Published in final edited form as:

J Surg Res. 2011 December ; 171(2): 769–776. doi:10.1016/j.jss.2010.05.035.

ROLE OF THE mTOR PATHWAY IN LPS-ACTIVATED MONOCYTES: INFLUENCE OF HYPERTONIC SALINE

Valérie Schaeffer, PhD^{*}, Saman Arbabi, MD, Iris A. Garcia, BS, Megan L. Knoll, BS, Joseph Cuschieri, MD, Eileen M. Bulger, MD, and Ronald V. Maier, MD
Harborview Medical Center, 325 9th Ave, Seattle, WA 98104.

Abstract

Background—As heightened protein synthesis is the hallmark of many inflammatory syndromes, we hypothesize that the mammalian target of rapamycin (mTOR) pathway, which control the cap-dependent translation initiation phase, was activated by lipopolysaccharide (LPS). In addition, we studied the effect of hypertonic saline solution (HTS) on the mTOR cascade in peripheral blood mononuclear cells (PBMCs).

Materials and methods—PBMCs were isolated from healthy volunteers and treated with LPS. Cells were pretreated with phosphatidylinositol 3-kinase (PI3K) and mTOR inhibitors, or with HTS. Supernatants were harvested 20hrs following LPS treatment, and interleukin-10 (IL-10), interleukin-6 (IL-6) and tumor necrosis α (TNF α) were analyzed by ELISA. Immunoblot experiments were performed for components of the PI3K/Akt/mTOR pathway at various time points. RNA was extracted after 90 min for real-time RT-PCR quantification.

Results—The mTOR pathway is activated in PBMCs within an hour of LPS stimulation. Pre-treatment with rapamycin, a specific inhibitor of mTOR, resulted in a significant decrease of IL-10 and IL-6 translation and expression but did not affect the LPS-induced TNF α production. Both the mTOR pathway and the LPS-induced IL-6 production were down-regulated by HTS pre-treatment.

Conclusions—The PI3k/Akt/mTOR cascade modulates LPS-induced cytokines production differentially. IL-10 and IL-6 expression are both up-regulated by activation of the mTOR pathway in response to LPS in PBMCs, while TNF α is not controlled by the mTOR cascade. Meanwhile, pre-treatment of PBMCs with a HTS solution suppresses mTOR activity as well as LPS-induced IL-6, suggesting a more central role for mTOR as a regulator of the immuno-inflammatory response.

Keywords

innate immuno-inflammatory response; mTOR pathway; cytokines; LPS; hypertonic saline

INTRODUCTION

A leading cause of late death of trauma patients is due to the multiple organ dysfunction syndrome (MODS), resulting from the deregulation of the inflammatory response as injury activates the innate immune system in an attempt to restore homeostasis. Following major trauma, numerous pro- and anti-inflammatory cytokines are released by activated monocytes/macrophages (reviewed in (1)). In a healthy patient with a minor injury, equilibrium is restored quickly and the inflammatory response remains local. In case of a major insult however, the inflammatory response may spread throughout the whole body and leads to diffuse organ injury including the acute respiratory distress syndrome (ARDS), sepsis, septic shock and MODS with a concomitant significant morbidity and mortality for the injured patient.

The anti-inflammatory cytokine interleukin-10 (IL-10) and the proinflammatory cytokines tumor necrosis factor α (TNF α) and interleukin-6 (IL-6) play important roles in the immunoinflammatory response. TNF α is a potent pro-inflammatory cytokine with potential detrimental effects when present at excessive levels, and injection of TNF α in humans reproduces the symptoms of sepsis and septic shock (2). IL-6 is a major pro-inflammatory mediator that coordinates the acute-phase response to trauma and infection. Elevated serum levels of IL-6 have been associated with poor outcome in a number of inflammatory conditions such as burn injury, trauma and sepsis (3-5). IL-10 is a pleiotropic cytokine with effects in immunoregulation and inflammation. In animal model of endotoxemia, plasma levels of IL-10 are elevated to prevent tissue damage by inhibiting pro-inflammatory cytokines such as IL6 and TNF α (6). The importance of the IL-10 response has been demonstrated in burn patients (7) and in animal model of sepsis (8,9). IL-10, TNF α and IL-6 are tightly controlled at several levels, including gene transcription and translation.

