Published in final edited form as:

Mol Immunol. 2010 ; 48(0): 294–304. doi:10.1016/j.molimm.2010.07.014.

Mammalian target of rapamycin (mTOR) regulates TLR3 induced cytokines in human oral keratinocytes

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Abstract

Recent studies implicate the mammalian target of rapamycin (mTOR) pathway in the control of inflammatory responses following Toll-like receptor (TLR) stimulation in myeloid cells but its role in non-myeloid cells such as human keratinocytes is unknown. Here we show that TLR3 signaling can induce robust cytokine secretion including interleukin 1 beta (IL-1 β), tumor necrosis factor alpha (TNF α), IL-12p70 and interferon beta (IFN- β), and our data reveal for the first time that inhibiting mTOR with rapamycin, suppresses these TLR3 induced responses but actually enhances bioactive IL-12p70 production in human oral keratinocytes. Rapamycin inhibited the phosphorylation of the 70-kDa ribosomal protein S6 kinase (p70S6K) and the 4E binding protein 1 (4EBP-1), and suppressed the mitogen activated protein kinase (MAPK) pathway by decreasing phosphorylation of c-Jun N-terminal kinase (JNK). We also show that TLR3 induces interferon regulatory factor 3 (IRF3) activation by Akt via an mTOR-p70S6K-4EBP1 pathway. Furthermore, we provide evidence that Poly I:C induced expression of IL-1 β , TNF α , IL-12p70 and IFN- β was blocked by JNK inhibitor SP600125. TLR3 preferentially phosphorylated IKK α through mTOR to activate nuclear factor kappa beta (NF- κ B) in human oral keratinocytes. Taken together, these data demonstrate p70S6K, p4EBP1, JNK, NF- κ B and IRF3 are involved in the regulation of inflammatory mediators by TLR3 via the mTOR pathway. mTOR is a novel pathway modulating TLR3 induced inflammatory and antiviral responses in human oral keratinocytes.

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AUTHOR CONTRIBUTIONS:

MRB, MM & DFK conceived the idea and designed the experiments. JZ, MRB, KBH did the experiments, MRB, JZ, KBH, JCG, MM and DFK analyzed results. MRB, JZ and DFK wrote the paper.

Keywords

Human oral keratinocytes; TLR3; mTOR; JNK; NF- κ B; IRF3

1.0 INTRODUCTION

Host defense against pathogens involves innate and adaptive immune responses (Rimoldi et al., 2005). Recognition of pathogens occurs through pattern recognition receptors (PRR) or Toll-like receptors (TLRs) which differentiate microbial molecular patterns and play a central role in the induction of host responses (Akira and Takeda, 2004; Beutler, 2004). TLRs are expressed predominantly in first-line defense cells, such as neutrophils, monocytes/macrophages, and dendritic cells (DCs), as well as in human oral keratinocytes (from now on keratinocytes) (Akira et al., 2006; Kinane et al., 2008). Keratinocytes serve as a first line of defense in the oral cavity, to invading bacteria (Benakanakere et al., 2009; Guggenheim et al., 2009; Kinane et al., 2008) and viruses (Slots et al., 2006) and regulating these responses is important in maintaining homeostasis. Recent reports implicate the mammalian target of rapamycin (mTOR) signaling pathway, in the control of pro-inflammatory cytokine production induced by various bacterial stimuli in myeloid cells such as monocytes, macrophages, DCs and other immune cells (Ohtani et al., 2008; Thomson et al., 2009; Weichhart et al., 2008; Weichhart and Saemann, 2009). However, mTOR signaling in the inflammatory response of these keratinocytes is unknown as is how TLR3 signaling is regulated by mTOR in modulating interferon regulated factor 3 (IRF3) to induce an anti-viral response. The prototypic mTOR inhibitor rapamycin is a bacterial macrolide that exhibits immunosuppressive and antitumor activity (Weichhart et al., 2008) by inhibiting the target of rapamycin (TOR) proteins, a highly conserved group of serine/threonine kinases (Hidalgo and Rowinsky, 2000; Jacinto and Hall, 2003). mTOR regulates phosphorylation of at least two proteins important for translation: 70-kDa ribosomal protein S6 kinase (p70S6K1) and eIF4E-binding protein 1 (4E-BP1) (Inoki et al., 2005). Polyinosinic-polycytidylic acid (poly I:C), a synthetic analog of viral double-stranded RNA (dsRNA), is recognized by TLR3 and has been used to study the effects of viral infection on several cell types (Kumar et al., 2006; Takahashi et al., 2006). In the present study, we investigated the role of mTOR in keratinocytes in response to the TLR3 agonist poly I:C. We show that mTOR positively regulates interleukin 1 beta (IL-1 β), tumor necrosis factor alpha (TNF α) and interferon beta (IFN- β) expression, but negatively regulates IL-12p70 expression in keratinocytes induced by a TLR3 ligand. These pathways have hitherto not been previously described. We further demonstrate that mTOR regulates inflammatory mediators at least, partly by reducing c-Jun N-terminal kinase (JNK) and NF- κ B activation via IKK α . These results indicate that the mTOR pathway critically modulates TLR3 dependent induction of inflammatory cytokines and type I interferon (IFN) in keratinocytes.

2.0 MATERIALS AND METHODS

2.1 Cell isolation and culture

The gingival tissue is obtained with Institutional Review Board approval from healthy patients after the third molar extraction. The tissue was treated with 0.025% trypsin and

0.01% EDTA overnight at 4°C, and keratinocytes were isolated, as previously described (Kinane et al., 2006). Briefly, the cell suspension was centrifuged at $120 \times g$ for 5 min, and the pellet was suspended in K-SFM medium (InvitrogenCA) containing 10 µg/ml insulin, 5 µg/ml transferrin, 10 µM 2-ME, 10 µM 2-aminoethanol, 10 mM sodium selenite, 50 µg/ml bovine pituitary extract, 100 U/ml penicillin/streptomycin, and 50 ng/ml fungizone (complete medium). The cells were seeded in 60-mm plastic tissue culture plates coated with type I collagen, and incubated in 5% CO₂ and 95% air at 37°C. When the cells reached subconfluence, they were harvested and subcultured as described (Stathopoulou et al., 2009).

