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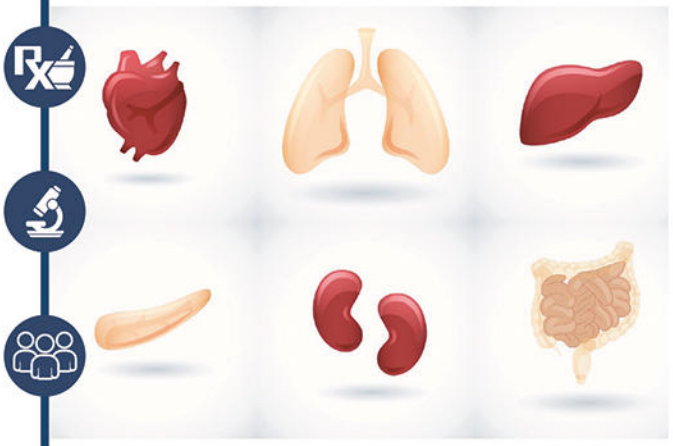

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CMV Late Phase-Induced mTOR Activation Is Essential for Efficient Virus Replication in Polarized Human Macrophages

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Human cytomegalovirus (CMV) remains one of the most important pathogens following solid-organ transplantation. Mounting evidence indicates that mammalian target of rapamycin (mTOR) inhibitors may decrease the incidence of CMV infection in solid-organ recipients. Here we aimed at elucidating the molecular mechanisms of this effect by employing a human CMV (HCMV) infection model in human macrophages, since myeloid cells are the principal *in vivo* targets of HCMV. We demonstrate a highly divergent host cell permissiveness for HCMV with optimal infection susceptibility in M2 but not M1 polarized macrophages. Employing an ultrahigh purified HCMV stock we observed rapamycin-independent viral entry and induction of IFN-β transcripts, but no proinflammatory cytokines or mitogen-activated protein kinases and mTOR activation early after infection. However, in the late infection phase, sustained mTOR activation was observed in HCMV-infected cells and was required for efficient viral protein synthesis including the viral late phase proteins pUL-44 and pp65. Accordingly, rapamycin strongly suppressed CMV replication 3 and 5 days postinfection in macrophages. In conclusion, these data indicate that mTOR is essential for virus replication during late phases of the viral cycle in myeloid cells and might explain the potent anti-CMV effects of mTOR inhibitors after organ transplantation.

Key words: Cytomegalovirus, interferon-beta, macrophages, rapamycin, renal transplantation

Abbreviations: ELISA, enzyme-linked immunosorbent assay; HCMV, human cytomegalovirus; IE-1, immediate early protein-1; IFN-β, interferon-beta; MNGCs, multinucleated giant cells. mTOR, mammalian target of rapamycin; PFU, plaque forming unit; PI3K, phosphoinositide-3 kinase; TSC2, tuberous sclerosis complex 2.

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Introduction

In transplantation medicine, human cytomegalovirus (HCMV) infection is associated with increased acute and chronic allograft rejection, opportunistic infections and reduced graft and patient survival (1–4). Without antiviral prophylaxis the majority of renal transplant patients would develop CMV infections (5–7). The high seroprevalence of CMV makes reactivation from latency more frequent than *de novo* infections (8,9).

CMV displays a broad target cell tropism including epithelial cells, endothelial cells, fibroblasts and different hematopoietic cells (10,11). The tropism of CMV for myelomonocytic cells is essential for the viral immune escape mechanisms mediated by effects on various immunological effector functions (12–16). Further, monocytes and macrophages are tightly involved in the reactivation of CMV from latency, which is dependent on the state of macrophage differentiation (17). While monocytes are targets for latent CMV infection, macrophages sustain viral replication *in vitro* and *in vivo* (18–20). Macrophages are found in a variety of different phenotypes in the body playing a fundamental role in immune-mediated renal disease, host defense, but also renal allograft responses (21). Macrophage differentiation depends on the cellular microenvironment resulting in distinct phenotypes ranging from proinflammatory M1-type to tissue-remodeling M2-type macrophages (22). Recent evidence suggests that HCMV reprograms monocytes to acquire a proinflammatory M1 phenotype to facilitate viral spread (23). Currently, however, it is unclear, whether macrophage subsets differ in their susceptibility to CMV infection.

mTOR exists in two complexes, mTORC1 and mTORC2, whereas inhibition of mTORC1 is part of current

immunosuppressive protocols. Recent evidence from various studies consistently demonstrated reduced CMV infections with mTORC1 inhibitor-based immunosuppressive therapy both when employed *de novo* as part of an immunosuppressive regimen and after conversion to an mTOR inhibitor (24–26). However, the detailed molecular mechanisms responsible for these beneficial antiviral effects have not been elucidated so far.

CMV has a low replicative cycle and must deal with the inhibitory effects of the stress response associated with viral entry. Hence CMV activates the PI3K-Akt-mTOR pathway including the mTORC1 downstream effectors S6 ribosomal protein and the translation factor 4E-BP-1 (27,28). Further, CMV was reported to encode mTORC1-activating proteins like pUL38 blocking TSC2, which negatively regulates mTOR, thereby providing survival signals for infected cells (29,30). Additionally, CMV induces a substrate-specific alteration of mTORC1 activity, since CMV-infected fibroblasts display a resistance against the inhibitory effects of rapamycin enabling hyperphosphorylation of 4E-BP1, while S6 phosphorylation remains rapamycin sensitive (28). Recently, Raptor, an essential component of the mTORC1 complex, was demonstrated to be essential for viral protein synthesis in fibroblasts (27), while in other cell types HCMV even blocked the PI3K-Akt-mTOR pathway possibly to avoid detrimental effects for the virus (31). In addition, rapamycin was reported to be ineffective in the inhibition of CMV replication and protein synthesis in fibroblasts (11,28,32).

Here we report that HCMV is able to productively infect human M2 polarized macrophages in an mTOR-dependent manner, while M1 polarized macrophages do not represent suitable host cells to support HCMV replication *in vitro*. Activity of mTOR was induced at late but not early time points by HCMV infection and was an essential prerequisite for successful CMV replication in human M2 macrophages. Given the general importance of the monocyte/macrophage system for HCMV persistence, suppression of viral replication by mTOR inhibition at late phases of the viral cycle might explain the beneficial effects of mTOR inhibitors on CMV incidence after allogeneic transplantation.

