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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 $\text{CD}4^+\text{IFN}\gamma^+$ and $\text{CD}4^+\text{IFN}\gamma^-$ 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+ $\text{CD}4^+\text{IFN}\gamma^+$ and $\text{CD}4^+\text{IFN}\gamma^-$ 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 $\text{CD}4^+\text{IFN}\gamma^-$ T cells, and IFN γ -producing and cytotoxic $\text{CD}4^+\text{IFN}\gamma^+$ T cells. *In vitro*, mTORi increased proliferation, viability, and CMV-induced IFN γ 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 $\text{CD}4^+\text{IFN}\gamma^-$ T cells the mTORi effect was related to increased TCR signaling.

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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 $\alpha\beta$ and $\gamma\delta$ 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 $\alpha\beta$ 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 $\alpha\beta$ and $\gamma\delta$ 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+ $\alpha\beta$ and $\gamma\delta$ 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 $\gamma\delta$ T cells, and IFN γ -producing and cytotoxic $\alpha\beta$ T cells. *In vitro*, mTORi increased proliferation, viability, and CMV-induced IFN γ 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 $\gamma\delta$ 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+ $\alpha\beta$ 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 IFN γ 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

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3 been recently associated to a decreased number of CMV events after solid organ transplantation
4 (19-21) but this association remains unexplained. Direct antiviral action of mTORi was reported
5 but has only been observed *in vitro* and not in all type of CMV-infected cells (22). Alternatively,
6 it could be due to an effect on CMV-specific immunity. Whereas mTORi have a strong anti-
7 proliferative effect on naive cells (23), they also increase CD8 memory T-cells maintenance
8 after LCMV infection in mice (24). However, data on mTORi effect on human T-cells remains
9 elusive particularly on highly differentiated CMV-specific T-cells.

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

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15 T-cell response against CMV involves both CD8+ $\alpha\beta$ and $\gamma\delta$ T-cells (26). Only V δ 2^{neg} $\gamma\delta$ T-
16 cells are involved in the control of CMV, as demonstrated in many different studies (for
17 review(27)), and their response is very similar to that of CMV-specific CD8+ $\alpha\beta$ T-cells. They
18 share similar kinetics, acquire the same late effector memory CD45RA+ (TEMRA) phenotype
19 and antiviral functions (cytotoxicity, IFN γ production) (28, 29). However, V δ 2^{neg} $\gamma\delta$ T-cells do
20 not recognize viral peptides presented by HLA molecules, but CMV-induced “stress-self-
21 antigens” at the surface of infected cells. Very few of these antigens have been described (30).

22
23 In the present study, our first aim was to assess if specific attributes of CMV-specific $\alpha\beta$ and
24 $\gamma\delta$ T-cells in kidney recipients before transplantation could be associated with the risk of
25 developing CMV reactivation after transplantation. Then, we investigated *in vitro* and *in vivo*,
26 the effect of mTORi on the function and phenotype of CMV-specific effector $\alpha\beta$ and $\gamma\delta$ T-cells
27 to understand why mTORi are associated with a better control of CMV infection in patients.
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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 negatvation 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 δ 2^{neg} $\gamma\delta$ 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-

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

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23 For $\gamma\delta$ T-cells, PBMC were cultured in complete medium (RPMI medium supplemented with
24 2mM glutamine and 10% human serum) with 1000 IU/ml recombinant human IL-2 (rIL2,
25 n°200-02), (all from Peprotech, France) for 28 days with 0; 0.5 or 10nM of the mTORi
26 everolimus (HY-102018, Medchemexpress EU). In some experiments 10 ng/ml recombinant
27 human IL-15 (rIL15, n°200-15) was added.
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32 For CMV-specific $\alpha\beta$ T-cell cultures, PBMC were cultured in complete medium with 10 IU/ml
33 recombinant human IL-2 for 16 days with 0 or 0.5nM of everolimus. 0.6 nmol/ml of peptivator
34 CMV pp65 (130-093-438, Miltenyi) was added at day 0 and day 9.
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38 Additional experiments were performed as previously described for $\gamma\delta$ T-cells and CMV-
39 specific $\alpha\beta$ T-cell cultures with the addition of 0/25/75/200ng.mL⁻¹ ciclosporin (Novartis
40 Pharma, Basel, Switzerland) with or without either 0.5nM everolimus or 1000ng.mL⁻¹
41 mycophenolate sodium (Cellcept, Roche, batch B8003).
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48 **Proliferation and viability assay for $\gamma\delta$ and CMV-specific $\alpha\beta$ T-cells**

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3 Proliferation within TEMRA cells was first assessed by CFSE assay. PBMC were labelled with
4 5 μM 5(6)-Carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE, n°V12883, eBioscience,
5 France) and cultured with or without 1000 U/ml rIL-2 and 10 ng/ml rIL-15. At day 7 of culture,
6 2×10^5 cells were incubated with monoclonal antibodies anti-pan TCR δ (PE), TCRV $\delta 2$ -PC-7
7 and anti-CD3 (BV510), anti-CD45RA (BV786), CD27 (BV650), washed and processed on the
8 BD LSRFortessa. Proliferation was also assessed by counting on a Neubauer cell counting
9 chamber and phenotyping with monoclonal antibodies anti-CD3 (V450), CD27
10 (APC), CD45RA (FITC), pan δ (PE), V $\delta 2$ (PC7) and processed with a BD Canto II (BD
11 biosciences).

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

20 Absolute numbers are the percentages of CMV-specific $\alpha\beta$ and V $\delta 2^{\text{neg}}$ T-cells among PBMC
21 multiplied by the total PBMC count.

22 Cell viability was measured by incubating 2×10^5 cultured PBMC with the viability marker
23 FVS575.

24 **Expression of co-receptors and transcription factors for $\gamma\delta$ and CMV-specific $\alpha\beta$ T-cells**

25 For V $\delta 2^{\text{neg}}$ $\gamma\delta$ T-cells, 5×10^5 PBMC at day 21 of culture were stained with either the viability
26 marker FVS575, then with antibodies anti-CD3 (BV510), PD-1 (BV650), TIM3 (BV711),
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3 DNAM-1 (BV786), pan δ (PE), V δ 2 (PC7) and KLRG1 (FITC); or the viability marker
4 FVS780, then with antibodies anti-CD3 (BV510), V δ 2 (pacific blue) and either pan δ (APC)
5 with Blimp1 (PE), T-bet (PC5.5), or EOMES (Pe-Cy7) alone or pan δ (PE) with Hobit (alex-
6 fluor 647) (BD bioscience). PBMC were permeabilized with the FOXP3 transcription factor
7 staining buffer (Fisher scientific). For CMV-specific $\alpha\beta$ T-cells, 5×10^5 PBMC cultured as
8 described above in 96-wells plates, were stained with FVS575, then either with antibodies anti-
9 PD-1 (BV650), KLRG1 (FITC), pan- $\alpha\beta$ (APC), CD3 (V450), CD69 (PE) and intracellular
10 staining with anti-IFN γ (BV786) or anti-pan- $\alpha\beta$ (PE), CD3 (V450), CD69 (Alexa Fluor 700)
11 antibodies , then anti-EOMES (PC7), Hobit (alex-fluor 647) and IFN γ (BV786) antibodies
12 (processed on the BD LSRFortessa)
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24 **S6 and Akt phosphorylation.**

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28 2×10^5 cells from the V δ 2^{neg} $\gamma\delta$ cultures at day 21, were washed and incubated in 400 μ L of RPMI
29 (2mM glutamine, 8% FCS alone or with 0.5nM everolimus) and activated with an anti-V δ 1
30 mAb (10 μ g/ml) for the indicated period of time. As previously described for phosphorylated
31 proteins staining (35) , cells were stained in the phosphoflow buffer with monoclonal antibodies
32 anti-CD3 (V450), V δ 2 (FITC) and pan δ (APC) and with either anti-pS6 (PeCy7), S6 (PE), pAkt
33 T308 (PE), pAkt S473 (FITC), Akt (PE) (processed on the Canto II).
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42 **pSLP-76, p-ERK and p38 expression T-cells**

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45 At day 21 of culture with rIL2, V δ 2^{neg} $\gamma\delta$ T-cells were purified after magnetic negative sorting
46 with pan T-cell isolation kit; $\alpha\beta$ -biotin and anti-biotin (Miltenyi Biotec), V δ 2 FITC (Beckman)
47 and anti-FITC (Miltenyi Biotec). Purity was controlled by staining sorted cells with anti-CD3
48 (V450), pan δ (PE), and V δ 2 (PC7) resulting in a 85-96% purity. Cells were activated with
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3 10µg/ml of purified UCHT1 (Beckman). Intracellular staining was performed as previously
4 described (35) (processed on Canto II).
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10 ***Ex vivo* phenotyping of $\gamma\delta$ T cells and CMV-specific CD8⁺ T cells.**

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14 For complete reference of antibodies, see supplemental Methods.
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17 Flow cytometry phenotyping of patients' V δ 2^{neg} $\gamma\delta$ T cells was performed on whole blood as
18 previously described (36) using the following monoclonal antibodies: anti-CD45-APC, anti-
19 pan δ -PE, anti-V δ 2-FITC, anti-CD27 Pe-Cy7 and anti-CD45RA-ECD and with the Lysing
20 Solution IOTest®3 10X Concentrate (Beckman). The samples were processed on a NAVIOS
21 flow cytometer (Beckman coulter).
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26 One million frozen PBMC were used for each multi-color flow cytometry analysis, with the
27 same viability marker FVS575, the same monoclonal antibodies against CD3 (PerCP), V δ 2
28 (PC-7), pan-delta (PE or APC), CD8 (either BV510 or PE-texas-red) and IFN- γ (BV786) and
29 CD69 (AF-700) for the staining of CMV-specific CD8⁺ T cells after overnight pp65
30 stimulation with a protein transport inhibitor. Then, the staining included either CD45RA
31 (FITC), and CD27 (BV786); or CD85j (FITC), CD161 (BV650), CD16 (BV786), and KLRG1
32 (PE-Vio 615); or granulysin (FITC), perforin (APC) and granzyme (BV421); or PD-1 (BV650),
33 TIM-3 (BV711), LAG3 (BV421), and DNAM-1 (BV786). CMV-specific CD8⁺ T cells were
34 gated as double positive cells for IFN γ and CD69 as previously described (34).
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45 PBMC stained for intracellular markers were permeabilized, fixed using
46 Fixation/Permeabilization Solution (BD biosciences), and processed on the **BD LSRFortessa**
47 **cytometer (BD biosciences).**
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50 **IFN γ production in co-cultures with CMV-infected cells**