2.2 Cell challenge assays

Keratinocytes at the 3rd passage were harvested, seeded at a density of 0.5×10^5 cells/well on to 6 well culture plate coated with type I collagen, and maintained in 2 ml of complete medium. When they reached 80% confluence, the cells were washed twice with fresh medium, and 2 ml of plain medium was added. When cells reached ~100% confluence the cells were incubated with either rapamycin (200 nM, Calbiochem) or LY290042 (10µM, Calbiochem) or SP600125 (50uM, Calbiochem) or DMSO (Sigma-Aldrich, St.Louis) for 2 h and then cells were challenged with 1 µg/ml FSL-1 (TLR2 ligand), 5 µg/ml of poly I:C (TLR3 ligand) and 1 µg/ml *E. coli* LPS (TLR4 ligand), (Invivogen, CA) determined after initial dose response and agonist screening. Culture supernatants were collected at the end of the experiment and stored at -80°C until being assayed. Production of IL-1β, TNFα, IL-12-p70, IL-12p40 and IL-10 (BD Biosciences, CA) and IFN-α/β (PBL Interferonsource, NJ) was determined by enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions. None of the agonist stimuli affected cell viability as determined by trypan blue exclusion.

2.3 RNAi

siRNA SMARTpool mTOR, siTLR3 ON-TARGETplus SMARTpool and ON-TARGETplus Non-targeting siRNA were from Dharmacon. Keratinocytes were transfected using siPort NeoFx transfection reagent (Ambion) according to the manufacturer's instructions. Briefly, 100 nM final concentration of siRNA was used to transfect cells at 60%–70% confluency. 24 h post transfection, cells were challenged with poly I:C before harvest. Cells were harvested 48 h after siRNA transfection.

2.4 Real-time PCR

Total RNA was extracted from cultured cells by using TRIzol reagent (Invitrogen, Carlsbad, CA). The isolated total RNA samples were used to perform first strand cDNA synthesis (Applied Biosystems, Foster City, CA). Real-time PCR was performed by using 50ng of cDNA with IL-12p35, IL-12p40, IFN-β as primers and probes and GAPDH as endogenous control on ABI 7500 Fast system (Applied Biosystems) in the presence of TaqMan DNA polymerase as previously described (Benakanakere et al., 2009). Quantitative TaqMan PCR-Array was custom designed based on previously published microarray data on keratinocytes (Kinane et al., 2006). Keratinocytes were grown to confluence and challenged with LPS (1 µg/ml), FSL-1 (1 µg/ml) and Poly I:C (5 µg/ml) for 4 hours. The cDNA conversion and real

time PCR were carried out as mentioned above. The fold increase was calculated as compared to untreated control sample according to $\Delta\Delta CT$ method (Livak and Schmittgen, 2001). Mean fold increase data was used to derive heatmap with two-way hierarchical clustering using MeV v4.1 software.

2.5 Western blot analysis

Cells were washed with cold PBS and then lysed on ice for 30 min in 100 μ l RIPA lysis buffer (Sigma-Aldrich, St. Louis) containing protease (Roche, IN) and phosphatase inhibitors (Sigma-Aldrich, St. Louis). The whole cell lysate was passed ten times through a 30-gauge needle and then incubated on ice for an additional 30 min. Cell debris was pelleted by centrifugation, and the supernatants were collected and stored at -80°C until assayed. Of total cellular protein, 50 μ g was suspended in lithium dodecyl sulfate (LDS) buffer, heated for 10 min at 70°C , resolved by LDS-PAGE and then transferred to PVDF membranes using the NuPAGE system (Invitrogen, CA). All Western blotting reagents were procured from Invitrogen, CA except for the ECL Plus western blotting detection system (GE Health Care, NJ). Primary antibodies [anti-phosphorylated mTOR (Ser2448), 4E-BP1 (Thr37/46), extra cellular signal regulated kinases 1 and 2 (ERK1/2) (Thr202/204), SAPK/JNK (Thr183/Tyr185), Akt (Ser473), c-JUN (Ser63 & Ser73), IRF3 (Ser396), IKK α (Ser176/180)/IKK β (Ser177/181), TBK-1 (Ser172), I κ B α (Ser32/36), RIG-1 and anti-total beta-Actin] and secondary antibody anti-rabbit/mouse IgG were from Cell Signaling technology, MA and p70S6K (Thr389) antibody was from Millipore, CA. The antibodies were used at 1:1000 for primary and 1:2000 for secondary respectively. Probing and visualization of immunoreactive bands were performed by exposing the membrane to X-Ray film and developed in the dark room using Konica Medical Film Processor.

2.6 Statistics

Statistical analysis (analysis of variance and Tukey multiple comparison test) was done using GraphPad Pism 5.0 and GraphPad InStat 3.0 (San Diego, CA). Statistical differences were considered significant at the $p < 0.05$ level and indicated by an asterisk ($p < 0.05$ (*))

3.0 RESULTS

3.1 TLR3 activation induces robust inflammatory response in HGEC

To identify the impact of mTOR activity on cellular inflammatory response generated through TLRs, we first assessed the effects of different TLR ligands, such as TLR2/6 ligand FSL-1, TLR3 ligand Poly I:C and TLR4 ligand *E. coli* LPS on keratinocytes based on our previous observations (Benakanakere et al., 2009; Kinane et al., 2008; Kinane et al., 2006), and after 4 h stimulation the cells were assessed for gene expression using customized qPCR-Arrays (Supplemental table 1) and after 24 h stimulation, cytokine levels were analyzed from cell culture supernatants. Unexpectedly, TLR3 activation by Poly I:C in keratinocytes led to a potent up-regulation of inflammatory genes at 4 h (Fig. 1A) and cytokine production after 24 h challenge including IL-1 β , TNF α and IL-12p70 relative to other TLR ligands tested (Fig. 1B). Two-way hierarchical clustering revealed similarities between TLR2 and TLR3 stimulation (Fig. 1A) but differed in terms of level of gene expression (Supplemental Table 1). In contrast to TLR3, either TLR2 or TLR4 did not