Materials and Methods

Cell isolation and culture

Human monocytes were purified from peripheral blood mononuclear cells as described (33). RPMI 1640 (Gibco BRL, Carlsbad, CA, USA) supplemented with 2 mM L-glutamine, 100 μg/mL streptomycin, 100 U/mL penicillin and 10% fetal calf serum (FCS; Hyclone, South Logan, UT, USA) was used as culture medium. Primary human foreskin fibroblasts (HFF) were used for experiments between passages 5 and 12 and were cultured in Dulbecco's Modified Eagle Medium (Gibco BRL, Grand Island, NY, USA) supplemented with 1.75 g glucose/500 mL, 2 mM L-glutamine, 100 μg/mL streptomycin, 100 U/mL penicillin and 10% FCS. Macrophages were cultured in serum-free medium (SFM; Macrophage SFM, Gibco) supplemented with 2% FCS, 100 μg/mL streptomycin and 100 U/mL penicillin. Rapamycin (Sigma Deisenhofen, Germany) treatment was performed for 90 min at a concentration of 100 nM with DMSO as a solvent control in all untreated samples.

Macrophage differentiation and polarization

CD14+ monocytes were suspended in RPMI 1640 medium supplemented with 10% FCS at a density of 7.5×10^5 cells/mL. Recombinant human M-CSF (Peprotech, Rocky Hill, NJ, USA) was added to a final concentration of 50 ng/mL, and 4 mL of the cell suspension (3×10^6 cells) were seeded per six wells. Cells were allowed to differentiate for 6 days. After the differentiation period, cells were gently resuspended and washed once with prewarmed macrophage SFM. Cell density was adjusted to 1.7×10^5 cells/mL before seeding to different well sizes at a density of 300 μL/cm^2. After allowing cells to settle and adhere for 1 h at 37°C, polarization was induced by setting final concentrations of 1 μg/mL LPS (*Escherichia coli* 0111:B4, Sigma) in combination with 20 ng/mL IFN-γ (R&D #285-IF) (M1 polarization) or 20 ng/mL IL-4 (Peprotech #200-04) (M2 polarization). After 24 h, polarization medium was removed and adherent cultures were washed once with prewarmed macrophage SFM followed by addition of fresh prewarmed macrophage SFM medium. After another 24 h of resting, cells were infected with virus as described.

Macrophage infection

For short time incubations (up to 48 h), virus stock dilutions were added to rapamycin or solvent-treated cultures to reach 10 plaque forming units (PFUs)/cell followed by subsequent incubation for the indicated periods. For long-term infections, virus was added to the cultures at 10 PFUs/cell and removed after an adsorption phase of 2 h at 37°C by washing twice with prewarmed macrophage SFM. Finally, macrophage SFM supplemented with 2% FCS followed by addition of 100 nM rapamycin or solvent was added.

Virus propagation and virus stock preparation

HCMV strain TB-40/E was kindly provided by U. Koszinowski and propagated for a maximum of 10 cycles in HFF cells by infecting 80% confluent HFF cultures with an M.O.I. (Multiplicity of Infection) of 0.1 PFU/mL. Virus stocks were obtained by employing a protocol adapted from Chen and Pagano (34) to reach a sufficient high purity for macrophage experiments. After the cytopathic effect in the propagation culture reached 100% between day 10 and 12, supernatant was precleared from cellular debris by 1 h centrifugation at 4°C and 4500 g. Supernatant was carefully applied onto a precooled 30% iodixanol cushion ($\rho = 1.178$ g/mL) in ultracentrifuge tubes (SW-28, Beckman Coulter, Indianapolis, IN, USA). The iso-osmotic density cushion was obtained by dilution of Visipaque™ (Amersham Pharmacia Biotech, Uppsala, Sweden) in PBS. Following centrifugation for 1 h at 4°C (23 000 rpm, 100 000 g), culture medium was removed by aspiration followed by an additional overlay of the iodixanol cushion with PBS in order to prevent potential contaminations of the virus stock with culture medium components. After completely removing PBS and the iodixanol cushion by aspiration, the virus pellet was resuspended in 2 mL of macrophage SFM. Single use aliquots were snap frozen in liquid nitrogen and stored at –80°C until use. Virus stock inactivation was performed either by 100 kDa molecular weight cut-off filtration or by exposure to UV light for 5 min using a transilluminator.

An isolate of HCMV was obtained from a renal transplant patient with HCMV disease and freshly used for the infection of macrophage cultures without passaging in fibroblasts. Viral genomes were quantified by reverse-transcriptase polymerase chain reaction (RT-PCR) yielding 5×10^6 copies/mL plasma.

Virus titer assays

PFUs per milliliters were determined by infecting HFF monolayers with serial dilutions of virus stocks and culture supernatants in 96-well triplicates as described (35).

ELISA

Cytokines were measured from cell-free supernatants by Luminex assays from R&D Systems (Minneapolis, MN, USA). IFN-β was determined with an ELISA kit (VeriKine Human IFN Beta Elisa Kit, PBL).

Quantitative RT-PCR

Macrophages were cultured in 6-well plates and pretreated as indicated followed by infection with TB-40/E or inactivated virus. Total RNA was extracted in TRIzol 6 h postinfection and cDNA was generated by Superscript II (Invitrogen, Carlsbad, CA, USA) as suggested by the manufacturer. mRNA levels were determined by TaqMan Gene Expression Assays (Applied Biosystems, Carlsbad, CA, USA) on an ABI Prism 7000 in a multiplex PCR. IFN-β mRNA levels were normalized to 18S rRNA.

The number of HCMV genomes per milliliter of culture supernatant was determined by a quantitative real-time PCR assay, as previously published (36).

Western blot analysis

Extract preparation and immunoblotting was done as described (33). Antibodies used were against IE-1 (NEA-9221), pUL-44 (CMV ICP36 Monoclonal Antibody, Virusys, Taneytown, MD, USA), GAPDH, TF-II/D (TBP (N-12), sc-104, Santa Cruz, Santa Cruz, CA, USA), Histone-H3 (Cell Signal, Danvers, MA, USA), S6- (54D2, #2317, Cell Signal), p-S6(240/244) (#2215, Cell Signal), p-ERK1/2 ((Tyr204) (sc-7383, Santa Cruz), p-p38 (Thr180/Tyr182) (#9211, Cell Signal), IκBα (#9242 Cell Signal), according to recommendations on datasheets.

