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Effect of everolimus-based drug regimens on CMV-specific T-cell functionality after renal transplantation: 12-month ATHENA subcohort-study results

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Post-transplant cytomegalovirus (CMV) infections and increased viral replication are associated with CMV-specific T-cell anergy. In the ATHENA-study, de-novo everolimus (EVR) with reduced-exposure tacrolimus (TAC) or cyclosporine (CyA) showed significant benefit in preventing CMV infections in renal transplant recipients as compared to standard TAC + mycophenolic acid (MPA). However, immunomodulatory mechanisms for this effect remain largely unknown. Ninety patients from the ATHENA-study completing the 12-month visit on-treatment (EVR + TAC n = 28; EVR + CyA n = 19; MPA + TAC n = 43) were included in a posthoc analysis. Total lymphocyte subpopulations were quantified. CMV-specific CD4 T cells were determined after stimulation with CMV-antigen, and cytokine-profiles and various T-cell anergy markers were analyzed using flow cytometry. While 25.6% of MPA + TAC-treated patients had CMV-infections, no such events were reported

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in EVR-treated patients. Absolute numbers of lymphocyte subpopulations were comparable between arms, whereas the percentage of regulatory T cells was significantly higher with EVR + CyA versus MPA + TAC ($p = 0.019$). Despite similar percentages of CMV-specific T cells, their median expression of CTLA-4 and PD-1 was lower with EVR + TAC ($p < 0.05$ for both) or EVR + CyA ($p = 0.045$ for CTLA-4) compared with MPA + TAC. Moreover, mean percentages of multifunctional CMV-specific T cells were higher with EVR + TAC (27.2%) and EVR + CyA (29.4%) than with MPA + TAC (19.0%). In conclusion, EVR-treated patients retained CMV-specific T-cell functionality, which may contribute to enhanced protection against CMV infections.

Keywords: CMV · CD4 T cells · CTLA-4 · Everolimus · PD-1

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Cytomegalovirus (CMV) infection is a common opportunistic infection following renal transplantation. Apart from causing direct symptoms, CMV infection or disease can indirectly affect long-term allograft function, morbidity, and mortality [1–4]. CMV-specific T cells are known to show a phenotype of replicative senescence characterized by a loss of CD28 and CD27 expression [3]. In renal transplant recipients, symptomatic CMV infection is associated with loss of T-cell functionality characterized by a restricted cytokine-expression profile and an increased expression of inhibitory receptors on CMV-specific CD4 T cells such as programmed cell death protein-1 (PD-1) [3, 5]. Likewise, increased expression of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and T-cell immunoglobulin and mucin-domain containing protein 3 (TIM-3) on pathogen-specific T cells has been associated with active infections [6–10]. NK cells, another subset of effector lymphocytes involved in innate immunity, reveal an activated phenotype in renal transplant recipients based on decreased surface expression of CD16 in $CD56^{dim}$ NK cells [11–13].

Immunosuppressive regimens containing the mammalian target of rapamycin (mTOR) inhibitors everolimus (EVR) or sirolimus have been shown to be associated with significantly lower rates of CMV infection compared to regimens containing mycophenolic acid (MPA) or mycophenolate mofetil (MMF) and can also protect against CMV-reactivation and disease [2, 14–23]. Given the mechanistic differences in interfering with signaling in transcription and proliferation of T cells, respectively, both drugs may differentially affect specific immunity toward the virus [24]. In a small study on renal transplant patients, conversion to EVR after 6 months was associated with a significant increase in CMV-specific effector-type CD8 T-cell numbers in the long term [25]; however, no long-term data on CMV-specific immune responses in patients after de novo EVR treatment are available.

ATHENA was a large, randomized trial conducted in 612 de novo renal transplant recipients to evaluate and directly compare EVR in combination with two calcineurin inhibitor (CNI) candidates (tacrolimus [EVR + TAC] and cyclosporine A [EVR + CyA]) versus a standard-of-care regimen of mycophenolic acid with TAC [MPA + TAC]). The study showed that renal function and efficacy outcomes were comparable between the EVR arms and standard-of-care regimen [26]. In addition, both EVR-based arms had at least threefold lower incidence of CMV infections relative to the MPA + TAC regimen, which may in part be due to a direct antiviral activity of mTOR inhibition by interference with the mTOR signaling pathway, which mainly affects late viral protein synthesis [2, 27]. Although findings from multiple randomized controlled trials, including the ATHENA study, have validated the fact that de novo EVR-based regimens are associated with a reduced risk of CMV infections [14, 15, 19, 26], the immune responses underlying this protective effect remain largely unknown.

In this posthoc analysis from the ATHENA study, we sought to evaluate the effect of the type of immunosuppressive regimen on major lymphocyte subpopulations. As CMV-specific CD4 T cells and their properties were shown to be associated with control of CMV, we hypothesized that the lower incidence of CMV infections during EVR treatment may be associated with a preserved state of CMV-specific CD4 T-cell functionality.

Results

Patient population

Out of 612 randomized patients, 511 completed the study (171 in EVR + TAC; 159 in EVR + CyA and 181 in MPA + TAC). Of these, 111 patients (39 in EVR + TAC, 26 in EVR + CyA, and 46 in MPA + TAC), all from German centers, completed the 12-month visit for the substudy analyses and comprised the full analysis set (FAS). Among these, 90 patients completed the 12-month visit on-treatment (OT) and were included in this posthoc analysis (28 in EVR + TAC, 19 in EVR + CyA, and 43 in MPA + TAC). We limited the majority of the analyses to the OT

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Table 1. Demographics and baseline characteristics (12-month analysis; on-treatment population)

Parameter	EVR + TAC N = 28	EVR + CyA N = 19	MPA + TAC N = 43
Recipient characteristics			
Age in years, mean (SD)	51.4 (12.02)	48.0 (11.67)	53.5 (11.01)
Male sex, n (%)	23 (82.1)	13 (68.4)	25 (58.1)
Caucasians, n (%)	26 (92.9)	19 (100)	42 (97.7)
BMI, kg/m^2, mean (SD)	26.0 (4.00)	26.4 (3.94)	26.5 (4.72) (n = 42)
CMV serostatus, n (%)			
D+/R+	10 (35.7)	8 (42.1)	12 (27.9)
D+/R-	7 (25.0)	5 (26.3)	12 (27.9)
D-/R+	5 (17.9)	0 (0.0)	12 (27.9)
D-/R-	6 (21.4)	6 (31.6)	7 (16.3)
% of patients with PRA, n (%)			
PRA 0%-≤10%	27 (96.4)	19 (100)	42 (97.7)
PRA >10%-≤20%	1 (3.6)	0 (0.0)	1 (2.3)
HLA-A mismatches			
0	10 (35.7)	7 (36.8)	18 (41.9)
1	14 (50.0)	10 (52.6)	18 (41.9)
2	4 (14.3)	2 (10.5)	7 (16.3)
HLA-B mismatches			
0	10 (35.7)	8 (42.9)	18 (41.9)
1	12 (42.9)	6 (31.6)	14 (32.6)
2	6 (21.4)	5 (26.3)	11 (25.6)
HLA-DR mismatches			
0	7 (25.0)	9 (47.4)	23 (53.5)
1	16 (57.1)	9 (47.4)	20 (46.5)
2	5 (17.9)	1 (5.3)	0 (0.0)
Cold ischemia time, hours, mean (SD)	8.9 (5.05)	9.6 (5.51)	10.2 (5.92)
Donor characteristics			
Age in years, mean (SD)	49.0 (13.73)	47.8 (15.07)	52.9 (13.95)
Male sex, n (%)	11 (39.3)	8 (42.1)	19 (44.2)
Donor category			
Deceased heart beating	23 (82.1)	15 (78.9)	35 (81.4)
Living related	5 (17.9)	4 (21.1)	4 (9.3)
Living unrelated	0 (0.0)	0 (0.0)	4 (9.3)