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3 After 21 days of culture with rIL-2 and rIL-15, V δ 2^{neg} $\gamma\delta$ T-cells were negatively purified as
4 described above. 50.10⁴ cells were incubated *per* 96 wells-plate with RPMI 8% FCS, 2mM
5 glutamine and 50ng/ml rIL18 (MBL international, B003-5, Massachusetts-US) (see (37)) with
6 or without everolimus alone, or with either non-infected or CMV-infected fibroblasts during 24
7 hours at 37°C. For CMV-specific $\alpha\beta$ T-cells, PBMC were cultured as described above but
8 without pp-65 stimulation and after 7 days, cells were incubated alone or with peptivator CMV-
9 pp65 0.6nmol/mL or PMA 25ng/ml/ionomycine 1 μ g/ml for 37°C during 24 hours. An extra
10 well was added to perform an IFN γ and CD69 staining after overnight pp65 stimulation with a
11 protein transport inhibitor.
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14 Then, IFN γ was measured in supernatants using the Human IFN- γ ELISA development kit
15 (Mabtech, n°3420-1H-6).
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19 **Preparation of CMV-infected fibroblasts**

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26 Human foreskin fibroblasts (HFF, kindly provided by Dr H. Rezvani, INSERM, U1035,
27 Bordeaux), grown in Dulbecco's Modified Eagle Medium (DMEM) containing 8% FCS and
28 2mM glutamine, were infected with the TB42/E strain of human CMV at a multiplicity of
29 infection (MOI) of 0.1. After virus adsorption overnight at 37°C, cells were washed and covered
30 with fresh growth medium. Co-cultures were performed when cytopathic effects were \geq 90%, 4
31 days after infection. Non infected (NI) cells grown in parallel were mock-infected using
32 medium alone.
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45 **Preparation of free CMV**

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48 To produce free CMV (TB42/E strain), human foreskin fibroblasts were infected at a MOI of
49 0.1 and incubated at 37°C in culture medium DMEM, 8% bovine serum and glutamine for 10
50 days or until cytopathic effects were \geq 90%. The supernatant was stored at -80°C. The
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3 preparation had a titer of $2,5 \cdot 10^6$ PFU (plaque-forming unit)/ml, the titration was performed as
4 previously described ¹. All virus stocks and cells were tested negative for the presence of
5 *mycoplasma*.
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8 9 10 11 12 **Viability of V δ ^{neg} $\gamma\delta$ T cells during co-culture with and without blocking anti-CD3** 13 **antibody analyzed with DAPI** 14

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17 V δ ^{neg} $\gamma\delta$ T cells after 21 days of culture with 0 and 0.5 nM everolimus were negatively
18 sorted with magnetic beads and were cultured in medium alone (either with 0 or 0.5 nM
19 everolimus), non-infected (NI), CMV-infected fibroblasts with or without blocking anti-CD3
20 mAb (10 μ g/ml) during 24 hours and cells were stained with 1 μ M DAPI (4',6-Diamidino-2-
21 Phenyindole, Dihydrochloride) and after 15 minutes of incubation at room temperature,
22 cells were analyzed by flow cytometry Canto II.
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32 ***Ex vivo* quanti-FERON-CMV.** 33

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35 QuantiFERON-CMV V (n°0350-0201, Qiagen) was performed as previously described (38)
36 from frozen plasma and read with the QUANTA-Lyser® 2 Inova Diagnostics; at day 14 (32
37 mTORi-treated and 43 MPA-treated patients) and month 6 post transplantation (24 mTORi-
38 treated and 39 MPA-treated patients). Results were analyzed with the CMV v3.03 software.
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48 **Statistics** 49

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51 The Mann-Whitney U test, the χ^2 test or the Fisher-test, the unpaired t test were used when
52 appropriate. Alternatively, paired-t test was used for paired data. Paired tests were used when
53 two sets of data from the same patients were compared whereas unpaired tests were used to
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3 compare data from two different group of patients. $p < 0.05$ was considered statistically
4 significant. Analyses were performed with conventional statistical methods using GraphPad
5 Prism. Figures were obtained with FlowJo software (V.10) and GraphPad Prism.
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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 < 999 IU/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=3762 IU/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 < 999 IU/ml). Late-differentiated CD45RA⁺ T effector cells (TEMRA) were highly represented in $\gamma\delta$ 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 CD8 $\alpha\alpha$ and CD16 for $\gamma\delta$ 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, CD8 $\alpha\alpha$ 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-

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3 controlled CMV (**Figure 1C**). We validated and extended these results by analyzing
4 concomitantly $\gamma\delta$ T-cells, total CD8⁺ T-cells (**Supplemental Figure 3B**) and also CMV-
5 specific CD8⁺ T-cells in an internal validation subgroup of 12 additional patients (belonging
6 to the 44 MPA-treated patients of the ancillary study): 7 with “severe CMV infection” of which
7 3 with CMV disease and 4 with CMV DNAemia for which antiviral drug has been introduced
8 by physician (peak viral load: mean=7930 IU/ml, standard deviation=3976 IU/ml); 5 with well-
9 controlled CMV (one patient without CMV DNAemia, 4 with CMV DNAemia spontaneously
10 cleared, peak viral load <999 IU/ml). We confirmed that patients with severe CMV infection
11 also displayed significantly higher percentages of PD-1⁺, and CD85j⁺ cells in CMV-specific
12 CD8⁺ T-cells (**Figure 1D**) and validated those markers in total CD8⁺ and $\gamma\delta$ T-cells
13 (**Supplemental Figure 3B**). At baseline, T-cells from CMV seropositive patients could thus
14 display a pre-existing dysfunctional profile, characterized by expression of inhibitory receptors,
15 correlating to severe CMV infection after transplantation. Finally PD1 and CD85j appeared to
16 be the more convincing markers differently expressed by both $\gamma\delta$ T cells and CMV-specific
17 CD8⁺ T cells. From a clinical perspective, only using two markers would be easier to
18 implement as a routine assay than a multicolor staining.
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35 **The proportion of functional T-cells is enhanced by mTORi treatment and correlates to a** 36 **subsequent lower incidence of CMV infection**

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38 To address the issue of mTORi effect on dysfunctional T-cells, 44 KTR treated with MPA were
39 compared to 27 KTR treated with mTORi (everolimus). As shown in **Table 1**, no major
40 difference was observed regarding age, sex, CMV serostatus of donor, rank of transplantation,
41 living donor, expanded criteria donor and acute rejection. We confirmed that mTORi treatment
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3 protects from CMV-infection since 26 % (7/27) of mTORi-treated patients displayed CMV
4 infection contrasting with 70 % (32/44) in MPA-treated patients ($p<0.001$) (**Figure 2A**).

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6 Between day 7 and day 14, total and TEMRA $\gamma\delta$ T-cell percentages increased significantly in
7 mTORi-treated patients whereas did not change in MPA-treated patients (prior to any CMV
8 replication) (**Figure 2B and 2C**). For CMV-specific $\alpha\beta$ T-cell immunity assessed by
9 QuantiFERON-CMV (**Figure 2D**), a significant difference between mTORi- and MPA-treated
10 patients already exists at day 7 but CMV-quantiferon significantly increased between day 7
11 and day 14 in mTORi- but did not in MPA-treated patients.
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18 Then, we compared the effect of MPA (n=7 patients) and mTORi (n=8 patients) on the
19 evolution of T cell phenotype before any CMV replication". mTORi treatment was associated
20 with a decreased percentage of PD-1+ and CD85j+ T-cells (in $\gamma\delta$ T-cells, CD85j was
21 unchanged) together with an increase of perforin+ T-cells while MPA was associated to
22 increased or stable PD-1+ and CD85j+ cell percentages (**Figure 2E**). Importantly, there was a
23 direct association between the decrease of CD85j- or PD1-expressing T-cells percentage during
24 the first month of transplantation and an absence or a low (<1000 IU/ml) CMV DNAemia
25 (**Figure 2F**).
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32 mTORi treatment increases the percentages of T-cells expressing a functional profile which is
33 associated to a low incidence of CMV infection post-transplantation.
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42 **mTORi treatment is associated with spontaneously cleared CMV replication that** 43 **correlate to an enhanced specific T-cell response** 44

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46 We then focused on the patients who developed post-transplantation CMV infection. The mean
47 of CMV infection occurrence was 51 days (standard deviation 37 days) post transplantation.
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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), $\gamma\delta$ T-cell expansion (**Figure 3C**) and increase of TEMRA proportion (**Figure 3D**) were higher in mTORi- than MPA-treated patients. Moreover, CMV-specific $\alpha\beta$ T-cell response was also improved since IFN γ 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 $\gamma\delta$ 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 $\gamma\delta$ 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.

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3 **Long-term *in vitro* treatment of T-cells with therapeutic doses of mTORi increase their**
4 **proliferation and viability**
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7 Proliferation and viability were assessed *in vitro* on R+ KTR PBMC comprising predominantly
8 TEMRA cells among both $\gamma\delta$ T-cells (more than 95%) and CMV-specific $\alpha\beta$ T-cells (up to
9 85%) (**Supplemental Figure 4**) at day 0.
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12 TEMRA $\gamma\delta$ T-cell can proliferate despite their late-differentiated phenotype, as shown through
13 CFSE experiments at day 7 of culture (**Supplemental Figure 4B**), and through the strong
14 increase of both percentages (**Supplemental Figure 4C**) and numbers (**Supplemental Figure**
15 **4D**).
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18 Then mTORi was added either at dose mimicking anti-proliferative effect (10 nM), or at dose
19 used in immunosuppressive treatment of solid organ transplantation (0.5 nM) for $\gamma\delta$ T-cells,
20 and only at the lowest dose for $\alpha\beta$ T-cells.
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23 As expected and shown in **Figure 4A**, high dose of mTORi inhibited $\gamma\delta$ T-cell proliferation.
24 By contrast, culture performed with low dose mTORi (0.5 nM) resulted in a better proliferation
25 than in the absence of mTORi (**Figure 4A and C**). Yet, the dose of 0.5 nM was as potent as
26 that of 10 nM to inhibit S6 phosphorylation in TEMRA $\gamma\delta$ T-cells while not affecting the
27 expression of total S6 protein level (**Figure 4B**). Interestingly, low and high doses of mTORi
28 were associated with an increased viability of the cells during the late period of the culture (day
29 28) (**Figure 4D**). Like $\gamma\delta$ T-cells, low dose mTORi-treated CMV-specific $\alpha\beta$ T-cells show a
30 better proliferation (**Figure 4E**) and viability (**Figure 4F**). Hence, low dose mTORi improves
31 the proliferation and the viability of TEMRA T-cells consistently with our previous
32 observations in mTORi-treated patients (**Figure 2**).
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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+ $\gamma\delta$ 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 $\gamma\delta$ 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 IFN γ , *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 IFN γ production (40). Low dose mTORi maintained, whereas high dose decreased, Tbet expression (**Figure 5B**). Like $\gamma\delta$ T-cells, CMV-specific $\alpha\beta$ 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. $\gamma\delta$ T-cells treated with low dose mTORi produced higher amount of IFN γ 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 $\alpha\beta$ T-cells, PBMC activated with CMV peptides after 7 days of mTORi culture comprised a similar quantity of CMV-specific $\alpha\beta$ T-cells (IFN γ +CD69+ $\alpha\beta$ T-cells) (**Supplemental Figure 6**) but an increased IFN γ production (**Figure 6B**) compared to PBMC cultured without mTORi.

