Homeostasis and function of T cells in healthy individuals and renal transplant recipients
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EVEROLIMUS TREATED RENAL TRANSPLANT RECIPIENTS HAVE A MORE ROBUST CMV-SPECIFIC CD8+ T-CELL RESPONSE COMPARED TO CICLOSPORIN OR MYCOPHENOLATE TREATED PATIENTS

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ABSTRACT

Background. In renal transplant recipients, mTOR inhibitors have been reported to protect against CMV disease. Here, we questioned whether mTOR inhibitors specifically influence human CMV induced T-cell responses.

Methods. We studied renal transplant recipients treated with prednisolone (P), ciclosporin A (CsA) and mycophenolate sodium (MPS) for the first 6 months after transplantation, followed by double therapy consisting of either P and the mTOR inhibitor Everolimus (EVL) (n=10), P/CsA (n=7) or P/MPS (n=9). All patients were CMV-IgG positive prior to transplantation. CMV-reactivation was detectable in the first 6 months after transplantation and not thereafter. None of the patients included in this study suffered from CMV-disease. Both CD27-CD8+ and CD27-CD28-CD4+ effector-type T-cell counts, known to be associated with CMV-infection, were measured before and at 6 and 24 months after transplantation. Additionally, we determined both number and function of CMV-specific CD8+ T-cells at these time points.

Results. The number of total CD8+ T-cells, CD27-CD8+ T-cells and CD28-CD4+ T-cells increased significantly after switch to therapy with P/EVL, but not after switch to P/CsA or P/MPS. Specifically, CMV-specific CD8+ T-cell counts significantly increased after switch to therapy with P/EVL. Furthermore, the mTOR inhibitor sirolimus strongly inhibited allo-responses in vitro, whereas it did not affect CMV-specific responses.

Conclusion. We observed a significant increase in (CMV-specific) effector-type CD8+ and CD4+ T-cell counts in EVL treated patients. These findings may at least in part explain the reported low incidence of CMV related pathology in EVL treated patients.
Mammalian target of rapamycin (mTOR) inhibitors belong to a class of immunosuppressive drugs which is currently used as part of multi-drug regimens in solid organ transplantation 1. Their immunosuppressive effects have long been considered to be due to interference in the cell cycle, leading to inhibition of T-cell proliferation 2. More recently, both in vitro and in vivo experiments have demonstrated that rapamycin (sirolimus) can selectively promote the expansion of regulatory T-cells (Tregs) 3-5, which may partly explain the tolerogenic properties of sirolimus in solid organ transplantation.

Besides immunosuppressive characteristics, mTOR inhibitors also possess other immunomodulatory properties. In several animal models, mTOR inhibition has been demonstrated to play an important role in memory T-cell differentiation. In a murine model of acute lymphocytic choriomeningitis virus infection, sirolimus treatment administered during the expansion and contraction phase of an acute T-cell response increased both quantity and quality of the memory T-cells 6 7. In nonhuman primates, sirolimus treatment appeared to enhance the magnitude, duration and quality of the antigen-specific CD8+ T-cell response after vaccinia virus vaccination 8. Furthermore, we have shown that immunosuppressive therapy, consisting of prednisolone and another mTOR inhibitor everolimus, preserves humoral immune responses and leaves secondary T-cell dependent immune responses after vaccination intact 9.

Clinical observations in renal transplant recipients suggest that, as compared to other immunosuppressive drugs, mTOR inhibitors can protect against CMV-reactivation and –disease 10 11. In the current study, we questioned why patients treated with mTOR inhibitors are relatively protected against CMV disease, whereas graft rejection seems to be adequately prevented. Recently, we performed a randomized prospective trial in which immunosuppression was tapered to double therapy, consisting of prednisolon (P) combined with everolimus (EVL), ciclosporin (CsA) or mycophenolate sodium (MPS) 12. In these three patient groups, we analyzed total and CMV-specific T-cell responses before and at 18 months after the switch to double therapy. Furthermore, we compared the influence of these different immunosuppressive drugs on allogeneic and CMV-specific T-cell responses in vitro.

