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# SEPSIS

**Sepsis associated platelet dysfunction**

**&**

**Rapamycin sensitive pathway of lipopolysaccharide  
signaling**

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## Abstract

Sepsis remains a serious disease without an effective pharmacotherapy. The disease is caused by an exaggerated immune response to an infection, which is often triggered by gram-negative bacteria. Lipopolysaccharide in the bacterial cell wall can elicit an immune response and lipopolysaccharide-challenged animals exhibit signs of sepsis. The mTOR signaling pathway influences protein translation, especially involving cell metabolism. In addition, mTOR has been shown to affect the response to LPS and inhibition of mTOR, with the drug rapamycin, enhances survival in lipopolysaccharide-challenged mice. Platelets have a central role in sepsis, because they interact closely with other immune cells. The purpose of this master thesis has been to study platelet function and evaluate the influence of the mTOR pathway during sepsis. Platelet function was assessed by activation after treatment with LPS and rapamycin, both in vivo and in vitro. The influence of the mTOR pathway on LPS signaling was analyzed in the murine model for sepsis, as well as, in macrophages-like cells and primary macrophages. Our data demonstrate that platelets from septic mice are hypo-reactive and that rapamycin affects platelet function. Inhibition of mTOR affects the LPS signaling both in vivo, seen as decreased cytokine expression, and in vitro. The conclusion is that platelets from septic mice are hypo-responsive and that mTOR is, in some way, involved in platelet function. Rapamycin pre-treatment lowers the cytokine expression in vivo and LPS signals thru the same signaling pathway as TNF- $\alpha$ ; activating the mTOR signaling pathway through phosphorylation of IKK.

## Introduction

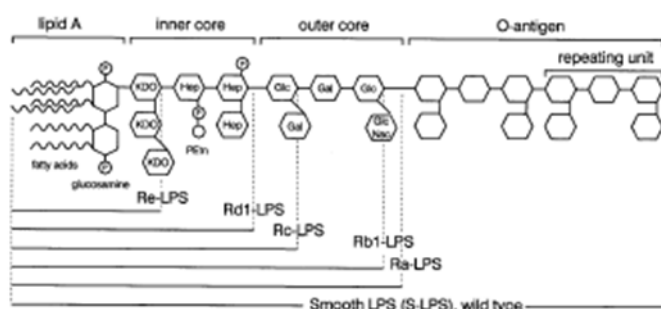
The immune system is a double-edge sword; besides its ability to effectively detect and mount an attack to eradicate pathogens, the immune system may also cause damage to the body, and even lead to death.

### Sepsis

While sepsis remains a serious cause of morbidity and mortality in the world, its pathophysiology is not completely understood. Sepsis is a complex, multi-factorial syndrome that can evolve into conditions of varied severity<sup>1,2</sup>. Infection is defined as the invasion of normally sterile tissue with microorganisms and sepsis constitutes of the systemic inflammatory response to that infection; severe sepsis is sepsis complicated by dysfunction of one or more organs, most commonly involving the lungs, liver, and kidneys<sup>2-4</sup>. Recently, a new classification system for sepsis, the PIR0 staging system, was introduced<sup>5</sup>. The system characterizes the disease based on predisposition, the insult infection, the response of the host system, and organ dysfunction<sup>6</sup>. The system divides the disease into five different subgroups; systemic inflammatory response sndrome (SIRS), sepsis (SIRS with evidence of infection), severe sepsis and septic shock, in which organ dysfunction and other complications are involved, and multiple organ dysfunction sndrome (MODS), in which the homeostasis cannot be maintained without intervention<sup>6</sup>. The incidence of sepsis has been rising during the past decades<sup>1,4,6</sup>, and the increasing septicemia rates are most likely caused by the increasing use of catheters or other invasive equipment, chemotherapy, and immunosuppressant therapy<sup>4</sup>. Despite much effort, severe sepsis is associated with a high mortality rate of 30-70%<sup>2,7,8</sup>, and an effective pharmacologic therapy for this disease remains elusive<sup>9</sup>.

