amount of Hobit+ T-cells, known to be long-lived effector cells in the context of CMV (8-10).

While mTORi effect have been well-studied in ~~In~~ naive cells and notably in allogenic naive cells, ~~mTORi have been shown to display anti-proliferative effects and to decrease response to activation, underlining the rationale behind using mTORi to prevent graft rejection~~ (23),

~~However,~~ it has been poorly evaluated in effector T-cells. Our *in vitro* results suggest that late-differentiated effector cells present high basal level of p-S6 that limit their capacity for further S6-phosphorylation during activation, that could be involved in their low IFNγ production after TCR engagement. Conversely, when basal level was decreased by mTORi pre-treatment, phosphorylation of S6 and IFNγ production was much more inducible during activation. Altogether, these results suggest that mTORi are able to dampen basal S6 phosphorylation in highly differentiated T-cells allowing them to respond more efficiently upon activation.

Finally, we also observed that mTORi treated T-cells improved TCR signaling. This is of particular interest since late effector memory T-cells during chronic infection are mostly dependent on TCR signaling rather than cytokine signals to persist (49, 50). Chronic antigen activation could lead to increased basal mTOR levels in T-cells, hampering their capacity to respond properly during a new viral challenge. Decreasing the basal level of mTOR pathway activation could sensitize effector T-cells to better respond to a new TCR activation.

Altogether, those findings have highlighted the involvement of dysfunctional T-cells in CMV infection risk after transplantation. CD85j and PD-1 expression offers a promising perspective in term of markers to better manage prevention strategies in CMV seropositive patients. We believe that these results provide a clinical and mechanistic understanding of mTORi effect on the restoration of CMV-specific late effector T-cells, leading to a better control of CMV after transplantation. These new insights could guide better use of mTORi in CMV seropositive patients and contribute to improve outcomes for the most frequent opportunistic infection in solid organ transplantation .

Author contributions

HK was involved in designing research studies, conducting experiments, acquiring data, analyzing data, and writing the manuscript. NY, GM, MJN, AZ, VP, SL, XG, IG, BP were involved in acquiring and analyzing data, and writing the manuscript. LC, IP, RT, RD, MMM, MC were involved in analyzing data and writing the manuscript. PM and JDM were involved in supervising research, designing research studies, in analyzing data, and writing the manuscript.

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Disclosure of Conflicts of Interest

L. Couzi reports Consultancy Agreements with Novartis, Biotest, Hansa; Research Funding from Novartis; and Honoraria from Hansa, Biotest, and Novartis. J. Dechanet-Merville reports Scientific Advisor or Membership with American Gene Technologies. P. Merville reports Consultancy Agreements: BMS; Astellas; Research Funding: Astellas; Honoraria: Sanofi, CSL Behring; Scientific Advisor or Membership: Novartis, BMS; and Other Interests/Relationships: Agence Biomédecine. M. Mamani-Matsuda reports Other Interests/Relationships: Reviewer for the For Woman in Sciences program of the L'Oreal-Unesco Fundation (fellowships). The remaining authors have declared that no conflict of interest exists.

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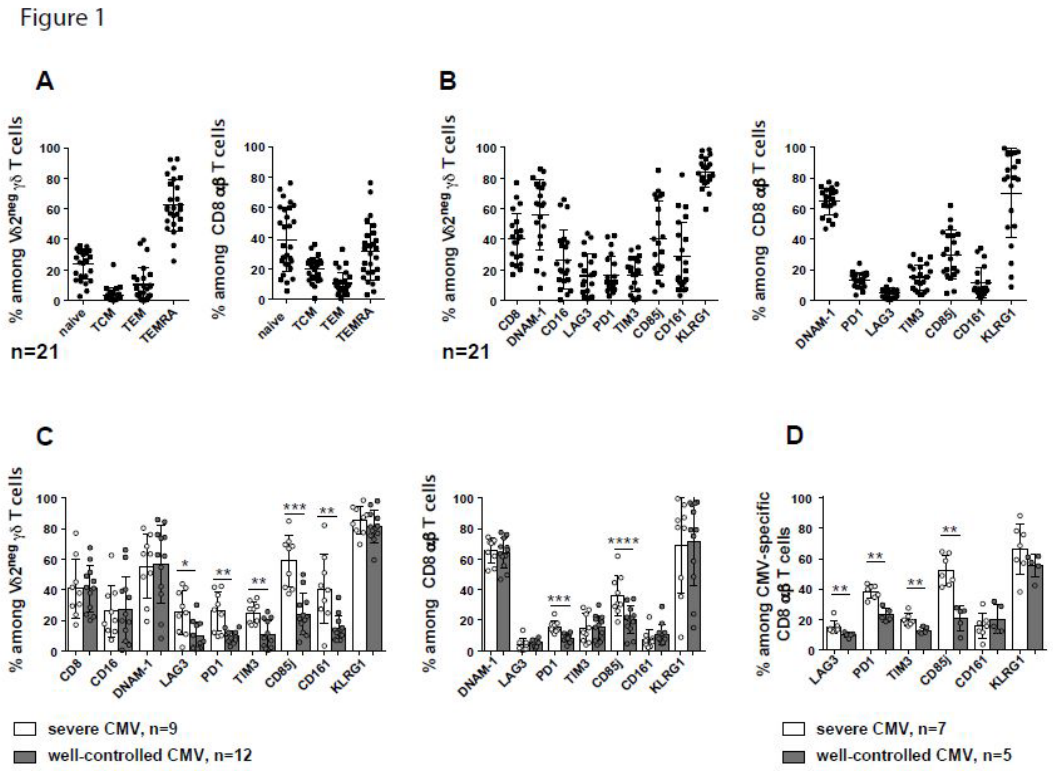
Table 1. Clinical parameters of patients

	EVR (n =27)	**MPA (n =44)**
Recipients		
Age (years), mean (SD)	64.2 (15.5)	62.6 (13.5)
Male, n (%)	15 (64)	32 (73)
Donors		
CMV Seropositive donors, n (%)	14 (51.8)	23 (52.6)
Living donor, n (%)	5 (14.8)	3 (6.8)
Rank of transplantation, first, n(%)	1 (3.7)	2 (4.5)
Expanded criteria deceased donor, n (%)	16 (59.2)	23 (52.2)
Ciclosporin whole blood trough concentrations day 7; mean (SD)	172.1 (71.4)	163.3 (51.2)
Ciclosporin whole blood trough concentrations day 14; mean (SD)	210.7 (65.2)	223.8 (55.6)
Ciclosporin whole blood trough concentrations month 6; mean (SD)	88.7 (34.3)	117.2 (30.9)
Everolimus whole blood trough concentration day 7; mean (SD)	4.2 (1.8)	
Everolimus whole blood trough concentration day 14; mean (SD)	6.3 (2.3)	
Everolimus whole blood trough concentration month 6; mean (SD)	4.9 (1.3)	
Biopsy proven acute rejection (12	8 (29,6)	11 (25)

months of follow-up), n (%)

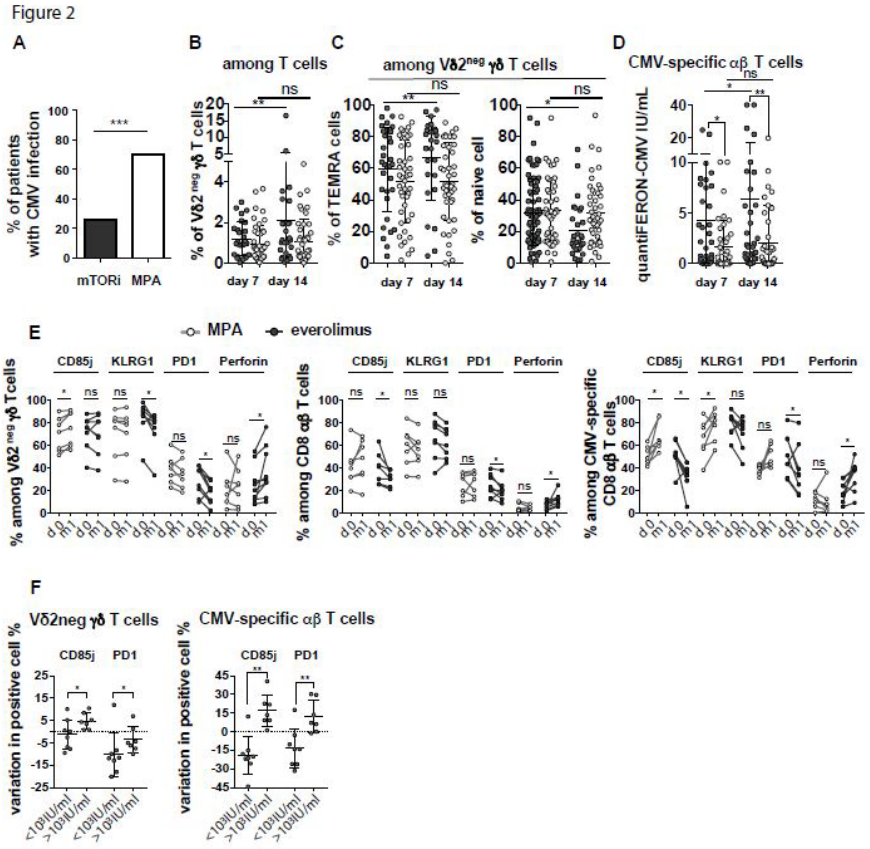
Figure Legend

Figure 1 Vδ2^{neg} γδ T cells and CD8+ T cells express inhibitory receptors at baseline in patients with severe CMV infections



Vδ2^{neg} γδ T cells and total CD8+ T cells were analyzed for their expression of CD27 and CD45RA (A) and their co-stimulatory and co-inhibitory receptors (B), in all CMV seropositive MPA treated patients (n=21) (A-B) or separating patients with well-controlled CMV (n=12) versus patients severe CMV (n=9) (C). Finally, phenotypes were extended to CMV-specific CD8+ T cells in an internal validation cohort of patients with well-controlled CMV (n=5) versus patients with severe CMV (n=7) (D). Each symbol represents an individual donor; large horizontal lines indicate the mean and small horizontal lines indicate the standard deviation, 0.05>p>0.01*; **0.01>p>0.001; ***p<0.001; as determined by the Mann-Withney U test.