Highly regulated proteins, with critical biological activities, such as cytokines, are often regulated at the translational level. mTOR is a well-conserved kinase of the phosphatidylinositol 3-kinase (PI3K)-related kinase (PIKK), which regulates protein synthesis through the phosphorylation and inactivation of the repressor of mRNA translation, eukaryotic initiation factor 4E-binding protein (4E-BP1) (Fig. 1). The TOR pathway is conserved from yeast to mammals and has been shown to control cell growth, cell cycle, neurite plasticity and metabolism, in addition to translation initiation (10). At the same time, dysregulation of the mTOR pathway has been implicated in many disorders, such as tumorigenesis, metastasis (11), metabolic diseases (12,13), cardiac hypertrophy (14), sepsis (15,16) and more recently after traumatic brain injury (17). As activation of mTOR has been shown to facilitate translation initiation, the rate-limiting step in protein synthesis, we hypothesized that the mTOR pathway, which control the cap-dependent translation initiation phase, was activated by lipopolysaccharide (LPS) and responsible for cytokine production. In addition, we hypothesized that some of the beneficial effects of hypertonic saline solution (HTS) on cytokine production, could be by the way of its action on the mTOR cascade in peripheral blood mononuclear cells (PBMCs).

MATERIALS AND METHODS

This study was approved by the Institutional Review Board of the University of Washington, Seattle, WA, USA under the number 18426. Informed consent was obtained from all volunteers.

PBMC preparation and treatment

Venous blood (60 ml) was collected from healthy human volunteers in syringes containing 5 ml of 3.6% Sodium Citrate and processed as described previously (18). Briefly, each aliquot

of 15 ml of whole blood was diluted with 20 ml of DPBS (Dubecco's PBS without calcium or magnesium, BioWhittaker, Walkersville, MD) and underlaid with 15 ml of Ficoll-Paque Plus (Amersham Bioscience, Piscataway, NJ). Tubes were then centrifuged at room temperature for 30 min at 1200 rpm, the buffy coat was retrieved and washed with RPMI (Gibco/Invitrogen, Carlsbad, CA) supplemented with 0.1 mg/ml of gentamycin (BioWhittaker, Walkersville, MD). PBMCs were then allowed to adhere on tissue culture plates by incubation at 37°C for 2 hrs. The cells were then rinsed 2 times with RPMI-gentamycin and incubated for 30 min at 37°C with RPMI-gentamycin + 10% ABS (Donor Adult Bovine Serum, HyClone, Logan, UT).

If indicated the following treatments were applied to the PBMCs: LY294002 a specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one, (19) (Biosource, Camarillo, CA, 25 μ M, (20)), rapamycin (Cell Signaling, Danvers, MA, 10 nM, as suggested by the manufacturer), NaCl (HTS, 40 mM-120 mM, (21)), starting one hour prior to the LPS (Lipopolysaccharides from *Escherichia coli* 0111:B4, Sigma, St Louis, MO) addition (10 ng/ml, (18)), except for HTS treatment that was started 4hrs prior to LPS addition (21).

Western blot

$3\text{--}5\times 10^6$ cells were lysed at the indicated time after LPS treatment, in 200 μ l of lysis buffer (20 mM Tris pH8.0, 137mM NaCl, 2mM EDTA, 10% glycerol, 1% Triton X100, 10 μ l/ml Protease Inhibitor Cocktail II (Sigma, St Louis, MO), 10 μ l/ml Phosphatase Inhibitor cocktail II (Sigma, St Louis, MO), 0.2 mM PMSF, 0.5mM DTT). If so indicated, treatment with inhibitors was started one hour prior to the LPS treatment with LY294002 or rapamycin. Lysates were then vortexed and incubated 15 min on ice prior to being cleared by centrifugation (5 min, 4°C, 12000 rpm). Protein concentrations of the supernatants were measured using the BCA protein assay (Pierce, Rockford, IL). 2X loading buffer was added to the supernatants. Samples were boiled for 5 min and stored at -70°C. 20-40 μ g of total cellular proteins were separated onto a SDS-PAGE gel and transferred onto Hybond-Nitrocellulose membrane (Amersham Pharmacia, Piscataway, NJ) for 2 hrs. Immunoblotting was performed as directed by the manufacturer of the primary antibodies: Phospho-Akt-Thr308 (pAkt), total Akt, phosphomTOR-Ser2448 (pmTOR), total mTOR and phospho-4EBP1-Ser65 (p4E-BP1) were obtained from Cell Signaling, Danvers, MA; and total 4E-BP1 from AbD Serotec, Raleigh, NC. Equal loading was confirmed using an antibody to ERK2 (Santa Cruz Biotechnology, Santa-Cruz, CA) or to β actin (AbD Serotec, Raleigh, NC). Anti-rabbit IgG labeled with horseradish peroxidase (Santa Cruz Biotechnology, Santa-Cruz, CA) was used as the secondary antibody and signal visualized using the Super Signal West Pico detection system (Pierce, Rockford, IL). Densitometry was performed using ImageJ 1.33u (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://rsb.info.nih.gov/ij/>, 1997-2005).

