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A Versatile Role of Mammalian Target of Rapamycin in Human Dendritic Cell Function and Differentiation

Michael Haidinger,* Marko Poglitsch,* Rene Geyerregger,† Sudhir Kasturi,‡ Maximilian Zeya,§ Gerhard J. Zlabinger,¶ Bali Pulendran,‡ Walter H. Hörl,* Marcus D. Säemann,* and Thomas Weichhart*

The mammalian target of rapamycin (mTOR) regulates cell growth and survival and exists as rapamycin-sensitive mTOR complex (mTORC) 1 and as rapamycin-insensitive mTORC2. Although mTOR is a well-known regulator of diverse immune cells, its detailed role in human dendritic cell (DC) function and differentiation is only incompletely understood. In this study, we demonstrate divergent roles of mTOR during activation and differentiation of myeloid DCs (mDCs) and monocyte-derived DCs (moDCs). Inhibition of mTORC1 in mDCs activated with TLR-dependent or -independent stimuli increased proinflammatory cytokines and NF-κB signaling. In differentiating moDCs, the PI3K/Akt-dependent mTOR pathway was constitutively activated by GM-CSF to induce DC differentiation in an mTORC1-dependent manner. Inhibition of mTORC1 or mTORC1/2 during moDC differentiation decreased moDC survival and markedly hampered its immunostimulatory phenotype. Analyzing the fate of DCs in vivo, we found that kidney transplant patients treated with rapamycin displayed an increased immunostimulatory potential of mDCs compared with patients treated with calcineurin inhibitors. Furthermore, rapamycin did not interfere with mDC differentiation in these patients. Collectively, mTOR exerts divergent immunoregulatory functions during DC activation and differentiation depending on the DC type that lead to opposing T cell responses, which might be of clinical importance in transplantation, cancer, and also for novel vaccination strategies. The Journal of Immunology, 2010, 185: 3919–3931.

Dendritic cells (DCs) are professional APCs that play a critical role in the initiation and regulation of adaptive immune responses (1). Mouse and human blood DC preparations contain several phenotypically and functionally distinct subpopulations (2) for which the functions and lineages of origin still require detailed clarification. The human myeloid DC (mDC) or conventional CD11c+CD123+CD14− DC subset is contrasted with the CD11c−CD123hi lymphoid or plasmacytoid DC (pDC) population (3). Whereas mDCs, as professional APCs, can potently activate naive T cells to induce efficient Ag-specific T cell immunity, pDCs are important mediators of antiviral immunity through their ability to produce large amounts of type I IFNs upon viral infection (1, 4). The cytokine profile expressed by DCs orchestrate adaptive immune responses in part by stimulating the differentiation of naive CD4+ T cells into Th effector cells, such as Th1, Th2, Th17, and regulatory T cells (5).

Because DCs comprise only ~0.3% of PBMCs, in vitro differentiation protocols were developed to allow the production of DCs in large quantities. Most commonly, monocytes or bone marrow-derived myeloid cells are employed as progenitor cells that are cultured in the presence of IL-4 and GM-CSF. Such protocols provide excellent yields of cells bearing phenotypic and functional characteristics of DCs, which have been named monocyte-derived DCs (moDCs) or sometimes inflammatory DCs (6, 7). Furthermore, it has been proposed that moDCs are equivalents to the TNF-α and inducible NO synthase producing DCs in mice that emerge post-inflammation (8). In this regard, blood monocytes emigrate into tissues and differentiate into DCs under inflammatory conditions via a GM-CSF–dependent pathway in humans (9). Generally, GM-CSF exerts its functions postinteraction with the corresponding GM-CSF receptor to induce a variety of signal transduction pathways, such as the MAPKs, the PI3K/Akt pathway, and the Jak2/STAT5 signaling pathway (10). However, the critical signaling mechanisms, which are activated by GM-CSF and IL-4 to induce and allow moDC differentiation, are surprisingly ill defined.

The mammalian target of rapamycin (mTOR) is a serine-threonine kinase that is a pivotal downstream mediator of the PI3K/Akt signaling pathway and a key regulator of cell growth and proliferation (11, 12). mTOR is contained in two distinct complexes: the mTOR complex (mTORC) 1 regulating the phosphorylation of the ribosomal protein S6 and translation-initiation inhibitor 4E-BP1, whereas mTORC2 induces phosphorylation of serine 473 of Akt (13). The novel ATP-competitive inhibitor Torin1 inhibits mTORC1 and mTORC2 by direct binding to the catalytic domain of mTOR,
whereas rapamycin is a specific mTORC1 inhibitor with potent immunosuppressive properties (14). mTORC1 inhibitors are currently used in solid organ transplantation as a possible alternative for calcineurin inhibitors to avoid chronic renal allograft damage. Furthermore, mTOR inhibitors are evaluated in various diseases including carcinomas (15, 16), tuberous sclerosis (17, 18), and polycystic kidney disease (19). Inhibition of mTOR in CD4+ T lymphocytes suppresses cytokine-driven cellular activation and impairs Th1, Th2, and Th17 cell generation (13, 20). In addition, rapamycin enhances the expansion of CD4+CD25+ regulatory T cells (13, 20, 21), and there is recent evidence that the mTOR pathway restrains the development of CD8+ memory cells in vivo (22).