Figure 2 High percentage of functional T cells in mTORi-treated patients correlated to a lower incidence of CMV infection



A. Incidence of CMV DNAemia in 27 mTORi-treated patients and in 44 patients treated with mycophenolic acid (MPA) at month 12 post-transplantation.

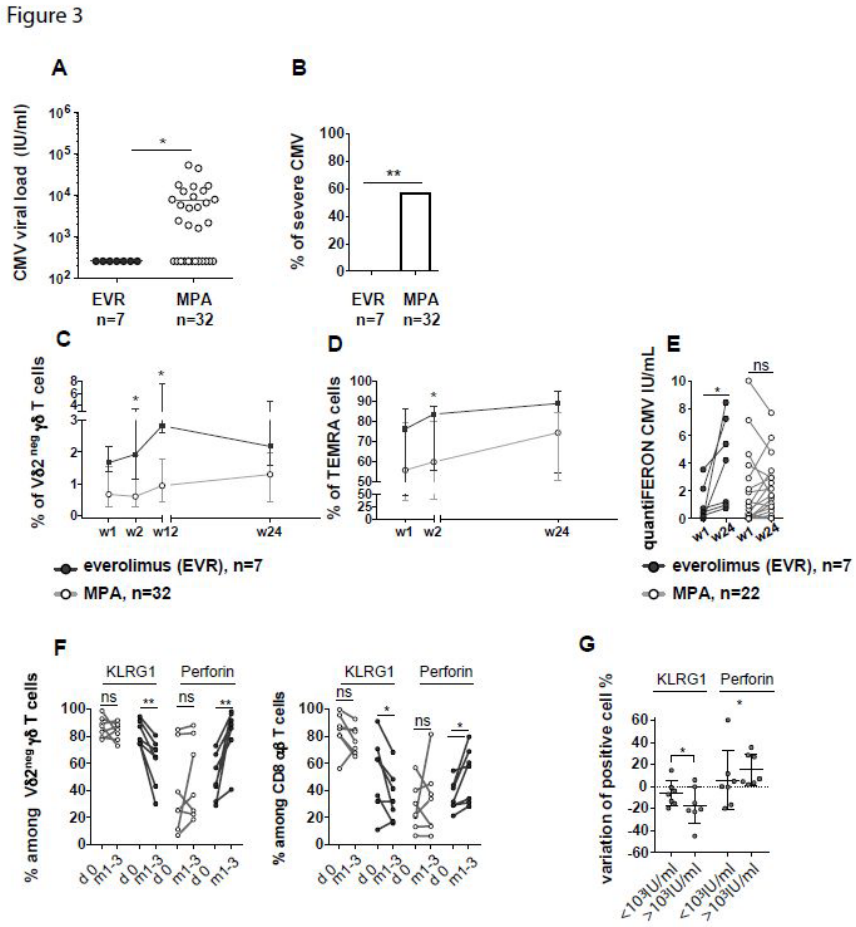
B.C. Whole blood staining of Vδ2^{neg} γδ T cells and their expression of CD27 and CD45RA. Frequencies of Vδ2^{neg} γδ T cells among T cells (B), of TEMRA (CD27 neg CD45RA pos) and naive (CD27 hi CD45RA pos) cells among Vδ2^{neg} γδ T cells (C) at day 7 and 14 post-transplantation (n=27 evero, n=44 MPA). Each symbol represents an individual donor; large horizontal lines indicate the mean and small horizontal lines indicate the standard deviation.

D. CMV-specific αβ T cells were analyzed with QuantiFERON-CMV (IU/mL) at day 7 (n=27 evero, n=41 MPA) and 14 post-transplantation (n=27 evero, n=43 MPA). Each symbol represents an individual donor, large horizontal lines indicate the mean and small horizontal lines indicate the standard deviation.

E. Proportions among Vδ2^{neg} γδ T cells (left), among total CD8+ T cells (middle) and CMV-specific CD8+ T cells (right) of CD85j+, perforin+, KLRG1+ and PD-1+ cells, compared between day 0 (d0) and month 1 (m1) post-transplantation in mTORi (n=8) and MPA (n=7)-treated patients. Each symbol represents an individual donor.

F. Difference of CD85j and PD-1+ cell percentage between month 1 and day 0 (value m1- value day0) was calculated and compared in patient groups with a post-transplantation CMV viral load > or <1000IU/ml (including 0IU/ml). 0.05>p>0.01*; **0.01>p>0.001; ***p<0.001; as determined for all unpaired data by the Mann-Withney U test and for paired data by Wilcoxon test.

Figure 3 Better response of T cells in mTORi-treated patients than in MPA-treated patients correlated to a lower severity of CMV infection



A. Maximal CMV viral load (IU/ml) in mTORi (n=7) and MPA (n=32)-treated patients. Each symbol represents CMV viral load value for an individual patient; large horizontal lines indicate the mean.

B. Proportion of MPA (n=38) and mTORi (n=7) treated patients with CMV DNAemias, 0.05>p>0.01*; as determined by the Fisher's exact test.

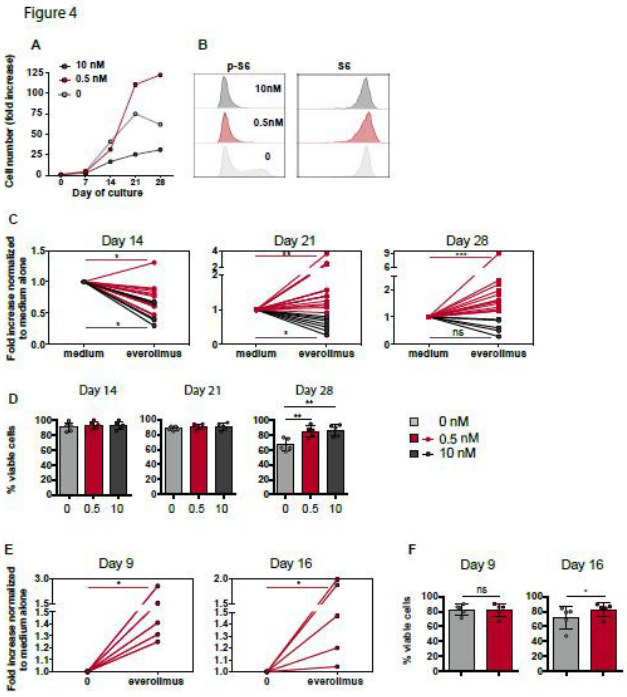
C.D. Whole blood staining of Vδ2^{neg} γδ T cells and their expression of CD27 and CD45RA. Proportion of Vδ2^{neg} γδ T cells among T cells (C) and of TEMRA (CD27 neg CD45RA pos) among Vδ2^{neg} γδ T cells at week 1, 2, 12 and 24 post-transplantation in mTORi (n=7) and MPA (n=32)-treated patients. Each symbol represents the median-value for each group of patients associated with interquartile ranges.

E. Quantiferon-CMV (IU/ml) at week one and week 24 in mTORi(n=7) and MPA-treated patients (n=22) with CMV DNAemia post transplantation. Each symbol represents an individual donor.

F. Proportions among Vδ2^{neg} γδ T cells (left) and among total CD8+ T cells (right) of perforin+ and KLRG1+ cells, compared between day 0 and during CMV DNAemia (either month 1 or month 3) in mTORi (n=7) and MPA (n=7) treated patients. Each symbol represents an individual donor.

G. Difference of KLRG1 and perforin+ Vδ2^{neg} γδ T cell percentages between month 1 and day 0 were calculated and compared in patient groups with a post-transplantation CMV viral load > or = 1000 IU/ml ns, not significant, 0.05>p>0.01*; **0.01>p>0.001 as determined for paired-data by Wilcoxon test and for unpared data by Mann-Withney U test.

Figure 4 Impact of long-term *in vitro* mTORi treatment on proliferation and viability of Vδ2^{neg} γδ T cells and CMV-specific αβ T cells



PBMC from CMV seropositive KTR were incubated with IL-2 with or without IL-15 for Vδ2^{neg} γδ T cells and IL-2 alone for CMV-specific αβ T cells and with indicated doses of everolimus. Proliferation and viability of Vδ2^{neg} γδ T cells at day 14, 21 and 28 of culture and of CMV-specific αβ T cells at day 9 and 16 of culture were performed.