BMI, body mass index; CyA, cyclosporine A; D, donor; EVR, everolimus; MPA, mycophenolic acid; PRA, panel reactive antibodies; R, recipient; TAC, tacrolimus.

population because this population comprised patients who were on the assigned treatment regimen throughout the study and would better represent the effect of immunosuppression on T-cell anergy and functionality patterns. Recipient (R) and donor (D) baseline characteristics were balanced between the three arms (Table 1).

Overall, 11 patients in the EVR + TAC arm, 7 in the EVR + CyA arm, and 3 in the MPA + TAC arm switched treatment during the study. Here, recipients in both EVR groups at baseline were predominantly seropositive (EVR + TAC, 7/11 [63.6%]; EVR + CyA, 5/7 [71.4%], while only one switcher in the MPA + TAC was seropositive (D-/R+); Supporting information Table S1). Adverse events were the main reason for switching in the EVR groups and the only reason in the MPA + TAC group. Only one patient in the EVR + TAC group switched treatment because of transplant rejection (Supporting information Table S1).

CMV primary infections and reactivations

Interestingly, in the OT population, no laboratory confirmed CMV infection or CMV disease as treatment emergent adverse events occurred in the EVR + TAC and EVR + CyA arms, while 11 of 43 patients (25.6%) on MPA + TAC experienced CMV infections (Fig. 1A, $p < 0.05$, four primary infections, seven reactivations). Among the 21 patients who switched treatment during the study, 5 had a CMV infection (Fig. 1B, 2/11 in EVR + TAC arm, 2/7 in EVR + CyA arm, and 1/3 in MPA + TAC arm). Although a trend toward a lower incidence of CMV infections with EVR-based regimens compared to MPA + TAC was also evident in the switcher populations, these differences were not significant, yet suggest a benefit of continuation on EVR-based regimen. This beneficial effect is further underlined by the fact that CMV infections in the EVR arms only occurred

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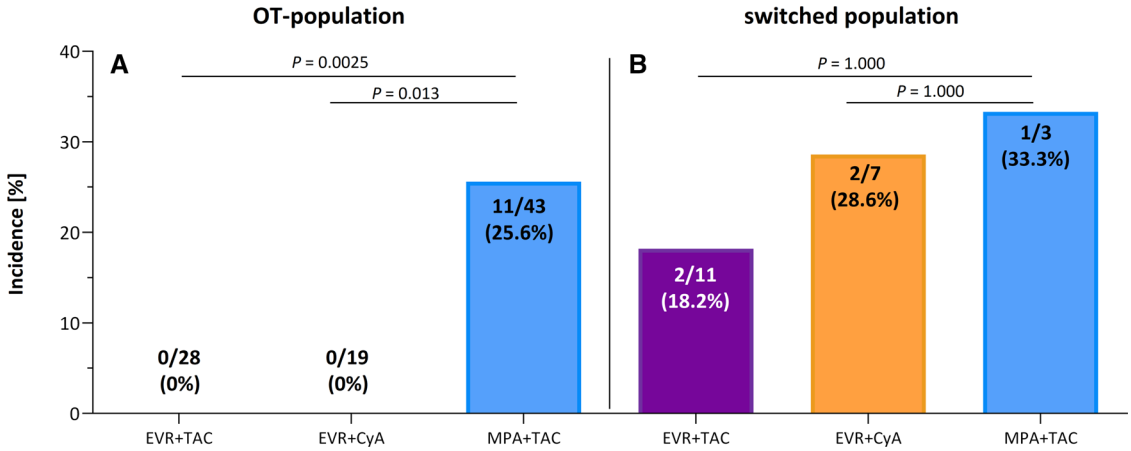

Figure 1. Incidence of CMV infection in the study population. Incidence of CMV infection was determined in the (A) on-treatment and (B) switcher populations. All patients including donor (D)/recipient (R) seronegative individuals are shown, despite low risk of infection. D-/R- patients in the on-treatment population did not develop any seroconversion, whereas one patient who switched from EVR + TAC seroconverted (included in panel B). Bars depict the incidence (%) of CMV infection in the various treatment groups for (A) the OT-population (EVR + TAC: n = 28, EVR + CyA: n = 19, MPA + TAC: n = 43) and B) switched population (EVR + TAC: n = 11, EVR + CyA: n = 7, MPA + TAC: n = 3). *p* values were computed by chi-square test and by two-sided Fisher's exact test for further comparison of EVR + TAC versus MPA + TAC and EVR + CyA versus MPA + TAC. CMV, cytomegalovirus; CyA, cyclosporine A; EVR, everolimus; FAS, full analysis set; MPA, mycophenolic acid; TAC, tacrolimus.

in the switcher population (4/18), and not in the OT population (Fig. 1).

Distribution of major lymphocyte subpopulations at month 12

We evaluated the impact of the immunosuppressive regimens on various lymphocyte populations under unstimulated condition in the OT population (Fig. 2). Total lymphocyte counts did not differ in the three treatment arms (median interquartile range [IQR] cells/μl EVR + TAC: 1480 [1000-1856], EVR + CyA: 1154 [873-1499], MPA + TAC: 1158 [828-1870]; Fig. 2A). Median numbers of CD4 T cells, CD8 T cells, and T_{reg} were also comparable between treatment arms, indicating no influence of treatment regimens on these major lymphocyte subpopulations (Fig. 2B-D). Similarly, no significant treatment-dependent differences were found for median numbers of NK cells (Fig. 2E) or for the four major NK cell subsets $CD56^{+++}CD16^{-}$, $CD56^{+++}CD16^{+}$, $CD56^{dim}CD16^{-}$, $CD56^{dim}CD16^{+}$ (Supporting information Fig. S1). Also, no significant differences were observed between treatment arms for median percentages of lymphocyte populations, except for T_{reg}, which were significantly higher in the EVR + CyA versus MPA + TAC arm (Supporting information Table S2, $p = 0.019$).