RESULTS

Patient characteristics and viral reactivation

In CMV-seropositive patients the diagnosis CMV-reactivation is based on the presence of CMV-DNA in the peripheral blood. CMV-disease is defined by CMV-reactivation combined with clinical symptoms. In the current study we analysed renal transplant recipients who were randomized to double treatment with either P/EVL (n=10) or P/CsA (n=7) or P/MPS (n=9) six months after transplantation. We only included patients who were CMV-seropositive prior to transplantation (table 1). These patients do not belong to the high risk-group (D+R-) for developing CMV-disease and were
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Pre-emptively treated with anti-viral therapy once they developed viremia. Patients were screened weekly for the presence of CMV-DNA in the peripheral blood. We noted a high percentage of patients developing CMV-reactivation (mean 92%). Except for one patient, in whom viral reactivation was detected after the withdrawal of CsA and continuation on P/MPS, all patients developed CMV-reactivation prior to the switch to double therapy (mean 59 days SD 68 days). None of the patients analysed in the present study developed CMV-disease.

Table 1. Baseline Characteristics of the studied patients

<table>
<thead>
<tr>
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<th>P/EVL(N=10)</th>
<th>P/CsA(N=7)</th>
<th>P/MPS(N=9)</th>
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<td>Age (yrs. mean ±)</td>
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<tr>
<td>Male gender</td>
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<td>66%</td>
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<td>CMV D-/R+</td>
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<tr>
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<td>100%</td>
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<tr>
<td>Living donor</td>
<td>30%</td>
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</tbody>
</table>

In P/EVL treated renal transplant patients, effector-type T-cell counts significantly increase in time after transplantation.

To investigate whether patients treated with different immunosuppressive reagents, mount different T-cell responses, we studied CD8+ and CD4+ T-cell subset numbers. Before transplantation and randomization to double therapy, we observed no differences in numbers of total and effector-type CD8+ T-cells between the three patient groups (figure 1A and C). Following randomization, we found a significant enlargement of the circulating CD8+ T-cell pool in the P/EVL treated patients (figure 1B) which was not observed in the P/CsA or P/MPS treated patients. Analyzing the different CD8+ T-cell subsets, we found that the increase of the total CD8+ T-cell pool was mainly caused by accumulation of CD27+ effector-type cells (figure 1D). The absolute numbers of CD27+CD45RA+ naive and CD27+CD45RA- memory CD8+ T-cells remained stable in time after transplantation and did not differ within the three groups (data not shown). Total CD4+ T-cell numbers did not change in time following transplantation and were similar in the three patient groups (figure 1E). However, circulating CD28-CD27-CD4+ T-cells significantly increased after the switch to P/EVL double therapy (figure 1F). When comparing the T-cell numbers 24 months after transplantation, we found no significant difference between CD27-CD8+ and CD28-CD27-CD4+ effector-type T-cells in P/EVL as compared to P/CsA and P/MPS treated patients. In summary, CD27-CD8+ and CD28-CD27-CD4+ effector-type T-cells, that have previously been associated with CMV-seropositivity 13-15, significantly increased after the switch to P/EVL.
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Figure 1) In the P/EVL treated recipients the effector-type and total CD8+ T-cells significantly increase in time after transplantation. (A) Analysis of the absolute number of CD8+ T-cells in patients treated with P/EVL (black circles), P/CsA (grey squares) and P/MPS (open triangles), before transplantation (preTX), at 6 months after transplantation, i.e. before randomization to double therapy (6M). Error bars indicate SD. (B) Paired analysis of the absolute number of CD8+ T-cell at 6 months (6M) compared to 24 months (24M) after transplantation. (C) Analysis of the absolute number CD27- effector-type CD8+ T-cells and (D) paired analysis of the absolute number CD27- effector-type CD8+ T-cells at 6 months (6M) compared to 24 months (24M) after transplantation. Longitudinal analysis of the absolute number of (E) total and (F) CD28-CD27- CD4+ T-cells. Statistical analysis was done by Mann-Whitney U test. P < 0.05 = *, P<0.01 = **, P<0.005=***
Renal transplant patients treated with P/EVL show a significant increase in the amount of circulating CMV-specific effector-type CD8\(^+\) T-cells, but not in the percentage of allo-reactive cells.