The effect of rapamycin on moDC maturation has been extensively studied (23–25), whereas the role of mTOR for moDC differentiation has been less well described (26). In moDCs, rapamycin inhibits Ag uptake and reduces DC recovery by increasing the rate of apoptosis, whereas rapamycin does not induce apoptosis in monocytes (25, 27, 28). Moreover, long-term inhibition of mTOR in moDCs suppresses the expression of costimulatory molecules and the production of inflammatory cytokines, such as IL-12 (23, 27, 28). Hence, rapamycin has been proposed to exert anti-inflammatory effects via DCs in vivo. Interestingly, however, inflammatory diseases, such as pneumonitis (29), glomerulonephritis (30, 31), and anemia of the chronic inflammatory state (32), have been more frequently observed in mTOR inhibitor-treated patients, indicating a potent proinflammatory capacity of rapamycin in some patients in vivo.

Recently, we and others (33–35) reported that inhibition of mTOR in freshly isolated human monocytes and murine macrophages stimulated with LPS enhances the production of IL-12 and IL-23, whereas IL-10 is blocked. This proinflammatory action of rapamycin is mediated by the transcription factors NF-κB and STAT3, as the inhibition of mTOR enhances proinflammatory cytokine production via NF-κB but blocks the release of IL-10 via STAT3 in human monocytes (34). The involvement of mTOR in the cellular mechanisms regulating pro- and anti-inflammatory responses in human blood DCs is poorly defined. Therefore, it was the aim of the current study to further explore and characterize the role of mTOR for both differentiation and function in peripheral mDCs and moDCs.

Materials and Methods

Reagents

LPS (Escherichia coli 0111:B4), IFN-γ, and anti-Flag were purchased from Sigma-Aldrich (Munich, Germany). Flagellin (from Salmonella typhimurium), Staphylococcus aureus, and rabbit IgG (Molecular Probes, Invitrogen). mDCs were directly analyzed or cultured in 50 ng/ml human rGM-CSF and 10 ng/ml human rIL-4 for 5–7 d.

Flow cytometry

Cells were pretreated for 90 min with the indicated concentrations of rapamycin and then activated with different stimuli in 24-well plates (moDCs) or 96-well round-bottom plates (mDCs), respectively. Cell-free supernatants were collected 24–48 h postaddition of the stimulus. Cytokines were measured by sandwich ELISA using matched-pair Abs to human IL-12p40, IL-12p70, IL-10, and IL-6 (all from R&D Systems, Minneapolis, MN). Abs to human TNF-α were from BD Pharmingen (San Diego, CA), and TNF-α standard was from R&D Systems.

Cell isolation and culture

PBMCs were isolated from acid-citrate dextrose buffy coats of healthy donors from the Department of Transfusion Medicine from the Medical University of Graz (Graz, Austria) by density gradient centrifugation using Ficol-Paque (Pharmacia, Uppsala, Sweden). mDCs were isolated from monocyte-depleted PBMCs using the CD1c isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). mDCs were directly analyzed or cultured (2 × 10^5 cells/mL) for 2 d in culture medium consisting of RPMI 1640 supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine (all from Invitrogen, Carlsbad, CA), and 10% FBS (HyClone, Logan, UT). To analyze cellular purity, DCs were stained for the lineage markers CD3, CD14, CD16, CD19, CD20, CD56, and CD11c. The purity of the DCs was consistently >95%. Monocytes were isolated by magnetically activated cell sorting using CD14 MicroBeads (Miltenyi Biotec). Purity postisolation was between 85 and 98%. For moDC differentiation, CD14-enriched monocytes were cultured in 50 ng/ml human rGM-CSF and 10 ng/ml human rIL-4 for 5–7 d.

Measurement of cytokine production

For evaluation of surface marker expression, cells were incubated with FITC- or PE-conjugated mAb for 20 min at 4°C. Cells were analyzed on a FACSCanto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ). FITC-conjugated lineage mixture as well as PE-labeled anti-CD80 (L307.4), anti-CD123, and anti-CD69 (IT2.2) mAbs were from Becton Dickinson. PE-labeled anti-CD11c was from Immunotech (Montreal, Quebec, Canada), and anti-programmed death ligand-1 (PD-L1; anti-CD274) was from Biolegend (San Diego, CA). To analyze cell differentiation and maturation of DCs, we analyzed several differentiation and maturation markers (CD1a, CD14, CD40, CD80, CD86, HLA-DR, CD206, and CD83), all from Becton Dickinson. PE-conjugated IL-12p40/70 from Becton Dickinson was used for intracellular cytokine staining.

Endocytosis assay

To determine mannose receptor-mediated endocytosis, 10^6 cells/ml was incubated in medium with FITC-labeled dextran [m.w. (M) 40,000; Sigma-Aldrich] at a concentration of 1 mg/ml. After an incubation period of 60 min at 37°C or on ice as a control, cells were washed extensively with ice-cold PBS. Fluid-phase endocytosis was measured via cellular uptake of lucifer yellow (LY; Sigma-Aldrich). Uptake events were analyzed with an FACS Canto II flow cytometer (Becton Dickinson). Phagocytic activity was expressed as percentage of mean fluorescence intensity of cells (calculated as mean fluorescence intensity of cells at 37°C – mean fluorescence intensity of cells at 4°C).