Characterization of CMV-specific T cells at month 12

Of 90 patients in the OT population, 47 were CMV-seropositive and 43 were CMV-seronegative at baseline. Among these, 11 underwent seroconversion by month 12. A higher number of these conversions were in the MPA + TAC arm (n = 7). Of note, although four seroconversions occurred in the EVR arms (3 in EVR + TAC, 1 in EVR + CyA), these occurred without detectable viral replication and/or clinical symptoms (Fig. 1A). The OT subgroups who were seropositive by month 12 were included for further analysis of CMV-specific T cells (n = 58). The absolute numbers and percentages of lymphocytes and subpopulations in these subgroups were comparable between treatment arms (Supporting information Table S2). All seronegative patients did not have any detectable CMV-specific T cells.

Quantitative analysis of CMV-specific T cells at month 12

CMV-specific T cells were quantified after stimulation with a CMV-lysate based on the induction of the activation marker CD69 and IFN-γ expression (Fig. 3). Stimulation with a control antigen served as negative control, and the superantigen *Staphylococcus aureus* enterotoxin B (SEB) was used as a polyclonal stimulus to characterize T-cell effector function largely unrelated to CMV. Typical dotplots are shown in Fig. 3A. Sufficient T cells for CMV-specific T-cell analyses were available in 51 of 58 patients. Interestingly, the percentages of CMV-specific CD4 T cells did not differ in the three treatment arms (Fig. 3B), indicating no specific effect of the immunosuppressive regimen on quantitative levels of CMV-specific T cells. Likewise, median percentages of SEB-reactive CD4 T cells did not differ in the three treatment arms (Fig. 3C). Finally, T-cell levels in seroconverters (shown as filled symbols) did not differ from the other seropositive individuals.

Expression of T-cell anergy markers at month 12

We also characterized CMV-specific CD4 T cells for the effect of immunosuppression on expression of CTLA-4, PD-1, and TIM-3,

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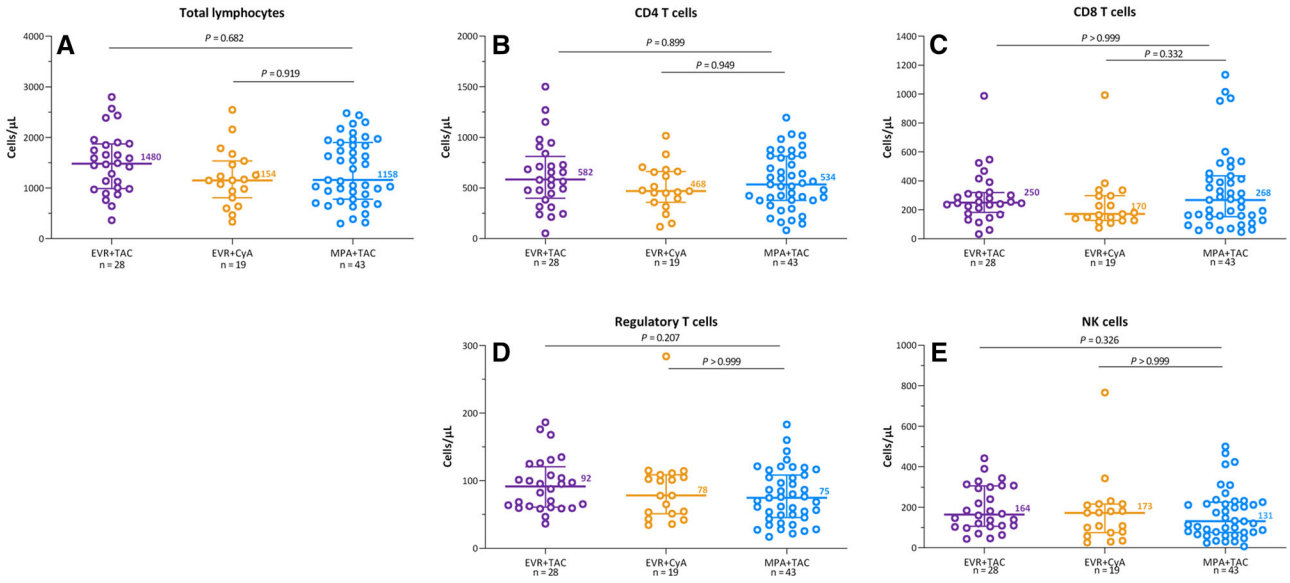

Figure 2. Distribution of lymphocyte subpopulations. Absolute numbers of (A) total lymphocytes, (B) CD4 T cells, (C) CD8 T cells, (D) regulatory T cells (as $CD4^+CD127^{low}$), and (E) NK cells in the on-treatment population were analyzed by flow cytometry under unstimulated conditions at month 12. Numbers were determined based on leukocyte blood counts and are represented as cells/µL blood with median and IQR. Data are pooled from single experiments of specimens from individual patients. In all panels, the number of patients in each group were as follows: EVR + TAC: n = 28, EVR + CyA: n = 19, MPA + TAC: n = 43. *p* values were determined by the Kruskall–Wallis test followed by Dunn´s post-test for the comparisons between EVR + TAC or EVR + CyA and MPA + TAC arms. A *p* value of 0.05 was considered statistically significant. CyA, cyclosporine; EVR, everolimus; IQR, interquartile range; MPA, mycophenolic acid; TAC, tacrolimus.

which are markers associated with T-cell anergy and/or exhaustion (Fig. 4). Typical dotplots are shown in Fig. 4A. Median CTLA-4 expression on CMV-specific T cells was significantly lower in both EVR arms as compared to MPA + TAC ($p = 0.043$ for EVR + TAC versus MPA + TAC, and $p = 0.045$ for EVR + CyA versus MPA + TAC). This effect was CMV-specific, as CTLA-4 expression on SEB-reactive T cells did not differ (Fig. 4B). Median PD-1 expression levels were significantly lower in EVR + TAC versus MPA + TAC in CMV-stimulated CD4 T cells ($p = 0.030$, Fig. 4C). However, no significant difference in PD-1 expression was observed between the EVR + CyA and MPA + TAC arms. This also held true for SEB-reactive CD4 T cells. Regarding TIM-3, expression was very low on both CMV- and SEB-reactive T cells in all three arms with no difference between the groups (Fig. 4D). Interestingly, despite the small numbers of seroconverters (shown as filled symbols) on EVR, PD-1 expression levels of these patients appear to be low (Fig. 4C), whereas TIM-3 expression seems high (Fig. 4D), which may be interesting given the fact that TIM-3 expression has been shown to be closely linked to specific T cells in the context of primary infection [8].