### Murine model of sepsis

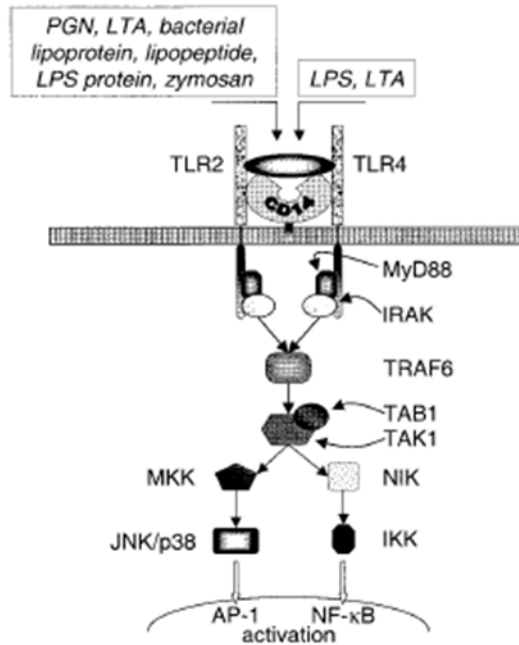
Septic condition is often due to a disseminated infection in the bloodstream, which often is caused by gram-negative bacteria<sup>7</sup>. Bacteria can elicit a tremendous immune response mainly because of the lipopolysaccharide, LPS, that is produced in the outer membrane of the bacteria<sup>4,8-10</sup>.



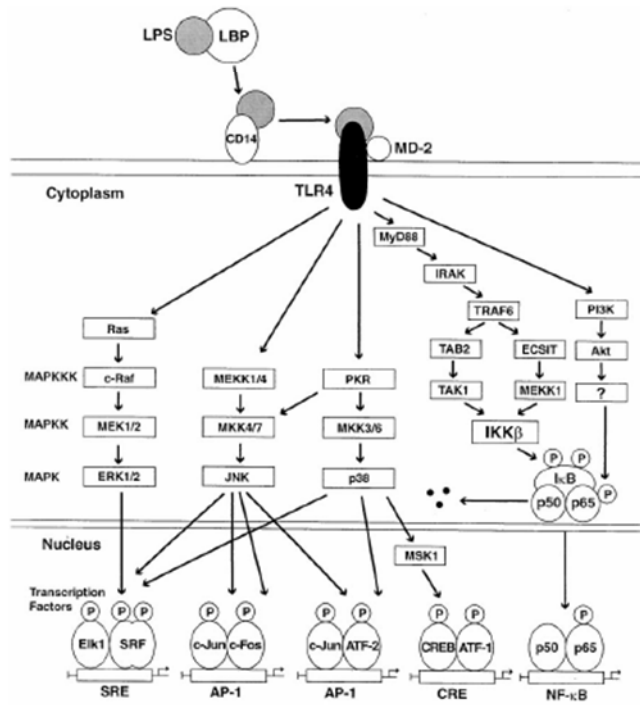
**Figure 1**  
The LPS molecule with its toxic moiety— Lipid A.<sup>4</sup>

The LPS molecule is not toxic as long as it is incorporated into the bacterial outer membrane, but after release from the bacterial wall, its toxic moiety, lipid A, is exposed to immune cells, thus evoking an inflammatory response<sup>4,11</sup>. LPS is released from the bacteria when they multiply and also when the bacteria dies or lyses. LPS activates the innate immune response through binding to a receptor in the toll-like receptor gene family, TLR4<sup>1,4,9</sup>. To be able to bind to TLR4, the LPS molecule first binds to LPS-binding protein, LBP, in the plasma and then the complex presents LPS for the CD14 receptor in the cell membrane<sup>9,11</sup>. The binding of LPS to TLR4 is enhanced by CD14, which presents

the LPS molecule to the TLRs<sup>1,9,11</sup>. CD14 also circulates in the blood and enhances LPS activation of CD14-negative cells, such as endothelial cells and epithelial cells<sup>9</sup>. Once activated, TLR4 conveys the signal and initiates the transduction pathway, resulting in transcriptional regulation and stimulated immune function<sup>1,9</sup>.



**Figure 2**  
The TLR4 signaling pathway.<sup>4</sup>



**Figure 3**  
The LBP binds to LPS and transfers LPS to CD14, which in its turn presents LPS to TLR-4.<sup>7</sup>

The downstream signaling pathway involves activating the IL-1 receptor associated kinase, IRAK, through the MyD88 adaptor protein and signaling through TRAF-6 and protein kinase cascades to activate nuclear factor kappa B, NF-κB. NF-κB is present as a latent, inactive, IκB-bound complex in the cytoplasm. In response to an inflammatory stimulus, IκB proteins are phosphorylated by IKK, and subsequently degraded. The free NF-κB-complex translocates to the nucleus where it initiates the transcription of genes encoding pro-inflammatory cytokines and tissue factor, among other substances.<sup>1,4,9,10,12</sup>