Cytokine measurements

Cell supernatants (from $1\text{--}2\times 10^6$ PBMCs/conditions) were collected after 20hrs of LPS (10 ng/ml) treatment and frozen at -80°C. TNF α , IL-6 and IL-10 concentrations were measured by using the BD OptEIA ELISA kits (BD Bioscience, San Jose, CA) specific to each cytokines using the manufacturer's protocol.

Real-time RT-PCR

5×10^6 PBMCs were lysed and total RNA was extracted with an RNeasy kit (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol. Reverse transcription was performed using an Omniscript RT kit (Qiagen Inc., Valencia, CA). Gene expression quantification was done using the TaqMan Gene Expression Assays listed below and a

TaqMan Universal PCR Master Mix (No Amperase UNG) from Applied Biosystems (Foster City, CA). PCR experiments were carried out as described previously. The following TaqMan gene expression assays were used: Hs99999905_m1 (GAPDH), Hs99999903_m1 (β actin), Hs00174131_m1 (IL-6), Hs00174086_m1 (IL-10) and Hs00174128_m1 (TNF α). PCR results were analyzed with the SDS 2.1 software (Applied Biosystems, Foster City, CA).

Cell viability

Total cellular protein content extracted from the final washed monolayers for western blotting was similar for all conditions. Cell viability was also confirmed with trypan blue exclusion and > 95%.

Statistics

All values represent mean and S.E.M. Group means were compared by ANOVA with post-hoc testing by Bonferroni using Prism 4 for Macintosh (GraphPad software, San Diego, CA).

RESULTS

LPS activates mTOR and the translational inhibitor 4E-BP1 via Akt in PBMCs of healthy volunteers

LPS, a major component of the outer membrane of gram-negative bacteria, induces a prototypical inflammatory response in immune cells such as PBMCs. To investigate the consequences of LPS stimulation of PBMCs of healthy volunteers on mTOR signaling, we examined the phosphorylation status of Akt, mTOR and 4E-BP1. As expected, Akt is phosphorylated at Thr 308 within 15-30 min upon LPS treatment (up to 2.5 fold change over control at 30 min, Fig. 2A). LPS led to an increase in mTOR phosphorylation (up to 1.75 fold change over control at 60 min, Fig. 2B) and 4E-BP1 phosphorylation (3 to 4 fold change over control for up to an hour, Fig. 2C) in PBMCs, indicating that the activity of the mTOR pathway is enhanced during the inflammatory process created by LPS treatment.

LY294002 and rapamycin inhibit the 4E-BP1 Ser 65 phosphorylation

Insulin and other growth factors have long linked the activation of PI3K/Akt cascade to the stimulation of translation initiation through the mTOR target, 4E-BP1. We asked if the same was the case for LPS stimulation. We used LY294002 (25 μ M, 1 hr), a PI3K inhibitor, and rapamycin (10 nM, 1 hr), a specific mTOR inhibitor, and examined the PI3K/Akt/mTOR cascade activation. LY294002 treatment significantly downregulated the PI3K/Akt/mTOR cascade as it abrogated phosphorylation of Akt, (data not shown) mTOR (Fig 3A) and 4E-BP1 (Fig. 3B). On the other hand, rapamycin only inhibited the phosphorylation and activation of 4E-BP1 (compare Fig. 3A and 3B). These results demonstrate that LPS stimulates the PI3K/Akt/mTOR pathway to activate and phosphorylate the mTOR target, the translational inhibitor 4E-BP1.

Regulation of IL-10, IL-6 and TNF α by rapamycin, a specific mTOR inhibitor

We then tested the functional consequence of inhibiting the mTOR pathway in PBMCs on the mRNA and protein levels of IL-10, IL-6 and TNF α . LPS-induced IL-10 and IL-6 protein levels are affected by rapamycin while their mRNA levels do not change, suggesting that mTOR acts at the translational level on these two mRNAs (Fig. 4A, 4B and 4C and 4D). IL-10 protein production is reduced by 75% (Fig 4A) and IL-6 protein levels are reduced by 60% (Fig 4B) respectively. However, LPS-induced TNF α protein production doesn't seem to be affected by rapamycin (Fig 4E), neither at the mRNA level nor at the protein level (Fig.

4F). These findings demonstrate that the mTOR cascade selectively regulates LPS-induced cytokine expression by the PBMCs in response to LPS.