Immunocytochemistry

Macrophages were cultured in 8-well Permanox™ chamber slides (Lab-Tek Chamber Slide System 177402). Staining was performed as described [36]. Antibodies against p-S6(240/244) (Cell Signal), IE-1 (NEA-9221) and pp65 (Clonab-CMV) were used at 1:50 dilutions. Secondary antibodies (GAR Alexa Fluor 488 F(ab')$_2$ fragment of goat antirabbit IgG (H+L) 2 mg/mL Invitrogen, GAM Alexa Fluor 594 F(ab')$_2$ fragment of goat antirabbit IgG (H+L) 2 mg/mL Invitrogen) were used at a 1:200 dilution. Images were taken on an Olympus Provis AX-70 fluorescence microscope equipped with a digital camera (Olympus XC-50).

Infection efficacy of CMV in macrophages

Macrophage cultures were stained for IE-1 antigen and Hoechst-33342. Infection efficacy was determined by counting the number of IE-1-positive cells in a field of vision containing at least 100 cells. The number of IE-1-positive cells per field of vision was related to the number of total cells (Hoechst-33342 positive) giving the percentage of infected cells per field of vision. Three fields of vision were counted followed by calculation of the arithmetic mean for an individual experiment.

Statistics

Cytokine levels, RNA levels and virus titers were compared using Student's *t*-test. In all experiments, differences were considered statistically significant at $p < 0.05$.

Results

Macrophage subtype differentiation defines susceptibility to HCMV infection

Monocyte-derived macrophage subsets play a fundamental role in the allograft response, however, it is unknown whether different subsets influence CMV dissemination and persistence. Hence, we generated M1 (LPS plus IFN-γ) and M2 (IL-4) macrophages and evaluated their permissiveness for CMV TB-40/E infection by assessing viral immediate early protein expression (IE-1). While M2 macrophages could be efficiently infected, a significantly decreased IE-1 expression was seen in M1 macrophages (Figures 1A–C). Interestingly, a strong difference in HCMV susceptibility was already existent only 4 h after macrophage polarization (Figure 1D). We used UV-inactivated virus as well as molecular weight cut-off filtrated virus stock as negative controls for our experiments. Both inactivation procedures completely abrogated CMV infection (Figure 1D and Figure S1).

To further examine M2 macrophage-specific CMV infection by a virus stock that was not propagated from human fibroblasts, we coincubated fresh plasma from a CMV-infected viremic renal transplant patient with M2 macrophages at 10 genomes per cell. Remarkably, after 3 weeks of culture, CMV-positive cells were identified by typical morphological changes and nuclear inclusions demonstrating that patient-derived CMV successfully infects and replicates in M2 macrophages (Figure 1E).

HCMV does not induce cytokine responsiveness and virus entry-associated signaling in M2 macrophages

As CMV was reported to activate inflammatory cytokine and chemokine expression in several innate immune cell types, we assessed IL-6, IL-10, IL-12 and TNF-α production in monocytes and macrophages. As both, monocytes and macrophages express functional Toll-like receptors (TLR), LPS stimulation was used as a positive control for functional cytokine responses in our cultures. In contrast to LPS stimulation, after HCMV infection for 24 h neither monocytes nor M2 macrophages produced significant amounts of any of the cytokines tested (Figures 2A and B). In line with the cytokine phenotype we could not identify an induction of mitogen-activated protein kinases or NF-κB, important mediators of cytokine signaling (Figure 2C). Similarly, we found no activation of the mTOR pathway as assessed by phosphorylation of the ribosomal protein S6 during the early phase of HCMV infection in M2 macrophages (Figure 2C).

HCMV infection of M2 macrophages culminates in an mTORC1-independent type-I interferon response

Next, we investigated whether the type-I interferon response, which is the prototypical antiviral immune response, is activated in M2 macrophages; HCMV potently induced IFN-β in M2 macrophages while UV-inactivated virus failed to induce an IFN-β response (Figures 3A and B) suggesting that only live HCMV induces a type-I interferon response in M2 macrophages. Interestingly, rapamycin neither affected IFN-β mRNA transcription nor IFN-β protein expression (Figures 3A and B). There was no induction of IFN-α1 detected following HCMV infection of M2 macrophages (data not shown). These results

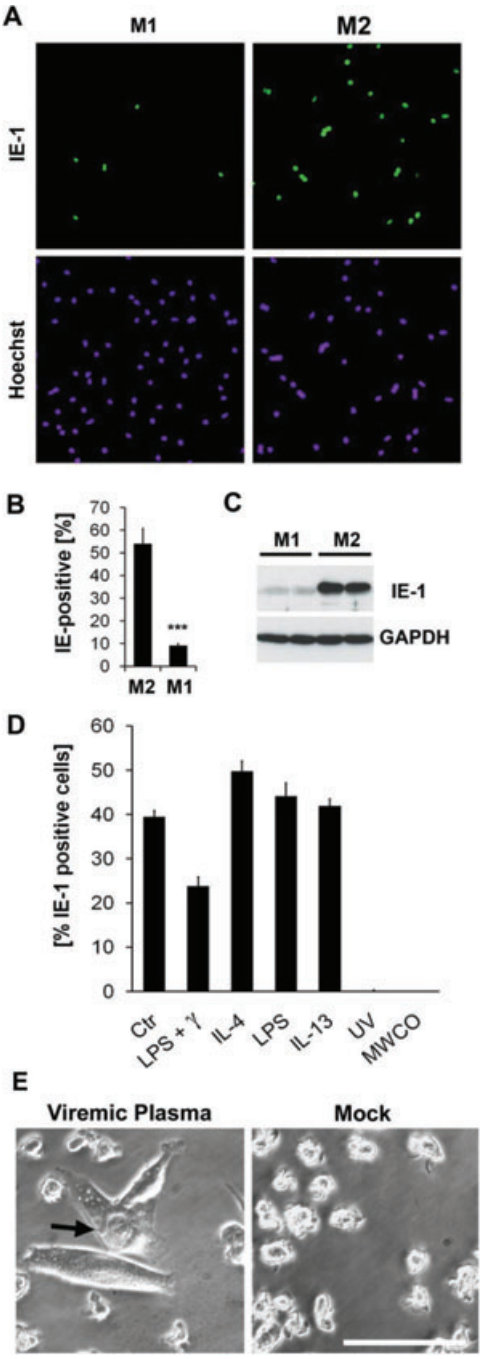

Figure 1: Infection susceptibility of M1 versus M2 macrophages by CMV. (A) M-CSF differentiated macrophages were generated as described in Materials and Methods. Representative image showing differences in TB-40/E infection efficacy between M1 and M2 macrophages with 10 PFU/mL 24 h postinfection. (B) The percentage of IE-1-positive cells of CMV-infected M1 and M2 macrophages was counted and the mean ±SEM is shown for four independent experiments. (C) CMV-infected M1 and M2 macrophages were analyzed 24 h postinfection for IE-1 expression by immunoblot. A representative experiment out of three is depicted. (D) M-CSF differentiated macrophages were either co-incubated with LPS (1 μg/mL), IL-4 (20 ng/mL), IL-13 (20 ng/mL) or LPS+IFNγ (1 μg/mL + 20 ng/mL). After 4 h, 10 PFU/cell of TB-40/E was added per well and allowed to attach to cells for 2 h followed by a change of culture medium; 24 h postinfection, cells were fixed and three independent experiments were analyzed for the percentage of IE-1-positive cells as described in the Methods section. (E) Photomicrographs showing M2 macrophages 3 weeks after 1 h of co-incubation with plasma drawn from a CMV viremia patient and control plasma (Mock) diluted 1:10 in serum-free culture medium or control macrophages (white bars: 50 μm).

collectively indicate that M2 macrophages selectively produce an mTORC1-independent type-I interferon but not a typical proinflammatory cytokine response upon HCMV infection.