induce bioactive IL-12p70 protein expression in keratinocytes (Fig. 1B). Hence we selected TLR3 ligand to study and characterize the effect of mTOR pathway in these cells. It has already been shown that TLR3 can activate PI3K (Sarkar et al., 2004) and activated PI3K phosphorylate Akt (Martin et al., 2005). Akt activates mTOR by phosphorylation at Ser2448 (Nave et al., 1999). However, involvement of mTOR in TLR3 activation has not been investigated at least in non-myeloid cells such as keratinocytes. In order to test the role of PI3K in TLR3 stimulated keratinocytes, we adopted pharmacological inhibition of PI3K by LY294002 pretreated initially for 2 h before challenging with Poly I:C and after 24h post challenge, the supernatant was tested for IL-1 β , a primary pro-inflammatory cytokine in keratinocytes (Eskan et al., 2008a), TNF α and IL-12p70. Poly I:C treatment led to a robust IL-1 β and TNF α increase, however the inhibition of PI3K by LY294002 significantly decreased IL-1 β and TNF α secretion but increased IL-12p70 secretion in these cells (Fig. 1C). This data is showing the activation of PI3K through TLR3 stimulation in keratinocytes confirming the previous observation in HEK293 cell line (Sarkar et al., 2004). Furthermore, we noted the phosphorylation of I κ B α (Ser32/36) in Poly I:C challenge (Fig. 1D). FSL-1 activated rapid I κ B α phosphorylation as early as 15 min, reached maximum at 60 min and then decreased in its activity over time. However, Poly I:C induced delayed and sustained I κ B α phosphorylation compared to FSL-1. This activity correlates with the higher cytokine release by keratinocytes challenged by Poly I:C for 24 h. Interestingly, LPS activated very minimal I κ B α phosphorylation and correlated with lower cytokine secretion at 24 h in these cells compared to either FSL-1 or Poly I:C. We also monitored the activation of JNK by these ligands in the cells (Fig. 1D). FSL-1 rapidly activated JNK as early as 15 min post challenge but decreased after 60 min. However, Poly I:C induced delayed JNK activation at 60 min with persisted phosphorylation until 6 h. As expected, LPS failed to activate significant levels of JNK phosphorylation in keratinocytes.

3.2 mTOR distinctly controls pro-inflammatory cytokines induced by TLR3

We investigated whether and how mTOR involved in the regulation of inflammatory mediators upon TLR3 stimulation by Poly I:C in keratinocytes. After determining the optimal dose of rapamycin to block mTOR activity, the cells were pre-incubated with vehicle or rapamycin for 2 h and challenged with Poly I:C. We noted the upregulation of both IL-12p35 and IL-12p40 transcripts at 12 h after rapamycin treatment (Fig. 2A). However, at 24 h the rapamycin treatment highly upregulated IL-12p35 but failed to induce IL-12p40 mRNA expression (Fig. 2B). 24 h post stimulation, supernatants were assayed for IL-1 β , TNF α , IL-10, IL-12p40 and IL-12p70 by ELISA. Rapamycin alone did not affect IL-1 β (Fig. 3A) and TNF α (Fig. 3B) expression, but increased IL-12p70 (Fig. 3C) expression. Poly I:C or rapamycin alone or Poly I:C and rapamycin together did not induce the production of IL-10 in keratinocytes. We could not detect IL-12p40 at 24 h post challenge. However, IL-12p40 induced at 16 h post challenge in the presence of rapamycin (Fig. 3D). The mRNA data at 4 h are contradictory to protein expression at 24 h. We noted upregulation of mRNA for IL-10 by poly I:C at 4 h but failed to detect protein expression starting from 1 h to 96 h post challenge in the presence or absence of SB216763, a GSK-3 inhibitor (Benakanakere et al., 2010). This data on IL-10 is intriguing and further analyses of post transcriptional and translational studies are underway. Surprisingly, inhibition of mTOR impaired Poly I:C induced release of IL-1 β and TNF α from keratinocytes (Fig.

3A&B). However, one exception was IL-12p70 which increased with rapamycin mediated mTOR inactivation in these cells (Fig. 3C). To strengthen our confidence in the ability of mTOR pathway to regulate inflammatory cytokine production by Poly I:C, we employed RNA interference to knock down mTOR. As expected, mTOR knockdown significantly reduced IL-1 β production (Fig. 4A), whereas IL-12p70 was increased upon Poly I:C stimulation (Fig. 4B) which is consistent with the inhibitory effect of rapamycin on Poly I:C stimulated inflammatory cytokines. Thus, our data demonstrates that inhibition of mTOR contributes to limiting cytokine production induced by Poly I:C, and show that activation of the mTOR pathway is required for the TLR3 mediated cytokine induction.