The immunological response to LPS mainly involves leukocytes and the production of cytokines, such as tumor necrosis factor alpha, TNF-α, and interleukins 1 and 6, IL-1 and IL-6<sup>4,6,9-11</sup>. TNF-α is one of the first cytokines released by the macrophages. TNF-α mRNA is constitutively transcribed in Kupffer cells, i.e. macrophages in the liver, allowing rapid release of TNF-α after an inflammatory challenge. Kupffer cells are also important in the clearance and detoxification of LPS from the circulation<sup>4</sup>. IL-1 and IL-6 are not constitutively expressed, but the mRNAs of these cytokines, as well as that of TNF-α, are immediately transcribed after a challenge, and maximum levels of mRNA are found at around one hour post-challenge<sup>4</sup>. Other cytokines are also released in response to an inflammatory challenge; IL-8, IL-12 and platelet activation factor, PAF, among others<sup>4</sup>. This response to LPS echoes the initial phase of sepsis, which is characterized by a high inflammatory response with increased levels of pro-inflammatory cytokines, released from macrophages, chiefly<sup>4,7,9,11,13,14</sup>. LPS also induces the anti-inflammatory cytokine IL-10, as well as, the two major macrophage growth and

differentiation factors; macrophage colony-stimulating factor, CSF-1, and granulocyte-macrophage colony-stimulating factor, GM-CSF<sup>11</sup>. The actions of CSF-1 is antagonized by interferon gamma, INF- $\gamma$ , which has an anti-proliferative effect and IL-10 forms part of the auto-inhibitory loop that controls the production of cytokines<sup>11,15</sup>.

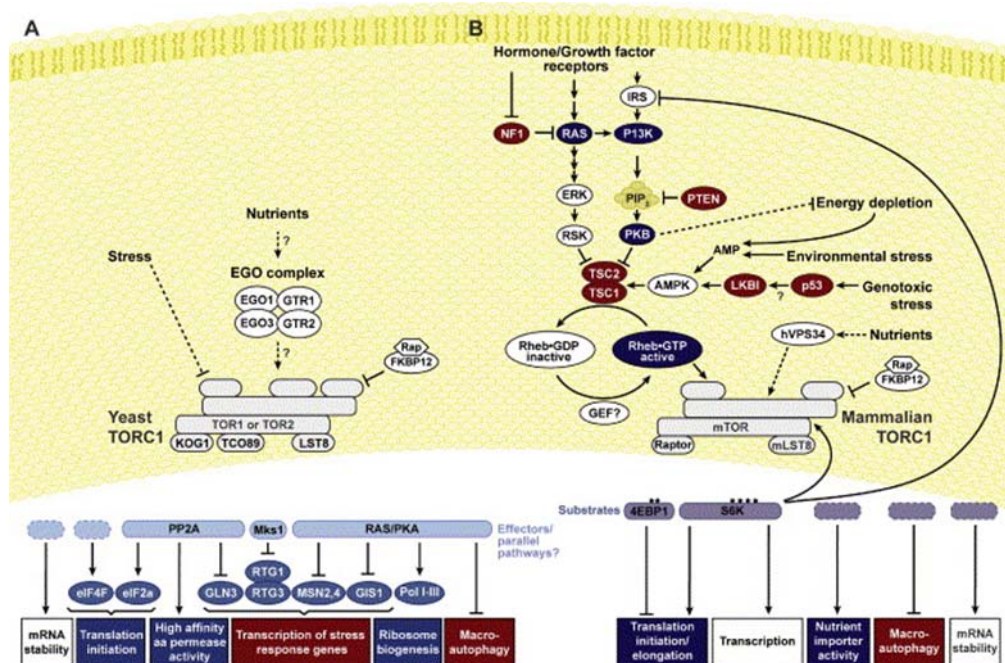
LPS mimics whole gram-negative bacteria in many respects. The effect of LPS treatment leads to symptoms such as fever, endothelial damage, capillary leakage, peripheral vascular dilation, coagulation disorders, microthrombi and myocardial depression. These phenomena may finally result in multiple organ dysfunction, shock, and death<sup>4</sup>. LPS treatment is therefore a well-recognized model for sepsis, and is widely used to study sepsis<sup>1,4,6,10,14,15</sup>.

### The mTOR signaling pathway

Within the Akt signaling pathway, the mammalian target of rapamycin, mTOR, is a central kinase. So called because the antifungal action of the lipophilic macrolide rapamycin was exploited to identify the kinase<sup>16</sup>. mTOR is a serine/threonine protein kinase conserved in all eukaryotes that associates with, and is inhibited by, the toxic complex formed between rapamycin and a conserved proline-isomerase<sup>16,17</sup>.