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3 In summary, a low mTORi dose improves functional fitness of T-cells with the reinforcement
4 of CMV-induced IFN γ production. mTORi drives an increased percentage of “Hobit^{high}/
5 EOMES^{low}/PD-1^{low}” cells, previously associated to an efficient antiviral potential (10, 12).
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14 **mTORi improvement of the T cell dysfunctional profile is maintained when combined**
15 **with Ciclosporin.**
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18 To mimic the patients immunosuppressive regimen, we also performed $\gamma\delta$ and CMV-specific
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22 $\alpha\beta$ T-cells T cells culture combining either 0/25/75/200ng.mL⁻¹ ciclosporin alone or with
23 0.5nM everolimus or with 1000ng.mL⁻¹ mycophenolate sodium. Mycophenolate sodium was
24 actually strongly inhibiting both $\gamma\delta$ and CMV-specific $\alpha\beta$ T cells proliferation. Ciclosporin
25 inhibits in a dose-dependent manner $\gamma\delta$ and CMV-specific $\alpha\beta$ T cells proliferation
26 (**Supplemental Figure 7A**). However, for the same concentration of ciclosporin, everolimus
27 kept a significant effect on increasing proliferation (**Supplemental Figure 7A**) and on
28 decreasing PD1 and CD85j expression (**Supplemental Figure 7B**). Consequently, our *in vitro*
29 observations about everolimus effects were confirmed together with ciclosporin and led us to
30 extrapolate what we observed *ex vivo* in patients.
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44 **Effect of mTORi on TCR engagement and signaling of $\gamma\delta$ T-cells**
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48 Since it has been previously demonstrated that dysfunctional T-cells are hypo-responsive to
49 TCR signaling (41, 42), we took the opportunity that high quantities of $\gamma\delta$ T-cells were
50 obtainable (compared to CMV-specific $\alpha\beta$ T-cells) to investigate their TCR-signaling
51 efficiency with or without mTORi. TCR neutralization significantly inhibited the production of
52 IFN γ by $\gamma\delta$ T-cells against CMV-infected cells (**Figure 7A**), and even more when $\gamma\delta$ T-cells
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3 were pre-incubated with mTORi, without viability issue (**Supplemental Figure 8**). mTORi
4 may amplify the intrinsic ability of $\gamma\delta$ T-cells to respond to the TCR engagement. In agreement
5 with this hypothesis, in mTORi-treated $\gamma\delta$ T-cells, IFN γ production T-cells after anti-TCR V δ 1
6 activation was increased (**Figure 7B**) together with a higher internalization of the TCR(**Figure**
7 **7C**). TCR signaling-induced phosphorylation of the kinase ERK was enhanced in mTORi-
8 treated $\gamma\delta$ T-cells (**Figure 7D**) while no difference was observed for MAPK-38 or of SLP-76
9 suggesting that mTORi was acting downstream of this very proximal event of TCR signaling
10 (**Supplemental Figure 9**).
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12 Moreover, while S6 phosphorylation was decreased at baseline in mTORi-treated $\gamma\delta$ T-cells
13 (**Figure 7E, left**), the induction of S6 phosphorylation upon TCR triggering was much more
14 important in mTORi-treated than in untreated cells, which was not associated to a feedback
15 activation of Akt (**Supplemental figure 10A and 10B**).
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17 Overall, inhibition of the mTOR pathway conditions $\gamma\delta$ T-cells to respond more efficiently to
18 an antigenic challenge, as occurs during CMV infection.
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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 $\alpha\beta$ and $\gamma\delta$ 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 IFN γ 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

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3 immunosuppressive treatment of their renal disease or to previous kidney transplantation. This
4 context could lead to a chronic higher level of viral load favoring the emergence of those
5 dysfunctional CMV-effector T-cells. Interestingly, documenting the presence of these
6 dysfunctional T-cells prior to the introduction of immunosuppressive drug regimen may be used
7 to predict the risk of CMV reactivation after transplantation in CMV seropositive patients to
8 avoid useless treatment. Here, PD-1 and CD85j, expressed on both CMV-specific CD8+ and
9 $\gamma\delta$ T-cells were the most discriminating markers in R+ patients for predicting severe CMV
10 infection. Especially, CD85j is a well-known inhibitory receptor induced on both $\alpha\beta$ (46) and
11 $\gamma\delta$ T-cells (47) during CMV infection, and binding with high affinity the CMV encoded UL18
12 protein (48).
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21 Secondly, we observed that mTORi improved the functional profile (decreased inhibitory
22 receptors, increased functionality) of effector T-cells together with a lower incidence and
23 severity of CMV infection after transplantation. In addition, during the course of CMV
24 infection, decreased proportion of T-cells expressing the inhibitory receptor KLRG1 and an
25 increased proportion of perforin-producing T-cells were directly associated to a better control
26 of CMV, independently of the immunosuppressive treatments and then these changes were
27 shown to be mostly induced by mTORi.
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33 *In vitro*, low dose of mTORi induced a long-lived cell profile (PD1 low, Tbet high and EOMES
34 low) which was associated with increased proliferation, viability and IFN γ production both on
35 $\gamma\delta$ and $\alpha\beta$ T-cells. Those characteristics were previously associated with improved effector T-
36 cells functionality (11, 12). Moreover, mTORi also increased the amount of Hobit+ T-cells,
37 known to be long-lived effector cells in the context of CMV (8-10).
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42 While mTORi effect have been well-studied in ~~in~~ naive cells and notably in allogenic naive
43 cells, ~~mTORi have been shown to display anti-proliferative effects and to decrease response to~~
44 ~~activation, underlining the rationale behind using mTORi to prevent graft rejection (23),~~
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3 However, it has been poorly evaluated in effector T-cells. Our *in vitro* results suggest that late-
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6 differentiated effector cells present high basal level of p-S6 that limit their capacity for further
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8 S6-phosphorylation during activation, that could be involved in their low IFN γ production after
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10 TCR engagement. Conversely, when basal level was decreased by mTORi pre-treatment,
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12 phosphorylation of S6 and IFN γ production was much more inducible during activation.
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14 Altogether, these results suggest that mTORi are able to dampen basal S6 phosphorylation in
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16 highly differentiated T-cells allowing them to respond more efficiently upon activation.
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18 Finally, we also observed that mTORi treated T-cells improved TCR signaling. This is of
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20 particular interest since late effector memory T-cells during chronic infection are mostly
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22 dependent on TCR signaling rather than cytokine signals to persist (49, 50). Chronic antigen
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24 activation could lead to increased basal mTOR levels in T-cells, hampering their capacity to
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26 respond properly during a new viral challenge. Decreasing the basal level of mTOR pathway
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28 activation could sensitize effector T-cells to better respond to a new TCR activation.

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

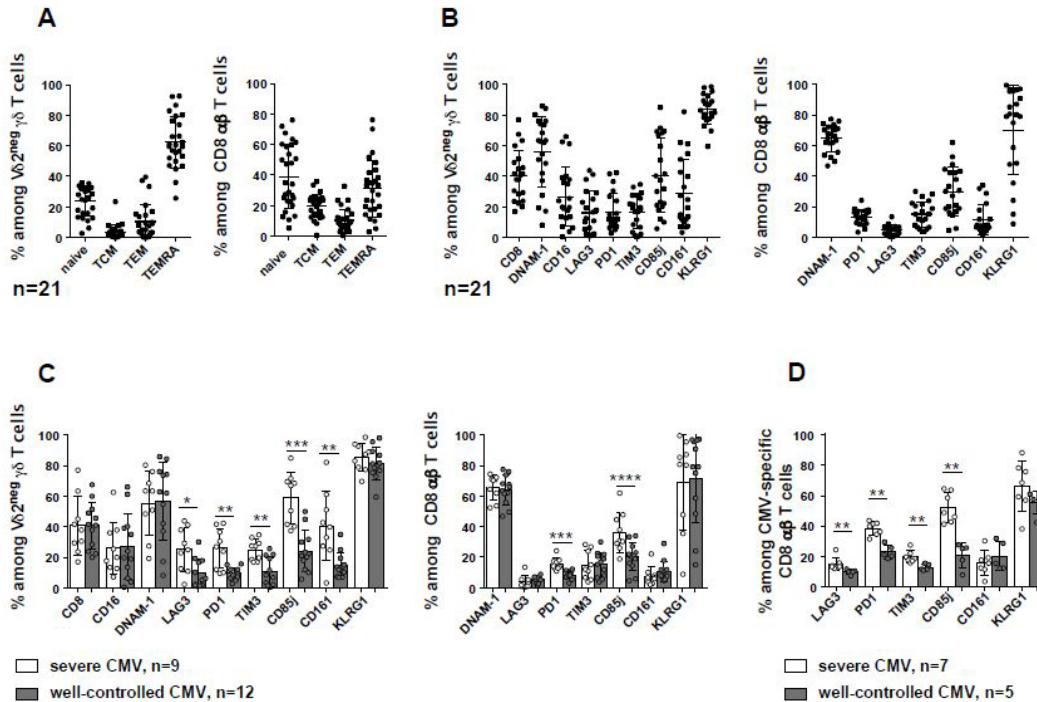
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months of follow-up), n (%)

Figure Legend

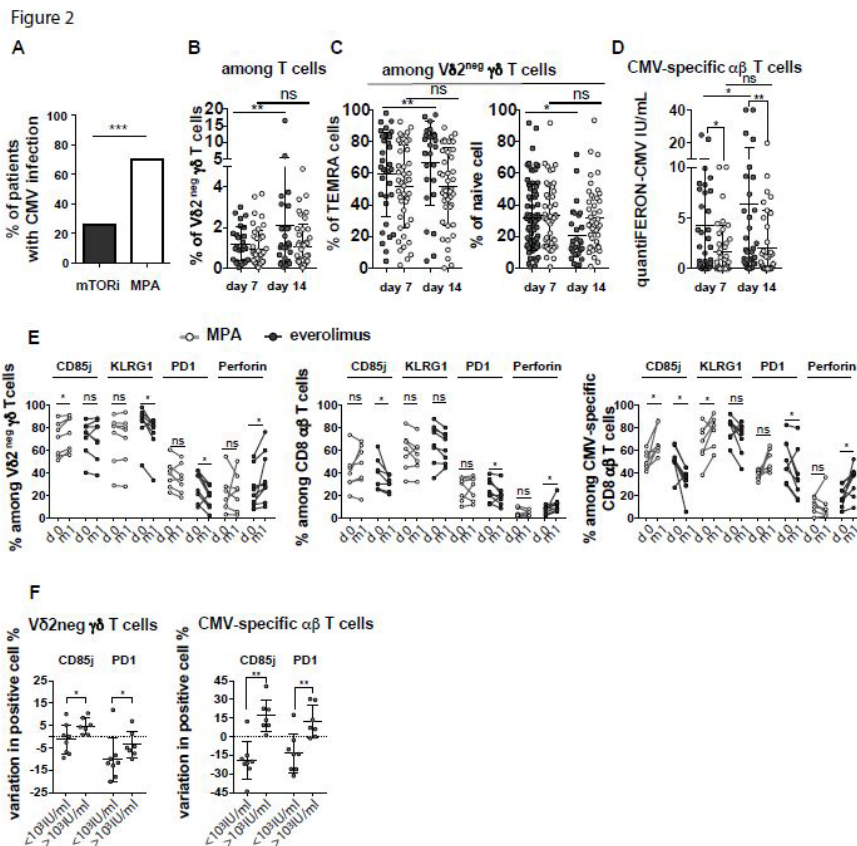
Figure 1 $V\delta 2^{neg}$ $\gamma\delta$ T cells and CD8⁺ T cells express inhibitory receptors at baseline in patients with severe CMV infections

Figure 1



$V\delta 2^{neg}$ $\gamma\delta$ 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-Whitney 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.