Quantitative RT-PCR

RNA from 2 × 10^5 mDCs or 1 × 10^6 moDCs was extracted in TRizol, and cDNA was generated by Superscript II (both from Invitrogen). mRNA levels were determined by TaqMan Gene Expression Assays (Applied Biosystems, Carlsbad, CA) on an ABI Prism 7000 (Applied Biosystems) and normalized to 18s rRNA. T cell stimulation

Allogeneic T cell activation was performed as described (36). Briefly, mDCs or moDCs were stimulated for 24 h with 100 ng/ml LPS with or without rapamycin and subsequently added at increasing cell numbers to 1 × 10^5 allogeneic T cells in 96-well culture plates in RPMI 1640 medium supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine, and 10% FCS (total volume 200 μl/well). After 5 d, cells were pulsed with 1 μCi (H)3 thymidine (ICN Pharmaceuticals, Irvine, CA) for 18 h. Cells were harvested on glass-fiber filters, and DNA-associated radioactivity was determined using a microplate scintillation counter (from Packard Instrument, Meriden, CT).

Immunofluorescence microscopy

Cells were applied to poly-L-lysine coated slides (Marienfeld Laboratory Glassware, Lauda-Königshofen, Germany), fixed with 4% paraformaldehyde, quenched with 100 mM glycine, permeabilized with methanol, blocked with 1% BSA, and incubated with pS6 (Ser 240/244) Ab (Cell Signaling Technology, Danvers, MA) overnight at 4°C. Cells were stained with Alexa Fluor 488-labeled goat anti-rabbit IgG followed by nuclear tracking using 0.5 μg/ml Hoechst-33342 (both from Invitrogen) and mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA).

Analysis of signal transduction events

Extract preparation and immunoblotting was performed as described (34, 36). p-STAT3 (Tyr705) and STAT3 Abs were from Cell Signaling Technology, For detection of p-STAT3 by flow cytometry, mDCs or moDCs were fixed with 2% formaldehyde, permeabilized with 0.1% saponin, and stained with anti-p-STAT3 followed by Alexa Fluor 488-labeled goat anti-rabbit IgG (Molecular Probes, Invitrogen).
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Reported gene assays

The reporter construct for NF-κB (pNF-κB-Luc) was from Stratagene (La Jolla, CA). A renilla luciferase construct was cotransfected for normalization of transfection efficiency. moDCs and THP-1 cells were transfected with Lipofectamine LTX and Plus Reagent (Invitrogen) for 24 h as described (34). Cells were then stimulated for 20 h. Lysates were prepared with passive lysis buffer according to the recommendation of the manufacturer (Promega, Madison, WI) and assayed for luciferase activity.

NF-κB p65 activity

Human mDCs or moDCs were pretreated for 90 min with 100 nM rapamycin or DMSO and then were incubated with medium, 100 ng/ml LPS, or 75 ng/ml S. aureus cells as indicated for 4 h. Cells were then collected, and nuclear extract was prepared with the NE-PER Nuclear and Cyttoplasmic Extraction Kit (Pierce Biotechnology, Rockford, IL). A total of 2 μg nuclear extract was assayed for NF-κB p65 activity with the TransAM NFκB Chemi kit (Active Motif, Carlsbad, CA).

Rapamycin encapsulation in poly(lactic-coglycolic) acid microparticles

Rapamycin was encapsulated in poly(lactic-coglycolic) acid microparticles as described previously (37). The rapamycin encapsulation level in the particles was ~12.5 μg rapamycin/mg, and 7.14 μg rapamycin or control particles/ml cell culture was added for the cell culture experiments (representing ~100 nM rapamycin).

mDC analysis in renal transplant recipients

All patients signed informed consent, and the study was approved by the local ethics committee. We compared kidney transplant patients on standard triple immunosuppression consisting of a calcineurin inhibitor (FK506 or cyclosporin A [CsA]), an antimitabolite (mycophenolate mofetil), and prednisone and compared it to patients receiving rapamycin instead of the calcineurin inhibitor. The numbers of the mDC (HLA-DR+Lin+CD11c+), and pDC (HLA-DR+Lin−CD123+) subpopulations were analyzed by immunofluorescence staining and cytometric analysis immediately after blood collection. In detail, heparinized blood was centrifuged for 15 min at 500 × g at 4°C. Postremoval of the supernatant, RBC lysis was performed, and the leukocytes were washed with PBS and stained with the respective Abs. In parallel, 2 ml heparinized whole blood was partitioned in 12-well plates and stimulated with 100 ng/ml LPS for 24 h. Afterwards, the cells were prepared for flow cytometry as described above. mDCs were stained with anti-HLA-DR, lineage mixture (anti-CD3, anti-CD14, anti-CD16, anti-CD19, anti-CD20, and anti-CD56), anti-CD11c (Immunotech, Marseille, France), anti-CD86, or anti–PD-L1, respectively. The samples were analyzed with an FACSCanto II flow cytometer using FACSDiva software (Becton Dickinson). A total of at least 100,000 events in the leukocyte gate were collected.