The Akt signaling pathway plays a critical role in controlling the balance between cell survival and apoptosis. Akt is indirectly activated by growth factors and upon activation, a cascade of ser/thr phosphorylation on different kinases and phosphatases takes place.<sup>18</sup>

mTOR integrates three types of inputs; nutrients, i.e. amino acids, growth factors and cellular energy status, and thereby controls the cells catabolic and anabolic processes to determine cell growth and metabolism<sup>19</sup>.



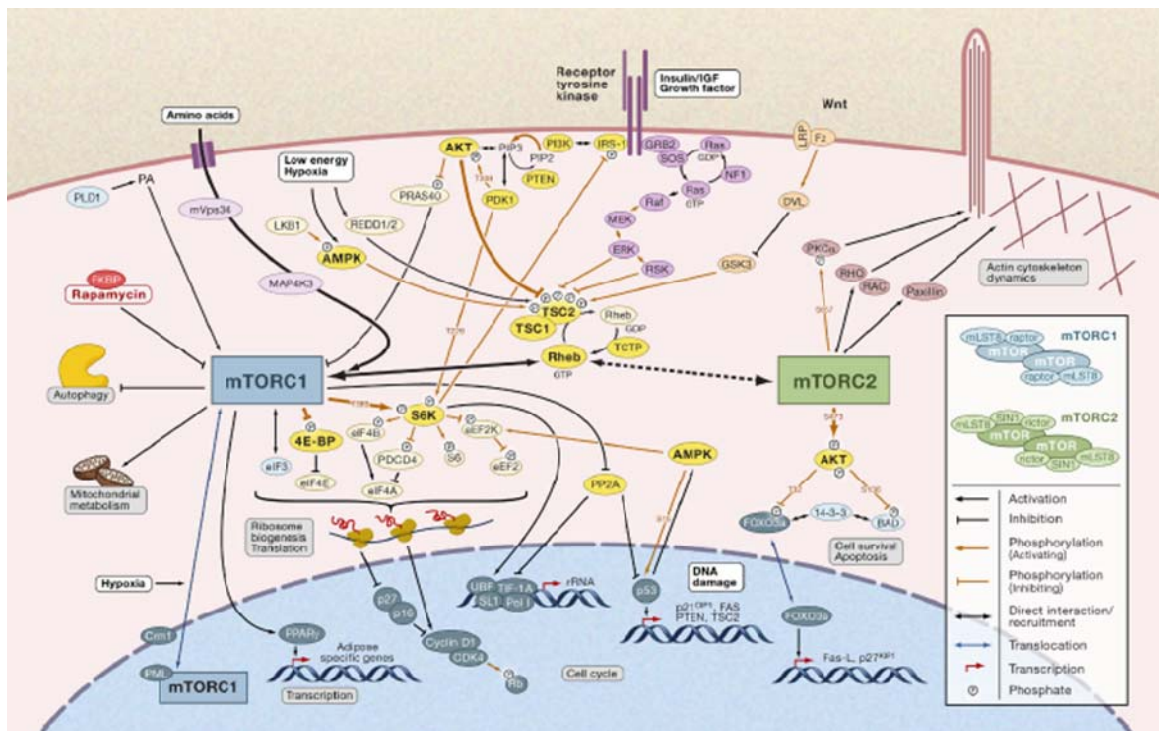
**Figure 4**  
**A: The TORC1 complex.**  
**B: mTOR activation leads to increased S6K-activity.<sup>15</sup>**

In response to growth factors, the Akt signaling pathway is activated through phosphorylation of Akt and this leads to inactivation of TSC1-TSC2, which is a heterodimeric GTPase-activating protein that

serves as a repressor of the mTOR pathway. This allows GTP-bound RHEB to activate mTORC1 and possibly mTORC2. Low energy status inhibits mTORC by activating AMPK, which in turn activates TSC1-TSC2.<sup>19,20</sup>

As already mentioned, mTOR forms two distinct multiprotein complexes, mTORC1 and mTORC2, which corresponds to the two branches in the signaling network<sup>19</sup>. mTORC2 promotes cell survival through direct phosphorylation of Akt<sup>19</sup>. mTORC1 controls protein synthesis by phosphorylating and inactivating the translational inhibitor 4E-BP and by phosphorylating and activating ser/thr protein kinase S6, S6K1<sup>19,20</sup>. Once activated, the S6K1 phosphorylates, and thereby activates, the S6 subunit on the ribosome, which means that the kinase regulates translation initiation and transcription<sup>16,18,21</sup>. S6K1 promotes the translation of TOP mRNAs, i.e. mRNAs that encode proteins important for cell growth and proliferation<sup>18</sup>.