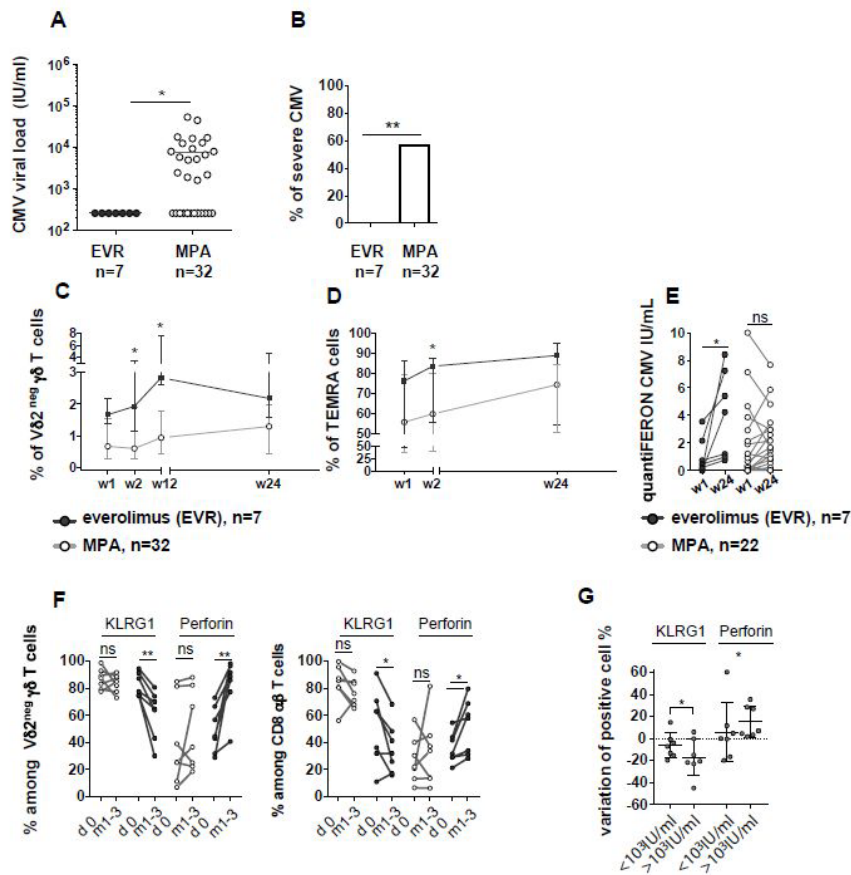
D. CMV-specific αβ T cells were analyzed with QuantiFERON-CMV (IU/mL) at day 7 (n=27 evero, n=41 MPA) and 14 post-transplantation (n=27 evero, n=43 MPA). Each symbol represents an individual donor, large horizontal lines indicate the mean and small horizontal lines indicate the standard deviation.

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-Whitney 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

Figure 3



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.

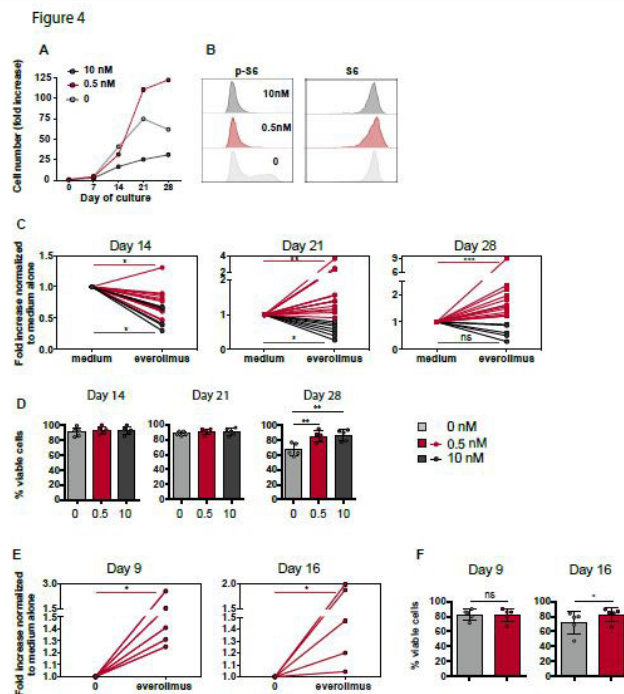
C.D. Whole blood staining of $V\delta 2^{neg} \gamma\delta$ T cells and their expression of CD27 and CD45RA. Proportion of $V\delta 2^{neg} \gamma\delta$ T cells among T cells (C) and of TEMRA (CD27 neg CD45RA pos) among $V\delta 2^{neg} \gamma\delta$ T cells at week 1, 2, 12 and 24 post-transplantation 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 $V\delta 2^{neg} \gamma\delta$ 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+ $V\delta 2^{neg} \gamma\delta$ T cell percentages between month 1 and day 0 were calculated and compared in patient groups with a post-transplantation CMV viral load $> \text{or} = 1000$ 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 unpaired data by Mann-Whitney U test.

Figure 4 Impact of long-term *in vitro* mTORi treatment on proliferation and viability of $V\delta 2^{\text{neg}}$ $\gamma\delta$ T cells and CMV-specific $\alpha\beta$ T cells



PBMC from CMV seropositive KTR were incubated with IL-2 with or without IL-15 for $V\delta 2^{\text{neg}}$ $\gamma\delta$ T cells and IL-2 alone for CMV-specific $\alpha\beta$ T cells and with indicated doses of everolimus. Proliferation and viability of $V\delta 2^{\text{neg}}$ $\gamma\delta$ T cells at day 14, 21 and 28 of culture and of CMV-specific $\alpha\beta$ T cells at day 9 and 16 of culture were performed.

A. Representative donor for $V\delta 2^{\text{neg}}$ $\gamma\delta$ T cells proliferation.

B. Representative flow cytometry staining of S6 and phospho-S6 (p-S6) among $V\delta 2^{\text{neg}}$ $\gamma\delta$ T cells at day 14 of culture.

C. Proliferation of $V\delta 2^{\text{neg}}$ $\gamma\delta$ 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; everolimus 10nM, n=8) and 28 (everolimus 0.5nM, n=12; everolimus 10nM, n=6).

D. $V\delta 2^{\text{neg}}$ $\gamma\delta$ T cell viability tested by flow cytometry live-dead staining (n=5).

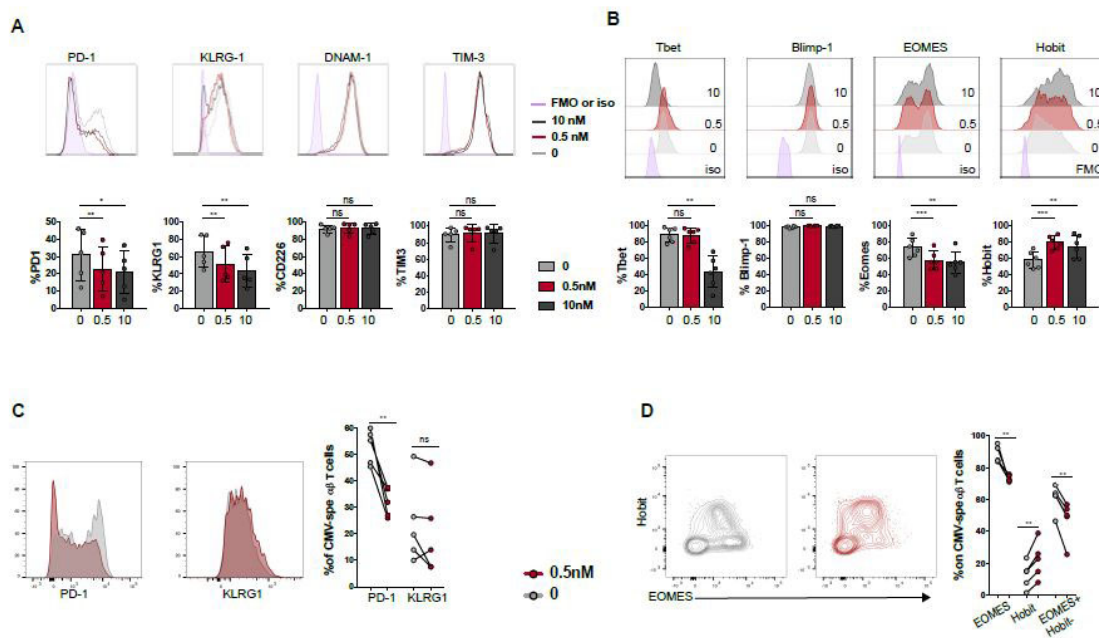
E. Proliferation of CMV specific $\alpha\beta$ T cells, represented as fold increases normalized to culture with medium alone (everolimus 0.5nM, n=5)

F. CMV-specific $\alpha\beta$ 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 $V\delta 2^{\text{neg}} \gamma\delta$ T cells and CMV-specific $\alpha\beta$ T cells

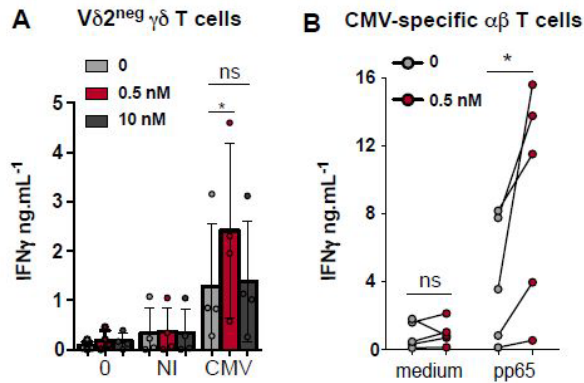
Figure 5



$V\delta 2^{\text{neg}} \gamma\delta$ T cells (after 21 days) and CMV-specific $\alpha\beta$ 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 $V\delta 2^{\text{neg}} \gamma\delta$ 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 $\alpha\beta$ 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 $V\delta 2^{neg}$ $\gamma\delta$ T cells and CMV-specific $\alpha\beta$ T cells response to CMV