To investigate the contribution of CMV to the enlarged effector-type CD8\(^+\) T-cell pool, we studied the amount of CMV-specific CD8\(^+\) T-cells by MHC class I tetramer staining. Prior to transplantation and before randomization to double therapy we observed no differences in the number of CMV-specific CD8\(^+\) T-cells between the three groups (figure 2A) but in P/EVL treated patients the CMV-specific CD8\(^+\) T-cells significantly increased after randomization (Figure 2B). A small significant increase was also observed in the P/MPS treated group. Phenotypical analysis demonstrated that in each treatment group CMV-specific CD8\(^+\) T-cells were largely CD27\(^-\) effector-type cells at all analysed time points (Figure 2C). To investigate whether the P/EVL treated patients have more proliferating CMV-specific effector-type CD8\(^+\) T-cells in vivo, we additionally analysed Ki-67 expression. The expression of Ki-67 on CMV-specific, effector-type and total CD8\(^+\) T-cells did not differ between the three patient groups (figure 2D).

Next, we questioned whether the percentages of alloreactive T cells change in time after transplantation. Therefore, we performed mixed lymphocyte cultures (MLC). CFSE labelled recipient cells from P/EVL, P/CsA and P/MPS treated patients at 6 and 24 months after transplantation were stimulated for 6 days with allogeneic fully mismatched third party donor cells. Unfortunately, allogeneic cells from the original transplant-donor were not available. In none of the three patient groups we found a significant alteration in the percentage of alloreactive CD4\(^+\) (figure 2E) or CD8\(^+\) T cells (figure 2F) over time. If anything, we observed a trend toward a decrease in alloresponsive cells in the P/EVL treated group.

mTOR inhibitors strongly inhibit proliferative responses upon allogeneic stimulation, but hardly inhibit proliferation upon virus-specific stimulation in vitro.

In order to assess the effect of the different immunosuppressive drugs on allogeneic versus virus-specific CD8\(^+\) T-cell responses, we performed mixed lymphocyte cultures (MLC) and CMV-peptide stimulations in vitro, in the presence of pharmacological concentrations of sirolimus, ciclosporin or mycophenolate. In a mixed lymphocyte reaction (MLR) naïve but also antigen experienced memory CD8\(^+\) T-cells can respond, whereas after stimulation with CMV-peptide, only the antigen experienced CMV-specific T-cells will proliferate. The inhibition of immunosuppressive drugs on primed memory T-cells and on naïve T-cells may differ. Therefore, we additionally analysed sorted non-naive CD8\(^+\) T-cells in a MLR. The percentage of divided, CFSE negative, total and non-naive CD8\(^+\) T-cells in the MLR was dose dependently reduced by the addition of ciclosporin or mycophenolate (Figure 3 A and B). However, sirolimus strongly inhibited the MLR already at the low concentration of 1ng/ml (Figure 3A and B). On the contrary, sirolimus was hardly capable of inhibiting virus-specific proliferation (Figure 3C). Ciclosporin and mycophenolate dose dependently reduced virus-specific proliferation, although less effectively as in the MLR (Figure 3C).

In summary, sirolimus strongly inhibits in vitro proliferative responses upon allogeneic stimulation, but not upon virus-specific stimulation.
Figure 2) In the P/EVL treated recipients the CMV-specific CD8^+ T-cells significantly increase in time after transplantation. (A) Analysis of the absolute number of CMV-specific, MHC class I tetramer-positive, CD8^+ T-cells in patients treated with P/EVL (black circles), P/CsA (grey squares) and P/MPS (open triangles) before transplantation (preTX), at 6 months after transplantation, i.e. before randomization to double therapy (6M). Error bars indicate SD. (B) Paired analysis of the absolute number of CMV-specific CD8^+ T-cells at 6 months (6M) compared to 24 months (24M) after transplantation. (C) The percentage of CD27^- effector-phenotype cells within the CMV-specific CD8^+ T-cells analysed before transplantation (preTX), at 6 months (6M) and at 24 months (24M) after transplantation. (D) Expression of Ki67 on total, effector-type and CMV-specific CD8^+ T-cells. (E) % of CFSE-negative CD4^+ and (F) CD8^+ T cells following mixed lymphocyte culture with PBMCs drawn at 6M compared to 24M after transplantation. Statistical analysis was done by Mann-Whitney U test. P<0.05 = * and P<0.01 = **
In the present study, we demonstrate that in renal transplant recipients treated with P/EVL, circulating CMV-specific and total CD27-effector-type CD8+ T-cells as well as CD28-CD27-CD4+ effector-type T-cells significantly increase in time after transplantation. Previously, we have shown that CD27-effector-type CD8+ T-cells and CD28-CD27-cytolytic CD4+ T-cells appear as a consequence of CMV infection. Assuming that these effector-type T-cells contribute considerably to the host’s defence against CMV, their increase offers a likely explanation for the low incidence of CMV-reactivation and –disease in mTOR treated patients.