Statistical analysis

Data are expressed as means ± SEM. Statistical significance of differences was determined by Student t test and considered significant at p < 0.05. Patient data (Fig. 6) was compared with one-way ANOVA and Dunnnett’s posttest.

Results

mTOR inhibition differentially regulates TLR- and T cell-mediated inflammatory cytokine production in human mDCs and moDCs

To analyze the role of mTOR in the regulation of inflammatory cytokines in DCs upon activation with different TLR-dependent and -independent ligands, highly purified human mDCs (Fig. 1A) and moDCs were stimulated with LPS (TLR4 agonist), flagellin (TLR5 agonist), S. aureus cells (TLR2 agonist), or CD40L (T cell-dependent activation). Whereas rapamycin strongly enhanced the production of IL-12p40 in mDCs with all ligands tested (Fig. 1B–E), rapamycin suppressed IL-12p40 production in LPS- or CD40L- activated moDCs (Fig. 1F, 1H). Of note, rapamycin promoted IL-12p40 expression in S. aureus cell-activated moDCs (Fig. 1F). Moreover, rapamycin augmented IL-6 production in LPS-treated mDCs, but blocked IL-6 in moDCs (Fig. 1B, 1F). TNF-α production was largely unaffected upon mTOR blockade in TLR-activated mDCs, whereas rapamycin significantly impaired TNF-α production in moDCs upon stimulation with all TLR agonists (Fig. 1B, 1I). Production of the anti-inflammatory cytokine IL-10 was diminished by rapamycin in both mDCs and moDCs. Unexpectedly, we observed that rapamycin increased the production of biologically active IL-12p70 in mDCs as well as moDCs upon stimulation with LPS plus IFN-γ (Fig. 1J). Analyzing IL-12p35 as an IFN-γ-dependent subunit of the IL-12 heterodimer, which functions as the rate-limiting subunit of bioactive IL-12, we found that rapamycin led to a significant increase of IL-12p35 production, whereas IL-12p40 production was concomitantly still repressed under mTOR inhibition in these cells (Fig. 1J). These data demonstrate that rapamycin regulates inflammatory cytokine production in TLR- and CD40L-stimulated mDCs differentially compared with moDCs.

Surface molecule expression but not Ag uptake capacity is regulated by mTOR in DCs

Ag uptake by phagocytosis, expression of costimulatory/adhesion molecules, and cytokine production by DCs are crucial to potently activate T cells. After short-term treatment with rapamycin, phagocytosis as evaluated by macrocinopcytosis via uptake of LY or receptor-mediated endocytosis via FITC-dextran was unaltered in mDCs as well as in moDCs (Fig. 2A). In agreement with these data, rapamycin delivered to mDCs in microparticles via endocytosis deviated the IL-12/IL-10 cytokine balance in a similar manner as the soluble mTOR inhibitor (Fig. 2B). We found that rapamycin enhanced the surface expression of CD25 as well as CD86 in LPS-stimulated mDCs, whereas it did not significantly affect CD80 expression (Fig. 2C). Moreover, expression of PD-L1, which negatively regulates T cell responsiveness (38), was significantly impaired in mDCs when mTOR was inhibited (Fig. 2C). In contrast, CD86 as well as CD80 surface expression were reduced in rapamycin-treated moDCs stimulated with LPS, whereas in moDCs, CD25 and PD-L1 were regulated as in mDCs (Fig. 2C, 2D). Analogous results were obtained with the TLR2 agonist S. aureus cells (Fig. 2E). These results suggest that inhibition of mTOR during TLR-mediated activation of peripheral mDCs specifically hyperactivates these cells, indicated by the reciprocal expression pattern of CD86 and PD-L1.

Inhibition of mTOR in mDCs augments their allostimulatory potential

The ability of rapamycin to enhance the production of proinflammatory cytokines and expression of costimulatory molecules in mDCs suggests an impact on subsequent T cell activation. By employing an allogeneic T cell activation model, we observed that rapamycin augmented the stimulatory capacity of LPS-stimulated mDCs (Fig. 2F). In sharp contrast, inhibition of mTOR in activated moDCs prevented allogeneic T cell stimulation (Fig. 2F). These data indicate that mTOR negatively regulates the capacity of human mDCs to induce T cell proliferation, whereas mTOR is essential for efficient T cell proliferation in moDCs.