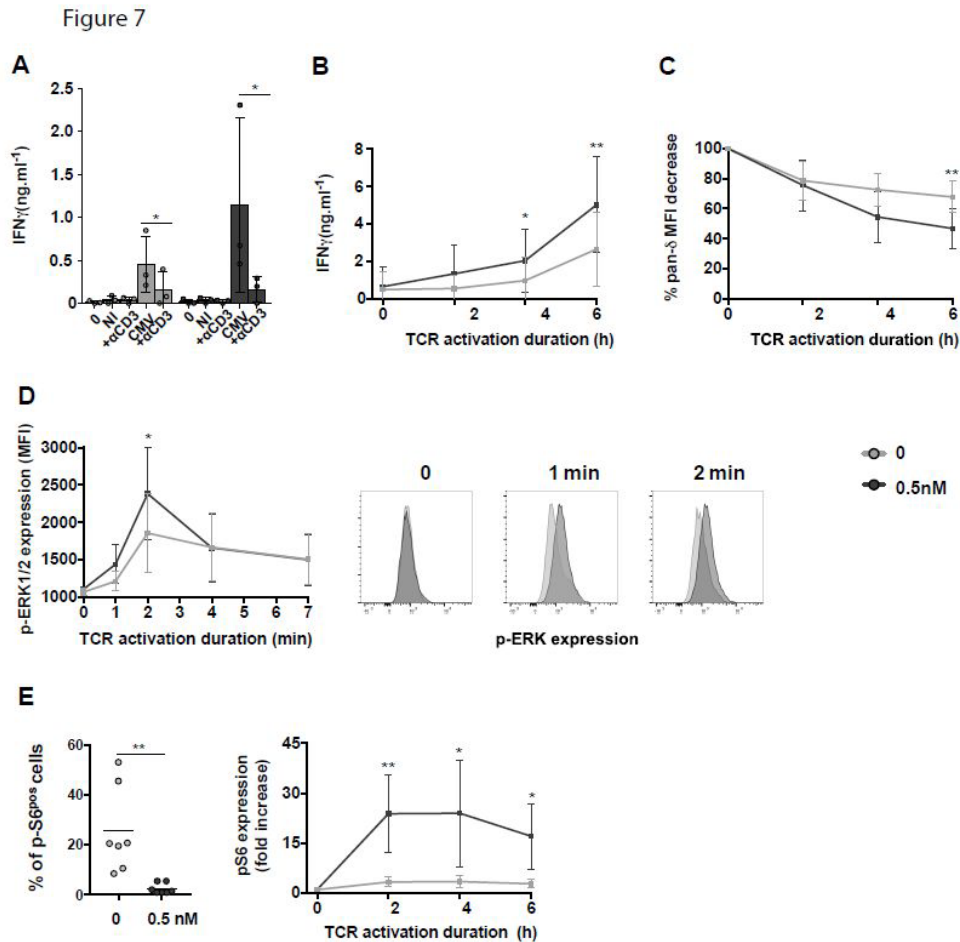
Figure 6



A. $V\delta 2^{neg}$ $\gamma\delta$ T cells were purified and cultured in medium alone or with non-infected (NI) or CMV-infected fibroblasts during 24 hours and ELISA IFN γ 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 IFN γ 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

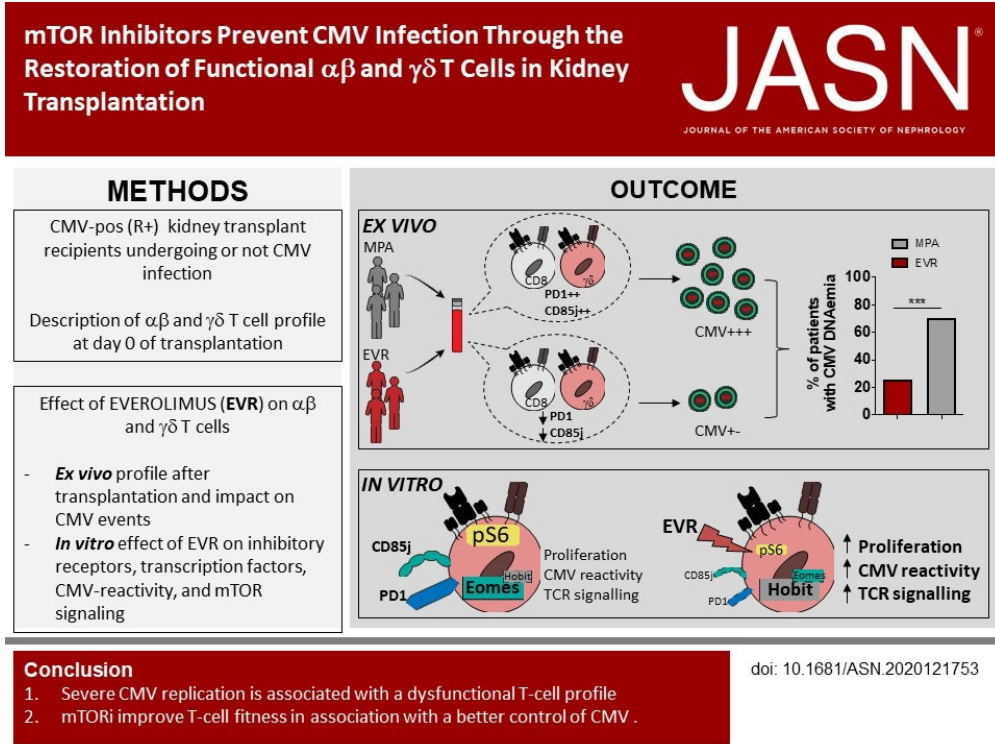


A. Vδ2^{neg} γδ T cells were purified and cultured in medium alone, with non-infected (NI) or CMV-infected fibroblasts with or without a blocking anti-CD3 mAb (10 μg/ml) during 24 hours and ELISA IFN γ was performed (n=4 donors).

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 IFN γ 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, 10 μg/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 (10 μg/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. Cyclosporin and everolimus whole blood concentrations were performed at day 7, day 14 and month 6 post transplantation.

Supplemental material Table of Contents

Supplemental Table 1: References of flow cytometry material

Supplemental Figure legends

Figure S1. Flow chart of the ancillary study

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

Figure S3 V δ 2^{neg} $\gamma\delta$ T cells and CD8⁺ $\alpha\beta$ T cells phenotype at baseline in CMV seropositive patients

Figure S4 Phenotype of V δ 2^{neg} $\gamma\delta$ T cells and CMV-specific $\alpha\beta$ T cells during in vitro culture

Figure S5 Low dose of mTORi decrease the number of V δ 2^{neg} $\gamma\delta$ T cells and CMV-specific $\alpha\beta$ T cell expressing CD85j.

Figure S6 mTORi does not affect CMV-specific $\alpha\beta$ T cell frequencies during in vitro culture

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2
3 Figure S7 mTORi improvement of the T cell dysfunctional profile is maintained when
4 combined with ciclosporin
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8 Figure S8 Blocking anti-CD3 mAb had no effect on V δ 2^{neg} $\gamma\delta$ T cell viability.
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10 Figure S9 mTORi does not affect SLP-76 and MAP kinase 38 signaling after TCR stimulation
11 of V δ 2^{neg} $\gamma\delta$ T cells
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15 Figure S10 No effect of mTORi on Akt phosphorylation after TCR stimulation of V δ 2^{neg} $\gamma\delta$ T
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60**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 V δ 2	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 V δ 2	Beckman coulter	IMMU389	5	B49310	0.04
FITC TCR V δ 2	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/cytopermtm	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				

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Supplemental Figures

Figure S1. Flow chart of the ancillary study

Samples came from patients initially included 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 $\gamma\delta$ T cell phenotype, and 12 were used for the second set of patients with CD8, CMV-specific CD8 and $\gamma\delta$ T cell phenotype.

Figure S3 $V\delta 2^{\text{neg}}$ $\gamma\delta$ T cells and CD8+ $\alpha\beta$ T cells phenotype at baseline in CMV seropositive patients

A. $V\delta 2^{\text{neg}}$ $\gamma\delta$ T cell and CD8+ $\alpha\beta$ 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. $V\delta 2^{\text{neg}}$ $\gamma\delta$ 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-Whitney U test.

Figure S4 Phenotype of V δ 2^{neg} $\gamma\delta$ T cells and CMV-specific $\alpha\beta$ 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 V δ 2^{neg} $\gamma\delta$ T cells, analyzed by flow cytometry.

B. One representative proliferation assay of TEMRA V δ 2^{neg} $\gamma\delta$ 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 V δ 2^{neg} $\gamma\delta$ T cells analyzed by flow cytometry.

D. Evolution of V δ 2^{neg} $\gamma\delta$ 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 V δ 2^{neg} $\gamma\delta$ T cells percentage by flow cytometry. Results are the mean \pm 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 $\alpha\beta$ T cells analyzed by flow cytometry.

Figure S5 Low dose of mTORi decrease the number of V δ 2^{neg} $\gamma\delta$ T cells and CMV-specific $\alpha\beta$ T cell expressing CD85j.

Expression of CD85j was analyzed in V δ 2^{neg} $\gamma\delta$ T cells and CMV specific $\alpha\beta$ 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 V δ 2^{neg} $\gamma\delta$ T cells and during 16 days for the culture

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3 of CMV specific $\alpha\beta$ T cells (stimulated overnight by pp65 peptides and gated on non- $\gamma\delta$ T cells
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5 positive for CD69 and IFN γ). Each symbol represents an individual donor; $0.05 > p > 0.01^*$;
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8 $**0.01 > p > 0.001$; as determined by Wilcoxon test.
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14 **Figure S6 mTORi does not affect CMV-specific $\alpha\beta$ T cell frequencies during in vitro**
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16 **culture**

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18 One representative phenotype of CMV-specific $\alpha\beta$ T cells after 7 days of PBMC culture with
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20 IL2 with or without 0.5nM everolimus. After overnight stimulation with CMV peptides, CMV-
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22 specific $\alpha\beta$ T cells were gated by flow cytometry through their expression of CD69 and IFN γ
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24 among non $\gamma\delta$ T cells (left). Cumulative results for 5 donors(right).
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31 **Figure S7 mTORi improvement of the T cell dysfunctional profile is maintained when**
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33 **combined with ciclosporin**

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36 (A) $V\delta 2^{\text{neg}}$ $\gamma\delta$ T cells (after 7, 14 and 21 days for expansion and 21 days for PD1 and CD85j
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38 expression) and (B) CMV-specific $\alpha\beta$ T cells (after 16 days) with 0/25/75/200mg/mL⁻¹ with
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40 either medium, 0.5nM everolimus or mycophenolate mofetil (MMF) were analyzed after *in*
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42 *vitro* culture of PBMC from CMV-seropositive KTR. Expansion are expressed as fold increase
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44 from day 0 of culture. PD-1 and CD85j are expressed as proportion among total $V\delta 2^{\text{neg}}$ $\gamma\delta$ T
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46 cells or CMV-specific $\alpha\beta$ T cells.
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51 Each symbol represents an individual donor. ns, not significant, $0.05 > p > 0.01^*$;
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53 $**0.01 > p > 0.001$; as determined by Wilcoxon test.
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60 **Figure S8 Blocking anti-CD3 mAb had no effect on $V\delta 2^{\text{neg}}$ $\gamma\delta$ T cell viability.**