Rapamycin is a registered drug in Europe; Rapamune®, used as an immune suppressant after kidney transplant and in-stent restenosis of cardiac arteries following angioplasty<sup>16,22</sup>. Rapamycin in complex with FKBP binds and inhibits mTORC1 but not mTORC2<sup>19</sup> and rapamycin treatment causes a dramatic down-regulation of cellular anabolic processes and correspondingly, an up-regulation of catabolic processes<sup>21</sup>.



**Figure 5**  
**The mTOR signaling pathway.<sup>17</sup>**

A hyper-metabolic state, where energy and oxygen consumption is elevated and water and salt are conserved, can be observed during sepsis<sup>13,23</sup>. Previous work, done in this research group [Lee, unpublished data], has shown that S6K1 plays a central role in the early hyper-metabolic state seen during endotoxemia, as shown by increased levels of the phosphorylated Akt protein in liver cells from animals treated with LPS. The elevated level of S6K1 is due to increased levels of phosphorylated mTOR and could therefore be blocked by rapamycin treatment. The work has also shown that S6K1 contributes to the pathology of sepsis, as rapamycin was shown to enhance survival of mice challenged with a lethal dose of LPS.

The activation of the mTOR pathway has also been shown by others<sup>21</sup>; the phosphorylation of mTOR on Ser2448, a residue in a domain that has been characterized as a repressor of mTOR function, was reduced in skeletal muscle but enhanced in the liver after LPS-treatment. The S6 kinase has been shown to be down-regulated in skeletal muscle during sepsis. Meanwhile the phosphorylation of S6K1 is enhanced in the liver, which means that the signaling through mTOR is increased in the liver but decreased in the skeletal muscle. The signaling transduction pathway that mediates the effect of LPS on S6K1 phosphorylation has not yet been identified in either muscle or liver.

As already mentioned above, LPS activates the NF- $\kappa$ B pathway, but other pathways are also involved. The phosphatidylinositol 3 kinase (PI3K) signaling pathway is involved in cell proliferation and survival. PI3K is also known to be involved in inflammatory responses but the exact molecular mechanisms are still unclear. Upon stimulation, PI3K activates the Akt signaling pathway by phosphorylation leading to IKK $\beta$  phosphorylation of p65 and the subsequent activation of NF- $\kappa$ B. The signaling pathways, NF- $\kappa$ B and PI3K, are overlapping and they both contribute to the LPS response.<sup>10</sup>