mTOR is persistently active and differentially affects NF-κB in moDCs

Inhibition of mTOR differentially activates essential DC features in mDCs, whereas it leads to a global dampening of critical functions in moDCs. The underlying molecular mechanisms, however, are still unclear. In primary human monocytes, murine embryonic fibroblasts, and endothelial cells, inhibition of mTOR directly augments NF-κB activation, a key transcription factor mediating proinflammatory events in innate immune cells (34, 39, 40). As mDCs are refractory to LPS stimulation posttransfection of a NF-κB reporter construct (data not shown), we used myeloid THP-1 cells as model system. In these cells, rapamycin significantly enhanced the NF-κB activity in LPS-stimulated cells (Fig. 3A). In contrast,
rapamycin failed to upregulate NF-κB signaling in moDCs compared with LPS alone (Fig. 3A). Moreover, DNA binding of the NF-κB subunit p65 was augmented in mDCs after 3 h, whereas it was reduced in moDCs (Fig. 3B). Interestingly, rapamycin increased DNA binding of p65 in moDCs when the cells were stimulated with S. aureus cells (Fig. 3B), compatible with the enhanced IL-12p40 production of these cells under mTORC1 blockade. LPS-stimulated moDCs did not upregulate IL-12p40 mRNA transcription under blockade of mTORC1 even with the addition of IFN-γ (Fig. 3C). Inhibition of IL-10 production by rapamycin is mediated via blockade of the transcription factor STAT3 in human monocytes (33, 34). We found a prominent inhibition of STAT3 phosphorylation after rapamycin treatment in both mDCs and moDCs (Fig. 3D). Analyzing the production of IL-10 mRNA, we observed that rapamycin suppressed IL-10 mRNA transcription in these cells (Fig. 3E).
We then explored the mTOR signaling pathway in mDCs and moDCs to obtain deeper insight into the molecular mechanisms involved upon mTOR inhibition. After a maturation stimulus, such as TLR4 or CD40L ligation, we detected a strong rapamycin-sensitive activation of the critical components of the mTOR signaling pathway in mDCs, including the phosphorylation of S6 and 4E-BP1 (Fig. 4). However, in moDCs, the mTOR pathway was constitutively active without stimulation on day 5, and LPS could only modestly enhance its stimulation (Fig. 4). Interestingly, rapamycin insufficiently blocked phosphorylation of S6 and 4E-BP1 (Fig. 4B). These data show that the mTOR pathway is strongly active under differentiating conditions in moDCs, associated with a relative resistance toward mTORC1 blockade.

The mTOR pathway is activated by GM-CSF during differentiation of moDCs

To closely assess the mTOR pathway during moDC differentiation, we treated monocytes with GM-CSF and IL-4 for 7 d and evaluated
the activation of the mTOR pathway during the entire differentiation process (Fig. 5A). We observed that the cells increased in size and augmented their cytoplasmic protein content as measured by the increase in GAPDH protein (Fig. 5A). Moreover, the levels of the mTOR pathway proteins were specifically upregulated during moDC differentiation (Fig. 5A). The mTOR pathway was dormant on day 0, but was potently activated by LPS (Fig. 5A). In sharp contrast, the mTOR pathway became activated during the moDC differentiation from day 1 onwards without LPS stimulation (Fig. 5A). In addition, LPS was only modestly able to superactivate the mTOR pathway, as shown by phosphorylation of S6, Akt, or 4E-BP1 (Fig. 5A). Assessing the LPS-induced production of IL-12p40 during the moDC differentiation process, we observed that the total amount of IL-12p40 substantially increased with ongoing DC differentiation (Fig. 5A). Whereas rapamycin was able to augment IL-12p40 production on day 0 and day 1, it failed to do so at later stages (Fig. 5A). As observed for IL-12p40, we found that the proinflammatory cytokine IL-6 was initially upregulated upon mTOR inhibition, but subsequently suppressed as the differentiation process proceeded (Fig. 5B). Interestingly, this loss of rapamycin’s ability to augment IL-12p40 and IL-6 production was associated with the expression and basal activation of the mTOR pathway in these cells after 1–3 d. In contrast, IL-10 was potently suppressed by rapamycin during all differentiation stages, and also, the total amount of IL-10 did not increase during the 7-d culture (Fig. 5C).

These results suggested that the mTOR pathway is induced upon activation with GM-CSF/IL-4. Hence, we analyzed whether GM-CSF and/or IL-4 are able to activate the mTOR pathway in myeloid cells. Surprisingly, we found that GM-CSF directly activated mTOR signaling within 30 min in monocytes to a level comparable to TLR signaling in a strictly rapamycin-sensitive manner (Fig. 5D, 5E). As it is known that GM-CSF activates PI3K, which might lead to mTOR activation in monocytes, we directly tested this possibility by employing the PI3K inhibitor wortmannin. Indeed, GM-CSF–induced activation of the mTOR pathway was abrogated by inhibition of PI3K (Fig. 5F). These results indicate that a specific differentiation program is started by GM-CSF, leading to a continuous activation of mTOR via PI3K.