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4 V δ 2^{neg} $\gamma\delta$ T cells were negatively sorted after 21 days of culture with 0 or 0.5 nM everolimus
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6 and were cultured in the same medium alone (either with 0 or 0.5 nM everolimus), with non-
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8 infected (NI), or with CMV-infected (CMV) fibroblasts with or without blocking anti-CD3
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10 mAb (10 μ g/ml) during 24 hours and cells were stained with 1 μ M DAPI (4',6-Diamidino-2-
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12 Phenylindole, Dihydrochloride) to assess their viability by flow cytometry.
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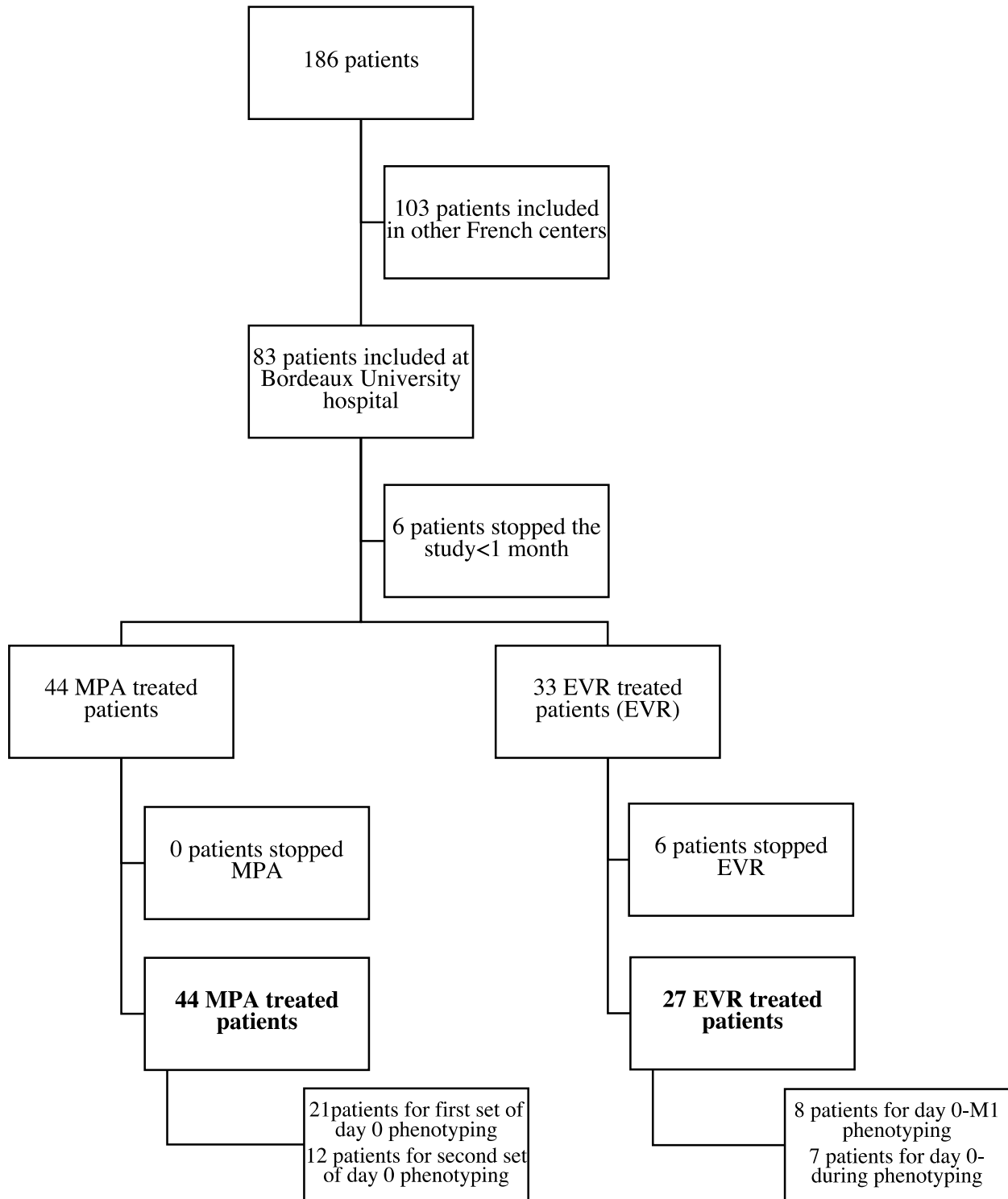
19 **Figure S9 mTORi does not affect SLP-76 and MAP kinase 38 signaling after TCR**
20 **stimulation of V δ 2^{neg} $\gamma\delta$ T cells**
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25 V δ 2^{neg} $\gamma\delta$ T cells were negatively sorted after 21 days of culture with 0 or 0.5 nM of everolimus
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27 , and stimulated with an agonist anti-CD3 mAb (UCHT1, 10 μ g/ml) for the indicated durations.
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29 SLP-76 phosphorylation (A) and MAP kinase 38 phosphorylation (B) were measured by flow
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31 cytometry in 4 donors (right, mean \pm ranges) and in a representative donor (left).
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38 **Figure S10 No effect of mTORi on Akt phosphorylation after TCR stimulation of V δ 2^{neg}**
39 **$\gamma\delta$ T cells**
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44 After 21 days of culture with 0 or 0.5 nM of everolimus, PBMC were stimulated using an
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46 agonist anti-V δ 1 mAb (10 μ g/ml) for 30 minutes, 2 and 4 hours and Akt phosphorylation was
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48 measured and analyzed by flow cytometry (T308 phosphorylation site, left; S473
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50 phosphorylation site, right) for 4 donors (mean \pm ranges).
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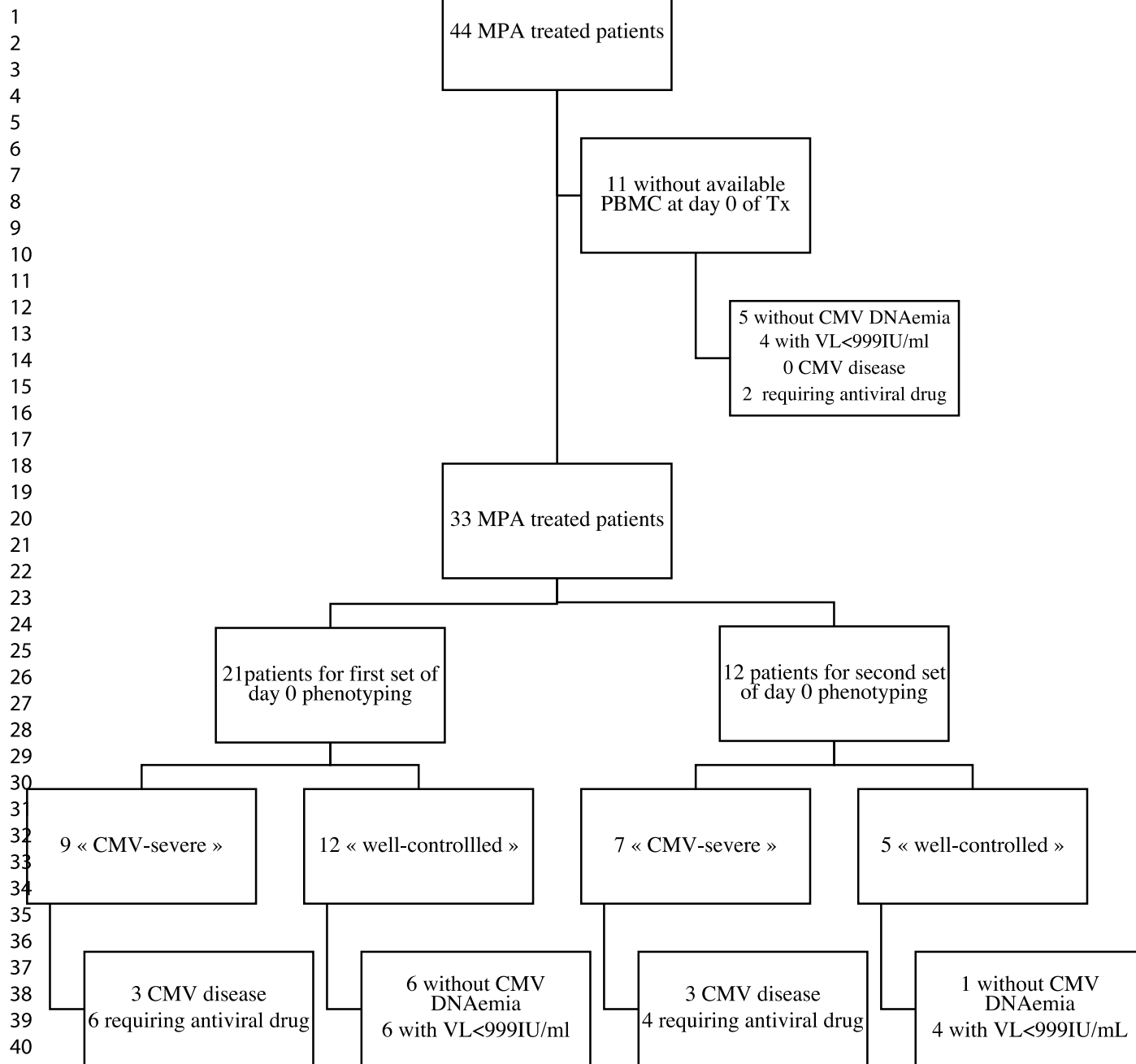
Figure S1



Supplemental Figure 1. Flow chart of the ancillary study

51 Samples came from patients initially included in a French multicenter study (n=186)
 52 from which 83 were included at Bordeaux University Hospital. Among those 83
 53 patients, 77 could be followed for more than 1 month, 44 were treated with
 54 mycophenolic acid (MPA-treated patients) and 33 were treated with everolimus
 55 (EVR-treated patients). Among EVR-treated patients, 6 have been switched to MPA
 56 and were excluded of analyses. Consequently the analyses of the study included 44
 57 MPA-treated patients and 27 EVR-treated patients.
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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 $\gamma\delta$ T cell phenotype, and 12 were used for the second set of patients with CD8, CMV-specific CD8 and $\gamma\delta$ T cell phenotype. VL:viral load

Figure S3

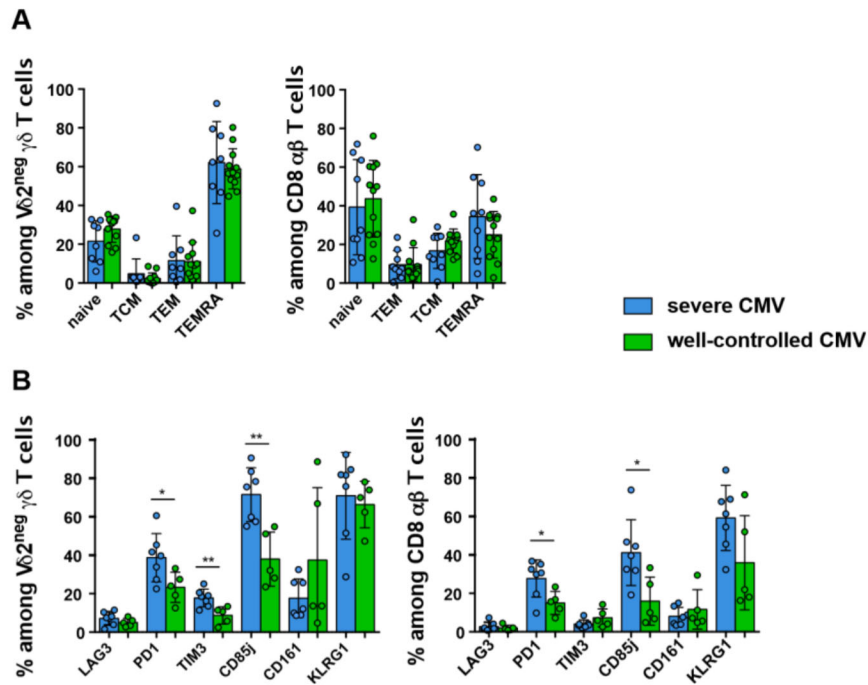


Figure S3: $V\delta 2^{\text{neg}} \gamma\delta$ T cells and CD8+ $\alpha\beta$ T cells phenotype at baseline in CMV seropositive patients

A. $V\delta 2^{\text{neg}} \gamma\delta$ T cell and CD8+ $\alpha\beta$ 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. $V\delta 2^{\text{neg}} \gamma\delta$ 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).