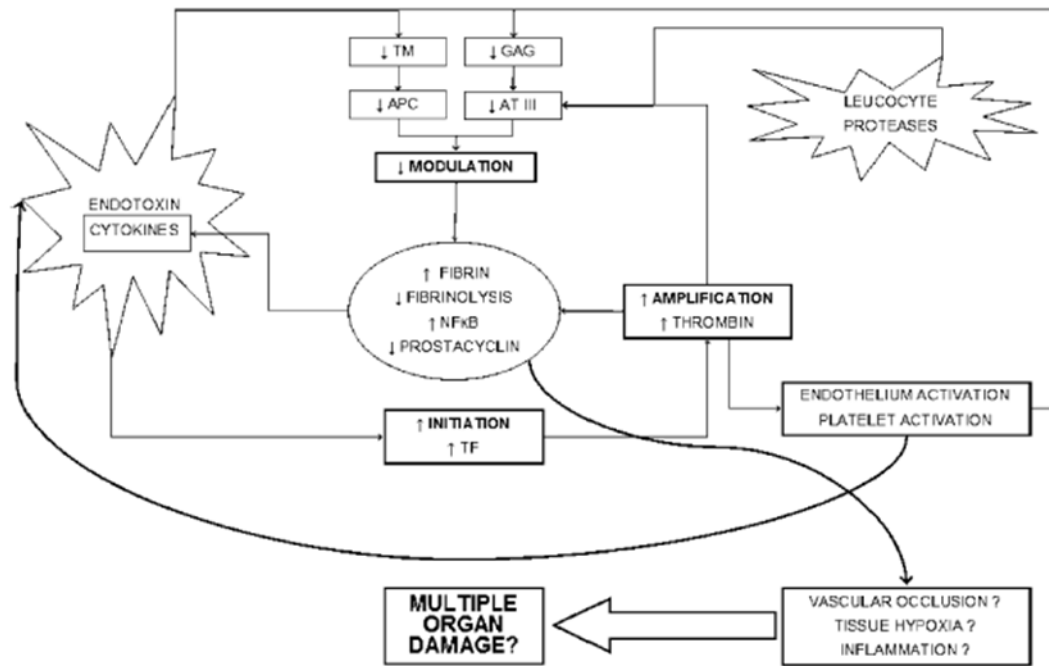
### Coagulation

Many inflammatory mediators are involved in the activation of the coagulation cascade and at the same time, many of the coagulation proteins are themselves actively involved in the inflammatory process. Thus, there is a strong link between inflammation and coagulation.<sup>24,25</sup>

The coagulation system is one of the systems that are frequently impaired during sepsis with resulting microthrombi and loss of microcirculation in vital organs.<sup>2,3,26,27</sup>

Activation of the coagulation system could occur through the intrinsic or the extrinsic pathways which are initiated by the activation of factor XII or factor VII, respectively. The extrinsic pathway is supposedly the main pathway through which sepsis activates the coagulation cascade. Factor VII is converted to its active form, VIIa, through the expression of tissue factor, TF, also known as factor III, by several cell types that are exposed to endotoxin or cytokines. TF binds to factor VIIa and the complex then activates factor X and factor IX. Factor Xa, associated with factor Va, forms the prothrombinase complex which subsequently converts prothrombin into thrombin. The thrombin formed, activates platelets, which release more coagulation factors that further amplify the thrombin formation. Thrombin cleaves fibrinogen to fibrin that is the base for blood clots. On the other hand, endotoxin or bacterial enzymes could directly activate FXII, i.e. the intrinsic pathway, which also leads to fibrin formation. The resulting pro-coagulant state leads to the formation of microvascular thrombi, which could disturb the organ microcirculation and thus promoting the development of organ dysfunction together with the damage caused by intra- and extravascular phagocytotic cells.<sup>3,4,24,26</sup>

The link between the coagulation system and inflammation is not yet completely understood but it is known that blood clotting not only leads to fibrin formation and platelet activation, but also it results in vascular cell activation, which contributes to leukocyte activation. On the other hand, inflammation could induce TF expression in monocytes, via NF- $\kappa$ B activation, with subsequent initiation of the coagulation.<sup>24</sup>



**Figure 6**  
The interaction between inflammation and coagulation.<sup>21</sup>

One other striking parallel between platelets, i.e. coagulation, and leukocytes, i.e. inflammation, can be seen at the molecular and cellular levels in the endothelium. The primary molecules responsible for initiation of platelet and leukocyte adhesion, von Willebrand factor and P-selection, respectively, are stored in the same granules and are thus released together. On cellular activation with either pro-thrombotic or pro-inflammatory substances, the granules fuse with the cell membrane and expose P-selectin and von Willebrand factor, which leads to cell rolling and adhesion of both cell types.<sup>8,28,29</sup>

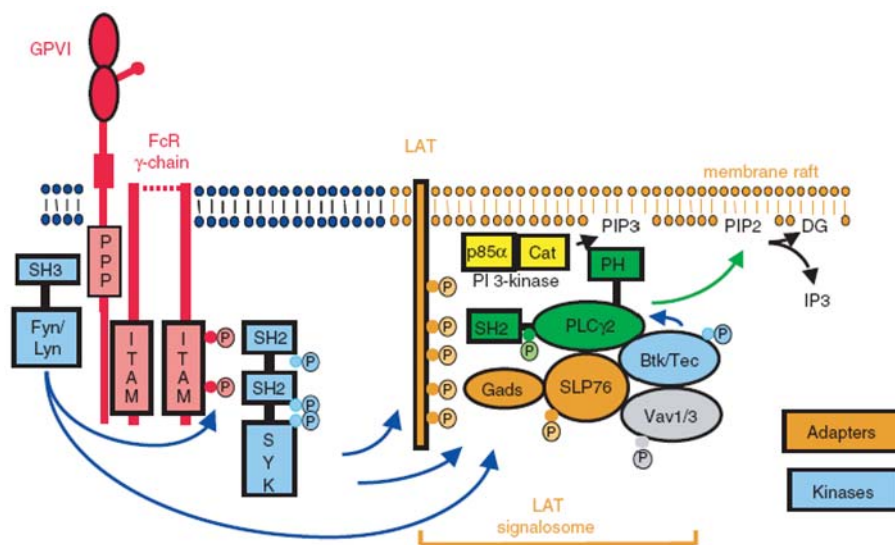
### Platelets

Platelets are the smallest blood cells and are derived from megakaryocytes through an endomitotic process rather than straightforward cellular duplication, which results in cells without a nucleus, but they contain preformed proteins as well as mRNA. The development is controlled by thrombopoietin, which is synthesized in smooth muscle and bone marrow. Thrombopoietin is eliminated by circulating platelets and hence, a fall in platelet counts results in increased levels of thrombopoietin.<sup>30,31</sup>