Rapamycin efficiently blocks CMV replication in M2 macrophages

Analyzing the effects of mTORC1 inhibition at later time points of the viral replication cycle, we found that rapamycin significantly suppressed CMV replication in M2 macrophages on days 3 and 5 postinfection, while CMV proliferation in fibroblasts was only moderately decreased by mTORC1 inhibition on day 5 but not on day 3 (Figure 4A). RT-PCR analysis of infected culture supernatant on day 5 post-CMV infection revealed that the inhibition of CMV proliferation reached a maximum already at 10 nM rapamycin (Figure 4A, right-hand side), which is in the range of physiologic levels reached during patient treatments with the immunosuppressant. HCMV-infected M2 macrophages remarkably increased their cell motility indicated by extensive cell polarization, which was decreased by rapamycin (Figure 4B). While CMV-positive cells could be identified via CMV-specific nuclear inclusions (Figure 4B, arrows), rapamycin strongly reduced number and size of these nuclear inclusions (Figure 4B). Furthermore, the expression of the late phase viral protein pUL44 was inhibited by rapamycin in a concentration-dependent manner, as indicated by western blot analysis of infected cultures on day 5 postinfection with CMV (Figure 4C, right-hand side). As shown in Figure 4C (left-hand side), rapamycin reduced the number of CMV-infected and adhering cells indicated by weaker signals for Histone-H3 and GAPDH at days 5 and 7 postinfection.

Early phases of CMV infection in macrophages are independent of mTORC1 activity

The suppressive effect of rapamycin on CMV replication suggested that mTORC1 might be critical for CMV infection. Therefore, we systematically assessed viral gene expression and mTORC1 activity during the course of M2 macrophage infection. mTORC1 activation was not associated with IE1 gene expression in infected cells 24 h and 48 h postinfection (Figure 5A). Infected cells, which could be easily identified by nuclear expression of the viral antigen IE-1, did not show increased levels of

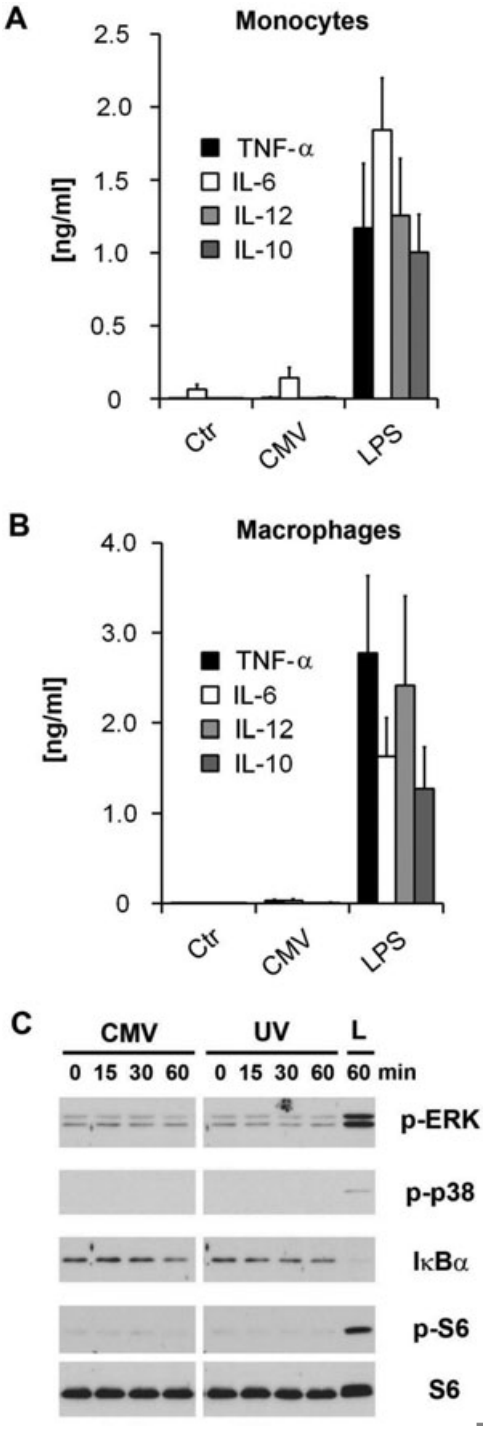

Figure 2: Effect of CMV infection on cytokine production and early signaling events in monocytes and M2 macrophages. CD14+ monocytes or M2 macrophages were infected with TB-40/E or stimulated with LPS for 24 h. TNF-α, IL-6, IL-12 and IL10 were determined in the supernatants by luminex analysis. The mean cytokine concentration ±SEM in cultures of monocytes (A, n = 4) and macrophages (B, n = 5) is shown. (C) M2 macrophages were incubated with CMV, UV-inactivated virus stock (UV) and LPS for the indicated time periods and whole-cell lysates were analyzed by immunoblotting. The experiment is representative of three independent donors.