Cytokine expression profiles at month 12

To assess functionality of CMV- and SEB-reactive T cells in the three treatment groups, the populations of cells expressing the cytokines IL-2, IFN-ɣ, and TNF-α alone or in combination were analyzed. As shown in Fig. 5, a numerically higher proportion of CMV-specific T cells from patients in the EVR + TAC and EVR-CyA arms were found to be multifunctional expressing all three cytokines compared with the MPA + TAC arm (black segments, mean percentage: 27.2 vs. 29.4 vs. 19.0%, Fig. 5A, $p = 0.069$ for EVR + TAC versus MPA + TAC, and $p = 0.101$ for EVR + CyA versus MPA + TAC, respectively). The difference in the percentage of multifunctional T cells after SEB stimulation was less pronounced and numerically rather similar in the three groups (mean percentage: 22.6 vs. 20.2 vs. 17.1%; Fig. 5B, $p = 0.054$ for EVR + TAC vs. MPA + TAC, and $p = 0.554$ for EVR + CyA vs. MPA + TAC, respectively). Although the differences did not reach statistical significance, this may indicate some loss in multifunctionality in the TAC-MPA group in CMV-reactive T cells and to a lesser extent also in SEB-reactive T cells. Apart from multifunctional cells producing all three cytokines, the majority of CMV-specific T cells in all groups simultaneously produced IFN-ɣ and TNF-α (dark grey segments), which was distinct from the general cytokine profile of SEB-reactive T cells.

Discussion

This posthoc analysis of a subcohort of the ATHENA study was one of the first to compare the effect of de novo EVR therapy with TAC or CyA versus a standard-of-care regimen comprising MPA + TAC on overall levels of lymphocyte subpopulations, as well as on CMV-specific T-cell levels and their functionality 12 months after transplantation. In line with a complete absence of CMV-related complications in the OT populations on mTOR inhibitors, CMV-specific CD4 T cells in these patients showed less

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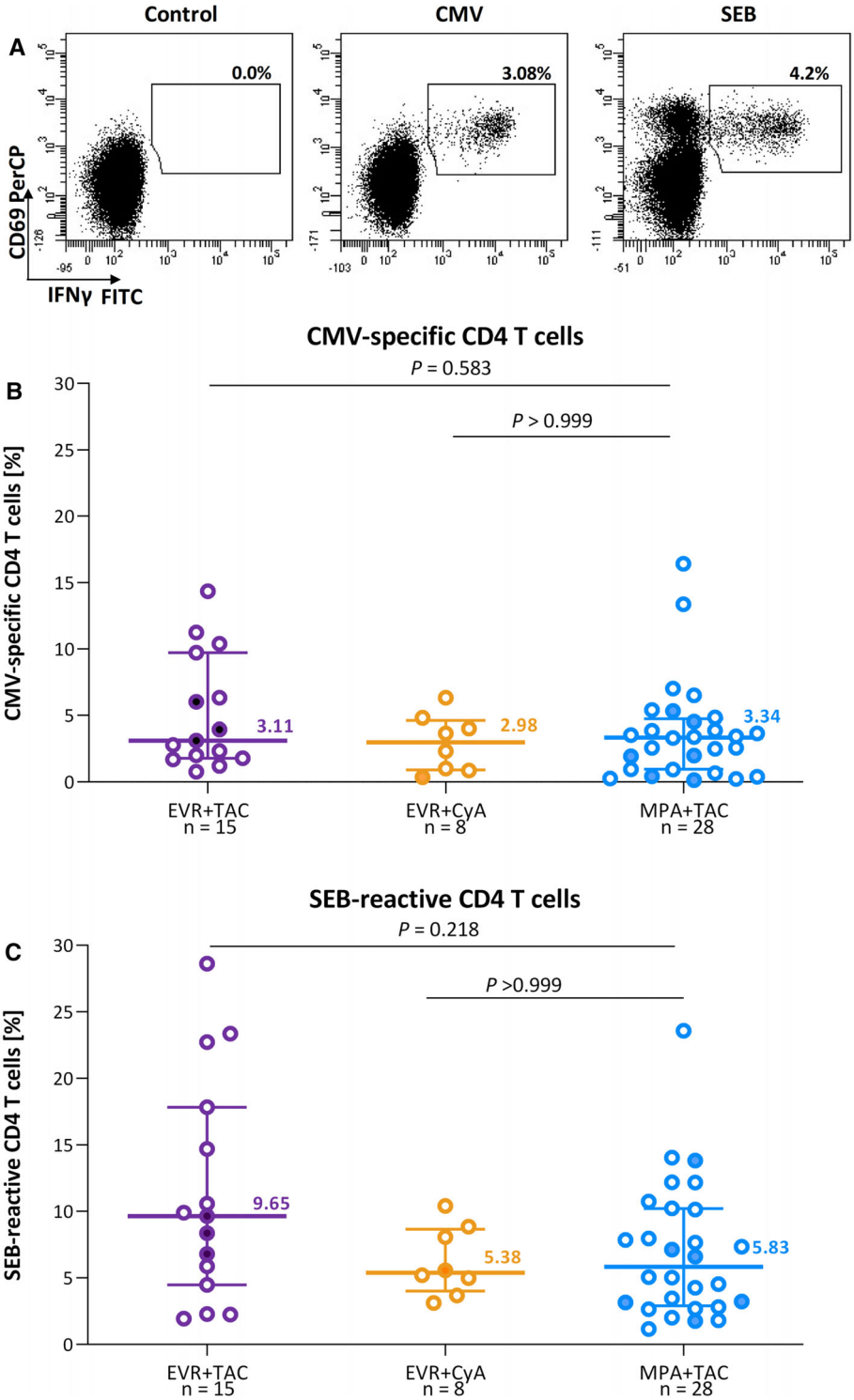

Figure 3. Quantification of CMV- and SEB-reactive $CD4^+$ T cells after specific stimulation. (A) Examples of dotplots of CD69/IFN-γ double positive CD4 T cells after stimulation with control antigen (control), CMV antigen (CMV), and SEB using flow cytometry. The percentage of IFN-γ-expressing CD4 T cells after stimulation with the control antigen was subtracted from the respective percentage after CMV stimulation. Percentages of IFN-γ-expressing CD4 T cells after stimulation with the (B) CMV-antigen and (C) SEB are shown for the on-treatment subgroups who were seropositive by month 12 and who had sufficient T cells for CMV-specific T-cell analyses (n = 51). Seropositive individuals who were seronegative prior to transplantation (D+/R-, seroconverters) are shown as filled symbols, seropositive individuals who were already seropositive prior to transplantation are shown as open symbols. Data in panels B and C are pooled from single experiments of specimens from individual patients. In all panels, the number of patients in each group were as follows: EVR + TAC: n = 15, EVR + CyA: n = 8, MPA + TAC: n = 28. Data are represented as median and IQR. *p* values were determined by the Kruskall–Wallis test followed by Dunn´s post-test for the comparisons between EVR + TAC or EVR + CyA and MPA + TAC arms. A *p* value of 0.05 was considered to be statistically significant. CMV, cytomegalovirus; CyA, cyclosporine A; EVR, everolimus; IFN-γ, interferon gamma; IQR, interquartile range; MPA, mycophenolic acid; SEB, *Staphylococcus aureus* enterotoxin B; TAC, tacrolimus.

signs of anergy and more preserved functionality. Thus, together with potential direct effects of mTOR inhibitors on viral replication by interference with mTOR kinase signaling [2, 28, 29], this immunomodulatory effect of EVR may contribute to a lower incidence of clinically apparent infections.