A. Representative donor for Vδ2^{neg} γδ T cells proliferation.

B. Representative flow cytometry staining of S6 and phospho-S6 (p-S6) among Vδ2^{neg} γδ T cells at day 14 of culture.

C. Proliferation of Vδ2^{neg} γδ T cells, represented as fold increases normalized to culture with medium alone, at day 14 (everolimus 0.5nM, n=13; everolimus 10nM, n=6), 21 (everolimus 0.5nM, n=15; everolmus 10nM, n=8) and 28 (everolimus 0.5nM, n=12; everolimus 10nM, n=6).

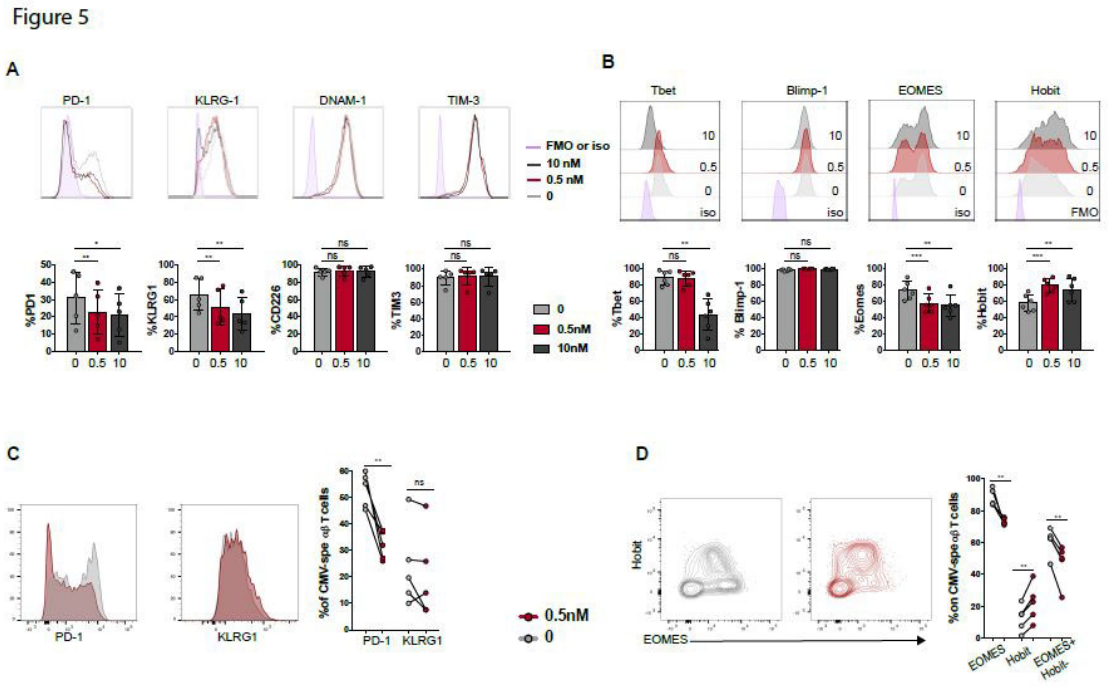
D. Vδ2^{neg} γδ T cell viability tested by flow cytometry live-dead staining (n=5).

E. Proliferation of CMV specific αβ T cells, represented as fold increases normalized to culture with medium alone (everolimus 0.5nM, n=5)

F. CMV-specific αβ T cells viability tested by flow cytometry live-dead staining (n=5).

For C, D, E and F, each symbol represents an individual donor. ns, not significant, 0.05>p>0.01*; **0.01>p>0.001; ***0p<0.001 as determined by Wilcoxon test.

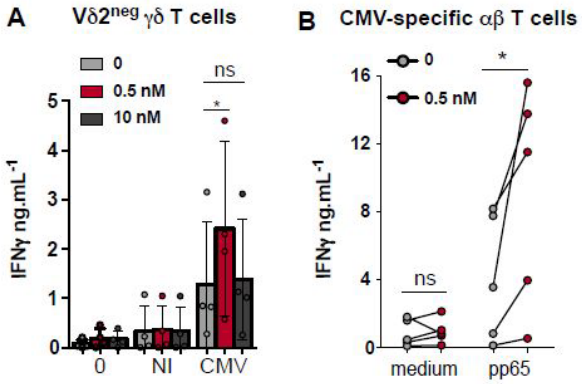
Figure 5 Low dose mTORi improves the functional profile of Vδ2^{neg} γδ T cells and CMV-specific αβ T cells



Vδ2^{neg} γδ T cells (after 21 days) and CMV-specific αβ T cells (after 16 days) were analyzed after *in vitro* culture of PBMC from CMV-seropositive KTR with or without everolimus. Frequencies of PD-1, KLRG1, DNAM-1 and TIM-3 (A) and Tbet, Blimp-1, EOMES and Hobit (B) among Vδ2^{neg} γδ T cells for one representative donor (top) and for 5 donors (bottom). Frequencies of PD-1 and KLRG1 (C) and of EOMES and Hobit (D) among CMV specific αβ T cells for one representative donor (left) and 5 donors (right). Each symbol represents an individual donor. ns, not significant, 0.05>p>0.01*; **0.01>p>0.001; as determined by Wilcoxon test.

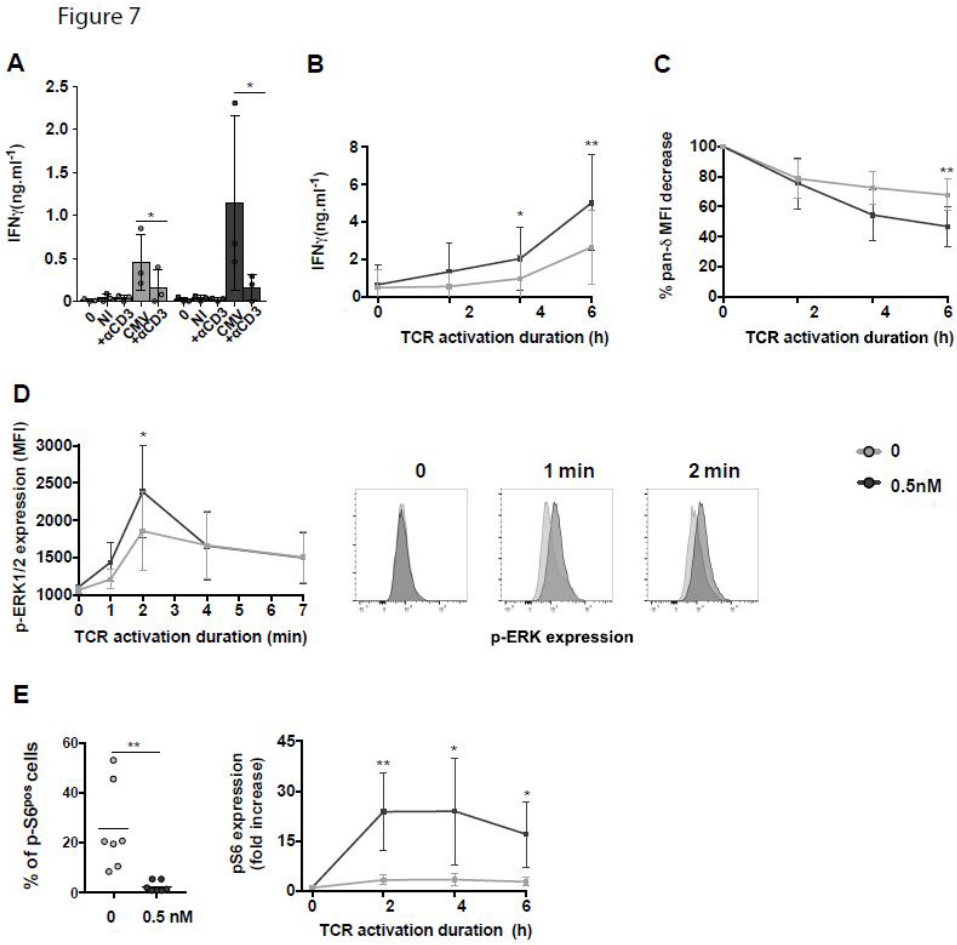
Figure 6 Low dose mTORi improves Vδ2^{neg} γδ T cells and CMV-specific αβ T cells response to CMV

Figure 6



A. Vδ2^{neg} γδ T cells were purified and cultured in medium alone or with non-infected (NI) or CMV-infected fibroblasts during 24 hours and ELISA IFNγ was performed (n= 4 donors).
B. PBMC from CMV seropositive donors were cultured during 7 days then maintained in medium alone or stimulated with 0.6 nmol/l of pp65 peptivator during 24 hours, and ELISA IFNγ was performed (n=5). Each symbol represents an individual donor and in Figure A, large horizontal lines indicate the mean and small horizontal lines indicate the standard deviation; ns, not significant, 0.05>p>0.01*; **0.01>p>0.001; as determined by Wilcoxon test.