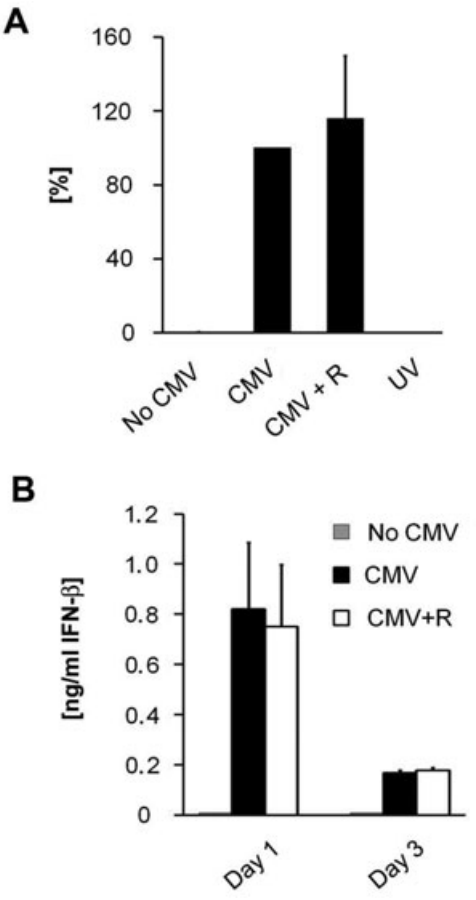

Figure 3: CMV-mediated type I-interferon induction in M2 macrophages. (A) mRNA was prepared from M2 macrophages following 6 h of co-incubation with HCMV, HCMV plus 100 nM rapamycin (CMV+R), UV-inactivated virus (UV) and medium alone. mRNA levels were analyzed by quantitative RT-PCR and are shown as the mean ± SEM relative to HCMV-infected sample (n = 3). (B) M2 macrophages were either infected with CMV or mock infected and cultured in the absence and presence of 100 nM rapamycin. Supernatants were analyzed for IFN-β secretion at the indicated time points. Results are expressed as the mean ± SEM of four independent donors (n = 4).

ribosomal protein S6 phosphorylation compared to uninfected cells in the cultures. Moreover, rapamycin did not affect the infection efficiency of macrophages as indicated by similar percentages of IE-1-positive cells at 24 h and 48 h postinfection in the presence and absence of rapamycin (Figure 5B). Immunoblot analysis further established that the expression of IE-1 is independent of mTORC1 and that a complete loss of IE-1 protein signals occurs between day 1 and day 4 after macrophage infection (Figure 5C).

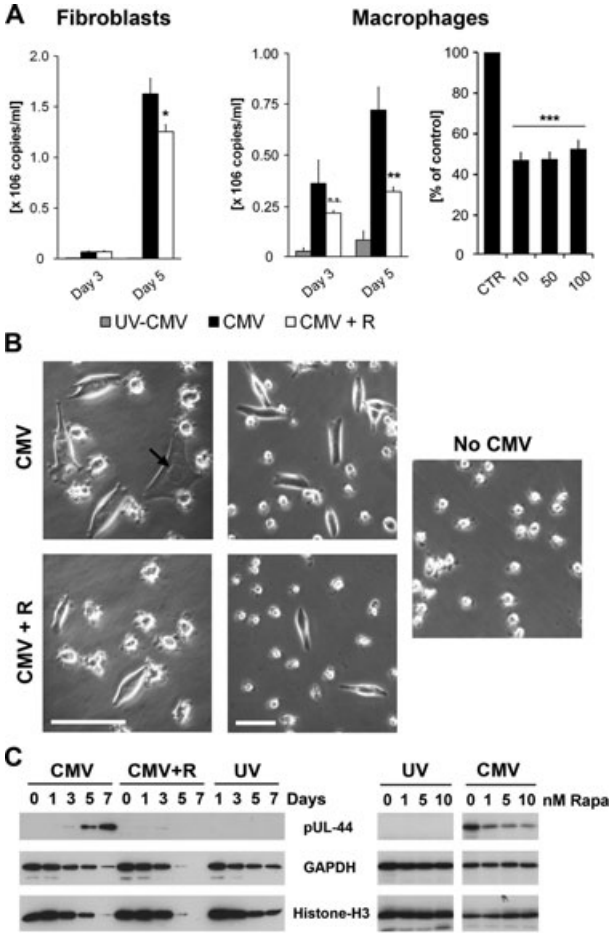

Figure 4: The effect of rapamycin on CMV replication and CMV-induced cytopathology in M2 macrophages. (A) M2 macrophages and fibroblast (HFF) cells were treated with UV-inactivated CMV (gray bars) or infected with TB-40/E in the absence (black bars) or presence (white bars) of 100 nM rapamycin. Supernatant aliquots were taken on day 3 and day 5 postinfection and virus copy numbers were determined by RT-PCR (n = 4) (left). Indicated concentrations of rapamycin were added to macrophage cultures immediately following infection with CMV. Supernatants were analyzed for virus copy numbers following 5 days of culture in the presence of rapamycin (right). (B) Photomicrographs of representative cultures of M2 macrophages infected with 10 PFU/cell TB-40/E after 5 days in the presence or absence of rapamycin are depicted in low (left) and high (right) magnification (white bars: 50 μm). (C) M2 macrophages were incubated with CMV, UV-inactivated virus stock (UV) and LPS and whole-cell lysates were analyzed by immunoblotting at the indicated time points (left). Expression of pUL-44, GAPDH and Histone-H3 in infected (CMV) and mock-infected (UV) cells were analyzed following treatment with indicated concentrations of rapamycin (right) for 5 days. The experiments are representative of three independent donors.

Similarly, in multinucleated giant cells (MNGCs) that develop in macrophage cultures, there was no correlation between IE-1 expression and cellular mTORC1 activity assessed by S6 phosphorylation (Figure 5D).