3.3 Rapamycin inhibits mTOR-mediated p70S6K and 4E-BP1 pathways

The effect of rapamycin on pro-inflammatory cytokine production demonstrated the involvement of the mTOR signaling pathway in the inflammatory responses of keratinocytes. However, the mTOR pathway has not been defined in keratinocytes so far. In the past, it has been shown that mTOR can be activated just by medium replacement before stimulation (Chang et al., 2007; Rosenbluth et al., 2008). Surprisingly mTOR and p70S6K were already activated in unstimulated keratinocytes measured by phosphorylation at Ser2448 and Thr389 respectively (Fig. 5A), although the medium was changed 24–48h prior to the addition of stimulant to avoid the effect of medium replacement on mTOR. This suggests that mTOR activation is mediated by both TLR3 dependent and independent pathways, the latter of which could involve environmental stresses. Phosphorylation of p70S6K is a prime indicator of mTOR activation (Ali and Sabatini, 2005). mTOR inhibitor rapamycin potently inhibits the phosphorylation of p70S6K (Thr389) (Choo et al., 2008). In order to test if rapamycin blocks p70S6K phosphorylation, we pre-incubated the cells with rapamycin for 2 h and challenged with Poly I:C at 0, 15, 30 and 60 min to check the phosphorylation state of p70S6K. As expected, rapamycin abrogated the phosphorylation of p70S6K at all time points (Fig. 5A); concurrently, rapamycin inhibited 4E-BP1 phosphorylation (Fig. 5A). Phosphorylation of mTOR at Ser2448 in keratinocytes was observed upon short-term treatment with Poly I:C in the presence of rapamycin (Fig. 5A), however, it significantly inhibited phosphorylation of mTOR and abrogated phosphorylation of p70S6K in keratinocytes over 24 h (data not shown). Taken together, mTOR activates p70S6K and 4E-BP1 to induce cytokines and upon rapamycin inhibition of mTOR, it can no longer phosphorylate p70S6K and 4E-BP1 to down modulate proinflammatory cytokine IL-1 β and TNF α . Our data on IL-1 β and TNF α is in contrast with myeloid cells such as human monocytes where TNF α found to be increased upon rapamycin treatment (Weichhart et al., 2008) and IL-1 β increased upon rapamycin treatment *in vivo* in mice (Schmitz et al., 2008). Further, we also wanted to test the effect of rapamycin on phospho-Akt because Akt is considered upstream of the mTOR pathway (Weichhart et al., 2008) and no change would be expected if mTOR is blocked. As expected, Poly I:C induced phosphorylation of Akt at ser473, however, rapamycin augmented phospho-Akt (ser473) phosphorylation (Fig. 5A). This data suggests a feedback loop for Akt whereby inhibition of mTOR by rapamycin activates the Akt survival pathway. Our data on Akt is consistent with that of human non-small cell lung cancer (NSCLC) cells (Sun et al., 2005).

3.4 mTOR modulates JNK to regulate Poly I:C induced cytokines

The production of proinflammatory cytokines can also be regulated by 3 major mitogen activated protein kinases (MAPKs), p38 MAPK, JNK, and ERK1/2 (Makela et al., 2009). We therefore determined the time course of p38, JNK and ERK1/2 activation in the presence or absence of rapamycin pretreatment in TLR3 stimulated keratinocytes. Rapamycin induced a time-dependent decrease in the phosphorylation of JNK, maximal inhibition of phosphorylated JNK by rapamycin was observed at 60 min (Fig. 5A). We noted no change with p38 activation after rapamycin treatment (data not shown). Surprisingly rapamycin increased ERK1/2 activity in keratinocytes (Fig. 5A). While rapamycin inhibited JNK phosphorylation in Poly I:C stimulated cells at early time points, prolonged (24h) incubation of cells with Poly I:C and rapamycin pretreatment also reduced JNK phosphorylation (data not shown), suggesting that rapamycin exerts its effects via JNK. To test this, we observed the effects of JNK inhibitor treatment on cytokines in Poly I:C stimulated keratinocytes. The cells were pretreated with SP600125, a pharmacological inhibitor of JNK for 2h and cultured with Poly I:C for 24 h whereby the supernatant was analyzed for proinflammatory cytokines. SP600125 substantially suppressed Poly I:C induced IL-1 β , TNF α , IFN- β and IL-12p70 production by keratinocytes (Fig. 5C). Furthermore, SP600125 also significantly inhibited secretion of IFN- β (Fig. 5C) suggesting that Poly I:C induced IL-1 β , TNF α , IL-12p70 and IFN- β production in keratinocytes is mediated by JNK signaling pathway. Rapamycin may positively regulate the transcription and translation of IL-12p35 and IL-12p40 to induce IL-12p70, while signaling inhibition of JNK might negatively regulate IL-12p70 in poly I:C treated keratinocytes. These results collectively show that rapamycin distinctly control cytokine production at least in part through JNK pathway. JNK phosphorylates AP-1 transcription factor c-JUN at Ser63 and Ser73 and promotes enhanced transcription activity (Davis, 2000; Pulverer et al., 1991). Hence we investigated the phosphorylation of c-JUN at Ser63/73 in TLR3 stimulated keratinocytes pretreated with rapamycin. As expected, the phosphorylation of c-JUN at ser63/73 sites was down-regulated by rapamycin (Fig. 5A). This indicates that AP1 transcription factor plays a crucial role in the induction of proinflammatory cytokines in keratinocytes.

3.5 mTOR modulates NF-kB pathway via IKK α

To investigate Poly I:C triggered NF-kB activation, we treated keratinocytes with poly I:C and determined NF-kB activation by assessing I κ B α phosphorylation at ser32/36. As shown in Fig. 5B, poly I:C treatment resulted in activation of NF-kB in a time-dependent manner. Keratinocytes treated with NF-kB inhibitor showed significant suppression of TNF α and type I IFN secretion upon stimulation with Poly I:C in comparison to the controls (data not shown). Rapamycin treatment decreased the phosphorylation of I κ B α (ser32/36) at 30 min of Poly I:C challenge (Fig 5B). Interestingly, we noted the activation of IKK α (Ser176/180) by Poly I:C more so than IKK β (Ser177/181) and rapamycin inhibited its activation (Fig. 5B). Thus IKK α , not IKK β is a target of TLR3 activation in keratinocytes and may follow non-canonical NF-kB pathway. There is evidence from an *in vitro* kinase assay suggesting the phosphorylation of CREB occurs directly by Akt (Du and Montminy, 1998) implicating Akt as a positive regulator of CREB. We also wanted to probe CREB phosphorylation as we observed the activation of Akt by rapamycin. Interestingly, Phospho-CREB (Ser133)

increased upon rapamycin treatment in keratinocytes (Fig. 5B). This increase may be due to the suppression of GSK-3 β by Akt increasing CREB activation.