Hypertonic saline solution downregulates mTOR cascade

As HTS administration has potential immuno-beneficial effects, we hypothesized that HTS pre-treatment will attenuate activation of the mTOR pathway in PBMCs, reduce cytokine production and thereby attenuate the ensuing immuno-inflammatory response. IL-6 has been shown to be a crucial player of the innate immune response and a major pro-inflammatory mediator that coordinates the acute phase response to trauma and infection (3-5). Pre-treatment with increasing concentration of HTS dramatically suppressed the LPS-induced IL-6 production by PBMCs (Fig. 5A, $p < 0.05$). We investigated the potential mechanism of hypertonic saline solutions and our data showed a clear suppression of mTOR cascade activation after HTS treatment, as shown by the immunoblot of p-mTOR (Fig. 5B), as well as of the translational inhibitor, p-4E-BP1 (Fig. 5C). Those results demonstrate that some of the immuno-beneficial effects of HTS could be explained by the effect on the mTOR pathway.

DISCUSSION

This study demonstrates a role for the mTOR cascade (Fig. 1) in the innate immune response to LPS in PBMCs. After LPS treatment, the mTOR kinase (Fig. 2 C, F) is activated through phosphorylation of the Akt kinase (Fig. 2 A, E), leading to the phosphorylation of the translational inhibitor 4E-BP1 (Fig 2 D, F). 4E-BP1 is phosphorylated in a sequential manner: phosphorylation at Thr 37/Thr 46 is followed by Thr 70 phosphorylation and Ser 65 is phosphorylated last (22). As it is the combination of all phosphorylation events that is responsible for 4E-BP1 activation, we measured 4E-BP1 phosphorylation at Ser 65. The PI3K inhibitor, LY294002, and the mTOR inhibitor, rapamycin, both inhibit the activation of the translational inhibitor 4E-BP1 and of its S65 phosphorylation (Fig. 3 B).

This study has several limitations: The relatively small number of volunteers included in the study (3 to 6) might not be enough to see a clear statistical effect especially in the immunoblotting experiments, as the individual inflammatory response can vary widely.

Sepsis has been shown to affect the rate of translation initiation in the heart, muscle and liver (15,23). In the event of a crisis, such as sepsis or a LPS challenge, the organism reorganizes protein synthesis to survive. It decreases muscle protein synthesis and up-regulate hepatic protein synthesis to produce acute phase proteins. Additionally, endotoxin has been shown to stimulate translation initiation by phosphorylation of 4E-BP1, therefore releasing eIF-4E to enhance translation in macrophages (24,25). In macrophages, phosphatidic acid accumulation also causes mTOR activation through the Akt kinase (26) and activated neutrophils have been shown to stimulate translation of the soluble IL-6 receptor α via the mTOR cascade (27).

The mTOR pathway is a central regulator of protein synthesis, cell growth, apoptosis and metabolism. Nutrients such as amino acids, growth factors (such as insulin) or the energy status of the cell can activate the cascade, which lead, among others, to the phosphorylation and inhibition of the translational inhibitor 4E-BP1, and the release of the cap-binding factor eIF4E. The dysregulation of the mTOR cascade has been implicated in numerous processes and disorders, ranging from cancers to metabolic disorders to aging (12). Several lines of evidence suggest that translational control through the mTOR checkpoint could be involved in the regulation of the innate immune inflammatory response (24,25). In the current study we demonstrated that mTOR pathway inhibition attenuated LPS-induced translation of IL-6 and IL-10, but it had no effect on the LPS-induced TNF expression (Fig. 4).

In different models, IL-6 expression has been shown to be both protective and harmful. Blockade of IL-6 signaling has improved survival in two mice sepsis models (28,29) and has proven to be highly effective in humans, for treating chronic inflammatory diseases, such as Crohn's disease or rheumatoid arthritis (30). At the same time, IL-6 exhibits anti-inflammatory properties and is able to stimulate the production of other anti-inflammatory cytokines (31). Since IL-6 plays a protective role in the immune response against gram-negative bacteria (32), it seems that it is the over-expression of IL-6 that is detrimental more than its mere presence (33). Rapamycin treatment attenuated LPS-induced IL-6 production by 60% in our system (Fig. 4A). In LPS-activated PBMCs, TNF α doesn't seem to be sensitive to rapamycin (Fig. 4E). These results are in accordance with Jorgensen *et al.* (34), who showed that Sirolimus strongly attenuates the LPS-induced IL-10 production in whole blood but had no statistically significant effect on LPS-dependent TNF- α production. However, in other cell types, such as bone marrow neutrophils, rapamycin treatment did decrease LPS-induced TNF α release (35), demonstrating that the sensitivity of TNF α production to inhibition of the mTOR pathway is cell-type and condition dependent.