Figure 7 Effect of mTORi on TCR engagement and signaling of Vδ2^{neg} γδ T cells

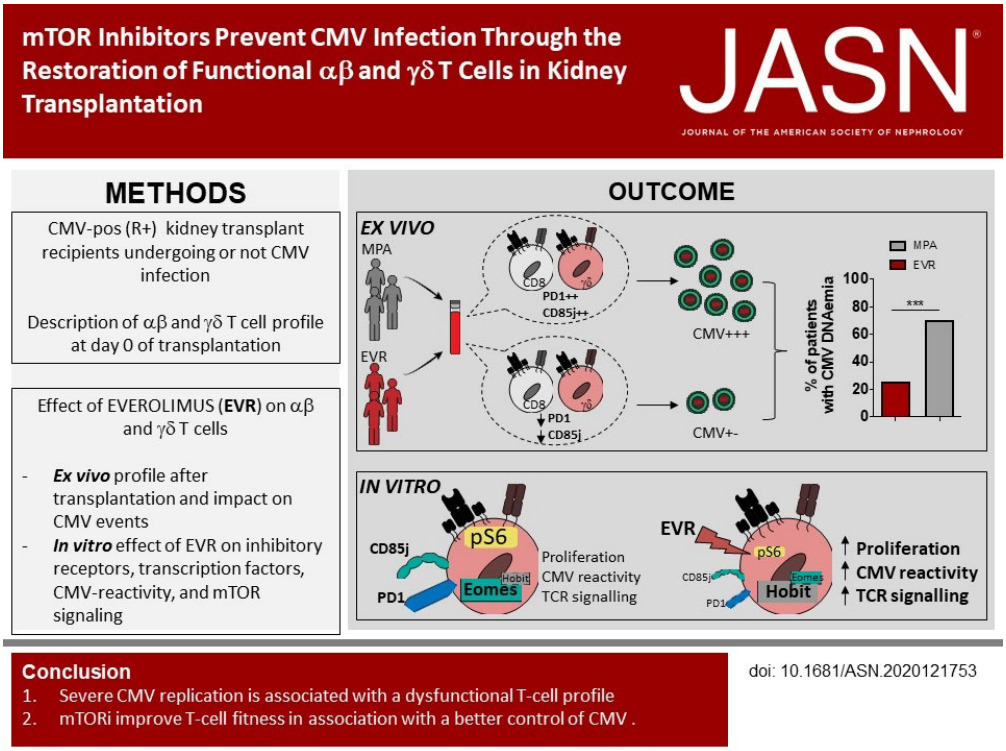


A. Vδ2^{neg} γδ T cells were purified and cultured in medium alone, with non-infected (NI) or CMV-infected fibroblasts with or without a blocking anti-CD3 mAb (10µg/ml) during 24 hours and ELISA IFNγ) was performed (n=4 donors).

B. C. Vδ2^{neg} γδ T cells among total PBMC were specifically stimulated via their TCR, by an anti-Vδ1 mAb (10 µg/ml), for 2, 4 and 6 hours, then ELISA IFNγ was performed (B), and cells were stained for gamma-delta TCR downregulation analysis by flow cytometry (C) (n=4 donors).

D. Vδ2^{neg} γδ T cells were purified and stimulated with an anti-CD3 antibody (UCHT1, 10µg/ml) for 0, 1, 2, 4 and 7 min. Erk 1/2 phosphorylation was measured by flow cytometry (one representative donor, right; in 4 donors (left).

E. Vδ2^{neg} γδ T cells among total PBMC were specifically stimulated via their TCR, by an anti-Vδ1 mAb (10µg/ml) for 2, 4 and 6 hours and S6 phosphorylation was measured by flow cytometry. Basal levels before stimulation are represented for 7 donors (left) and activation kinetics normalized on basal level for each condition (0 and 0.5 nM everolimus) (right) for 4 donors are represented. Each symbol represents an individual donor, large horizontal lines indicate the mean and small horizontal lines indicate the standard deviation in Figure A and E (left). Each symbol represents the median of the results for 4 donors and small horizontal lines represent the ranges in Figure B, C, D and E (right). ns, not significant, 0.05>p>0.01*; **0.01>p>0.001; as determined by Wilcoxon test.



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Supplementary materials

Immunosuppressive regimen of patients

For all patients, induction therapy was anti-IL2RA (20mg on day 0 and day 4) (SIMULECT®, Novartis Pharma SAS) and Methylprednisolone: 500 mg at day 0, 120 mg at day 1, then prednisone or equivalent, 20 mg/day from day 3. Maintenance immunosuppressive regimen was either based on everolimus 0.75mg bid, targeted to 3-8 ng/ml and Cyclosporin A (CsA) with target ranges 100-200 ng/ml from day 3 to month 2, 75-150 ng/mL from month 2 to month 4 and 25-50 ng/mL from months 6 to 12, either Csa with target ranges of 150–220 ng/mL from day 3 to month 2, 100–150 ng/mL from month 2 to month 12 and mycophenolic acid 1080 mg bid for one month, then 720 mg bid. Ciclosporin and everolimus whole blood concentrations were performed at day 7, day 14 and month 6 post transplantation.

Supplemental material Table of Contents

Figure S7 mTORi improvement of the T cell dysfunctional profile is maintained when combined with ciclosporin

Figure S8 Blocking anti-CD3 mAb had no effect on $V\delta2^{neg}$ γδ T cell viability.

Figure S9 mTORi does not affect SLP-76 and MAP kinase 38 signaling after TCR stimulation of $V\delta2^{neg}$ γδ T cells

Figure S10 No effect of mTORi on Akt phosphorylation after TCR stimulation of $V\delta2^{neg}$ γδ T cells

Supplemental table 1. References of flow cytometry material

	MANUFACTURER	CLONE	N°CATALOG	LAST BATCH	DILUTION
PER-CP CD3	BD biosciences	SP34-2	9091596	552851	0.4
FITC CD16	BD biosciences	3g8	8255938	560996	
BV421 CD154	BD biosciences	TRAP-1	9171764	566268	0.1
fixable viability stain 575V	BD biosciences		7020921	565694	0.0002
BV510 CD8	BD biosciences	SK-1	8003887	561617	0.1
BV786 CD27	BD biosciences	L128	8236716	563328	0.02
PE pan-δ	Miltenyi	REA 591	5190321373	130-113-512	0.02
PC-7 Vδ2	Miltenyi	REA 711	5190131222	130-111-012	0.1
BV650 CD161	BD biosciences	DX12	7235832	563864	0.1
BV786 CD16	BD biosciences	3G8	7139586	563690	0.1
FITC CD85J	BD biosciences	GHI/75	6214793	555942	0.4
PE VIO-615 KLRG1	Miltenyi	REA 261	5171115662	130-108-395	0.06
BV650 PD1	BD biosciences	EH12	7258845	564104	0.1
BV711 TIM3	BD biosciences	7D3	8310921	565566	0.1
BV786 DNAM	BD biosciences	DX11	8318701	742497	0.04
FITC Granulysin	BD biosciences	RB1	7089964	558254	0.04
APC pan-δ	Miltenyi	REA591	5190314461	130-113-508	0.06
PE-CF594 CD8	BD biosciences	RPA-T8	7150677	562282	0.04
V450 CD3	BD biosciences	UCHT1	8164556	560365	0.06
BV786 Interferon-γ	BD biosciences	4S-B3	7187947	563731	0.04
PE Granzym B	Molecular probes	GB12	1735130	MHB04	0.04
APC-H7 CD45RA	BD biosciences	HI 100	7226562	560674	0,1
APC Perforin	BD biosciences	dG9	7124573	563576	0,1
BV421 Granzym	BD biosciences	GB11		563389	0,1
APC CY7 CD16	BD biosciences	3G8	7075615	560195	0,1
FITC CD16	BD biosciences	3g8	8255938	560996	0.1
PC7 P-S6	Cell signalling	D57.2.2E	2	#34411	0,005
Alexa fluor 647 P-38 MAPK	Cell signalling	3D7	7	#14594	0,02
PE PAKT T308	BD biosciences	J1-223.371	9192801	558275	0,1
FITC PERK 1/2	BD biosciences	20A	720836	612592	0,1
FITC PAKT S473	BD biosciences	M89-61	9282654	560404	0.1
PE AKT	BD biosciences	55/PKBa/Akt	8310873	560049	0,1
PE S6	Cell signalling	54D2	1	55594S	0,005
Alexa fluor 647 HOBIT (ZNF683)	BD biosciences	sanquin-hobit/1	8201604	566250	0.06
PC5.5 Tbet	ebioscience	P3.6.2.8.1	4289808	45-4714-80	0.06
PC7 EOMES	ebioscience	WD1928	192396	25-4877-42	0.06
PE BLIMP1	BD biosciences	6D3	7290934	564702	0.02
Foxp3 transcription factor staining buffer	fisher scientific		2075534	115-000-597	
BV510 CD3	BD biosciences	UCHT1	8297756	563109	0.06
Pacific blue TCR Vδ2	Beckman coulter	IMMU389	5	B49310	0.04
FITC TCR Vδ2	Beckman coulter	IMMU389	2000040	I1464	0.04

FITC KLRG1	ebioscience	13F13F2	4345831	53948842	0.4
PE CD69	Beckman coulter	TP1.55.3	41	IM19930	0.1
Alexa fluor 700 CD69	BD biosciences	FN50	7258876	560739	0.1
PE-CY5.5 Panδ	Beckman coulter	IMMU510	30	A99021	01:50
live viability e fluor 780	ebioscience		4302692	65-0865-13	0.0002
BD cytofix/cytoperm^{tm}	BD biosciences			555028	
Protein transport inhibitor	BD biosciences		9011506	554724	
Phospho buffer perm III	BD biosciences		5260671	558050	
Lyse/fix buffer 5X	BD biosciences		7180900	558049	
Purified anti-CD3	Beckman coulter	UCHT1	200036	IM1304	
Purified anti-Vδ1	produced in the lab				

Supplemental Figures

Figure S1. Flow chart of the ancillary study

Samples came from patients initially includes in a French multicenter study (n=186) from which 83 were included at Bordeaux University Hospital. Among those 83 patients, 77 could be followed for more than 1 month, 44 were treated with mycophenolic acid (MPA-treated patients) and 33 were treated with everolimus (EVR-treated patients). Among EVR-treated patients, 6 have been switched to MPA and were excluded of analyses. Consequently, the analyses of the study included 44 MPA-treated patients and 27 EVR-treated patients.