A protective role of mTOR inhibitors for CMV-complications is supported by the absence of treatment emergent CMV-infections in all patients with EVR-based regimens in the OT population (n = 47). In the Callisto study, EVR was either given de novo or after a delay of 5 weeks. Interestingly, although sample size was low, CMV infections were less frequently observed in the de novo EVR group than in patients with delayed exposure (1.5% [1/65] versus 6.8% [5/74]) [30], but this did not reach statistical significance ($p = 0.215$). Nevertheless, the particular protective

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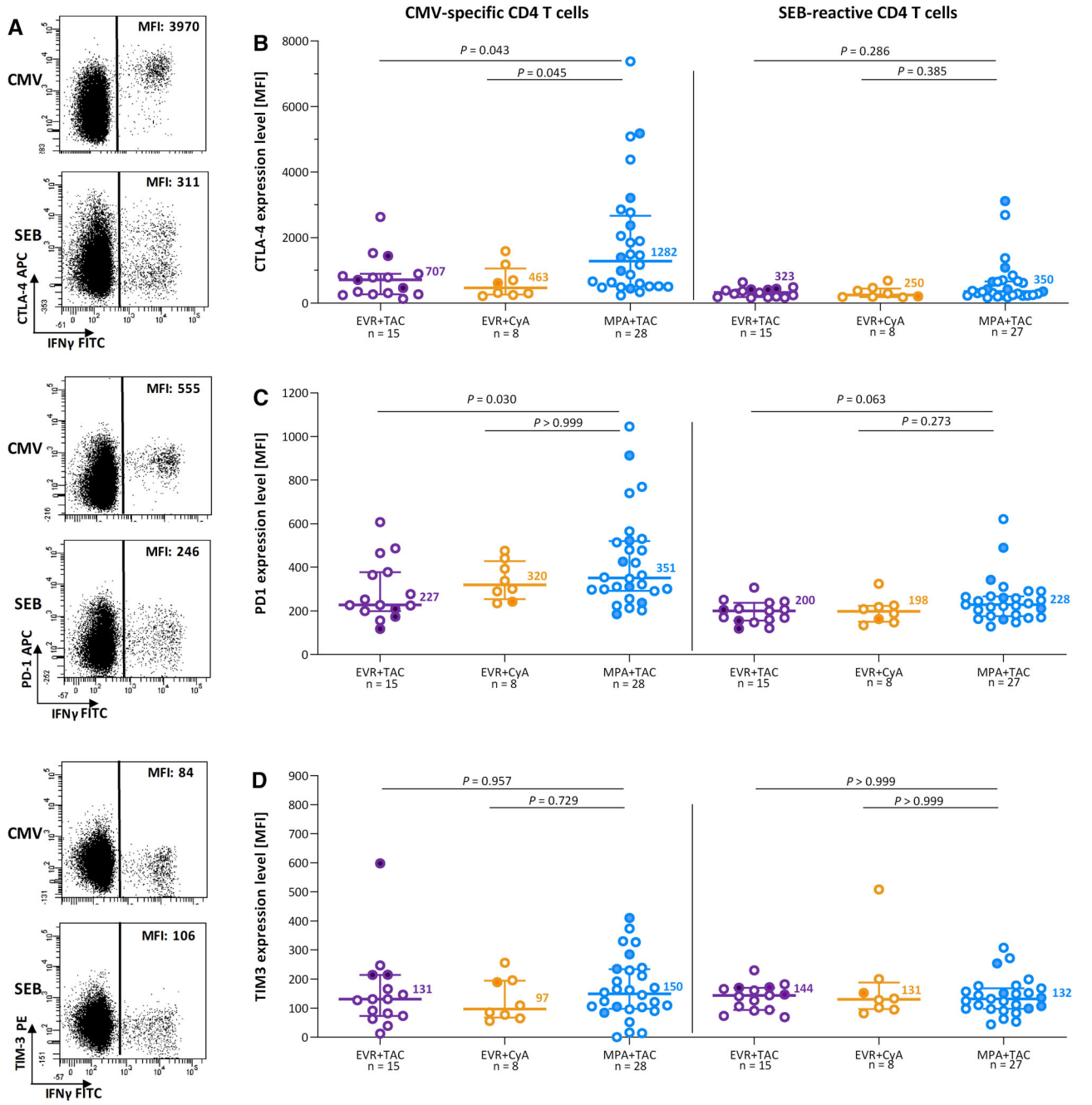

Figure 4. Expression of anergy markers on CMV- and SEB-reactive CD4 T cells. (A) Examples of dotplots of the expression patterns of CTLA-4, PD-1, and TIM-3 are shown for CMV- and SEB-reactive CD4 T cells determined after stimulation with the CMV-antigen or SEB. Expression levels of (B) CTLA-4, (C) PD-1, and (D) TIM-3 on CMV- and SEB-reactive CD4 T cells are results of the on-treatment subgroups who were seropositive by month 12 and who had sufficient T cells for CMV-specific T-cell analyses (n = 51). Seropositive individuals who were seronegative prior to transplantation (D+/R- seroconverters) are shown as filled symbols, seropositive individuals who were already seropositive prior to transplantation are shown as open symbols. Expression levels were determined as median fluorescence intensities (MFI) and displayed as median and IQR. Data in panels B-D are pooled from single experiments of specimens from individual patients. For CMV-specific CD4 T cells, the number of patients in each group for panels B, C, and D were: EVR + TAC: n = 15, EVR + CyA: n = 8, MPA + TAC: n = 28. For SEB-reactive CD4 T cells, the number of patients in each group for panels B, C, and D were: EVR + TAC: n = 15, EVR + CyA: n = 8, MPA + TAC: n = 27). *p* values were determined by the Kruskall–Wallis test followed by Dunn´s post-test for the comparisons between EVR + TAC or EVR + CyA and MPA + TAC arms. A *p* value of 0.05 was considered statistically significant. CMV, cytomegalovirus; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; CyA, cyclosporine; EVR, everolimus; IQR, interquartile range; MPA, mycophenolic acid; PD-1, programmed cell death protein 1; SEB, Staphylococcus aureus enterotoxin B; TAC, tacrolimus; TIM-3, T-cell immunoglobulin mucin-3.