Hemorrhagic shock is one of the leading causes of early death in the trauma patient. Resuscitation from traumatic blood loss activates the innate immune system potentially leading to ARDS and MODS. HTS solutions are proven to be safe and efficient fluids for resuscitation from hemorrhagic shock as well as reduce intra-cranial pressure in brain-injured patients (36) with some potentially beneficial immuno-modulating effects (37,38). HTS (7.5% saline) have been shown to modulate the whole body systematic immuno-inflammatory response after injury (39) as well as to diminish the cytokine production by monocytes and blunt neutrophil activation induced by shock (40-43). In this paper, we show that HTS decreased the activation of the mTOR kinase. These results are in accordance with experiments done in human kidney cells where hypertonic shock (150 mM NaCl, 1 hr) has been shown to cause a rapid inhibition of protein synthesis via the dephosphorylation of 4E-BP1 and the ribosomal protein S6, another mTOR read-out (44-46). We also demonstrate that LPS-induced IL-6 production is attenuated by HTS treatment. Therefore, some of the immunomodulatory effects seen with HTS resuscitation may be, in part, due to attenuation of the mTOR pathway.

CONCLUSION

In conclusion, we have demonstrated that the mTOR pathway seems to be a novel player in the innate immune inflammatory response that regulate the level of cytokine translation, and potentially influences the innate immune response to HTS resuscitation. The mTOR pathway activation status may be a marker to identify patients that have a high risk of developing systemic inflammatory response syndrome and post-traumatic complications.

Acknowledgments

We thank Staci Knudson for excellent technical assistance. This work was supported by grants from the national Institute of health (R01 GM45813 and T32 GM07037).

REFERENCES

1. Keel M, Trentz O. Pathophysiology of polytrauma. *Injury*. 2005; 36:691–709. [PubMed: 15910820]
2. Michie HR, Spriggs DR, Manogue KR, Sherman ML, Revhaug A, O'Dwyer ST, Arthur K, Dinarello CA, Cerami A, Wolff SM, et al. Tumor necrosis factor and endotoxin induce similar metabolic responses in human beings. *Surgery*. 1988; 104:280–286. [PubMed: 2456628]
3. Hack CE, De Groot ER, Felt-Bersma RJ, Nuijens JH, Strack Van Schijndel RJ, Eerenberg-Belmer AJ, Thijs LG, Aarden LA. Increased plasma levels of interleukin-6 in sepsis. *Blood*. 1989; 74:1704–1710. [PubMed: 2790194]