We observed a high percentage (mean 92%) of CMV-reactivation occurring shortly after transplantation, but before randomization to double therapy. Thus, at time of viremia the immunosuppression was equal and as expected there were also no significant differences in viral load between the three patient groups (data not shown). Because there were no differences in viral reactivation after randomization, we were able to study the influence of the three different drug regimens on the CMV-specific immune response independent of the viral-load. Like in our study, previous reports demonstrate that patients pre-emptively treated with anti-viral drugs, have an incidence of CMV-reactivation of up to 90%. None of the patients analysed here developed CMV-disease.
Ki67 is a protein that is expressed in the nucleus of dividing cells. In the mTOR treated patients we observed no increased expression of Ki67 on circulating CMV-specific and effector-type CD8+ T-cells. Thus, increased proliferation in the peripheral blood compartment does not explain the expansion of CMV-specific and effector-type CD8+ T-cells in the mTOR treated patients. Possibly, this expansion takes places in a different lymphoid compartment, for example in lymph nodes.

In animal experiments, the presence of sirolimus during an acute immune response has demonstrated to result in the generation of more memory-phenotype T-cells, which can mount a robust response upon secondary antigen challenge. On the contrary, we observed that CMV-specific CD8+ T-cells, in all three patient groups, have an effector phenotype, which is in agreement with earlier observations. This discrepancy can be explained by differences between the previously described animal models and our present study in man. In our study we analysed an ongoing immune response against a latent viral infection as opposed to the primary immune response studied in mice. It is likely that mTOR inhibition regulates memory cell differentiation during the early phase of the primary immune response. Nevertheless, we can not exclude that mTOR inhibition may influence homeostasis of pre-existing effector-type CD8+ T-cells, nor can we judge the quality and quantity of immune responses taking place in secondary lymphoid compartments. Previously, we have shown that CMV-specific CD8+ T-cells in the lymph nodes predominantly have a memory phenotype. We cannot exclude that mTOR inhibition influences these cells and their recruitment from the lymph nodes during viral reactivation.

The increase in pathogen-specific T-cell responses by mTOR inhibitors in mice and - as described here - also in humans seems in contrast to the effectiveness of these drugs in clinical transplantation. This paradox implies a difference in effect of mTOR inhibitors on pathogen- as compared to allo-antigen-specific T-cell responses. In an in vivo mouse model, sirolimus has been shown to enhance the antigen-specific T-cell response to OVA in the context of a bacterial infection but not to a skin transplant. Here, we extend these findings to the human situation demonstrating that sirolimus appears hardly capable of inhibiting CMV-specific CD8+ T-cell responses, while it strongly inhibits alloresponses in vitro. Furthermore, we showed a decrease in the percentage of alloreactive T cells after switch to double therapy as compared to before. Previous experiments have revealed that by increasing the strength of both the T-cell receptor (TCR) signal and co-stimulatory signals in vitro, anti-proliferative effects of mTOR inhibitors could be overcome. The difference in the effect of mTOR inhibitors on allo- versus CMV-specific responses in vitro may be partly explained by a difference in TCR-signal strength. The affinity of allo-specific T cells might be less than the affinity of CMV-specific T cells for their ligands, which can possibly lead to less potent TCR binding and signalling in the allo-specific T cells, making them more susceptible to mTOR inhibition. Additionally, one can also hypothesize that there are differences in the strength of the co-stimulatory signals in CMV- vs allo-specific T cell responses. Furthermore, as mentioned earlier, in sirolimus treated patients higher numbers of circulating Tregs have been found. These Tregs may specifically inhibit graft specific T-cell responses and to a lesser extent antigen-driven T-cell responses.
Apart from a quantitatively larger T-cell response against CMV, mTOR inhibitors may also influence the quality of this T-cell response. In recent years, the importance of so-called polyfunctional T-cells has been convincingly demonstrated\textsuperscript{24-26}. Here, we studied the effect of mTOR-inhibitors and other immunosuppressive drugs on polyfunctionality of T-cells ex-vivo and in vitro. We found only little influence of the mTOR inhibitor sirolimus on intracellular cytokine production in vitro, whereas ciclosporin exerted profound effects (data not shown).