Platelet function could be divided into four phases; activation, adhesion, aggregation and secretion. Platelets could become activated by various stimuli including thrombin, collagen, adenosine diphosphate, platelet activation factor, just to mention a few<sup>32</sup>. Once activated, the platelets change their shape from disk-like to a more spherical appearance with arm-like extensions that facilitate adhesion. Adhesion is mediated by platelet membrane glycoprotein I-IX, which interacts with components in the subendothelial matrix, such as collagen. Secretion of various substances occurs when  $\alpha$ -granules and dense granules are released during the interaction of platelet stimulators with platelet membrane receptors. The  $\alpha$ -granules contain factors involved in coagulation such as P-selectin, factor V and von Willebrand factor, whereas the dense granules contain adenosine diphosphate, calcium and serotonin. The cytoplasm can contain a number of other substances including nitric oxide and cytokines, which could also be released during activation. Thus, the release

of substances could result in a wide-range of effects; stimulation of further platelet activation, attracting neutrophils and leukocytes, which are key players in mediating inflammation, including sepsis.<sup>25,26,29-31,33</sup>

The activation of platelets could be initiated by the interaction of several platelet receptors with collagen and collagen-associated proteins in the extracellular matrix at the sight of vascular injury. Initially the platelets are tethered transiently to exposed collagen when the receptor GPIb $\alpha$  interacts with collagen-bound von Willbrand factor, VWF<sup>32</sup>. For stable platelet adhesion to occur, the immunoglobulin-like receptor GPVI must bind to collagen, triggering the tyrosine phosphorylation of the Fc receptor  $\gamma$ -chain ITAM, which is associated with the GPVI receptor, by the Src family kinases Fyn and Lyn. The subsequent signaling cascade is the same as used by immunoreceptors, and leads to activation of the tyrosine kinase Syk. This initiates a phosphorylation cascade of multiple signaling proteins, that culminate in the activation of a number of effector enzymes including phospholipase C- $\gamma$ 2, protein kinase C, the G protein regulator Vav and phosphoinositide-3 kinase<sup>34,35</sup>. In addition, the cascade includes the adaptor proteins LAT, SLP76 and SLAP-130<sup>35</sup>. As a result, platelets respond by inside-out activation of the platelet integrins  $\alpha_2\beta_1$  and  $\alpha_{IIb}\beta_3$ . Activated  $\alpha_2\beta_1$  binds tightly to a specific sequence in collagen to allow firm adhesion of the platelets to the site of injury and activated  $\alpha_{IIb}\beta_3$  mediates platelet aggregation. In addition to this, GPVI signaling stimulates an increase in Ca<sup>2+</sup>, shape change, and secretion of platelet granule contents to activate nearby circulation platelets and thus propagate thrombus formation.<sup>34,35</sup>