**Differentiation of moDCs is dependent on mTORC1**

The above results suggested that the chronic stimulation of the PI3K/mTOR pathway by GM-CSF in monocytes might be important for moDC differentiation. To directly assess this possibility, we
FIGURE 4. mTOR is constitutively active in moDCs. A, moDCs or moDCs (day 5) were treated with medium (med), CD40L plus anti-FLAG (200 ng/ml and 5 μg/ml, respectively), or 100 ng/ml LPS with or without 100 nM rapamycin (Rap) as indicated. Phosphorylation of S6 (pS6) was detected by immunohistochemistry 4 h poststimulation. Nuclei were stained with Hoechst. One representative experiment of three (from individual donors) is shown. B, moDCs and moDCs were stimulated as indicated for 30 min. Phosphorylation of S6 and 4E-BP1 was performed by immunoblotting. Arrows represent the individual phosphorylated isoforms of 4E-BP1. One representative experiment of three is shown.

differentiated monocytes into moDCs in the presence of rapamycin (Rap-DCs) or Torin1 (Torin1-DCs). Inhibition of mTORC1 with rapamycin or mTORC1/2 with Torin1 decreased the survival and enhanced the apoptosis of these cells, indicating that mTOR signaling is essential for survival during the differentiation process (Fig. 6A, 6B and data not shown). Moreover, the mTOR pathway was suppressed in Rap-DCs and Torin1-DCs in contrast to moDCs, which showed constitutive activation of 4E-BP1 and S6, as noted above (Fig. 6C). When testing for the ability to produce inflammatory cytokines, we observed that Rap-DCs or Torin1-DCs only produced small amounts of IL-12p40 (Fig. 6D). In addition, the surface expression of critical DC markers of these cells was severely altered, as they expressed lower levels of CD1a, CD40, CD86, HLA-DR, and CD206 compared with moDCs (Fig. 6E). This phenotype also persisted poststimulation with LPS (Fig. 6E). Moreover, we did not observe major differences between the mTORC1 inhibitor rapamycin and the mTORC1/2 inhibitor Torin1, indicating that mTORC2 might be of limited importance during moDC differentiation. These results imply that the mTORC1 pathway is pivotal for moDC differentiation, and inhibition of mTOR by rapamycin or Torin1 severely interferes with moDC differentiation and survival.

Hyperactivity of mDCs in rapamycin-treated patients without alteration of mDC numbers

So far, we observed that short-term inhibition of the mTOR pathway hyperactivates mDCs, whereas conversely, mTOR is a central regulator for successful DC differentiation in vitro. Therefore, we wanted to assess which function of mTOR is prevailing under physiological conditions of mTOR inhibition in vivo. We evaluated the number and phenotype of mDCs (Fig. 7A) in healthy controls and kidney transplant patients, who are on rapamycin triple therapy (concomitantly receiving the antimetabolite mycophenolate mofetil and the glucocorticoid prednisolone) and compared them to patients on conventional triple therapy receiving the calcineurin inhibitor tacrolimus (FK506) or CsA instead of rapamycin. The basic characteristics of the patients were similar (Table I). We found that the number of mDCs in rapamycin-treated patients was comparable to healthy controls or patients treated with CsA or FK506 (Fig. 7B). Moreover, the ratio of mDCs versus pDCs has been used to monitor DC levels in transplant recipients and may correlate with allograft rejection (41, 42). The mDC/pDC ratio was not lower in patients on rapamycin therapy, indicating that mDC development in patients treated with mTOR inhibitor is unaffected compared with patients receiving a calcineurin inhibitor or healthy controls (Fig. 7C). Next, we tested if the mDCs in rapamycin-treated patients can be hyperactivated as in their in vitro-stimulated counterparts. Therefore, we assessed the expression of CD86 and PD-L1 in mDCs under basal conditions and poststimulation of whole blood from healthy individuals and the kidney-transplanted patients with LPS. It is important to note that glucocorticoids inhibit the expression of IL-12p40 (43) and CD86 (44, 45), whereas they enhance PD-L1 expression in DCs (46, 47), rendering patients on CsA or FK506 suitable controls for patients on rapamycin, as all concomitantly take prednisolone. Interestingly, CD86 was significantly higher expressed in mDCs from rapamycin-treated patients upon stimulation with LPS compared with calcineurin inhibitor-treated patients or even healthy controls (Fig. 7D). Remarkably, the expression of the coinhibitory molecule PD-L1 was significantly decreased in rapamycin patients compared with CsA or FK506, both under basal conditions and poststimulation with LPS (Fig. 7E). High expression of CD86 and low expression of PD-L1 are indicative of a potent immunostimulatory potential of mDCs, and, indeed, the ratio of CD86 to PD-L1 has been correlated to a strong T cell activation potential previously (48). The calculated ratio of CD86/PD-L1 under steady-state conditions and after LPS stimulation was significantly higher in patients treated with rapamycin compared with CsA- or FK506-treated patients (Fig. 7F, 7G). Moreover, compared with CsA or FK506, rapamycin-treated patients produced significantly more IL-12p40 but less IL-10 in whole blood stimulated with LPS (Fig. 7H). Indeed, these results suggest that mTOR inhibition in human kidney transplant patients in vivo does not reduce mDC levels and leads to a strong immunostimulatory phenotype of mDCs under basal conditions and also in response to TLR4 ligation ex vivo.