Figure S2 Flow chart of MPA-treated patients of the ancillary study

Among the 44 MPA-treated patients of the ancillary study, 33 had available frozen PBMC at day 0 of transplantation. 21 were used to constitute the first set of patients with total CD8 and γδ T cell phenotype, and 12 were used for the second set of patients with CD8, CMV-specific CD8 and γδ T cell phenotype.

Figure S3 $V\delta2^{neg}$ γδ T cells and CD8+ αβ T cells phenotype at baseline in CMV seropositive patients

A. $V\delta2^{neg}$ γδ T cell and CD8+ αβ T cell expression of CD27 and CD45RA analyzed by flow cytometry separating patients with no CMV DNAemia or CMV DNAemia requiring no treatment (well-controlled CMV, n=12) versus patients requiring CMV antiviral treatment (severe CMV, n=9).

B. $V\delta2^{neg}$ γδ T cells andCD8+ T cells phenotypes for inhibitory receptors and KLRG1 expression were validated in an internal cohort of patients with well-controlled CMV (n=5) versus patients with severe CMV (n=7).

Each symbol represents an individual donor; large horizontal lines indicate the mean and small horizontal lines indicate the standard deviation, 0.05>p>0.01*; **0.01>p>0.001; ***p<0.001; as determined by the Mann-Withney U test.

Figure S4 Phenotype of Vδ2^{neg} γδ T cells and CMV-specific αβ T cells during in vitro culture

A. One representative phenotype at day 0 of CMV positive patient's PBMC used for *in vitro* IL2-IL15 culture of Vδ2^{neg} γδ T cells, analyzed by flow cytometry.

B. One representative proliferation assay of TEMRA Vδ2^{neg} γδ T cells analyzed by CFSE staining of CD45+ CD27- cells by flow cytometry.

C. One representative phenotype at day 7, 14 and 21 of CMV positive patient's PBMC during *in vitro* culture of Vδ2^{neg} γδ T cells analyzed by flow cytometry.

D. Evolution of Vδ2^{neg} γδ T cell number during 21 days of PBMC cultures with IL2 (n=4) and IL2-IL15 (n=4) ,obtained by Neubauer counting associated with analysis of Vδ2^{neg} γδ T cells percentage by flow cytometry. Results are the mean ± SD of the cell number fold increase from day 0, of PBMC cultures from those 4 different donors.

E. One representative phenotype at day 0 of CMV positive patient's PBMC used for *in vitro* culture of CMV-specific αβ T cells analyzed by flow cytometry.

Figure S5 Low dose of mTORi decrease the number of Vδ2^{neg} γδ T cells and CMV-specific αβ T cell expressing CD85j.

Expression of CD85j was analyzed in Vδ2^{neg} γδ T cells and CMV specific αβ T cells by flow cytometry after culturing PBMC of CMV seropositive donors (one representative, Figure A; cumulative results for 5 donors, Figure B) *in vitro* with or without a low dose (0.5nM) of everolimus during 21 days for the culture of Vδ2^{neg} γδ T cells and during 16 days for the culture

of CMV specific αβ T cells (stimulated overnight by pp65 peptides and gated on non-γδ T cells positive for CD69 and IFNγ). Each symbol represents an individual donor; 0.05>p>0.01*; **0.01>p>0.001; as determined by Wilcoxon test.

Figure S6 mTORi does not affect CMV-specific αβ T cell frequencies during in vitro culture

One representative phenotype of CMV-specific αβ T cells after 7 days of PBMC culture with IL2 with or without 0.5nM everolimus. After overnight stimulation with CMV peptides, CMV-specific αβ T cells were gated by flow cytometry through their expression of CD69 and IFNg among non γδ T cells (left). Cumulative results for 5 donors(right).

Figure S7 mTORi improvement of the T cell dysfunctional profile is maintained when combined with ciclosporin

(A) $V\delta2^{neg}$ γδ T cells (after 7, 14 and 21 days for expansion and 21 days for PD1 and CD85j expression) and (B) CMV-specific αβ T cells (after 16 days) with 0/25/75/200mg/mL^{-1} with either medium, 0.5nM everolimus or mycophenolate mofetil (MMF) were analyzed after *in vitro* culture of PBMC from CMV-seropositive KTR. Expansion are expressed as fold increase from day 0 of culture. PD-1 and CD85j are expressed as proportion among total $V\delta2^{neg}$ γδ T cells or CMV-specific αβ T cells.

Each symbol represents an individual donor. ns, not significant, 0.05>p>0.01*; **0.01>p>0.001; as determined by Wilcoxon test.

Figure S8 Blocking anti-CD3 mAb had no effect on $V\delta2^{neg}$ γδ T cell viability.

Vδ2^{neg} γδ T cells were negatively sorted after 21 days of culture with 0 or 0.5 nM everolimus and were cultured in the same medium alone (either with 0 or 0.5 nM everolimus), with non-infected (NI), or with CMV-infected (CMV) fibroblasts with or without blocking anti-CD3 mAb (10µg/ml) during 24 hours and cells were stained with 1µM DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) to assess their viability by flow cytometry.

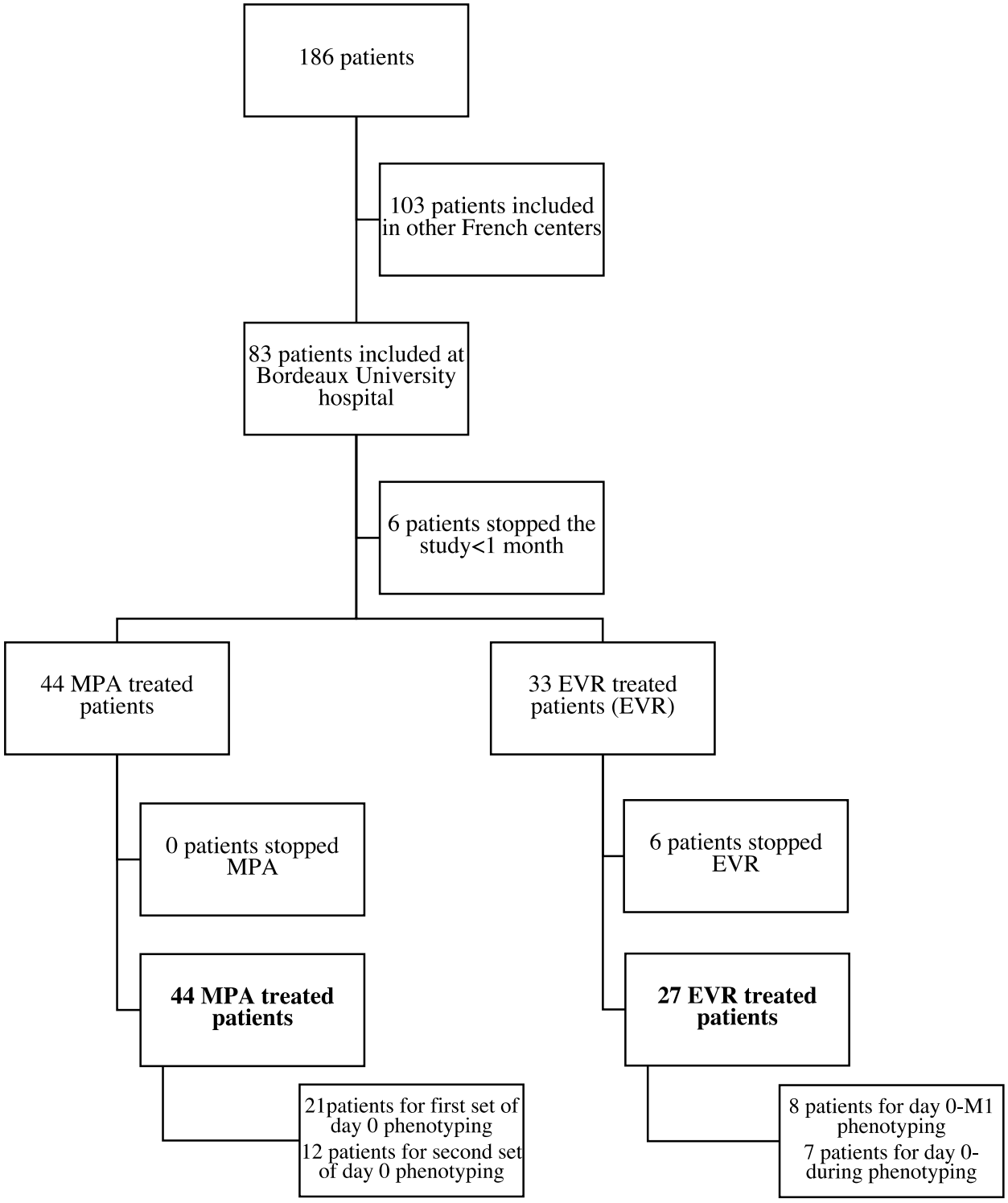
Figure S9 mTORi does not affect SLP-76 and MAP kinase 38 signaling after TCR stimulation of Vδ2^{neg} γδ T cells

Vδ2^{neg} γδ T cells were negatively sorted after 21 days of culture with 0 or 0.5 nM of everolimus , and stimulated with an agonist anti-CD3 mAb (UCHT1, 10µg/ml) for the indicated durations. SLP-76 phosphorylation (A) and MAP kinase 38 phosphorylation (B) were measured by flow cytometry in 4 donors (right, mean ± ranges) and in a representative donor (left).