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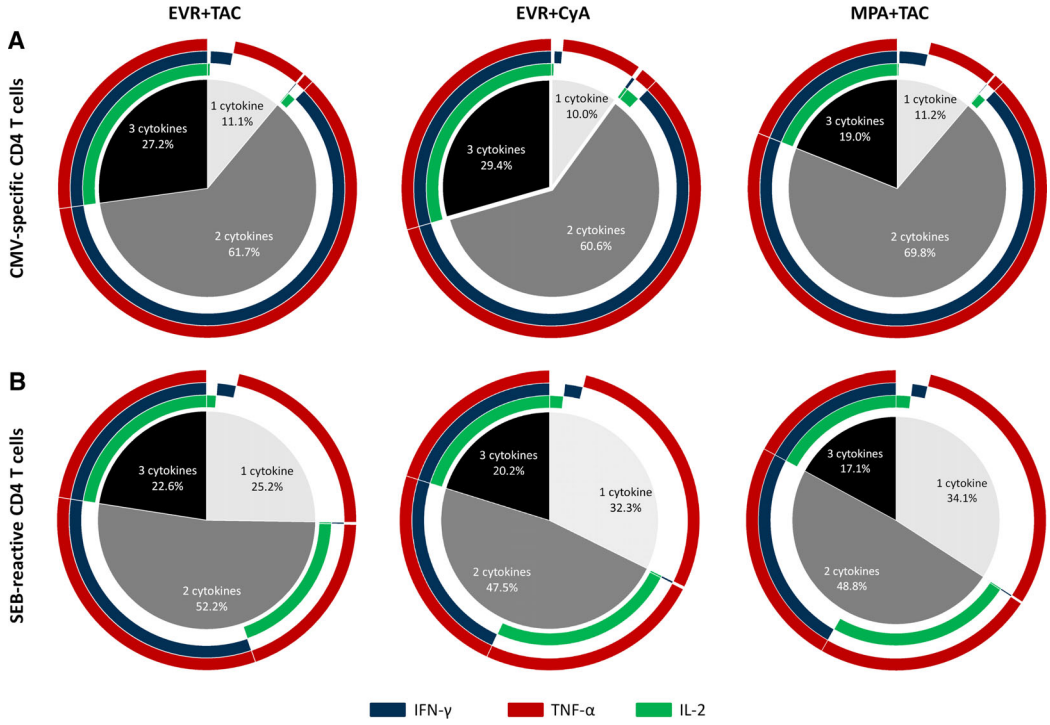

Figure 5. Cytokine expression profiling of CMV- and SEB-reactive CD4 T cells. CD4 T cells from EVR + TAC, EVR + CyA, MPA + TAC were analyzed for expression of IFN-γ, IL-2, and TNF-α after stimulation with (A) CMV antigen and (B) SEB by flow cytometry. Results are derived from the on-treatment subgroups who were seropositive by month 12 and who had sufficient T cells for CMV-specific T-cell analyses (n = 51). Data in panels A and B are pooled from single experiments of specimens from individual patients. Pies show the percentages of cells simultaneously expressing all three cytokines (black), two cytokines (grey) or one cytokine only (light grey). Colored lines denote percentages of cells expressing IL-2 (green), IFN-γ (blue), and TNF-α (red). CMV, cytomegalovirus; CyA, cyclosporine; EVR, everolimus; IFN, interferon; IL2, interleukin 2; MPA, mycophenolic acid; SEB, *Staphylococcus aureus* enterotoxin B; TAC, tacrolimus; TNF, tumor necrosis factor.

effect of EVR may be related to its de novo use, as delayed exposure or discontinuation may increasingly allow CMV infections to emerge. In our study, the majority of clinically apparent infections in patients who switched from EVR treatment occurred after switching, which further indicates that discontinuation of EVR may increase the susceptibility of patients to develop CMV infections. In line with our observation, a single-center retrospective study found that the 1-year incidence of late-onset CMV-disease among D+/R- patients after withdrawal of antiviral prophylaxis was significantly lower in patients receiving de novo EVR with reduced-dose CNI compared with patients on MPA in combination with standard dose CNI [20]. As in our study, the vast majority of CMV-disease cases in the EVR arm occurred after discontinuation of EVR [20]. This is in line with findings from the main ATHENA study cohort, where fewer treatment-emergent CMV infections occurred in the EVR-receiving patients compared with the MPA + TAC group, which held true for both seroconversions and reactivations [26]. Thus, EVR-treatment was not only associated with less CMV-reactivation events in seropositive recipients, but also with less seroconversion processes in CMV seronegative recipients. Our substudy even showed that seroconversions were clinically inapparent in all patients on mTOR inhibitors whereas this was the case in only 3 out of 7 patients on MPA + TAC.

At month 12 after transplantation, no significant differences were observed between treatment arms in the number of total lymphocytes or subsets such as CD4 T cells, CD8 T cells, or Treg. This also held true for CMV-specific T-cell levels, which may be conceivable, given the absence of clinical symptoms at the time of analysis. No comparable studies exist that have analyzed lymphocytes and CMV-specific T cells in patients after treatment with mTOR inhibitors de novo. In a small series of CMV-IgG positive patients who were treated with prednisolone, cyclosporine A and MPA for 6 months, patients were randomized to receive a double drug regimen consisting of prednisolone with either an mTOR inhibitor or cyclosporine A or MPA. No CMV-infection was observed after randomization, yet by month 24, a significant increase in the number of CD8 T cells and CMV-specific CD8 T cells was observed in patients who had switched to an mTOR regimen, but not in those who were continued on cyclosporine or MPA [25]. Unfortunately, no data were reported at month 12 to allow direct comparison of lymphocyte dynamics with our study, where results may still be more directly influenced by an ongoing need for viral surveillance in closer temporal proximity to primary infection or reactivation episodes.

Although overall numbers of various T-cell populations were largely similar between treatment arms, the percentage of T_{regs} was significantly higher in patients on EVR + CyA and numerically higher with EVR + TAC in comparison with the MPA + TAC regimen, indicating a moderate expansion of T_{regs} with EVR, which is in line with studies in mice and humans in vitro and

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in vivo [31–33]. In contrast, treatment with CNIs in the absence of mTOR inhibition is known to result in a relative decrease in T_{reg} frequencies [34]. Studies in humans showed that renal transplant recipients who received sirolimus monotherapy as de novo or maintenance immunosuppression had an increased frequency of T_{regs} compared with patients receiving CyA monotherapy [31]. Furthermore, among liver transplant recipients receiving either EVR or CyA, a higher percentage of T_{regs} was observed with EVR [35]. As the CMV-status has been shown to have an impact on NK-cell subsets [36–38], we also investigated the number of total NK cells as well as major NK cell subsets including $CD56^{bright}CD16^{-}$ NK cells that harbor the CMV-associated $NKG2C^{+}$ NK-cell subset. The overall distribution of these NK cell subsets in our cohort confirmed previous observations of their dynamic changes in kidney transplantation [12, 39]. In line with previous findings [37, 38] remarkably high frequencies of $CD56^{bright}CD16^{-}$ NK cells were identified in all patients; however, no significant differences were detected between the treatment groups.