3.6 mTOR pathway regulates IRF3 to induce IFN- β

IRF3 has been shown to have a critical role in TLR3 induced responses through the TRIF-dependent pathway leading to IFNs (Fitzgerald et al., 2003). IRF3 is found in the cytoplasm as an inactive monomer and becomes activated by phosphorylation (Tsuchida et al., 2009). Recently the mTOR pathway has been implicated in type I IFN induction through IRF7 (Colina et al., 2008). We have previously shown that IRF3 is a crucial transcription factor for the induction of IFN- β in keratinocytes (Eskan et al., 2008b). Moreover, there are no reports showing the mTOR pathway modulating IRF3 to induce type I IFN. We wanted to test whether and how rapamycin affects type I IFN secretion in keratinocytes. To answer this question, we pre-treated the cells with vehicle or rapamycin for 2 h and challenged with Poly I:C for 24 h and then tested for IFN- α/β . Interestingly, rapamycin pretreatment downregulated TLR3 induced IFN- β secretion in keratinocytes (Fig. 6A). But, poly I:C or rapamycin alone or Poly I:C and rapamycin together could not able to induce the production of IFN- α in keratinocytes. Further, the cells were transfected with siTLR3 with mock siRNA as control to verify the involvement of TLR3 in recognizing Poly I:C in keratinocytes. After 24 h of transfection, the cells were challenged with Poly I:C for 24 h and IFN- β mRNA expression was detected. The cells transfected siTLR3 significantly downregulated IFN- β upon Poly I:C treatment (Fig. 6B). This clearly indicates the importance of TLR3 in recognizing dsRNA by keratinocytes. We wanted to determine if TLR2, TLR3 or TLR4 stimulation induces IRF3 phosphorylation in keratinocytes. To test this, we performed time course experiment by stimulating keratinocytes with LPS, FSL-1 and Poly I:C. To our surprise, only Poly I:C induced IRF3 phosphorylation and not LPS (Fig. 6C). This data correlates with IFN- β mRNA expression (Fig. 1A). Thus in keratinocytes, induction of IRF3 phosphorylation to induce IFN- β is mediated by TLR3 but not through TLR4 signaling. In the past, RIG-1 and MDA-5 has been shown to recognize dsRNA (Hirata et al., 2007; Kang et al., 2002; Kato et al., 2006; Yoneyama et al., 2004), so we wanted to test if Poly I:C induces RIG-1 and MDA-5 in keratinocytes. We performed time dependent stimulation of Poly I:C and noted no significant change with respect to RIG-1 induction with or without rapamycin pretreatment (Fig. 6D). MDA-5 expression was very low and we were not able to detect any changes compared to untreated cells (data not shown). Since we noted Akt activation upon poly I:C treatment, we wanted to test if Akt activation is blocked, whether it affects IRF3 phosphorylation or not. As expected, suppression of IRF3 phosphorylation was noted upon Akt inhibition (Fig. 6D). Taken together, PI3K-Akt activation is necessary to induce IRF3 phosphorylation in keratinocytes. Next we wanted to test IRF3 phosphorylation at Ser396 after Poly I:C challenge with or without rapamycin pretreatment. The immunoblot against phospho-IRF (ser396) showed significant decrease in phosphorylation after rapamycin pretreatment (Fig. 6E). This suggests the involvement of the mTOR pathway in modulating IFN- β induction via downregulation of IRF3 phosphorylation. Further, we examined the activation of TBK-1 after poly I:C treatment with or without rapamycin. The phosphorylation of TBK-1 (Ser172) is increased upon poly I:C treatment but augmented with rapamycin treatment (Fig. 6E). This data shows that mTOR modulates the induction of TBK-1. However, activated TBK-1 failed to induce IRF3

phosphorylation and IFN- β induction as revealed by mTOR inhibition. This data reveals the importance of PI3K-Akt-mTOR in the induction of IRF3 phosphorylation and IFN- β induction in keratinocytes.

4.0 DISCUSSION

The epithelial barrier of the oral mucosa prevents microbial invasion and can differentiate pathogenic and commensal microorganisms, thus eliciting innate immune defenses where necessary and avoiding tissue destruction (Kimbrell and Beutler, 2001). Previously it has been shown in TLR4 induced myeloid cells that rapamycin promotes production of TNF α , IL-6, and IL-12, concurrently preventing IL-10 production (Weichhart et al., 2008). However the physiological role of mTOR in non-myeloid cells such as keratinocytes remains unstudied. In this paper, we show for the first time that rapamycin can modulate TLR3 mediated cellular signaling pathway resulting in the suppression of pro-inflammatory cytokines IL-1 β , TNF α and Type I interferon IFN- β with activation of IL-12p70 production in oral keratinocytes. Rapamycin displayed both an anti-inflammatory activity by lowering IL-1 β and TNF α secretion and a pro-inflammatory property by enhancing IL-12p70, suggesting that the dominant effect of mTOR on keratinocytes is to modify the inflammatory responses. It has been previously demonstrated that TLR4 signaling pathway is typically a potent mediator of inflammatory cytokines in myeloid cells, and LPS was mainly employed to induce inflammatory cytokines (Martin et al., 2005; Ohtani et al., 2008; Weichhart et al., 2008). We compared the effects of TLR2, TLR3 and TLR4 ligands on release of inflammatory cytokines. We expected activation of cell surface TLR2 or TLR4 signaling to dominantly stimulate cytokines in keratinocytes as the first line of defense against plaque bacteria (Gram -ve or Gram +ve) in the gingival crevice which then initiates the chronic inflammatory condition known as periodontitis. However our initial ligand survey showed that TLR3 mediated more potent inflammatory responses than TLR2 or TLR4 in keratinocytes. Moreover, IL-12p70 protein was induced only with Poly I:C but not with LPS or FSL-1 although we noticed IL-12 mRNA expression in FSL-1 treated cells at 4 h.

Ohtani et al. (Ohtani et al., 2008) showed that rapamycin had no effect on LPS induced TNF α and IL-6 production in mouse bone marrow derived DCs (BMDCs), and Weichhart et al. (Weichhart et al., 2008) reported that rapamycin increased proinflammatory cytokines TNF α and IL-6 in human monocytes stimulated by LPS. In contrast, we found that TLR3 ligand stimulated IL-1 β , TNF α and IL-12p70 in keratinocytes but following incubation with rapamycin, there was suppression of IL-1 β and TNF α accompanied by enhancement of IL-12p70 production. Several investigations reported an increase in LPS-mediated IL-12p40 production and a decrease in IL-10 production upon rapamycin pretreatment in myeloid cells (Ohtani et al., 2008; Weichhart et al., 2008), however, we were unable to detect poly I:C induced IL-10 protein expression in keratinocytes with or without rapamycin treatment. But IL-12p40 was upregulated at 16 h in the presence of rapamycin. IL-12p40 could be early inducer protein as we noticed mRNA upregulation for IL-12p40 at 12 hours post challenge. At 24 h, we could not detect either protein or mRNA for IL-12p40. IL-12p40 seems to be converted to bioactive IL-12p70 at 24 h and hence we could not detect its

protein expression by ELISA. Thus mTOR appears to increase the transcription and translation of IL-12p40 and this perhaps is an early event compared to IL-12p35 expression.