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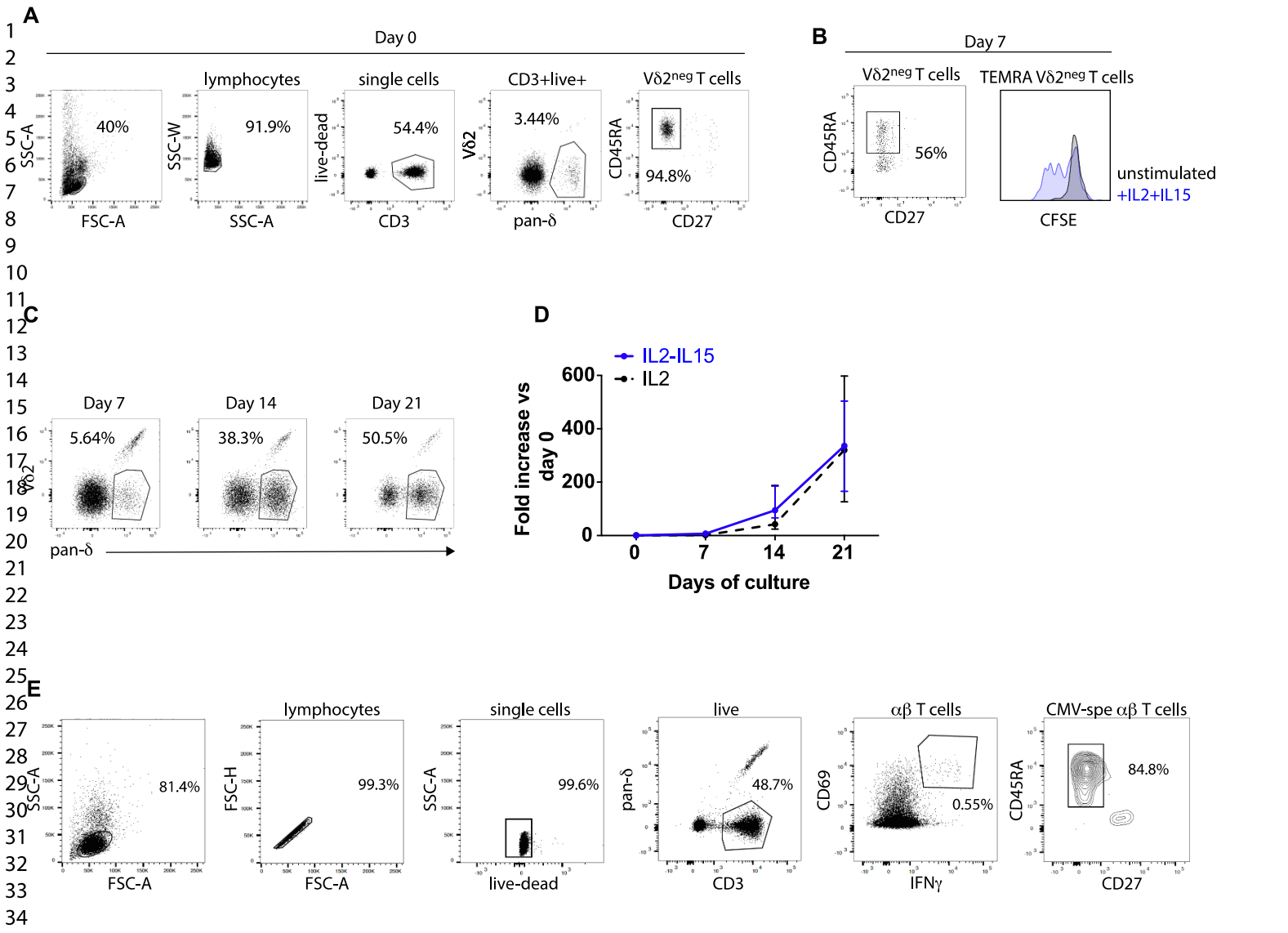


Figure S4: Phenotype of V δ 2^{neg} $\gamma\delta$ T cells and CMV-specific $\alpha\beta$ T cells during *in vitro* culture

A. One representative phenotype at day 0 of CMV positive patient's PBMC used for *in vitro* IL2-IL15 culture of V δ 2^{neg} $\gamma\delta$ T cells, analyzed by flow cytometry. B. One representative proliferation assay of TEMRA V δ 2^{neg} $\gamma\delta$ 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 V δ 2^{neg} $\gamma\delta$ T cells analyzed by flow cytometry. D. Evolution of V δ 2^{neg} $\gamma\delta$ 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 V δ 2^{neg} $\gamma\delta$ T cells percentage by flow cytometry. Results are the mean \pm 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 $\alpha\beta$ T cells analyzed by flow cytometry.

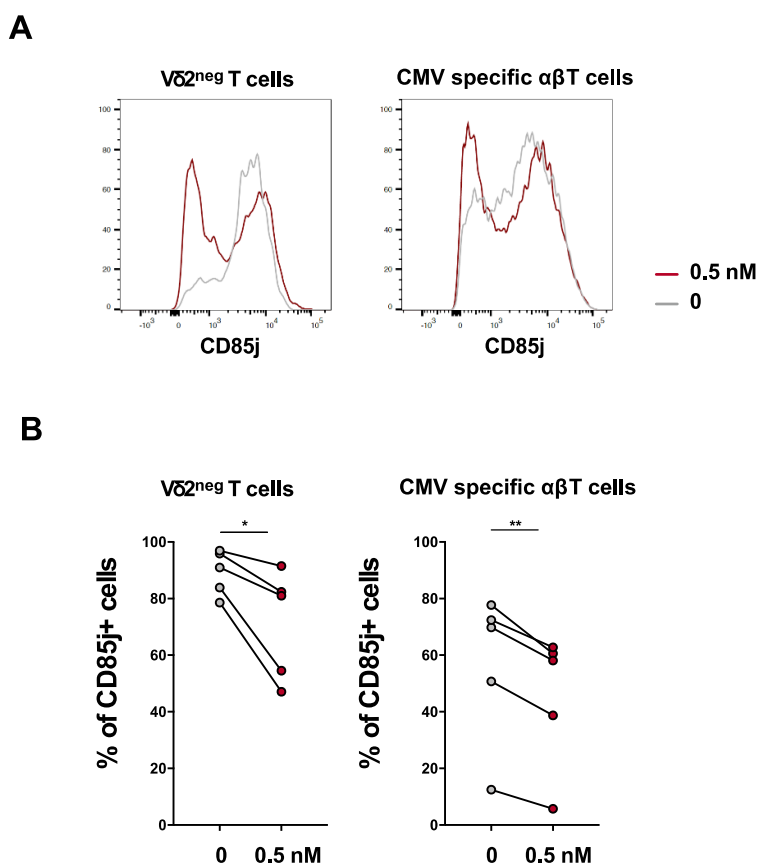


Figure S5: Low dose of mTORi decrease the number of Vδ2^{neg} γδ T cells and CMV-specific αβ T cell expressing CD85j.

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

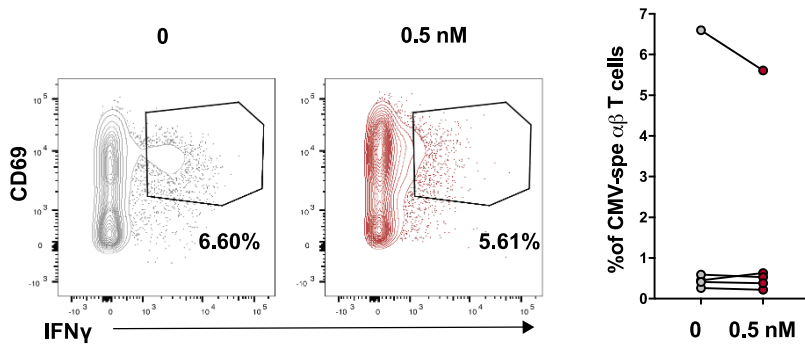
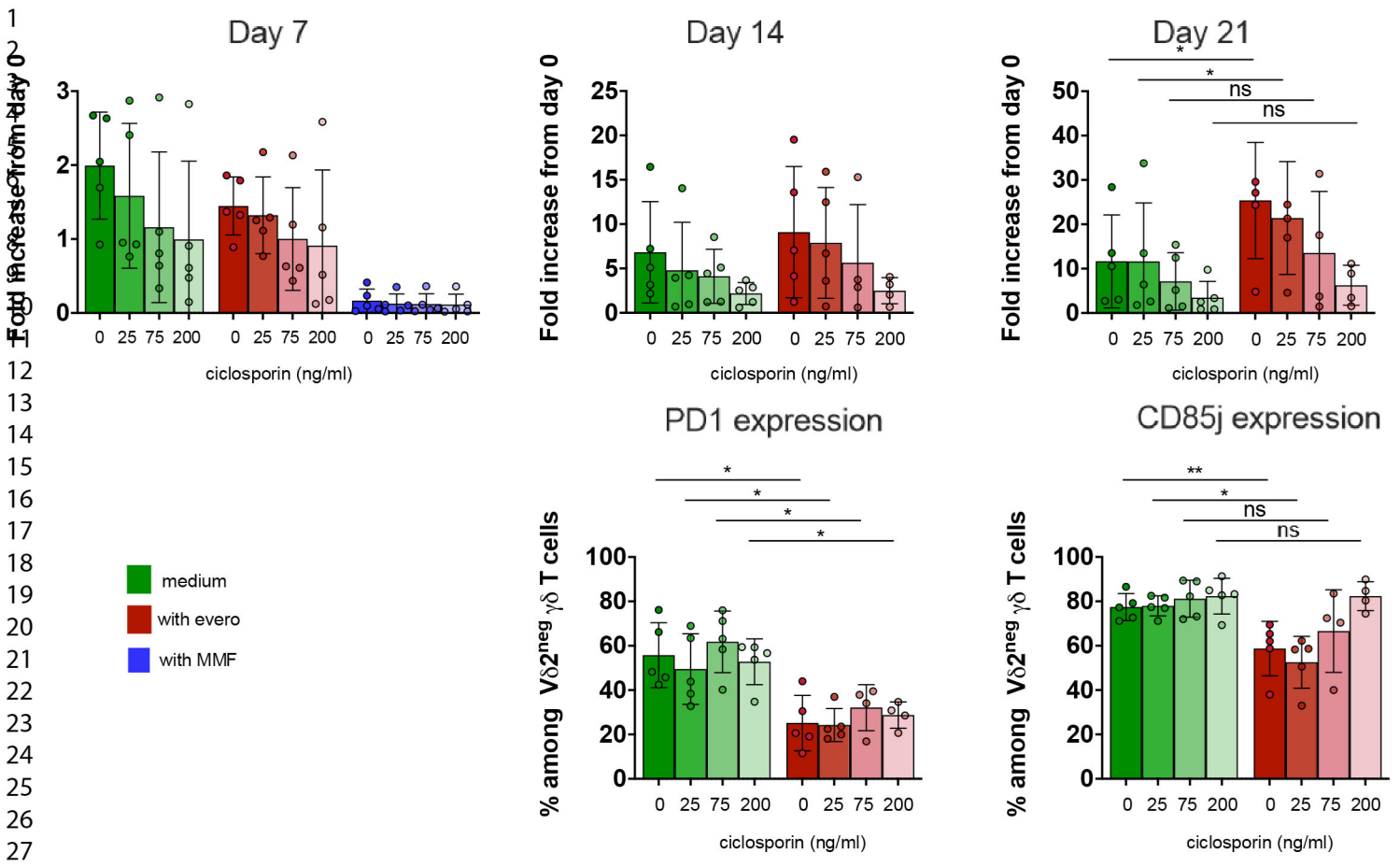