Studies in patients on a standard immunosuppressive drug regimen have shown that symptomatic CMV disease was associated with an overall loss in CMV-specific T cells that was preceded by a decrease in functionality characterized by an increased expression of inhibitory receptors, a restricted cytokine expression profile, and a decreased level of proliferation. This has been shown to particularly apply to CD4 T cells [3, 5, 40, 41]. To understand the immunomodulatory mechanisms by which EVR prevents CMV infection and reactivation in transplant patients, CMV-specific CD4 T cells in the three treatment arms were assessed for functionality and phenotypical signs of CMV-specific T-cell anergy and/or exhaustion. While the overall percentage of CMV-specific CD4 T cells or polyclonally activated SEB-reactive CD4 T cells were comparable between the three treatments arms, the percentages of multifunctional CMV-specific T cells were numerically higher in the two EVR-treatment arms as compared to the MPA + TAC arm. This may indicate a better preservation of CMV-specific T-cell functionality due to a potential direct antiviral effect of EVR in decreasing the incidence of infection, and/or may suggest a better protection from CMV replication by a combined inhibitory role of EVR and CNIs on T-cell exhaustion. Interestingly, a recent in vitro study performed on PBMCs obtained from healthy volunteers showed that EVR exhibited a moderate inhibitory effect on the secretion of IL2 and IFN-γ in the presence of low-dose CyA and TAC, but this moderate effect was lost at high TAC and CyA concentrations [42]. Mechanistic details whereby mTOR inhibition may preserve CMV-specific CD4 T-cell functionality in contrast to immunosuppression via MPA warrants further study. Interestingly, as exemplified for CD8 T cells, animal models have shown a specific effect of mTOR inhibitor treatment on enhancing pathogen-specific T-cell immunity, whereas alloimmunity was suppressed [43, 44]. This observation may be explained by the notion that pathogen-specific and alloreactive T cells differ in affinity toward their MHC-peptide complex. Thus, unlike an assumed general immunosuppressive effect of MPA, mTOR inhibitors seem to exert a differential effect on T cells with different specificity, as strong TCR signaling has been shown to overcome mTOR inhibitor-mediated inhibition of T-cell functionality [45].

Apart from a more preserved functionality, CMV-specific T cells from patients on EVR showed less phenotypical signs of functional anergy as compared to patients on MPA-TAC, who had a higher expression of CTLA-4 and PD-1. Although not shown in the present study due to limited sample volume, increased expression of PD-1 is associated with impaired proliferative responses [5]. Increased expression of CTLA-4 on antigen-specific T cells was previously shown to be associated with active pathogen replication during primary infection or reactivation [6, 41]. Accordingly, unlike in patients on MPA + TAC, CMV replication was clinically suppressed in the two EVR arms. Interestingly, the increased expression of CTLA-4 in the MPA + TAC group was specific for CMV-specific T cells, as CTLA-4 expression levels on SEB-reactive T cells were similar in the three treatment arms. PD-1 is a member of the CD28 family and represents a negative regulator of T-cell activation. We have previously reported a higher proportion of PD-1-positive CMV-specific CD4 T cells in viremic versus nonviremic transplant recipients [3, 5, 41]. In our study, a significantly higher expression of PD-1 was observed on CMV-specific T cells of patients on MPA + TAC as compared to patients on EVR + TAC, which may indicate that the EVR + TAC regimen possibly prevents CMV-specific T-cell exhaustion. As this effect was not observed in the presence of CyA, the potency of EVR in preventing T-cell exhaustion in combination with the two CNIs needs further investigation in a larger study population. Our results observed in patients in vivo are in line with recent observations of CMV-specific T-cells cultured in vitro in the presence of mTOR inhibitors, which showed evidence of better viability and more potent functionality as compared to nontreated cells [46, 47]. Unlike CTLA-4 and PD-1, TIM-3 expression in our study was comparable in all three treatment arms following stimulation with both CMV and SEB. This absence of difference may be due to the fact that TIM-3 is primarily upregulated upon active viral replication during primary viremia or reactivation [8, 48]. In our study, not all patients experienced primary infection and patients were analyzed at month 12, a time point by which viremia had already been controlled in all cases. Future studies of phenotypical and functional characteristics during primary infection and reactivation may allow assessment as to how EVR differentially impacts development of primary immunity and memory T cells.

A major limitation of the study was the relatively low number of patients available for the substudy evaluation at month 12 and the considerably low proportion of seropositive, OT patients in the EVR arms. Moreover, as per ethical approval, there was no sampling at the end of prophylaxis before onset of viremia and no long-term follow up beyond 12 months. Therefore, the effect of immunosuppression on dynamic changes of the phenotype of CMV-specific T cells before and during CMV reactivations and primary infections, and on long-term follow-up could not be evaluated. Finally, our analysis was restricted to CMV-specific CD4 T cells with a limited number of functional tests. Given the role of CD8 T cells for direct cytotoxicity, it would have been interesting to study a potential drug effect on these cells. However,

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this would have required stimulation by individual or overlapping peptides from immunodominant proteins (such as IE-1 or pp65), thereby resulting in reduced sensitivity and limited applicability to the whole study population. Therefore, longitudinal analyses of both seroconverters and seropositive individuals, including CD8 T cells, with larger sample size would present an interesting area for future research.

In conclusion, the study findings indicate that EVR-treated patients are able to retain CD4 T-cell functionality in CMV-specific T cells, which may explain the enhanced protection against CMV-infections observed in these patients compared with those treated with a standard-of-care regimen. In addition, less viral replication by direct inhibition of mTOR kinase signaling may result in less phenotypical and functional alterations of CMV-specific T cells. Thus, prevention of T-cell anergy and preservation of CMV-specific functionality could represent an additional immunomodulatory effect of EVR that extends beyond its direct interference with CMV replication.