In keratinocytes, Poly I:C induced release of IL-1 β was significantly reduced, whereas IL-12p70 was significantly increased by a transient knockdown of mTOR by siRNA. Thus, we identify the mTOR pathway as a crucial regulator of TLR3 signaling. We also found mTOR and p70S6K, to be constitutively activated in keratinocytes in the unstimulated state. A plausible explanation is that cell culture conditions do not mimic the tissue state and there are various stresses including changes in surroundings, temperature and nutrients. Even though the keratinocytes were cultured in serum free medium and starving the cells up to 24 to 48 h prior to challenging the cells induced mTOR activation at “0” h in unstimulated state. This basal mTOR activation may contribute to p70S6K phosphorylation event.

mTOR regulates cell growth and protein synthesis by activating p70S6 kinase (Wullschleger et al., 2006). Rapamycin decreased the phosphorylation of mTOR but completely abolished the phosphorylation of p70S6K in the presence of Poly I:C. Moreover, rapamycin blocked constitutively phosphorylated p70S6K suggesting it has a direct as well as indirect inhibition through mTOR. The same phenomenon was observed with small cell lung cancer cells where rapamycin inhibited the phosphorylation of constitutively active p70S6K (Sun et al., 2005). It has also been shown by site directed mutagenesis experiments that rapamycin can directly dephosphorylate p70S6K at rapamycin sensitive sites in 293 cells (Ferrari et al., 1993). It is evident from our data that rapamycin has profound effect directly on p70S6K more so than indirectly through mTOR in keratinocytes.

TLR3 recognizes viral dsRNA and its synthetic analog poly I:C that induces type I IFN, inflammatory cytokines, chemokines and DC maturation via the adaptor protein TRIF (Matsumoto and Seya, 2008). Other than TLR3, RIG-1 and MDA-5 has been shown to recognize dsRNA (Kang et al., 2002; Kato et al., 2006; Yoneyama et al., 2004). In our model, RIG-1 expression did not change compared to the control cells after Poly I:C stimulation. Moreover we noted very low levels of MDA-5 expression and observed no change compared to untreated in these cells (data not shown). However, when TLR3 was silenced by siRNA, the IFN- β expression was suppressed. This data clearly indicates the role of TLR3 in recognizing dsRNA in keratinocytes. Similar findings were observed in human mesothelial cells (Wornle et al.).

From the past literature, we know that TLR3 signaling leads to the activation of MAPKs, NF- κ B and IRF3. Activation of these pathways results in expression of various inflammatory mediators (Jiang et al., 2003; Vercammen et al., 2008), however the downstream signaling pathway of TLR3 to regulate MAPK, NF- κ B and IRF3 remain largely unknown. We also found that in the presence of rapamycin, Poly I:C activated JNK phosphorylation was inhibited. By comparison, Ohtani et al. (Ohtani et al., 2008) found that rapamycin had no effect on the LPS induced phosphorylation of JNK in DCs. In this study, JNK inhibitor SP600125 prevented the upregulation of IL-1 β , TNF α , IL12p70 and IFN- β upon stimulation with Poly I:C, suggesting that intracellular signaling by the JNK pathway contributes to regulation of proinflammatory cytokines and type I IFN. These results also indicate that mTOR regulates Poly I:C induced IL-1 β , TNF α , and IFN- β at least in part

through the JNK pathway. We found that rapamycin partially inhibited the phosphorylation of I κ B α (Ser32/36) in keratinocytes. Moreover, NF- κ B inhibitor reduced the expression of both inflammatory cytokine and type I IFN in our system (data not shown). Our data is consistent with PC3 cells where mTOR control the activation of NF- κ B and siRNA against mTOR significantly reduced NF- κ B activation (Dan et al., 2008). In our model, the TLR3 activation of NF- κ B is predominantly through the activation of IKK α but not IKK β . TLR3 also participates in PI3K signaling to activate IRF3 through phosphorylation at Ser396 residue (Sarkar et al., 2004). We have previously shown that IRF3 phosphorylation at Ser396 is critical to induce IFN- β in keratinocytes and its inhibition by RNAi led to decreased IFN- β production (Eskan et al., 2008b). It is reasonable to hypothesize the involvement of mTOR signaling in TLR3 mediated IRF3 phosphorylation. Rapamycin downregulated the secretion of IFN- β and also the phosphorylation of IRF3 (Ser396) in keratinocytes demonstrating the involvement of Akt-mTOR-p70S6K-IRF3 pathway in the regulation of Type I IFN in keratinocytes. Suppression of mTOR and p70S6K increased ERK1/2 activity in Keratinocytes. We also noted levels of phospho-Akt increase upon rapamycin treatment. This increase suggests the existence of feedback loop for Akt activation and may be dependent on increased signaling of IRS-1 as previously observed (Guertin and Sabatini, 2009; O'Reilly et al., 2006). Enhanced ERK1/2 phosphorylation may be due to increased phospho-Akt activation by inhibiting GSK-3 β leading to the suppression of phospho-RAC1 at Ser71 and subsequent phosphorylation of PAK1 at Ser199/204 and cRaf at Ser338 activating ERK1/2 as observed in human monocytes (Rehani et al., 2009). mTOR activation might thus cause inflammatory responses and participate in the generation of protective immunity against some viral infections.