Discussion

DCs are the most potent APCs and essential for the initiation and regulation of the immune response. However, the critical signaling pathways, which regulate the differentiation and immunostimulatory properties of DCs, are still incompletely defined. Recently, the PI3K/mTOR pathway has been identified as negative regulator of TLR signaling in human monocytes and murine bone marrow-derived macrophages (13, 49). The results obtained from the current study demonstrate that mTOR is a central signaling modifier of human mDCs. Hence, mTOR inhibition promotes proinflammatory responses, such as IL-12, production and prevents IL-10 secretion in freshly isolated mDCs postactivation with TLR-dependent stimuli. Moreover, we provide the first evidence, to our knowledge, that the mTOR pathway also controls cytokine production in mDCs.
after stimulation with the TLR-independent stimulus CD40L. Previous results showed that the PI3K/mTOR pathway negatively regulates NF-κB activity and IL-1β, IL-12, and IL-23 production, whereas mTOR mediates a positive signal to IL-10 via STAT3 in monocytes and macrophages (33–35, 50, 51). Our results demonstrate that in mDCs, STAT3 signaling in response to rapamycin is similarly inhibited, indicating that the induction of the major anti-inflammatory transcription factor STAT3 is under control of the mTOR pathway in all myeloid immune cells.

FIGURE 5. The mTOR pathway is activated by GM-CSF during differentiation of moDCs. A–C, Monocytes were differentiated into moDCs with 50 ng/ml GM-CSF and 10 ng/ml IL-4 for 7 d. Cells were stimulated with 100 ng/ml LPS (L) in the presence or absence of 100 nM rapamycin (R) as indicated and assessed by immunoblotting (30 min stimulation) or to measure IL-12p40, IL-6, and IL-10 by ELISA (24 h stimulation). For immunoblotting, 2.5 × 10^5 cells were loaded per lane and are controlled by Ponceau S staining of the histone bands. One representative experiment of three (from individual donors) is shown.

D and E, Monocytes were stimulated for 30 min with 50 ng/ml GM-CSF, 10 ng/ml IL-4, or 100 ng/ml LPS with or without rapamycin as indicated. Cell extracts were analyzed by immunoblotting. One representative experiment of two is shown. F, Monocytes were treated with 100 nM wortmannin (WM) as indicated and then stimulated with GM-CSF and IL-4 for 30 min. Phosphorylation of Akt and S6 was performed by immunoblotting. One representative experiment of two is shown.
globally decreases specific innate immune cell functions in moDCs leading to a reduced capacity to prime T cells (23, 27, 28, 33, 35, 52–54). In line with these experiments, we show that short-term inhibition of mTOR in moDCs leads to a decrease in their inflammatory cytokine production and their T cell stimulatory capacity after both TLR and CD40L activation. However, it is interesting to note that rapamycin actually enhanced IL-12p40 production in TLR2-activated moDCs. More surprisingly, rapamycin also increased bioactive IL-12 production in moDCs when treated with LPS plus IFN-γ due to the ability of rapamycin to foster IL-12p35 production as IL-12p40 expression was still blocked. At the molecular level, inhibition of mTOR enhances NF-κB signaling in endothelial cells, mDCs, and other myeloid immune cells (34, 40, 55), whereas rapamycin did not upregulate NF-κB activity in moDCs in response to LPS. These results may suggest that mTOR lacks its negative regulatory role regarding NF-κB signaling in moDCs, potentially leading to the differential phenotypes observed in mDCs and moDCs.

In addition, our data demonstrate that mTOR controls the expression of various surface molecules on mDCs. Most importantly, expression of CD86, a critical costimulatory molecule for efficient T cell priming, is enhanced in activated mDCs during mTOR inhibition. This phenotype was not observed in moDCs, in which surface expression of CD80 and CD86 was inhibited by rapamycin. In contrast, the negative T cell regulator PD-L1 expression was reduced in mDCs by rapamycin (38). In line, inhibition of mTOR in TLR4-activated mDCs enhanced their T cell stimulatory capacity. In addition, recent data show that rapamycin can augment adaptive Th1 immune responses via enhanced MHC Ag presentation in murine DCs by promoting autophagy (56). These data demonstrate that rapamycin can also foster T cell responses by acting solely on myeloid cells. Furthermore, we could show that inhibition of mTOR augments surface expression of the α-chain of the IL-2R (CD25) in TLR4-activated mDCs by promoting autophagy (56). Different subtypes of DCs have been shown to express CD25 and its ligand IL-2 plays an important role in the control of immune responses (57, 58). IL-2 is released in a variety of tissues in response to inflammatory stimuli, and the stimulation of DCs by IL-2 is known to result in the enhanced expression of proinflammatory cytokines (59).