4. Giannoudis PV, Hildebrand F, Pape HC. Inflammatory serum markers in patients with multiple trauma. Can they predict outcome? *J Bone Joint Surg Br.* 2004; 86:313–323. [PubMed: 15125116]
5. Faunce DE, Gregory MS, Kovacs EJ. Acute ethanol exposure prior to thermal injury results in decreased T-cell responses mediated in part by increased production of IL-6. *Shock.* 1998; 10:135–140. [PubMed: 9721981]
6. Howard M, Muchamuel T, Andrade S, Menon S. Interleukin 10 protects mice from lethal endotoxemia. *J Exp Med.* 1993; 177:1205–1208. [PubMed: 8459215]
7. Yeh FL, Shen HD, Fang RH. Deficient transforming growth factor beta and interleukin-10 responses contribute to the septic death of burned patients. *Burns.* 2002; 28:631–637. [PubMed: 12417156]
8. Kusske AM, Rongione AJ, Ashley SW, McFadden DW, Reber HA. Interleukin-10 prevents death in lethal necrotizing pancreatitis in mice. *Surgery.* 1996; 120:284–288. discussion 289. [PubMed: 8751594]
9. Rongione AJ, Kusske AM, Kwan K, Ashley SW, Reber HA, McFadden DW. Interleukin-10 protects against lethality of intra-abdominal infection and sepsis. *J Gastrointest Surg.* 2000; 4:70–76. [PubMed: 10631365]
10. Gingras AC, Raught B, Sonenberg N. mTOR signaling to translation. *Curr Top Microbiol Immunol.* 2004; 279:169–197. [PubMed: 14560958]
11. Graff JR, Zimmer SG. Translational control and metastatic progression: enhanced activity of the mRNA cap-binding protein eIF-4E selectively enhances translation of metastasis-related mRNAs. *Clin Exp Metastasis.* 2003; 20:265–273. [PubMed: 12741684]
12. Schaeffer V, Abrass CK. Mechanisms and Control of Protein Translation in the Kidney. *Am J Nephrol.* 2009; 31:189–201. [PubMed: 20029175]
13. Wullschlegel S, Loewith R, Hall MN. TOR signaling in growth and metabolism. *Cell.* 2006; 124:471–484. [PubMed: 16469695]
14. Young DA, Nickerson-Nutter CL. mTOR--beyond transplantation. *Curr Opin Pharmacol.* 2005; 5:418–423. [PubMed: 15955739]
15. Kimball SR, Orellana RA, O'Connor PM, Suryawan A, Bush JA, Nguyen HV, Thivierge MC, Jefferson LS, Davis TA. Endotoxin induces differential regulation of mTOR-dependent signaling in skeletal muscle and liver of neonatal pigs. *Am J Physiol Endocrinol Metab.* 2003; 285:E637–644. [PubMed: 12773308]
16. Lang CH, Frost RA. Sepsis-induced suppression of skeletal muscle translation initiation mediated by tumor necrosis factor alpha. *Metabolism.* 2007; 56:49–57. [PubMed: 17161226]
17. Chen S, Atkins CM, Liu CL, Alonso OF, Dietrich WD, Hu BR. Alterations in mammalian target of rapamycin signaling pathways after traumatic brain injury. *J Cereb Blood Flow Metab.* 2006
18. Schaeffer V, Cuschieri J, Garcia I, Knoll M, Billgren J, Jelacic S, Bulger E, Maier R. The priming effect of C5a on monocytes is predominantly mediated by the p38 MAPK pathway. *Shock.* 2007; 27:623–630. [PubMed: 17505301]
19. Vlahos CJ, Matter WF, Hui KY, Brown RF. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J Biol Chem.* 1994; 269:5241–5248. [PubMed: 8106507]
20. Cuschieri J, Bulger E, Biligren J, Garcia I, Maier RV. Vitamin E inhibits endotoxin-mediated transport of phosphatases to lipid rafts. *Shock.* 2007; 27:19–24. [PubMed: 17172975]
21. Cuschieri J, Gourlay D, Garcia I, Jelacic S, Maier RV. Hypertonic preconditioning inhibits macrophage responsiveness to endotoxin. *J Immunol.* 2002; 168:1389–1396. [PubMed: 11801680]
22. Gingras AC, Raught B, Gygi SP, Niedzwiecka A, Miron M, Burley SK, Polakiewicz RD, Wyslouch-Cieszyńska A, Aebersold R, Sonenberg N. Hierarchical phosphorylation of the translation inhibitor 4E-BP1. *Genes Dev.* 2001; 15:2852–2864. [PubMed: 11691836]
23. Lang CH, Pruznak AM, Frost RA. TNFalpha mediates sepsis-induced impairment of basal and leucine-stimulated signaling via S6K1 and eIF4E in cardiac muscle. *J Cell Biochem.* 2005; 94:419–431. [PubMed: 15534870]
24. Potter MW, Shah SA, Elbirt KK, Callery MP. Endotoxin (LPS) stimulates 4E-BP1/PHAS-I phosphorylation in macrophages. *J Surg Res.* 2001; 97:54–59. [PubMed: 11319880]