In our system, rapamycin caused Poly I:C to induce IL-12p70, thus positively regulating most cytokine responses, mTOR appears to suppress IL-12p70 production. Rapamycin may induce epigenetic changes and chromatin modifications to expose the promoter region which may induce increased transcription of IL-12p70 in this case. Suppression of bioactive IL-12p70 by mTOR in epithelial cells seems to make sense because the overproduction of IL-12 gives rise to strong cell-mediated immunity and organ specific autoimmune diseases via exaggerated Th1 cell differentiation, and it is critical that IL-12 levels be tightly controlled (Trinchieri, 2003). Rapamycin caused activation of TLR3 stimulated IL-12p70, whereas signaling inhibition of JNK causes suppression of IL-12p70 in TLR3 stimulated cells, so it is also possible that rapamycin enhances IL-12p70 production through the CREB dependent pathway as the phospho-CREB (ser133) was increased by rapamycin pretreatment. How the Akt induces CREB phosphorylation is still controversial and it is not clear whether Akt should positively or negatively affect CREB activation via GSK-3 β (Peltier et al., 2007). mTOR modulates TLR3 induced cytokines directly and indirectly, at least in part via JNK and NF- κ B. When mTOR is inhibited by rapamycin, TBK-1 induction was augmented. Nevertheless, IRF3 activation was limited. This clearly indicates that TBK-1 only partially activate IRF3 consistent with other findings (Sarkar et al., 2004; Sharma et al., 2003). Although TBK-1 partially activated IRF3, inhibition of either PI3K or Akt or mTOR inhibited IFN- β induction and marks the crucial role of mTOR in the activation of IRF3 to induce IFN- β in keratinocytes. Similarly two step phosphorylation of IRF3 involving TBK-1 and PI3K have been shown with insufficient IRF3 transcriptional

capability through TBK-1 (Sarkar et al., 2004). Based on our present study as well as other reports, we propose that signal transduction pathways downstream of TLR3 regulating cytokine production are composed of at least 3 components: mTOR, JNK, and NF- κ B in keratinocytes. We also propose a mechanism involved in IRF3 activation by Akt via mTOR and p70S6K and describe in this report i) the role of mTOR in keratinocytes ii) role of mTOR in TLR3 responses iii) Akt induces IRF3 activation via mTOR signaling iv) TLR3 preferentially activate IKK α to induce NF- κ B and v) IFN- β secretion in gingival epithelial cells is exclusively through TLR3 and not by TLR4.

The ability of mTOR to differentially regulate distinct cytokines in non-myeloid cells such as keratinocytes suggests an impact on subsequent T cell activation in viral infections. In summary, our work indicates mTOR signaling is indispensable and plays a significant role in modulation of TLR3 stimulated response through both direct and indirect mechanisms and thus could play an important immunomodulatory role in innate immune and inflammatory responses associated with viral infection of keratinocytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This work was supported by United States Public Health Service, National Institutes of Health, NIDCR grant DE017384 to DFK

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Figure 1. TLR3 stimulation of Keratinocytes leads to robust cytokine secretion

Keratinocytes were incubated with *E. Coli* LPS (1µg/ml), FSL-1 (1µg/ml) and Poly I:C (5 µg/ml) after determining optimal dose for each ligand for 4 h and total RNA was isolated, converted to cDNA and real time qPCR-Arrays were performed. The CT values were used to generate heatmap based on two-way hierarchical clustering with MeV v4.1 software (*rows*=genes, *columns*=samples). The *color scale* indicates relative expression: *yellow*, above mean; *blue*, below mean; and *black*, below background (A). The cells were treated with *E. Coli* LPS (1µg/ml), FSL-1 (1µg/ml) and Poly I:C (5 µg/ml) for 24 h and supernatant

was subjected to IL-1 β /TNF α / IL-12p70 ELISA. TLR3 stimulation by poly I:C induced robust cytokine induction (B). The cytokine induction by Poly I:C was significantly downregulated by PI3K pharmacological inhibition by LY294002 (10 μ M) (C). Time course experiment was done by stimulating epithelial cells with *E. Coli* LPS (1 μ g/ml), FSL-1 (1 μ g/ml) and Poly I:C (5 μ g/ml). The total protein was subjected to immunoblot against phospho-IkBa (Ser32/36) (i), Phospho-SAPK/JNK (Thr183/Tyr185) (ii) with β -actin (iii) as control (D). Media control cells received DMSO unless otherwise stated. Results are mean $\pm$ SEM and are representative of three independent experiments. Statistical comparisons are shown by horizontal bars with asterisks above them (* indicates p<0.01 determined by ANOVA and Tukey multiple comparison test).

Figure 2. IL-12p35 and IL-12p40 gene expression in keratinocytes

Keratinocytes were pretreated with rapamycin (200 nM) for 2 h before challenging with Poly I:C (5 µg/ml) for 12 and 24 hours. After challenge, the total RNA was collected and subjected to real-time PCR. IL-12p35 and IL-p40 mRNA expression was upregulated by rapamycin pretreatment in the presence of Poly I:C (A). We could only detect IL-12p35 at 24 h post challenge with rapamycin pre-treatment (B). Results are mean $\pm$ SEM and are representative of three independent experiments. Statistical comparisons are shown by horizontal bars with asterisks above them (* indicates $p < 0.01$ determined by ANOVA and Tukey multiple comparison test).

Figure 3. mTOR distinctly regulate cytokine induction by Keratinocytes

Keratinocytes were pretreated with rapamycin (200 nM) for 2 h before challenging with Poly I:C (5 µg/ml) for 24 h. The supernatant was subjected to IL-1β/TNFα/ IL-12p70 ELISA according to manufacturer's instruction. In another experiment, the pre-treated cells were challenged for 16 h in the presence of poly I:C and supernatant was subjected to IL-12p40 by ELISA. The cytokine induction of IL-1β (A) and TNFα (B) were significantly downregulated upon rapamycin pretreatment. However, the bioactive IL-12p70 secretion is significantly upregulated upon rapamycin pretreatment (C). IL-12p40 was upregulated by

rapamycin at 16 h (D). Media control cells received vehicle unless otherwise stated. Results are mean $\pm$ SEM and are representative of three independent experiments. Statistical comparisons are shown by horizontal bars with asterisks above them (* indicates $p < 0.01$ determined by ANOVA and Tukey multiple comparison test).