In an attempt to identify a molecular feature that distinguishes mDCs from moDCs and might explain the different immunoregulatory uses of the mTOR pathway in these cells, we found that the Akt/mTOR pathway is constitutively activated in moDCs in contrast to mDCs or monocytes. In addition, TLR engagement could only
modestly stimulate the mTOR pathway compared with unstimulated moDCs, indicating that mTOR signaling might be essential for moDCs under steady-state conditions. The protein levels of the mTOR pathway became upregulated from day 1 during moDC differentiation, and this overexpression was positively associated with an increased ability to produce IL-12p40 and IL-6, whereas it was negatively associated with the capacity of rapamycin to augment production of proinflammatory cytokines. Whether the reduced ability of differentiating moDCs to potently upregulate the mTOR pathway in response to LPS or the enhanced protein levels of the mTOR pathway is causative for this phenomenon remains to be investigated. Interestingly, binding of p65 to NF-κB was higher in unstimulated mDCs, which have low basal mTOR activity, whereas p65 binding to NF-κB was low in moDCs, which display a constant activation of the mTOR pathway (Fig. 3B).
differentiation. Indeed, inhibiting mTORC1 or mTORC1/2 during moDC differentiation 1) reduced the survival and quantitative outcome of moDCs, 2) reduced the overexpression of the mTOR pathway during moDC differentiation, 3) reduced cytokine production, and 4) altered the expression of critical costimulatory molecules and surface receptors. This is in line with the finding that high doses of rapamycin inhibit the differentiation and mobilization of mouse DCs in vivo in response to the administration of FLT3 ligand (23). Moreover, direct activation of the mTOR pathway by GM-CSF may represent the molecular basis for the observation that rapamycin induces apoptosis in vitro by interfering with GM-CSF signaling in moDCs (26). Furthermore, the suppressive effects of rapamycin on moDC maturation are well documented in vitro (23, 24, 27, 52, 61). As rapamycin potently inhibits STAT3 signaling in various cells (34, 62, 63), STAT3 may be one critical downstream regulator of mTOR to control moDC differentiation. Indeed, STAT3 is essential for FLT3L-dependent DC differentiation in mice and the mTOR/STAT3 pathway has been causally linked to regulate proliferation and differentiation (64, 65). These data together with our finding that GM-CSF/IL-4 activates the mTOR pathway suggest that the continuous activation of mTOR by GM-CSF is critical for moDC survival and differentiation.

To determine the relative importance of mTOR in DC differentiation versus activation for the outcome of an inflammatory immune response in vivo, we analyzed renal transplant patients treated with either rapamycin or calcineurin inhibitors. We observed that the numbers of mDCs were not different between the patient groups, indicating that mTOR inhibition at doses ranging from 5–10 ng/ml, which is far beyond the concentrations used for murine studies, does not compromise the mDC compartment in vivo. Strikingly, mDCs from rapamycin-treated patients displayed an exceptionally immunostimulatory profile compared with CsA and FK506 patients as characterized by the high expression of CD86 and the low expression of PD-L1, both at steady-state conditions, and especially poststimulation with LPS ex vivo. These data suggest that mTOR also controls the generation of an inflammatory immune response.

### Table 1. Clinical parameters of kidney-transplanted patients

<table>
<thead>
<tr>
<th>Healthy (n = 12)</th>
<th>CsA (n = 9)</th>
<th>FK506 (n = 11)</th>
<th>Rapamycin (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (% women)</td>
<td>45.5</td>
<td>33.0</td>
<td>45.0</td>
</tr>
<tr>
<td>Age (y)</td>
<td>35.7 (6.5)</td>
<td>54.1 (17.4)</td>
<td>55.1 (11.3)</td>
</tr>
<tr>
<td>IS levels (ng/ml)</td>
<td>90.4 (28.7)</td>
<td>5.8 (1.6)</td>
<td>5.9 (1.3)</td>
</tr>
<tr>
<td>Prednisolone (mg/dl)</td>
<td>3.8 (1.3)</td>
<td>3.1 (2.0)</td>
<td>3.3 (1.4)</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>1.7 (0.7)</td>
<td>1.5 (0.5)</td>
<td>1.7 (0.5)</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>29.9 (16.1)</td>
<td>27.2 (10.6)</td>
<td>32.3 (13.6)</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>1.5 (3.1)</td>
<td>0.6 (0.9)</td>
<td>1.5 (2.6)</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>12.2 (0.6)</td>
<td>13 (1.8)</td>
<td>12.1 (1.8)</td>
</tr>
</tbody>
</table>

Data are presented as numbers (SD) unless indicated otherwise. BUN, blood urea nitrogen; CRP, C-reactive protein; IS, immunosuppression of CsA.

FIGURE 8. Proposed model of the role of mTOR in human mDC and moDC differentiation and immunostimulatory function. See Discussion for details.
in human mDCs in vivo (33, 34). Interestingly, several distinct rapamycin-associated inflammatory diseases have emerged, such as pneumonitis (29), de novo glucorenlonopetidosis (30, 31, 66), systemic inflammatory response syndrome, and anemia of chronic disease (32). A negative regulatory role of mTOR on the inflammatory response in cells like monocytes, mDCs, or endothelial cells may provide a molecular explanation of these inflammatory events (13, 40).

Therefore, we propose a model in which mTOR has two different roles during the life cycle of a DC (Fig. 8). mTOR is important for the survival and proper differentiation process of monocytes to moDCs, because moDCs depend on the continuous GM-CSF–dependent survival and proper differentiation process of monocytes to moDCs, characterized the effects of mTOR inhibition for mDC and moDC generation, differentiation, and impact on the inflammatory and T cell response in these cells. This additional knowledge about the critical role of mTOR in the most potent APCs may lead to the design of novel therapeutic strategies using mTOR inhibitors as vaccine adjuvants against bacterial infections or cancer.

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Disclosures
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References