Figure S10 No effect of mTORi on Akt phosphorylation after TCR stimulation of Vδ2^{neg} γδ T cells

After 21 days of culture with 0 or 0.5 nM of everolimus, PBMC were stimulated using an agonist anti-Vδ1 mAb (10µg/ml) for 30 minutes, 2 and 4 hours and Akt phosphorylation was measured and analyzed by flow cytometry (T308 phosphorylation site, left; S473 phosphorylation site, right) for 4 donors (mean ± ranges).

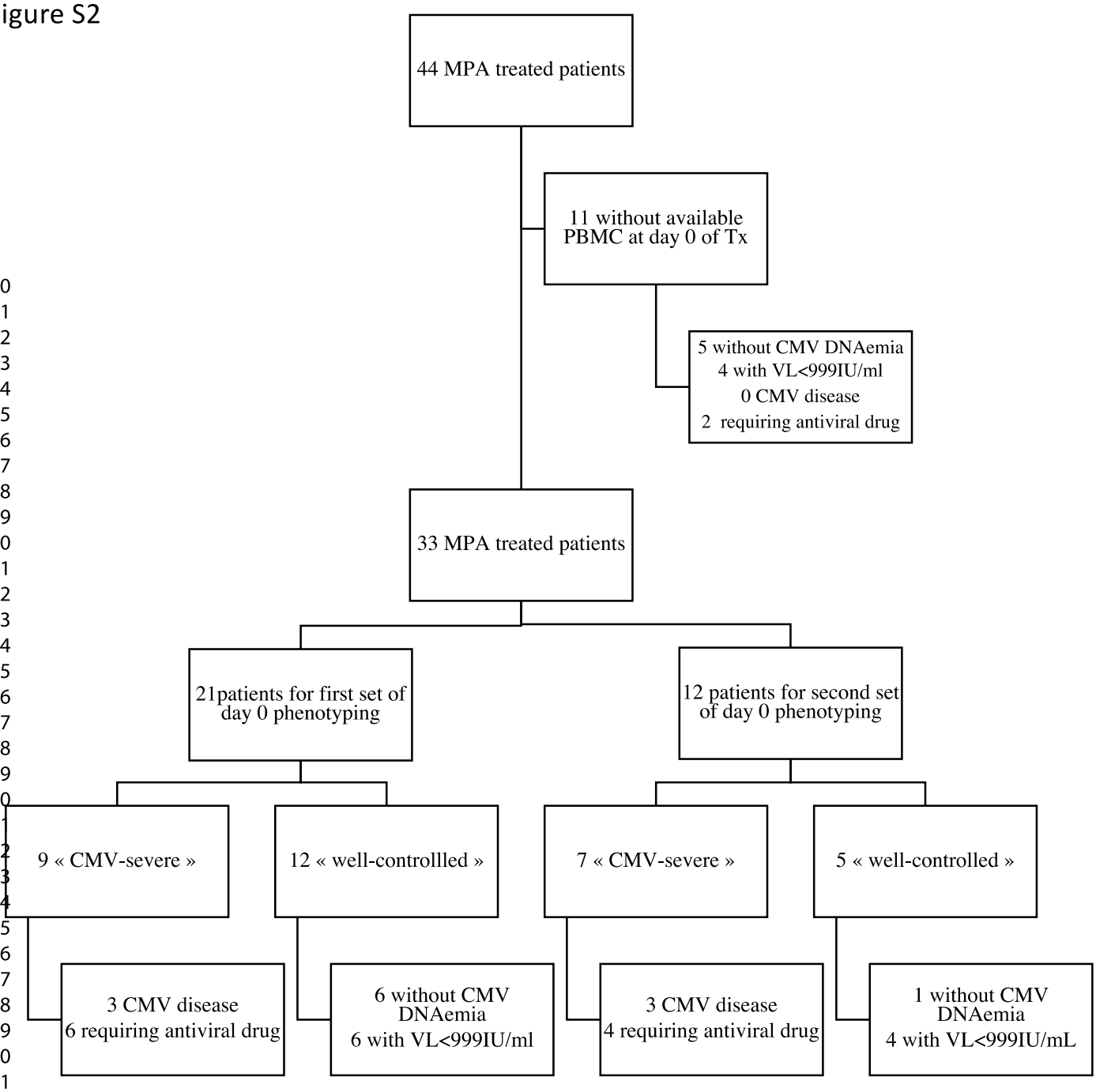
Figure S1



Supplemental Figure 1. Flow chart of the ancillary study

Samples came from patients initially includes in a French multicenter study (n=186) from which 83 were included at Bordeaux University Hospital. Among those 83 patients, 77 could be followed for more than 1 month, 44 were treated with mycophenolic acid (MPA-treated patients) and 33 were treated with everolimus (EVR-treated patients). Among EVR-treated patients, 6 have been switched to MPA and were excluded of analyses. Consequently, the analyses of the study included 44 MPA-treated patients and 27 EVR-treated patients.

Figure S2



Supplemental Figure 2. Flow chart of MPA-treated patients of the ancillary study

Among the 44 MPA-treated patients of the ancillary study, 33 had available frozen PBMC at day 0 of transplantation. 21 were used to constitute the first set of patients with total CD8 and γδ T cell phenotype, and 12 were used for the second set of patients with CD8, CMV-specific CD8 and γδ T cell phenotype. VL:viral load

Figure S3

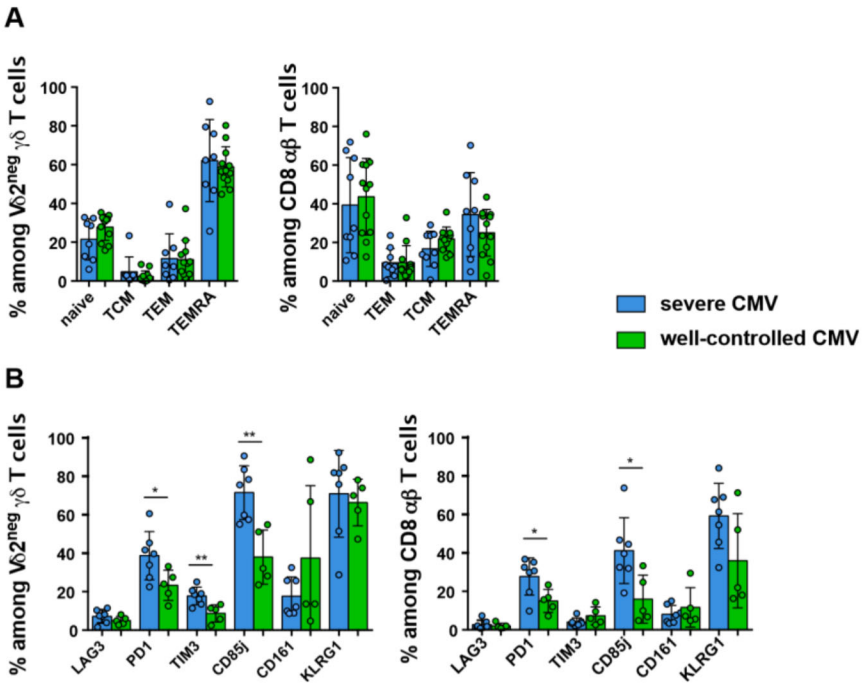


Figure S3: Vδ2^{neg} γδ T cells and CD8+ αβ T cells phenotype at baseline in CMV seropositive patients

A. Vδ2^{neg} γδ T cell and CD8+ αβ T cell expression of CD27 and CD45RA analyzed by flow cytometry separating patients with no CMV DNAemia or CMV DNAemia requiring no treatment (well-controlled CMV, n=12) versus patients requiring CMV antiviral treatment (severe CMV, n=9).

B. Vδ2^{neg} γδ T cells and CD8+ T cells phenotypes for inhibitory receptors and KLRG1 expression were validated in an internal cohort of patients with well-controlled CMV (n=5) versus patients with severe CMV (n=7).

Each symbol represents an individual donor; large horizontal lines indicate the mean and small horizontal lines indicate the standard deviation, 0.05>p>0.01*; **0.01>p>0.001; ***p<0.001; as determined by the Mann-Withney U test.