Materials and methods

Study design and patient population

ATHENA was a 12-month, multicenter, randomized, open-label study conducted in de novo renal transplant recipients (NCT01843348; EudraCT number: 2011-005238-21). Recipients of a primary or secondary transplant from a deceased or living donor were eligible for the study, unless loss of the primary graft was due to immunological reasons. Key exclusion criteria were an AB0-incompatible transplant, preexisting donor-specific antibodies, or cold ischemia time >30 h. The study design and endpoints have been described previously [26]. Eligible patients were randomized (1:1:1) pretransplant to receive either EVR + TAC, EVR + CyA, or MPA + TAC [26]. Patients who received study drug and continued on the randomized treatment until end of the study comprised the OT population. The switcher population included patients who discontinued study treatment due to adverse events or other reasons at any time point until month 12. CMV serology was determined at the time of transplantation, and at month 12 in the OT-population. CMV prophylaxis (preferably with valganciclovir) was mandatory for ≥3 months for all patients with CMV seropositive donors. As per protocol, a CMV event was either laboratory-defined (based on DNAemia and/or antigenemia) at month 1, 3, 6, 9, and 12 after transplantation, and/or defined by clinical symptoms (viral syndrome, i.e. fever for the last 2 days, neutropenia, leukopenia, or CMV disease with organ involvement) at any time after transplantation [49]. The incidence of CMV infections as treatment emergent adverse events was determined in all three treatment arms. An adverse event was defined as treatment emergent if it occurred after introduction of the first dose of study medication and within 28 days after last study drug intake. Clinical data from the main study were available for months 3, 6, 9 and 12. Further, as per ethical approval of the substudy, 4.7 milliliters of whole blood was available at 12 months post-transplantation. Blood samples were collected in lithium heparin containing tubes, placed in thermos flasks containing water at 4°C, and shipped to the central laboratory for analysis within 24 h. Recruitment into the substudy and analyses of samples collected for the substudy were performed under blinded conditions ensuring no selection bias.

Immunosuppression

All patients received induction therapy with basiliximab (20 mg on days 0 and 4). EVR was initiated within 24 h of transplantation and maintained at target trough concentration of 3-8 ng/mL throughout the study period. The target trough concentration of TAC in the EVR + TAC and MPA + TAC arms was 4-8 ng/mL until month 2 and 3-5 ng/mL thereafter. In the EVR + CyA arm, the target trough concentration of CyA was 75-125 ng/mL until month 2 and 50-100 ng/mL thereafter. All drugs were from Novartis except TAC which was from Astellas. MPA was used either as enteric coated mycophenolate sodium (Novartis) or Mycophenolate Mofetil (Roche).

Quantitation of T-cell populations

Quantitation of CD4, CD8, and T_{regs} was performed on 100 μL of whole blood as described before [46] using monoclonal antibodies toward CD4 (clone SK3), CD8 (clone RPA-T8), CD25 (clone M-A251, all from BD), and CD127 (clone eBioRDR5; eBiosciences). NK cells were identified using antibodies toward CD3 (clone SK7), CD16 (clone 3G8, all BD), and CD56 (clone N901, Beckman Coulter). Samples were incubated for 20 min. Thereafter, cells were treated with lysing solution (BD). Finally, cells were washed with FACS-buffer (PBS, 5% filtered fetal calf serum, 0.5% BSA, 0.07% NaN3), and analyzed using flow cytometry (FACS-Canto-II) and FACS-Diva-V6.1.3-software (BD). CD4 and CD8 T cells were quantified among lymphocytes, and T_{regs} were quantified as $CD127^{low}$ CD4 T cells expressing CD25. NK cells were quantified as CD3 negative/CD56 positive lymphocytes with CD16 positive/negative and $CD56^{bright/dim}$ subsets. Gating strategies for the identification of cellular subpopulations are provided in Supporting information Fig. S2. Absolute lymphocyte numbers were calculated based on differential blood counts.

Stimulation assays

Whole blood samples were stimulated with CMV-antigen (32 μL/mL; Virion/Serion) to induce antigen-specific activation and cytokine induction as described previously [5]. A noninfected control lysate served as negative control. Although the use of this stimulus mainly elicits CD4 T-cell immunity, it was chosen due to its ability to specifically induce cytokines in all seropositive individuals [50]. Cells were stimulated with 2.5 μg/mL SEB (Sigma) as a positive control for general characteristics of

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antigen-specific T cells. Stimulation was performed from whole blood for 6 h, with 10 μg/mL brefeldin A added after 2 h of incubation. After 6 h, samples were treated with 20 mM EDTA for 15 min; thereafter, cells were fixed using BD lysing solution, and stimulated cells were immunostained using anti-CD4 (clone SK3), anti-CD69 (clone L78), anti-IFNγ (clone 4S-B3), anti-IL2 (clone MQ1-17H12), anti-TNFα (clone MAb11), anti-PD-1 (clone MIH4), anti-TIM-3 (clone 344823), or anti-CTLA4 (clone BNI3). All stains except for PD-1 were performed after fixation. Cells were analyzed using flow cytometry (FACS-Canto-II) and FACS-Diva-V6.1.3-software (BD Biosciences). Gating strategies for the identification of CMV- and SEB-reactive CD4 T cells including their cytokine-profile are provided in Supporting information Fig. S3. Analyses of CMV-specific T cells were restricted to all samples where material was sufficient for all data sets.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism 8.0 software. Fisher's exact test was used to analyze differences between treatment arms for CMV incidence. The unpaired nonparametric Kruskall–Wallis test using Dunn´s post-test was used to compare differences between EVR + TAC or EVR + CyA and MPA + TAC arms for absolute levels of lymphocyte subpopulations, CMV- and SEB-reactive T-cell levels, expression of T-cell anergy markers PD-1, CTLA-4, and TIM-3, and for cytokine expression profiles. A p value of <0.05 was considered statistically significant.

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Author Contributions: SM performed all experiments and primary data analysis, NK-cell evaluation was done by CN and CSF. The main recruiters of patients were CS, BS, OW, FL, and FT. MS and IAH designed the ATHENA substudy. MP and ChS undertook statistical analyses. MS, SM, IAH, and ChS comprised the data analysis and writing team, and received input from the scientific steering committee of the ATHENA main study (BS, IAH, CS, DD, FT, and BN). All authors had access to the study data, assessed the analyses, critically reviewed the manuscript, and approved the final version for publication.

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Abbreviations: **CMV**: cytomegalovirus · **CNI**: calcineurin inhibitor · **CTLA-4**: cytotoxic T-lymphocyte-associated protein 4 · **CyA**: cyclosporine · **D/R**: donor/recipient · **EVR**: everolimus · **FAS**: full analysis set · **IQR**: Interquartile range · **MMF**: mycophenolate mofetil · **MPA**: mycophenolic acid · **mTOR**: mammalian target of rapamycin · **OT**: on-treatment · **PD-1**: programmed cell death protein-1 · **SEB**: *Staphylococcus aureus* enterotoxin B · **TAC**: tacrolimus · **TIM-3**: T-cell immunoglobulin and mucin-domain containing protein 3

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