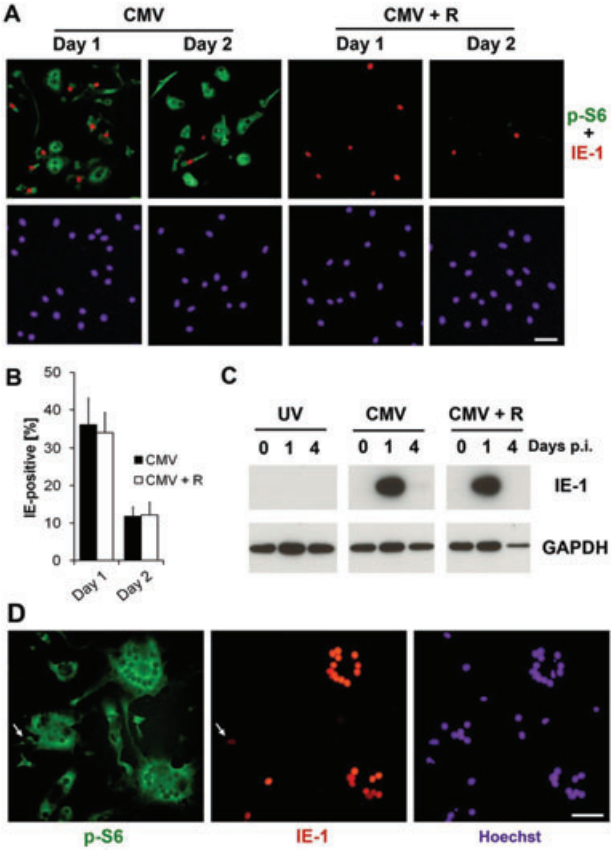

Figure 5: mTORC1 activity in early phases of macrophage infection by TB-40/E. (A) After treatment with rapamycin (CMV+R) or solvent control (CMV), M2 macrophages were infected with TB-40/E. Cultures were fixed at the indicated time points postinfection and double stained for p-S6 (green) and IE-1protein (red). Hoechst-33342 channel is shown below to control cell numbers in the fields of vision (white bar: 50 μm). (B) The percentages of IE-1-positive cells at day 1 and day 2 postinfection are given comparing solvent control (black) and 100 nM rapamycin (white)-treated M2 macrophage cultures by determination of the infection rate in three independent experiments. (C) Analysis of IE-1 expression by western blot on day 0, day 1 and day 4 postinfection of M2 macrophages with UV-inactivated TB-40/E (UV), living TB-40/E (CMV) and living TB-40/E in the presence of 100 nM rapamycin (CMV+R). (D) Representative immunocytochemistry staining for p-S6 (green) and IE-1 (red) on day 1 postinfection showing MNGCs developing in M2 macrophage cultures during polarization and infection (white bar: 50 μm).

Late phase CMV infection is linked to enhanced mTOR activation

Our experiments suggested that pUL-44 protein synthesis and HCMV replication are affected by rapamycin in M2 macrophages, while early phases of the HCMV infection/replication cycle were found to be independent of mTORC1 in M2 macrophages. mTORC1 is essential for various cellular functions including cell growth, protein synthesis and cell motility (37–39). Especially late phases

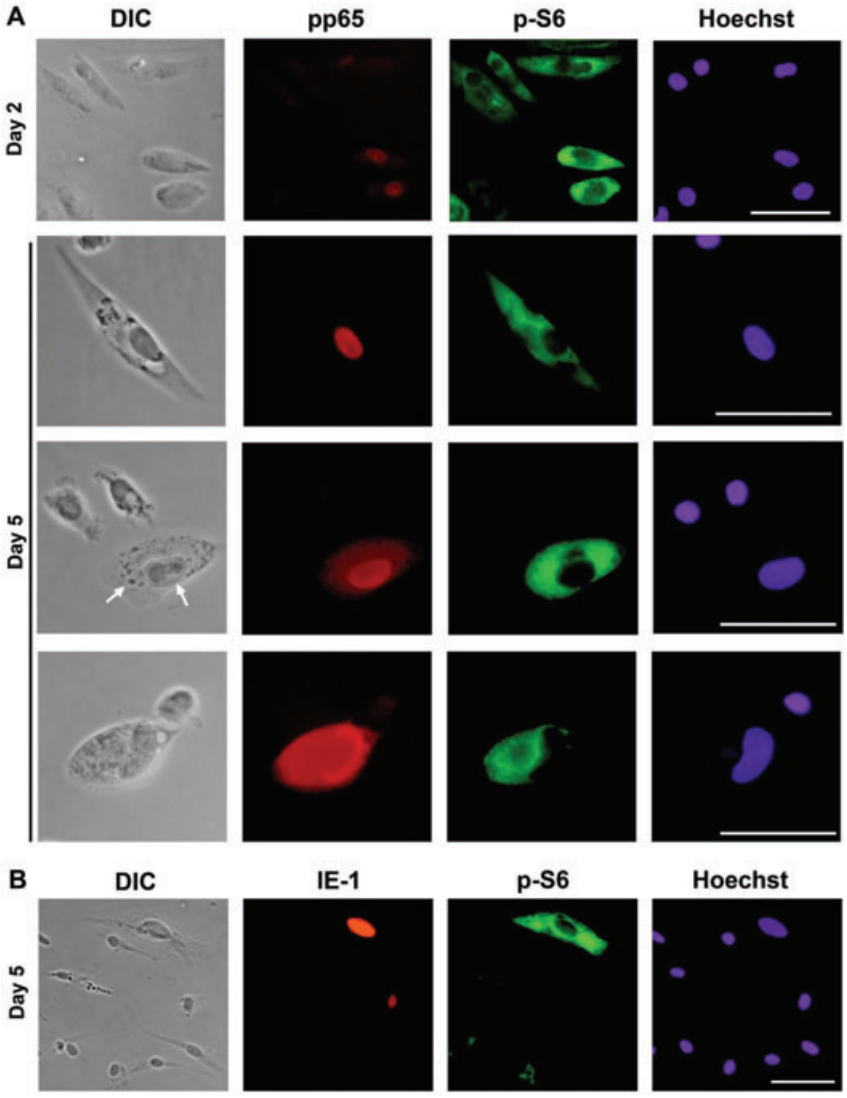

Figure 6: Ribosomal protein S6 activity in TB-40/E infected M2 macrophages at late phases of viral replication. (A) Photomicrographs of M2 macrophage cultures at day 2 and day 5 postinfection with TB-40/E are shown. Cells were fixed at indicated time points and stained for p-S6 (green) and pp65 (red). Morphology and nuclear structure are presented by differential interference contrast (DIC) images and Hoechst-33342 stain. White arrows indicate cytoplasmic and nuclear inclusions caused by productive CMV infection (white bars: 50 μm). (B) Cultures were treated as in (A) followed by immunofluorescence staining of p-S6 (green) in combination with IE-1 (red) and Hoechst-33342 (white bars: 50 μm).

of the viral replication require a high rate of protein synthesis, which is essential for efficient production of virus particles. Immunofluorescence triple stains for the viral tegument protein pp65 that increases with the rate of production of viral particles, phosphorylated ribosomal protein S6, and DNA were performed to investigate CMV-mediated mTORC1 activation at the single cell level. As depicted in Figure 6A, pp65 was expressed at low levels in the nuclei of infected cells along with increased S6 phosphorylation on day 2 postinfection. After three further days of culture pp65-positive cells were present in the culture while the localization of the viral tegument protein changed from an exclusively nuclear to a strong whole cell signal. The signal for nuclear pp65 gradually increased over time while morphological cytopathic effects including large nuclear and cytoplasmic inclusions appeared more frequently (Figure 6A, white arrows). The terminal phase of CMV infection is characterized by excessive cell rounding and a strong but diffuse pp65 signal signifying the presence of viral particles in the cytoplasmic compartment. Further, a nuclear transition from a condensed to a low-density state was observed between early and late pp65-positive cells and was also prominent in MNGCs in macrophage cultures (Figure S3). Only the pp65-positive macrophages and MNGCs at day 5 postinfection displayed S6 phosphorylation (Figure 6A). Furthermore, late phase-infected cells also displayed high levels of nuclear IE-1 (Figure 6B).