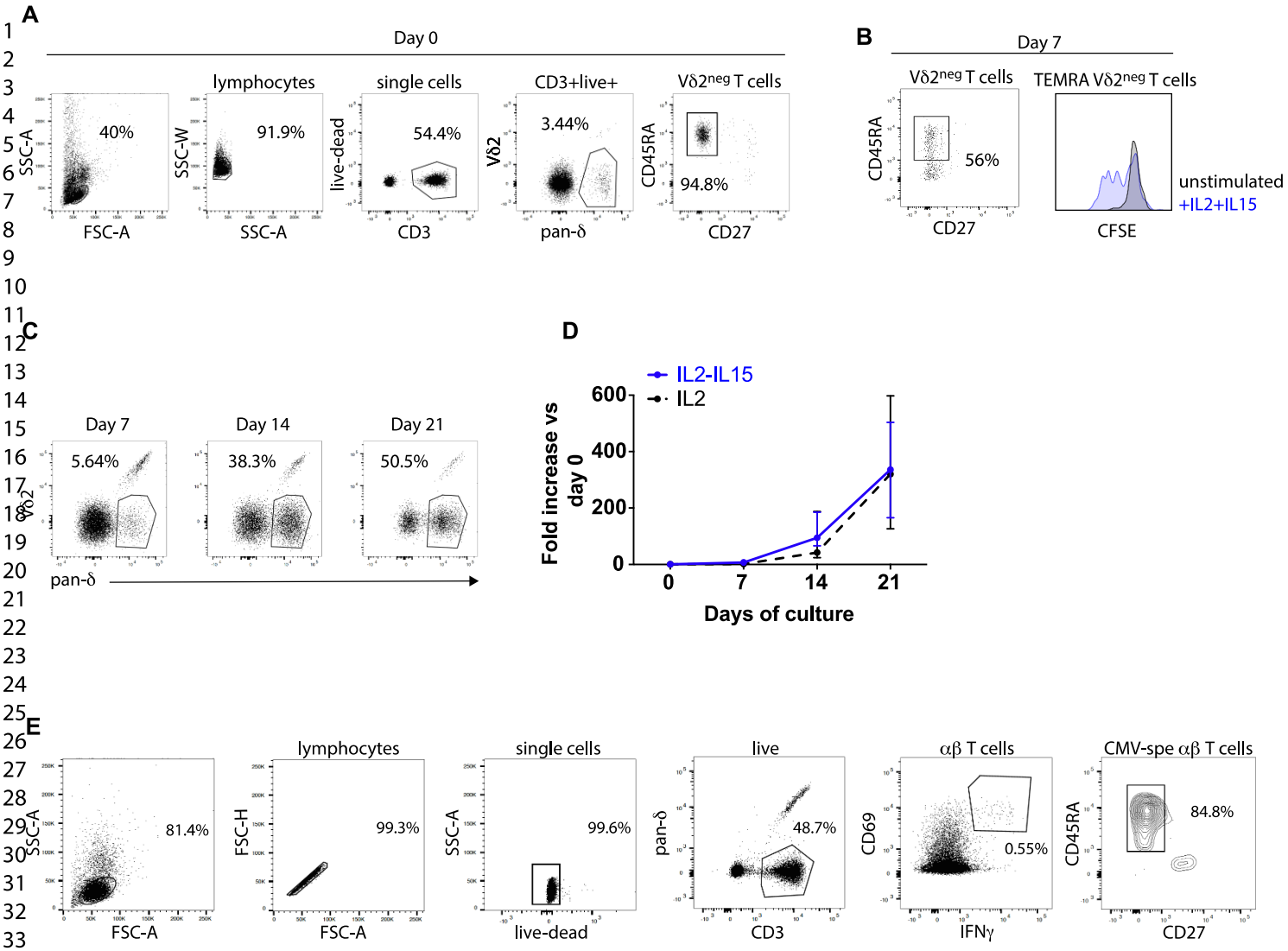
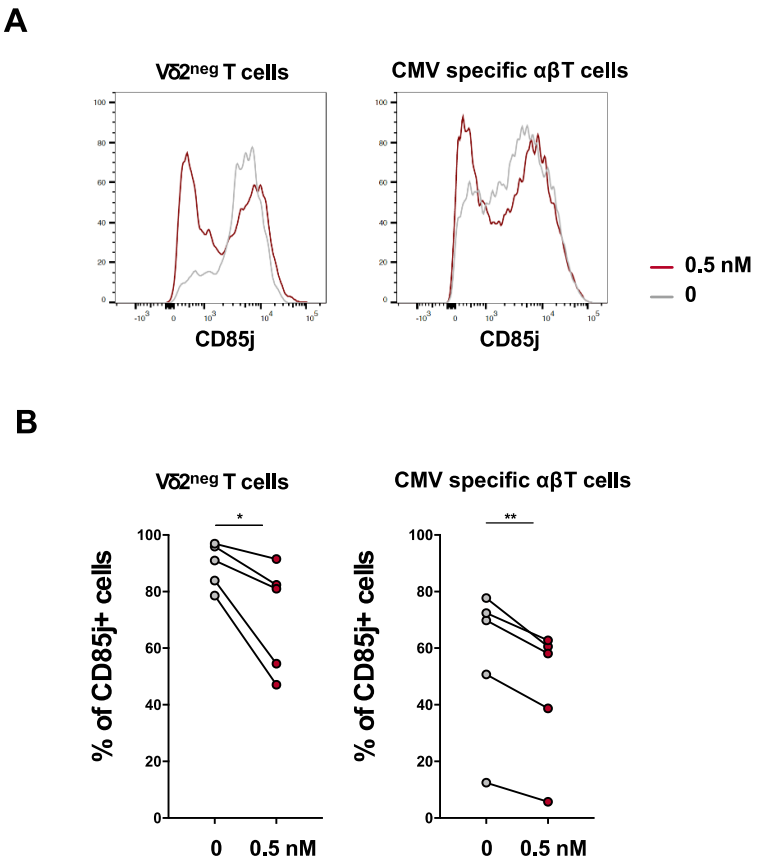


Figure S4: Phenotype of Vδ2^{neg} γδ T cells and CMV-specific αβ T cells during in vitro culture

A. One representative phenotype at day 0 of CMV positive patient's PBMC used for *in vitro* IL2-IL15 culture of Vδ2^{neg} γδ T cells, analyzed by flow cytometry.B. One representative proliferation assay of TEMRA Vδ2^{neg} γδ T cells analyzed by CFSE staining of CD45+ CD27- cells by flow cytometry. C. One representative phenotype at day 7, 14 and 21 of CMV positive patient's PBMC during *in vitro* culture of Vδ2^{neg} γδ T cells analyzed by flow cytometry. D. Evolution of Vδ2^{neg} γδ T cell number during 21 days of PBMC cultures with IL2 (n=4) and IL2-IL15 (n=4) ,obtained by Neubauer counting associated with analysis of Vδ2^{neg} γδ T cells percentage by flow cytometry. Results are the mean ± SD of the cell number fold increase from day 0, of PBMC cultures from those 4 different donors. E. One representative phenotype at day 0 of CMV positive patient's PBMC used for *in vitro* culture of CMV-specific aβ T cells analyzed by flow cytometry.

Figure S5



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Expression of CD85j was analyzed in Vδ2^{neg} γδ T cells and CMV specific αβ T cells by flow cytometry after culturing PBMC of CMV seropositive donors (one representative, Figure A; cumulative results for 5 donors, Figure B) *in vitro* with or without a low dose (0.5nM) of everolimus during 21 days for the culture of Vδ2^{neg} γδ T cells and during 16 days for the culture of CMV specific αβ T cells (stimulated overnight by pp65 peptides and gated on non-gδ T cells positive for CD69 and IFNγ). Each symbol represents an individual donor; 0.05>p>0.01*; **0.01>p>0.001; as determined by Wilcoxon test.

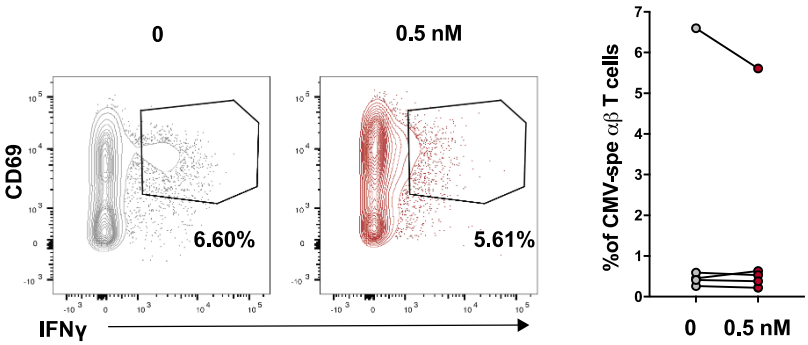

Figure S6: mTORi does not affec CMV-specific αβ T cell frequencies during in vitro culture