Figure S6: mTORi does not affect CMV-specific $\alpha\beta$ T cell frequencies during in vitro culture

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

A $V\delta^{neg} \gamma\delta$ T cells



B CMV-specific $\alpha\beta$ T cells

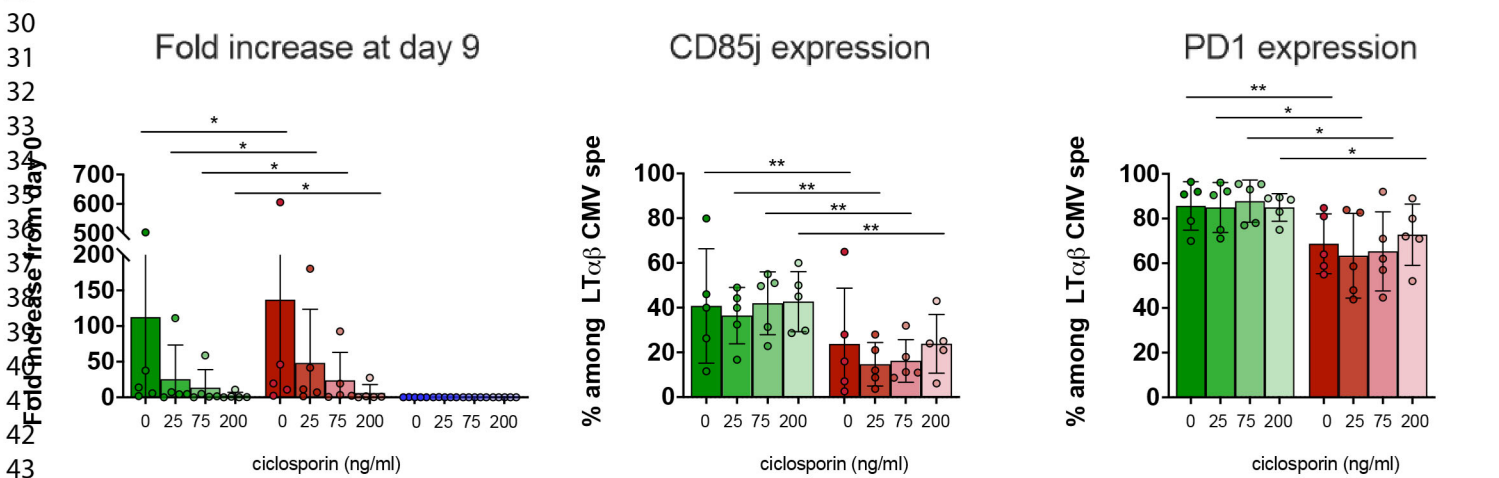


Figure S7: mTORi improvement of the T cell dysfunctional profile is maintained when combined with ciclosporin

(A) $V\delta^{neg} \gamma\delta$ T cells (after 7, 14 and 21 days for expansion and 21 days for PD1 and CD85j expression) and (B) CMV-specific $\alpha\beta$ 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 $V\delta^{neg} \gamma\delta$ T cells or CMV-specific $\alpha\beta$ 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

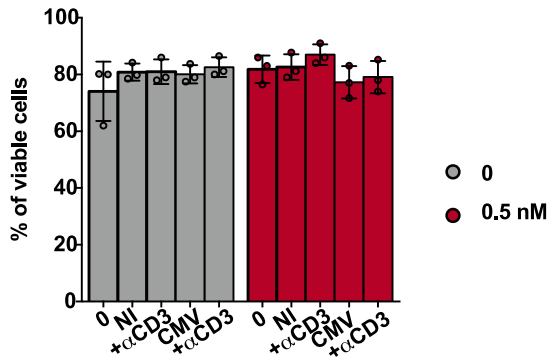


Figure S8: Blocking anti-CD3 mAb had no effect on Vδ2^{neg} γδ T cell viability.

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 (10 μg/ml) during 24 hours and cells were stained with 1 μM DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) to assess their viability by flow cytometry.

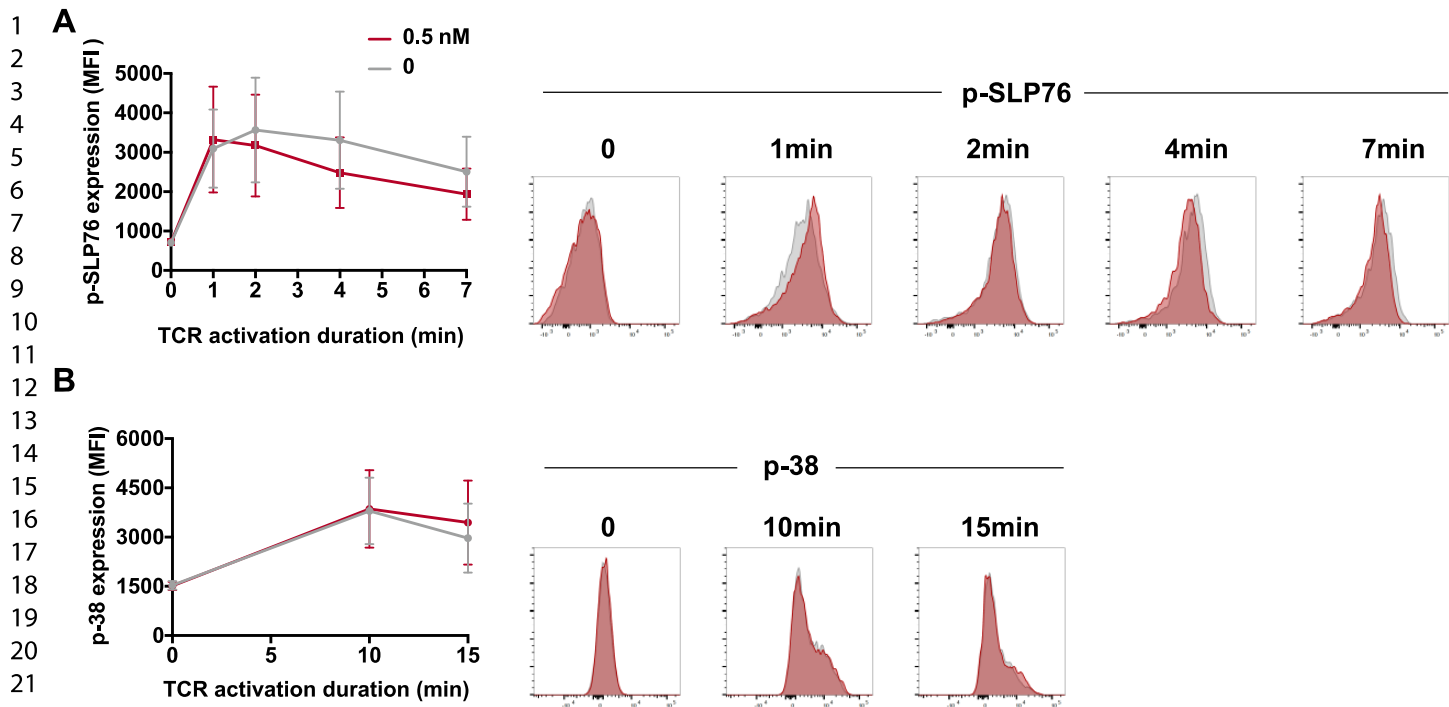


Figure S9: mTORi does not affect SLP-76 and MAP kinase 38 signaling after TCR stimulation of $V\delta 2^{neg}$ $\gamma\delta$ T cells

$V\delta 2^{neg}$ $\gamma\delta$ 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, 10 μ g/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 \pm ranges) and in a representative donor (left).

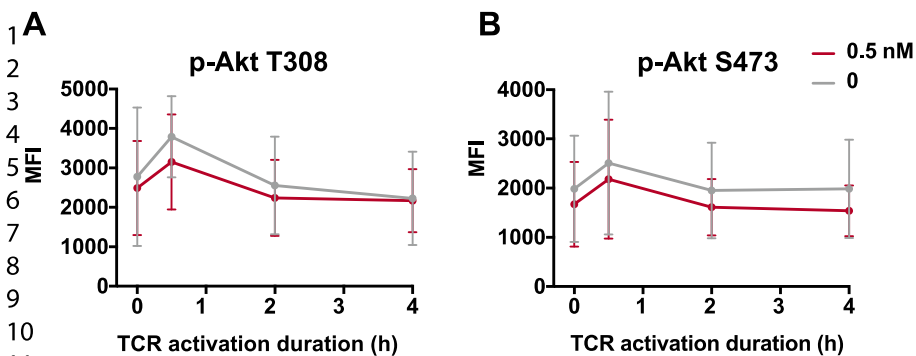


Figure S10: No effect of mTORi on Akt phosphorylation after TCR stimulation of V δ 2^{neg} γ δ T cells

After 21 days of culture with 0 or 0.5 nM of everolimus, PBMC were stimulated using an agonist anti-V δ 1 mAb (10 μ g/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 \pm ranges).