HCMV late phase-induced activation of ribosomal protein S6 in macrophages is mediated through mTORC1

CMV was reported to induce rapamycin-insensitive phosphorylation of S6 in fibroblast cultures (28). Our experiments demonstrated that rapamycin efficiently blocks CMV replication in M2 macrophages and that mTOR is

markedly hyperactivated in these cells in late phases during CMV infection. To investigate the rapamycin sensitivity of CMV induced mTOR activation, we employed western blot analysis of cultures, whereas different concentrations of rapamycin were added 48 h following CMV infection. CMV potently enhanced S6 phosphorylation in infected cultures in the absence of rapamycin. The effect was highly sensitive to inhibition by rapamycin as S6 phosphorylation was completely abrogated at 1 nM rapamycin. Simultaneously with the inhibition of S6 phosphorylation, the expression of the late phase viral protein pUL-44 was efficiently repressed in the presence of low concentrations of rapamycin (Figure 7A). Furthermore, we assessed the sensitivity of CMV-induced S6 phosphorylation for inhibition by rapamycin by immunofluorescence analysis of a CMV-infected macrophage culture treated with 100 nM rapamycin for 90 min on day 5 postinfection (Figure 7B, left panel). The solvent- and the rapamycin-treated cultures contained cells showing nuclear as well as whole cell localization of the viral tegument protein pp65. The selective increase of protein S6 phosphorylation in pp65-positive cells points to an infection-mediated increase in mTORC1 activity during late phases of viral replication. As depicted in the right panel of Figure 7(B), rapamycin completely suppressed CMV infection-induced S6 phosphorylation demonstrating that mTORC1 is essential for the enhanced S6 phosphorylation that is exclusively observed in pp65-positive, CMV-infected M2 macrophages.

Discussion

Our results demonstrate that suppressing mTORC1 activity potently blocks HCMV replication in polarized macrophages. While recent data found a critical role for mTORC1 for HCMV lytic replication in fibroblasts, our results imply an essential requirement of mTORC1 activity adjusting the substantially increased rate of ribosomal activity in immunocompetent cells with late viral gene expression during CMV infection. Since macrophages play a pivotal role in the propagation of CMV infection, our data indicate that the clinically observed anti-CMV benefits in transplant recipients treated with mTOR inhibitors may also occur by a direct blockade of HCMV replication at the level of myeloid immunocompetent cells.

CMV infection is one of the most important complications following solid organ transplantation and occurs in 44–85% of untreated transplant recipients, most commonly during the first 3 months posttransplantation when immunosuppression is most intense. Two mTOR inhibitors that have been investigated for use in the *de novo* renal transplantation setting are the macrocyclic lactone antibiotic sirolimus and its derivative everolimus. The ability of both sirolimus and everolimus to prevent acute rejection in renal transplant recipients has been demonstrated in several clinical investigations (40–45). Interestingly, it was found that mTOR inhibitors have strong anti-CMV effects in

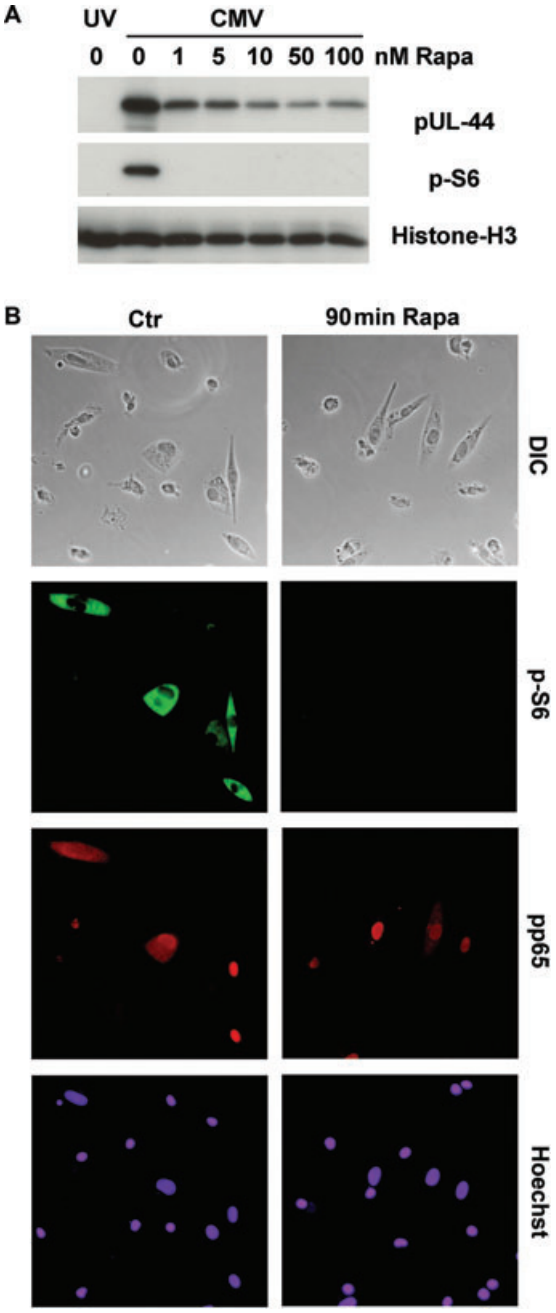

Figure 7: HCMV-mediated protein S6 activation is mediated through mTORC1. M2 macrophages were infected with TB-40/E (CMV) or UV-inactivated virus (UV): (A) Forty-eight hours following infection, either solvent (0) or indicated concentrations of rapamycin were added to the cultures. On day 5 postinfection, cells were recovered and analyzed for expression levels of the viral late phase protein pUL-44, phosphorylated protein S6 and Histone-H3. (B) On day 5 postinfection, solvent (left) or rapamycin (right) was added to CMV-infected cultures. After 90 min of incubation at 37°C, cells were fixed and immunofluorescence double stains for p-S6 (green) and pp65 (red) were employed. Images are shown together with DIC channel and nuclear staining using Hoechst-33342 (white bars: 50 μm).

kidney-transplanted patients (25). Thus far, however, only a limited number of preclinical studies have been conducted to analyze the anti-CMV mechanisms of action for the mTOR inhibitors.