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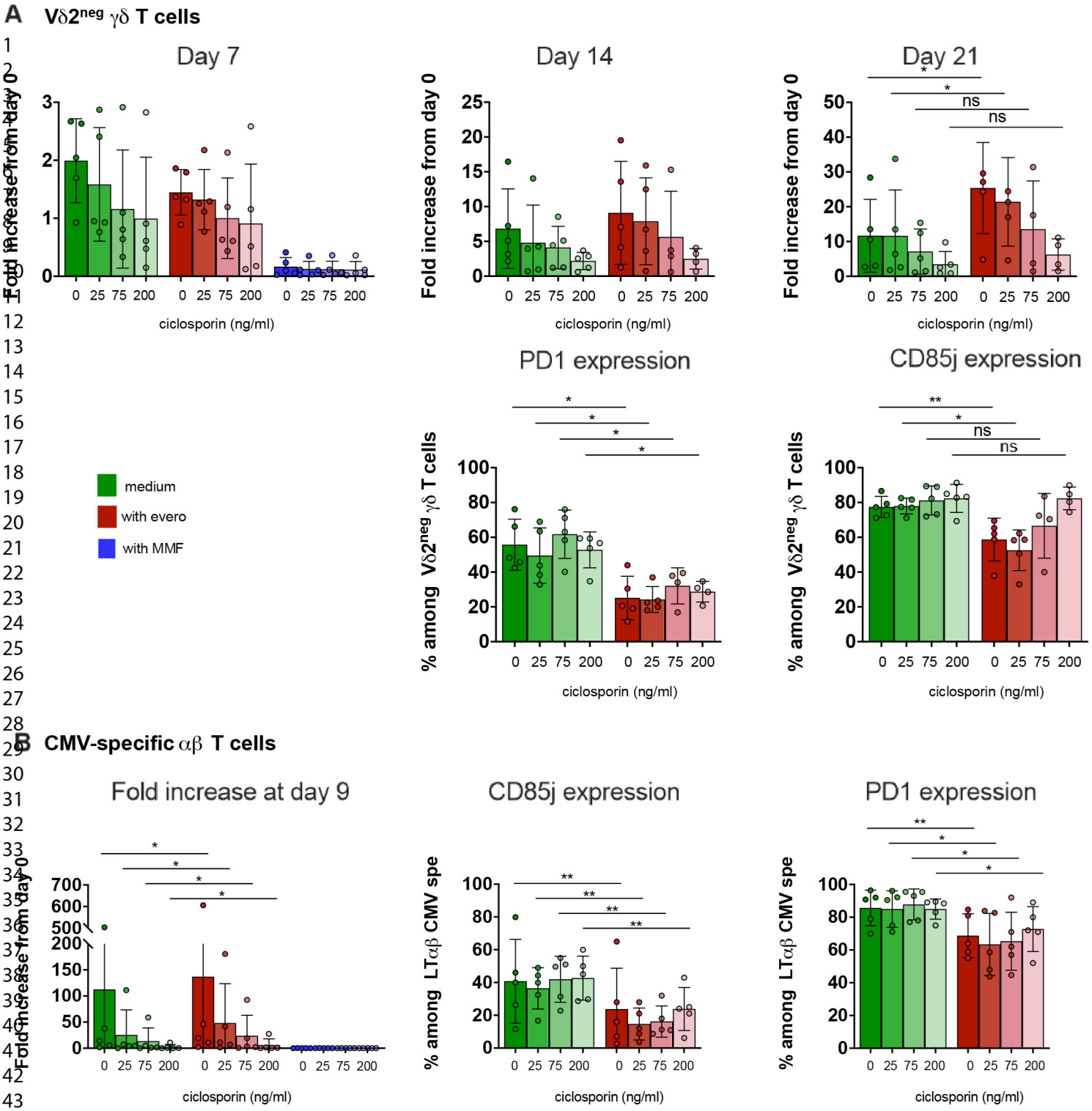


Figure S7: mTORi improvement of the T cell dysfunctional profile is maintained when combined with ciclosporin

(A) Vδ2^{neg} γδ T cells (after 7, 14 and 21 days for expansion and 21 days for PD1 and CD85j expression) and (B) CMV-specific αβ T cells (after 16 days) with 0/25/75/200mg/mL^{-1} with either medium, 0.5nM everolimus or mycophenolate mofetil (MMF, 1000ng.mL^{-1}) were analyzed after *in vitro* culture of PBMC from CMV-seropositive kidney transplant recipients. Expansion are expressed as fold increase from day 0 of culture. PD-1 and CD85j are expressed as proportion among total Vδ2^{neg} gδ T cells or CMV-specific αβ T cells. Each symbol represents an individual donor, ns: not significant, 0.05>p>0.01*; **0.01>p>0.001; as determined by Wilcoxon test.

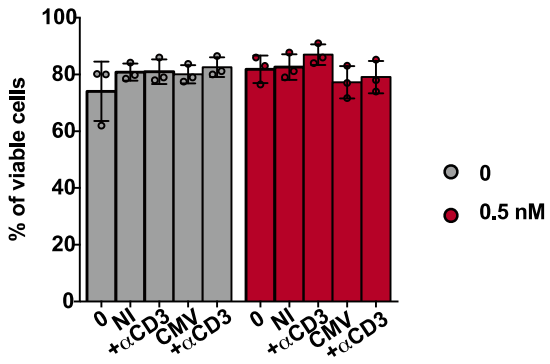


Figure S8: Blocking anti-CD3 mAb had no effect on Vδ2^{neg} γδ T cell viability.

Vδ2^{neg} γδ T cells were negatively sorted after 21 days of culture with 0 or 0.5 nM everolimus and were cultured in the same medium alone (either with 0 or 0.5 nM everolimus), with non-infected (NI), or with CMV-infected (CMV) fibroblasts with or without blocking anti-CD3 mAb (10μg/ml) during 24 hours and cells were stained with 1µM DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) to assess their viability by flow cytometry.

Supplemental figure S9

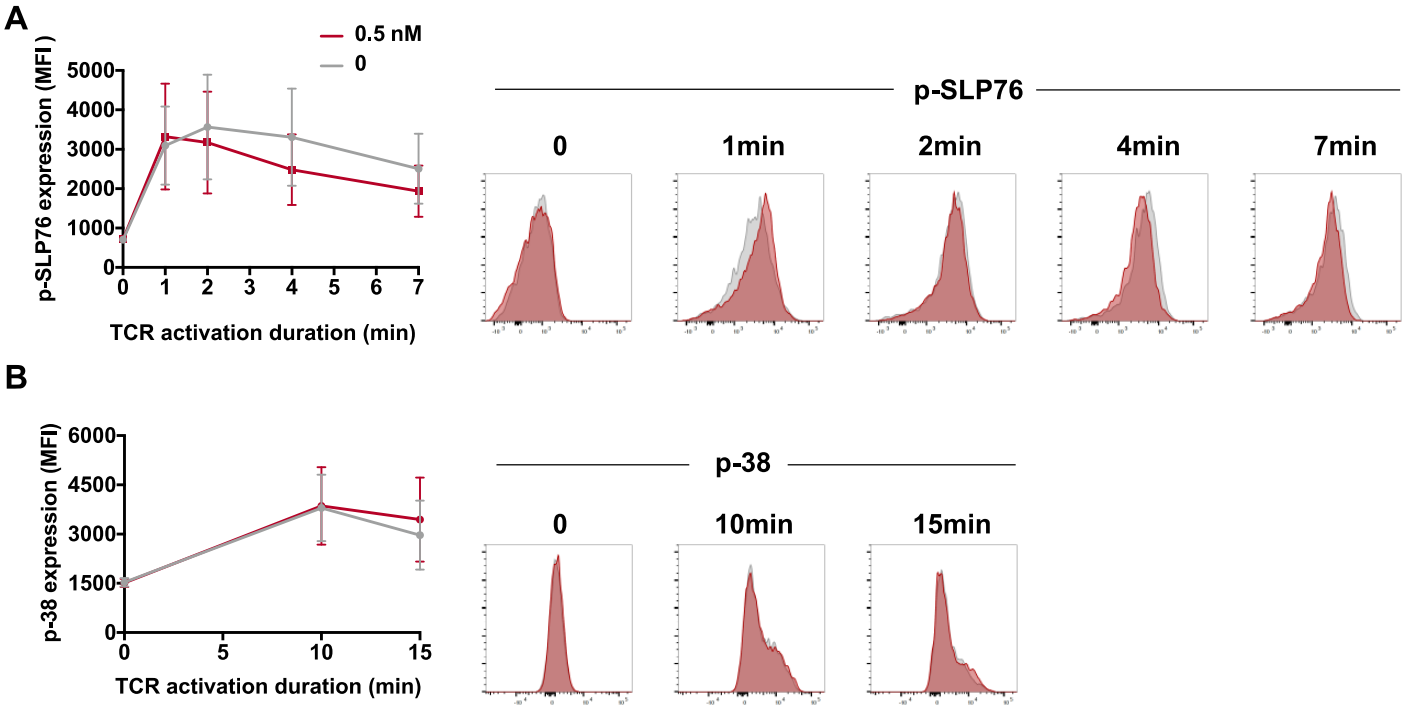
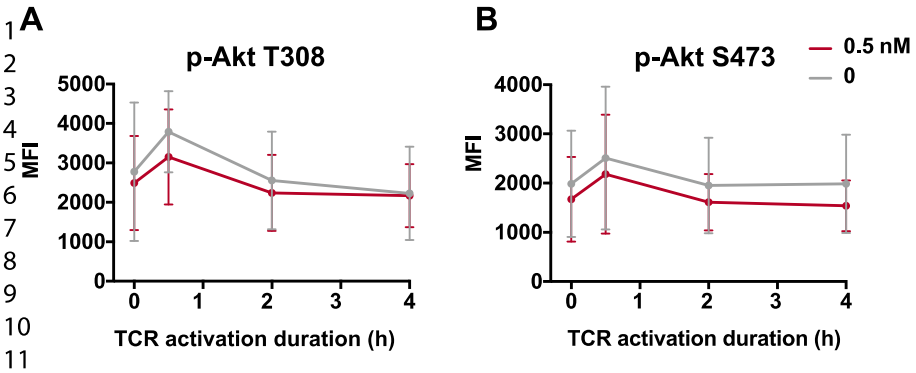


Figure S9: mTORi does not affect SLP-76 and MAP kinase 38 signaling after TCR stimulation of Vδ2^{neg} γδ T cells

Vδ2^{neg} γδ T cells were negatively sorted after 21 days of culture with 0 or 0.5 nM of everolimus , and stimulated with an agonist anti-CD3 mAb (UCHT1, 10µg/ml) for the indicated durations. SLP-76 phosphorylation (A) and MAP kinase 38 phosphorylation (B) were measured by flow cytometry in 4 donors (right, mean ± ranges) and in a representative donor (left).



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