25. Weinstein SL, Finn AJ, Dave SH, Meng F, Lowell CA, Sanghera JS, DeFranco AL. Phosphatidylinositol 3-kinase and mTOR mediate lipopolysaccharide-stimulated nitric oxide production in macrophages via interferon-beta. *J Leukoc Biol.* 2000; 67:405–414. [PubMed: 10733102]
26. Lim HK, Choi YA, Park W, Lee T, Ryu SH, Kim SY, Kim JR, Kim JH, Baek SH. Phosphatidic acid regulates systemic inflammatory responses by modulating the Akt-mammalian target of rapamycin-p70 S6 kinase 1 pathway. *J Biol Chem.* 2003; 278:45117–45127. [PubMed: 12960176]
27. Lindemann SW, Yost CC, Denis MM, McIntyre TM, Weyrich AS, Zimmerman GA. Neutrophils alter the inflammatory milieu by signal-dependent translation of constitutive messenger RNAs. *Proc Natl Acad Sci U S A.* 2004; 101:7076–7081. [PubMed: 15118085]
28. Gennari R, Alexander JW. Anti-interleukin-6 antibody treatment improves survival during gut-derived sepsis in a time-dependent manner by enhancing host defense. *Crit Care Med.* 1995; 23:1945–1953. [PubMed: 7497716]
29. Riedemann NC, Neff TA, Guo RF, Bernacki KD, Laudes IJ, Sarma JV, Lambris JD, Ward PA. Protective effects of IL-6 blockade in sepsis are linked to reduced C5a receptor expression. *J Immunol.* 2003; 170:503–507. [PubMed: 12496437]
30. Mihara M, Nishimoto N, Ohsugi Y. The therapy of autoimmune diseases by anti-interleukin-6 receptor antibody. *Expert Opin Biol Ther.* 2005; 5:683–690. [PubMed: 15934843]
31. Jones SA. Directing transition from innate to acquired immunity: defining a role for IL-6. *J Immunol.* 2005; 175:3463–3468. [PubMed: 16148087]
32. Dalrymple SA, Slattery R, Aud DM, Krishna M, Lucian LA, Murray R. Interleukin-6 is required for a protective immune response to systemic *Escherichia coli* infection. *Infect Immun.* 1996; 64:3231–3235. [PubMed: 8757858]
33. Cuschieri J, Bulger E, Schaeffer V, Sakr S, Nathens AB, Henessy L, Minei J, Moore EE, O'Keefe G, Sperry J, Remick D, Tompkins RG, Maier RV, The Inflammation and the Host Response to Injury Collaborative Research Program. Early elevation in random plasma IL-6 following severe injury is associated with development of organ failure. *Shock.* 2010 In press.
34. Jorgensen PF, Wang JE, Almløf M, Solberg R, Okkenhaug C, Scholz T, Thiemermann C, Foster SJ, Aasen AO. Sirolimus interferes with the innate response to bacterial products in human whole blood by attenuation of IL-10 production. *Scand J Immunol.* 2001; 53:184–191. [PubMed: 11169223]
35. Lorne E, Zhao X, Zmijewski JW, Liu G, Park YJ, Tsuruta Y, Abraham E. Participation of mammalian target of rapamycin complex 1 in Toll-like receptor 2- and 4-induced neutrophil activation and acute lung injury. *Am J Respir Cell Mol Biol.* 2009; 41:237–245. [PubMed: 19131641]
36. Himmelseher S. Hypertonic saline solutions for treatment of intracranial hypertension. *Curr Opin Anaesthesiol.* 2007; 20:414–426. [PubMed: 17873594]
37. Bulger EM, Jurkovich GJ, Nathens AB, Copass MK, Hanson S, Cooper C, Liu PY, Neff M, Awan AB, Warner K, Maier RV. Hypertonic resuscitation of hypovolemic shock after blunt trauma: a randomized controlled trial. *Arch Surg.* 2008; 143:139–148. discussion 149. [PubMed: 18283138]
38. Staudenmayer KL, Maier RV, Jelacic S, Bulger EM. Hypertonic saline modulates innate immunity in a model of systemic inflammation. *Shock.* 2005; 23:459–463. [PubMed: 15834313]
39. Poli-de-Figueiredo LF, Cruz RJ Jr. Sannomiya P, Rocha ESM. Mechanisms of action of hypertonic saline resuscitation in severe sepsis and septic shock. *Endocr Metab Immune Disord Drug Targets.* 2006; 6:201–206. [PubMed: 16787295]
40. Deitch EA, Shi HP, Feketeova E, Hauser CJ, Xu DZ. Hypertonic saline resuscitation limits neutrophil activation after trauma-hemorrhagic shock. *Shock.* 2003; 19:328–333. [PubMed: 12688543]
41. Chen Y, Hashiguchi N, Yip L, Junger WG. Hypertonic saline enhances neutrophil elastase release through activation of P2 and A3 receptors. *Am J Physiol Cell Physiol.* 2006; 290:C1051–1059. [PubMed: 16282197]
42. Choi SH, Lee SW, Hong YS, Jeun JM, Min BW. Selective inhibition of polymorphonuclear neutrophils by resuscitative concentration of hypertonic saline. *Emerg Med J.* 2006; 23:119–122. [PubMed: 16439740]