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Figure 4. RNAi mediated inhibition of mTOR differentially regulates cytokine induction

The cells were transiently transfected with siRNA against mTOR and after 24 h of post transfection, the cells were pretreated with or without rapamycin (200 nM) before challenging with Poly I:C (5 μ g/ml) for 24 h. After 24 h, the supernatant was subjected to IL-1 β and IL-12p70 ELISA. IL-1 β secretion in Keratinocytes was significantly reduced in cells transfected (siRNA mTOR) with rapamycin pretreatment (A). However, IL-12p70 was significantly upregulated upon mTOR knockdown (B). Media control cells received vehicle unless otherwise stated. Results are mean $\pm$ SEM and are representative of three independent experiments. Statistical comparisons are shown by horizontal bars with asterisks above them (* indicates $p < 0.01$ determined by ANOVA and Tukey multiple comparison test).

Figure 5. mTOR mediate JNK pathway to regulate cytokine induction in Keratinocytes

The cells were pretreated with or without rapamycin (200 nM) for 2 h and challenged with Poly I:C (5μg/ml) for 0, 15, 30 and 60 min. Total protein was subjected to immunoblot against phospho- mTOR antibody (Ser2448) and each time the antibody is stripped and re-probed with Phospho-p70S6K (Thr389), Phospho-4E-BP1 (Thr37/46), Phospho-ERK 1/2 (Thr202/Tyr204), Phospho-SAPK/JNK (T183/Y185), Phospho-c-JUN Ser63/73 and Phospho-Akt (Ser473) with β-actin as loading control (A). In a different experiment, the cells were pretreated with or without rapamycin (200 nM) for 2 h and challenged with Poly I:C (5μg/ml) for 0, 15, 30 and 60 min and total protein was subjected to immunoblot against Phospho-IKKα (Ser176/180)/IKKβ (Ser177/181), Phospho-IκBα (Ser 32/36) and Phospho-CREB (Ser133) with β-actin as loading control (B). Pre-incubation of rapamycin led to complete inhibition of Phospho-p70S6K (Thr389) and reduction in phospho- mTOR (Ser2448), Phospho-4E-BP1 (Thr37/46), Phospho-c-JUN Ser63/73, Phospho-SAPK/JNK (T183/Y185) phosphorylation levels. Surprisingly, Phospho-ERK 1/2 (Thr202/Tyr204) and Phospho-Akt (Ser473) were increased upon rapamycin pretreatment (A). The phosphorylation of Phospho-IKKα (Ser176/180) and Phospho-IκBα (Ser 32/36) was

reduced but Phospho-CREB (Ser133) activity increased upon treatment with rapamycin (B). The cells were pretreated with JNK inhibitor SP600125 for 2 h and challenged with Poly I:C for 24 h. The supernatant was subjected to IL-1 β /TNF α /IL-12p70/IFN- β ELISA. Pretreatment of SP600125 significantly downregulated IL-1 β /TNF α /IL-12p70/IFN- β production by Poly I:C in Keratinocytes (C). Media control cells received DMSO unless otherwise stated. Results are mean $\pm$ SEM and are representative of three independent experiments (for panel C). Statistical comparisons are shown by horizontal bars with asterisks above them (* indicates $p < 0.01$ determined by ANOVA and Tukey multiple comparison test).

Figure 6. Rapamycin regulate IRF3 and inhibits IFN-β in Keratinocytes

The cells were pretreated with or without rapamycin (200 nM) for 2 h and challenged with Poly I:C (5 μg/ml) for 24 h and supernatant was subjected to IFN-β ELISA. Rapamycin pretreatment significantly downregulated IFN-β expression in Keratinocytes (A). Keratinocytes were transfected with siTLR3 and Mock siRNA. 24 h post transfection, the cells were challenged with poly I:C (5 μg/ml) for 24 h and supernatant was subjected to IFN-β ELISA. siTLR3 transfected cells downregulated IFN-β compared to mock transfected cells (B) Total protein from time course experiment was subjected to immunoblot against phospho-IRF3

(Ser396) with β -actin as control. Poly I:C treatment induced the phosphorylation of IRF3 (Ser396) in Keratinocytes (C). In a different experiment, the total protein from indicated time point was immunoblotted against RIG-1 antibody with β -actin as control. No change with RIG-1 induction was noted upon Poly I:C treatment in the presence or absence of rapamycin (D). Keratinocytes were pretreated with Akt inhibitor and stimulated with poly I:C and total protein was subjected to immunoblot against phospho-IRF3 (Ser396) and β -actin as control. Akt inhibition significantly reduced phospho-IRF3 (Ser396) activation (E). The time course experiment was done to check the effect of rapamycin on IRF3 phosphorylation. The cells were pretreated with or without rapamycin for 2 h and challenged with Poly I:C at indicated time points. The total protein was subjected to immunoblot against Phospho-TBK-1 (Ser172), phospho-IRF3 (Ser396) with β -actin as loading control. Rapamycin pretreatment reduced the phosphorylation of IRF3 at Ser396 compared to control cells, however, increased TBK-1 (Ser172) phosphorylation (E). Media control cells received DMSO unless otherwise stated. Results are mean $\pm$ SEM and are representative of three independent experiments. Statistical comparisons are shown by horizontal bars with asterisks above them (* indicates $p < 0.05$ determined by ANOVA and Tukey multiple comparison test).

Figure 7. Model of mechanism involved in the regulation of mTOR pathway in Keratinocytes
 Stimulation of TLR3 by Poly I:C leads to activation of PI3K. Activation of PI3K by TLR3 recruits PKB/Akt. Akt is subsequently activated by the Ser473 phosphorylation by PI3K. Activated PI3K/Akt phosphorylates mTOR at Ser2448. Phosphorylated mTOR controls the activation of Phospho-SAPK/JNK at Thr183/Tyr185 and NF-κB via IKKα to induce proinflammatory cytokines and IRF3 phosphorylation at Ser396 to induce IFNβ in

Keratinocytes. Blockade of mTOR by rapamycin leads to downregulation IL-1 β and TNF α but induce IL-12p70 expression in Keratinocytes.

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