We and others could recently identify mTOR as a critical regulator of the innate immune system (33,46). Since CMV can effectively subvert the host immune response by several complex mechanisms, we initially postulated that CMV might engage mTOR at the myeloid cell level to alter the innate immune response for host evasion. However, we found that CMV did not activate intracellular cascades such as MAPK or NF-kB and no significant proinflammatory cytokine responses. The HCMV–macrophage interaction, however, was still productive since a significant IRF-3- and IRF-7-driven production of type-I IFN-β was detected albeit in an mTORC1-independent manner and CMV readily induced intense morphological alterations comparable to CMV-infected fibroblast cultures along with increased cell motility and widespread cell polarization (Figure 3 and data not shown). While a significant number of studies on the immunomodulatory properties of human CMV (HCMV) employed virus stocks obtained from lytic fibroblast infection without sufficient virus purification, we applied special effort on the preparation of a virus stock, which is free of immune modulating contaminants. Without ultracentrifugation through a density cushion, our HCMV preparations also triggered high levels of proinflammatory cytokines in polarized macrophages along with strong intracellular activation patterns (data not shown). However, we considered that the elimination of potent confounding signals generated by proinflammatory cytokines or intracellular danger molecules within the culture supernatant is a *conditio sine qua non* to diligently dissect potential immunomodulatory properties of HCMV from intracellular signaling events in the context of mTOR activation. Further, it is known that the tropism of a CMV laboratory strain can change during *in vitro* propagation due to changes in the viral genome (47); hence we also aimed to employ CMV preparations directly from CMV diseased renal transplant patients which confirmed the principle finding that HCMV can successfully infect M2 macrophages.

Our experiments revealed that pUL-44 protein synthesis and CMV replication are strongly affected by rapamycin in M2 macrophages, while early phases of the CMV infection/replication cycle in M2 macrophages including IE1 protein expression were found to be independent of mTORC1. These data are in agreement with recent findings by Moorman and Shenk, who demonstrated that both rapamycin and the direct serine/threonine inhibitor of mTOR torin1 could not affect HCMV entry and IE1 accumulation in fibroblasts (32). In line, assessment of polarized macrophages at later time points revealed exquisite sensitivity to mTORC1 inhibition, while in accordance with previous results we found that mTORC1 inhibition only moderately affected viral replication in fibroblasts (28,32). As evidenced by strongly reduced CMV-specific nuclear inclusions and also by completely abolished pUL-44 signals at least 5 days postinfection, mTORC1 is crucially involved in the maintenance of viral replication during later phases of the infectious cycle. Especially late phases of the viral replication require a high rate of protein synthesis essential for efficient production of virus particles. The remarkable increase in cell size and motility of CMV-infected M2 macrophages further supports the involvement of mTORC1 in late phases of CMV infection (Figure 4B). These data indicate a substantially increased rate of ribosomal activity in CMV-infected M2 macrophages being tightly associated with late viral gene expression.

Several recent reports demonstrated that CMV induces mTOR activity presumably to increase translation of viral proteins (32,48). Furthermore, it has been shown by Moorman et al. that HCMV encodes a protein, pUL38 that binds to and inhibits TSC1/2, the principal mTORC1 negative regulator, allowing for constant mTORC1 activation (29). However, HCMV induces a distinct set of molecular changes in the canonical mTOR signaling pathway, hence rapamycin still efficiently blocks phosphorylation of the mTORC1 effector S6, but it can no longer prevent phosphorylation of the other effector 4EBP. This rapamycin-resistance of at least CMV-infected fibroblasts still lacks a molecular explanation, but was hypothesized to be present to ensure efficient and robust viral replication (32). Our data are in line with the findings of a critical role of mTORC1 for virus replication: while several data on herpesvirus replication show that the PI3-K-mTOR signaling axis becomes activated early after infection and may also be critical for infectious efficiency, our data rather suggest an importance of mTORC1 activity during later time points after infection. Interestingly, the number of IE-1-positive cells was found to decrease significantly between 24 and 48 h postinfection with CMV. While a small percentage of IE-1-positive cells continued in the viral life cycle as indicated by expression of the viral late phase protein pp65, the majority of IE-1-positive cells shut down IE-1 expression again, entering a state of latency.

Differentiation and functional specification is a central feature of macrophages critically affecting their immunological features including the allograft response. Unlike M1 macrophages that show enhanced production of proinflammatory cytokines (TNF-α, IL-6 and IL-12) and increased generation of reactive oxygen species such as NO that may also mediate allograft rejection, M2 macrophages are generally characterized by low levels of proinflammatory cytokines and high expression of antiinflammatory cytokines (IL-12^{low} and IL-10^{high}) (49,50). Hence, M2 macrophages have immunoregulatory and immunosuppressive functions. Previous studies demonstrated that reactivation of CMV from latently infected CD14 monocytes is dependent on cellular differentiation induced by allo-cytokines that are able to induce macrophage

differentiation and virus production (17). We found that monocyte-derived M2-type macrophages are highly susceptible to CMV infection in contrast to M1 macrophages (Figure 1) indicating that allo-cytokine differentiation may promote an M2-like macrophage phenotype. Together, these findings suggest a potential involvement of M2 macrophages in CMV reactivation following solid-organ transplantation. The local cytokine milieu in the graft may lead to M2-type macrophage differentiation of recruited macrophages which in turn drives CMV reactivation from latency and enhances the permissiveness of *de novo* CMV infection.

In conclusion, we have demonstrated that HCMV efficiently infected human polarized M2 macrophages, while M1 macrophages were resistant toward infection. Counterintuitively, we found that HCMV did not exert early signaling events along with absent inflammatory cytokine induction, whereas IFN-β production was triggered at significant amounts upon viral entry. The complete independence of mTORC1 activity was superseded by a strong dependence of viral replication on mTORC1 activity at later time points of the viral life cycle with a profound suppression of viral replication by rapamycin. As monocytes and macrophages cells are pivotal for host persistence and continuing viral replication of CMV as part of their immunoevasive strategies, our data argue for a direct antiviral effect of currently employed mTOR inhibitors rather than an interference with potent immunomodulatory effects exerted by CMV. Molecular analysis of the various antiviral mechanisms engaged by mTORC1 inhibitors within prospective trials in organ transplant patients might finally resolve the final role of mTORC1 as an essential component for CMV infection and disease.

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Disclosure

The authors of the manuscript have no conflicts of interest to disclosure as described by the *American Journal of Transplantation*.

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Supporting Information

Additional information may be found in the online version of this article:

Figure S1: Virus inactivation for mock infections.

Figure S2: Spreading of IE-1 viral protein across MNGC nuclei.

Figure S3: Ribosomal protein S6 activity in TB-40/E infected MNGCs at late phases of viral replication.

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