43. Hashiguchi N, Lum L, Romeril E, Chen Y, Yip L, Hoyt DB, Junger WG. Hypertonic saline resuscitation: efficacy may require early treatment in severely injured patients. *J Trauma*. 2007; 62:299–306. [PubMed: 17297316]
44. Novoa I, Carrasco L. Cleavage of eukaryotic translation initiation factor 4G by exogenously added hybrid proteins containing poliovirus 2Apro in HeLa cells: effects on gene expression. *Mol Cell Biol*. 1999; 19:2445–2454. [PubMed: 10082510]
45. Morley SJ, Naegel S. Phosphorylation of eukaryotic initiation factor (eIF) 4E is not required for de novo protein synthesis following recovery from stress in human kidney cells. *J Biol Chem*. 2002; 277:32855–32859. [PubMed: 12138083]
46. Naegel S, Morley SJ. Molecular cross-talk between MEK1/2 and mTOR signaling during recovery of 293 cells from hypertonic stress. *J Biol Chem*. 2004; 279:46023–46034. [PubMed: 15292274]
47. Nave BT, Ouwens M, Withers DJ, Alessi DR, Shepherd PR. Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation. *Biochem J*. 1999; 344(Pt 2):427–431. [PubMed: 10567225]

Figure 1. Schematic representation of the PI3K/Akt/mTOR pathway

Activation of PI3K by extracellular signals leads to the production of the lipid second messenger PIP3 (phosphatidylinositol-3, 4, 5-tri-phosphate) in the membrane. Among other things, PIP3 translocates Akt to the membrane where it becomes phosphorylated in its activation loop at Thr 308. Activated Akt is able to phosphorylate mTOR at Ser2448 (47). Targets of mTOR include the p70 S6 kinase (p70S6K) and the translational repressor 4E-BP1 (reviewed in (12,13)). LY294002 is an inhibitor of the PI3K kinase and rapamycin is a specific inhibitor of the mTOR kinase.

Figure 2. Activation of the PI3K/Akt/mTOR cascade by LPS

Adherent PBMCs were treated with LPS (10 ng/ml) for 0-60 minutes as indicated (n=4). Cells extracts were prepared and analyzed by Western blotting as described in Materials and Methods. (A): p-Akt, Akt, (B): p-mTOR, mTOR, (C) p-4E-BP1, 4E-BP1. Graphs represent densitometric data; represented as mean $\pm$ SEM, normalized to the loading control, expressed as fold increase from the controls (No LPS). Results were analyzed using oneway ANOVA with an appropriate post-test (Bonferroni).

Figure 3. Effect of LY294002 and rapamycin on the LPS activation of the PI3K/Akt/mTOR pathway

Adherent PBMCs were treated with or without inhibitor, as indicated, for an hour prior to LPS treatment (n=3). (A): p-mTOR, mTOR; (B): p-4E-BP1, 4E-BP1. Graphs represent densitometric data; represented as mean $\pm$ SEM, normalized to the loading control, expressed as fold increase from the controls (No LPS). Results were analyzed using one-way ANOVA with an appropriate post-test (Bonferroni).

Figure 4. Protein and mRNA levels of IL-10, IL-6 and TNFα

PBMCs were treated with or without inhibitor for an hour prior to LPS addition and supernatants were collected after 20 hours of LPS stimulation. Data are represented as mean $\pm$ SEM. Results were analyzed using repeated measure ANOVA with an appropriate post-test (Bonferroni) (protein levels) and one-way ANOVA with an appropriate post-test (Bonferroni) (RNA levels) A) IL-6 protein levels, (** p<0.001, * p<0.05, n=6) B) IL-6 mRNA levels (n=3), C) IL-10 protein levels (** p<0.001, n=6) D) IL-10 mRNA levels (n=3) E) TNFα protein levels (** p<0.001, n=6) F) TNFα mRNA levels (n=3). (LPS with or without RAP different from control with or without RAP, except were specified; p<0.05).

Graphs represent densitometric scan data (protein) or qPCR quantification (RNA). Statistical differences as indicated by horizontal line between bars or versus controls.

Figure 5. Effect of HTS treatment on the mTOR cascade

: Adherent PBMCs were treated with HTS (40-120mM) for an hour prior to LPS addition (n=4). (A) Supernatants were collected after 20hrs of LPS stimulation and assayed for IL-6 levels. Data are represented as mean $\pm$ SEM. Results were analyzed using one-way ANOVA with an appropriate post-test (Bonferroni). Statistical differences as indicated by horizontal line between bars. (B) Adherent PBMCs were treated with HTS (40-120mM) one hour prior to LPS (10 ng/ml) as indicated. Cells extracts were prepared and analyzed by Western blotting as described in Materials and Methods. Membranes were probed with the respective phospho-antibody: (B) p-mTOR, mTOR, (C) p-4E-BP1, 4E-BP1. All membranes were stripped and re-probed with a loading control. Graphs represent densitometric data; represented as mean $\pm$ SEM, normalized to the loading control, expressed as fold increase from the controls (NO LPS). Graphs represent densitometric scan data.