In conclusion, patients treated with P/EVL mount a quantitatively larger T-cell response against CMV, which offers at least in part an explanation for the reported low incidence of CMV viremia and infection in these patients.

MATERIAL AND METHODS

Patients and study design
We studied T-cell responses in kidney transplant patients who were included in a multicenter randomized trial. This trial was initiated to study the effects of withdrawal of ciclosporin A (CsA) from a triple immunosuppressive regimen containing CsA, Prednisolone (P) and Mycophenolate sodium (MPS) early after transplantation. The immunosuppressive regimen during the first 6 months after transplantation was similar for all patients and consisted of two doses of 20 mg of basiliximab intravenously (IV) before transplantation and on day 4; two doses of 50 mg of prednisolone (IV) during the first 48 hr, followed by 10 mg of oral prednisolone (P) daily; two doses of 720mg Mycophenolate sodium (MPS) from the first postoperative day; and CsA (targeting an AUC\textsubscript{12} of 3250 ug.h/L). After 6-months, patients were randomized in a 1:1:1 ratio to CsA (Target AUC\textsubscript{12} 3250 ug.h/L), MPS (target AUC\textsubscript{12} 35 mg.h/L) or everolimus (EVL) (target AUC\textsubscript{12} 150 mg.h/L). After randomization, all patients continued on 10 mg of prednisolone daily. Pre-transplantation, before conversion to double-therapy and at 24 months after transplantation we analyzed PBMCs from 10 patients who were switched to double treatment with P/EVL, 7 patients with P/CsA and 9 with P/MPS (Figure 1). Table 1 shows their characteristics. All patients gave written informed consent, and the study was approved by the local medical ethics committee.

Viral diagnostics
Quantitative polymerase chain reaction (PCR) for CMV was performed in EDTA whole blood samples, as described earlier \textsuperscript{27}. To determine CMV serostatus, CMV immunoglobulin G (IgG) antibodies were measured in serum using the AxSYM microparticle enzyme immunoassay (Abbott Laboratories, Abbott Park, IL, USA) according to the manufacturer's instructions. Measurements were calibrated relative to a standard serum.

Cell isolation
PBMCs were isolated using standard density gradient centrifugation technique and subsequently cryopreserved until analysis.
Tetramer complexes

HLA-peptide tetramer complexes were provided by A. ten Brinke (Sanquin, Amsterdam, the Netherlands). We used 8 different tetramers, loaded with pp65- and IE 1-derived CMV peptides: HLA-A1 tetramer was loaded with CMV pp65–derived YSEHPTFTSQY, HLA-A2 with CMV pp65–derived NLVPMVATV peptide, HLA-B7 with CMV pp65-derived TPRVTGGGAM peptide, HLA-B35 with CMV pp65-derived IPSINVHHY peptide, HLA-A2 with CMV IE 1-derived VLEETSVML peptide, HLA-B8 with CMV IE 1-derived ELKRKMIYM peptide, HLA-B8 with CMV IE 1-derived ELRRKMMYM peptide, HLA-B8 with CMV IE 1-derived QIKVRVDMV peptide.

Immunofluorescent staining and flowcytometry

500,000 PBMCs were incubated with APC-labeled tetrameric complexes for 30 minutes at 4°C, protected from light. Monoclonal antibodies were added for 30 minutes. The following antibodies were used: CD3 PE AF610, CD8 PE AF610 (Invitrogen, Paisley, UK), CD3 PE Cy7, CD4 AF700, CD8 PE, CD45RA PE Cy7 (BD-Pharmingen, San Diego, CA, USA), CD27 APC AF750, CD127 PerCP Cy 5.5(eBioscience Inc, San Diego, CA, USA). Samples were acquired on a BD FACSCanto. Analysis was done using FlowJo Mac.

Detection of polyfunctional T-cells

Cytokine release after peptide or PMA/Ionomycin stimulation was performed as described by Lamoreaux et al 28, PBMCs and LNMCs were thawed and rested overnight in suspension flasks (Greiner) in RPMI supplemented with 10% FCS, penicillin and streptomycin (culture medium). Two million cells were stimulated with PMA/Ionomycin or with the viral peptides in culture medium in the presence of CD107a FITC (eBioscience), αCD28 (15E8;2μg/ml), αCD29 (TS 2/16;1 μg/ml), brefeldin A (Invitrogen) (10μg/ml) and Golgistop (BD) in a final volume of 200ul, for 4 hours (PMA (10ng/ml)/Ionomycin(1μg/ml)) or 6 hours (peptide) at 37°C 5% CO2. Stimulations were performed in non-treated round bottom 96-well plates (Corning). Subsequently, cells were incubated with the appropriate tetramers followed by incubation with CD3 V500, CD8 V450, CD4 PE-Cy5.5 and Live/Dead fixable red cell stain for 30 min at 4°C. Then, they were washed twice, fixed and permeabilized (Cytofix/Cytoperm reagent, BD) and subsequently incubated with the following intracellular mAbs: IFNγ APC-Alexa Fluor 750 (Invitrogen), TNFα Alexa Fluor 700, IL-2 PE and MIP-1β PE-Cy7 (BD) for 30 min at 4°C. Cells were washed twice and measured on a LSR Fortessa flow cytometer and analyzed with FlowJo software.

Cell sorting

One day prior to cell sorting, PBMCs were thawed and labelled with CFSE. For isolation of nonnaive CD8+ T-cells and CD8- cells, PBMCs were labelled with CD3 PE, CD27 APC (eBioscience), CD8 PerCP-Cy5.5, and CD45RA PE-Cy7 (BD Biosciences). Purity of the obtained sorted cells was verified by flowcytometry and was at least 97%.
Cell cultures in vitro

CFSE labeled PBMCs or sorted cells from healthy donors were cultured in IMDM (Life Technologies, Gaithersburg, MD) containing 10% fetal calf serum, 100U/ml sodium penicillin G (Brocades Pharma BV, Leiderdorp, the Netherlands), 100ug/ml streptomycin sulphate (Invitrogen) and 0.0035% 2-ME (Merck). Allo-responsive T-cells were identified in mixed-lymphocyte culture (MLC) after addition of irradiated stimulator cells from unrelated third party donors in a one-to-one ratio. To the MLC with sorted non-naive CD8\(^+\) T-cells, we added CD8\(^-\) cells from the same donor. CMV-specific CD8\(^+\) T-cells were stimulated with their cognate peptides in the presence of CMV-Ag.

Addition of immunosuppressive drugs to the cell cultures

At day 0 of cell cultures, sirolimus (Rapamune, Wyeth Laboratories, Philadelphia, USA) was added in final concentrations of 1, 10 or 100 ng/ml; ciclosporin (Novartis Pharma AG, Basel, Switzerland) was added in final concentrations of 10, 100 or 1000ng/ml, and mycophenolate (Cellcept, Roche, New Jersey, USA) was added in final concentrations of 100, 1000 or 10000ng/ml. The inactive ingredients in sirolimus solution are Phosal 50 PG, in ciclosporin solution macrogolglycerolricinoleaat and mycophenolate mofetil powder polysorbaat 80. Mycophenolate mofetil powder was dissolved in 5% glucose for intravenous infusion. These inactive ingredients do not influence cell proliferation. Due to practical reasons we used sirolimus instead of everolimus in the in vitro experiments. Because both mTOR inhibitors have very similar working mechanisms this will not have made a difference to the results.

Statistical analysis

Statistical analysis of paired samples was performed by two tailed Wilcoxon signed rank test with a 95% confidence interval. Non-paired samples were analyzed with two tailed Mann-Whitney test with a 95% confidence interval.

REFERENCE LIST

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