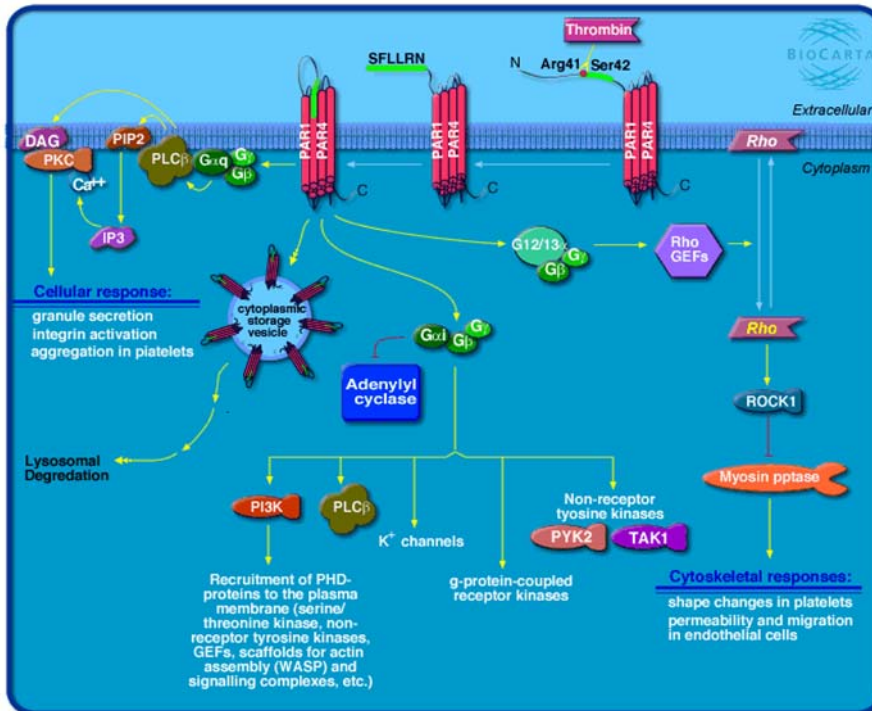


**Figure 7**  
**The GPVI signaling pathway.<sup>27</sup>**

The quaternary structure of collagen is required to activate platelets through GPVI, but synthetic collagen-related peptides, CRPs, that mimic the structure can also activate platelets through GPVI. CRPs contain the specific GPVI recognition motif GPO, a tri-peptide with proline, glycine, and hydroxyproline in a helical conformation.<sup>32,34-36</sup>

Platelets can also be activated by the coagulation protease thrombin<sup>37</sup>. The thrombin signaling is mediated, at least in part, by a family of G protein-coupled protease-activated receptors, PARs<sup>37,38</sup>. The PAR receptors are activated when thrombin cleaves their NH<sub>2</sub>-terminal exodomain to unmask a new receptor NH<sub>2</sub>-terminus. This new terminus then serves as a tethered peptide ligand, binding intra-molecularly to the body of the receptor to effect transmembrane signaling<sup>37-39</sup>. PARs are thus in essence peptide receptors that carry their own ligands, which remain silent until unmasked by site-

specific receptor cleavage. Signaling through PAR4, the functional receptor in mice, couples to Rho signaling pathway and leads to change in cytoskeletal structure and cell shape. PAR-4 also induces signaling through IP3 and DAG, which leads to secretion, aggregation, and integrin activation. Since the activation of PARs by protease cleavage is irreversible, the primary mechanism for down-regulation of the PAR signaling cascade appears to be internalization and degradation of PAR receptors.<sup>37</sup>



**Figure 8**  
The thrombin signaling pathway.

Synthetic peptides mimicking this tethered ligand act as PAR agonists and activate their receptors independently of protease and receptor cleavage<sup>37,39</sup>. Mouse-TRAP is such a peptide, constituting of the following amino acids; AYPGKF. PAR4 seems to be the functional receptor in mouse platelets, as compared to PAR1 in humans and PAR3 seems to be functioning in both mouse and human platelets<sup>38,39</sup>.

Platelets are involved in the pathogenesis of sepsis; in part by the fact that marked thrombocytopenia is a common feature of sepsis. The thrombocytopenia could be due to two different reasons; impaired platelet production or, more likely, platelet consumption due to generation of thrombin.<sup>2,26,30</sup> Also disseminated intravascular coagulation, DIC, is a frequent complication of sepsis and platelets play a major role in the pathogenesis of DIC<sup>8,40</sup>.

One other reason why platelets seem to be important in the pathogenesis is that platelets seem to occupy one of the crossroads in the complex interaction between inflammation and coagulation, not only by facilitating and propagating thrombin generation, but also by acting as an important mediator of growth factor and adhesion molecule activity<sup>26</sup>. Platelets can interact with leukocytes and macrophages, activating them by either direct cell-cell adhesion or by release of chemokines. The interaction also works in the opposite direction, leukocytes can enhance platelet function<sup>30</sup>. Platelets also play a role in the innate immune system, since they express toll-like receptors; platelets recognize bacterial components, such as LPS. Thereby, platelets have the ability to respond to

bacterial infections, by the release of cell surface molecules and cytokines, and this suggests that platelets may play a role in the inflammatory response.<sup>8,31,41</sup>

**The purpose of this master thesis project**

As it is becoming increasingly apparent that platelets play a complex role in sepsis the purpose of this project was to study if platelet activation was affected during endotoxemia and also to continue to evaluate the consequences of rapamycin treatment during sepsis, in regard to cytokine release and signaling pathways, both in vivo and in vitro.

## Material and Methods

### 1. Murine Model of Sepsis

To induce sepsis, 3 C57/BL6 male mice from Charles River Laboratories (Wilmington, MA, USA), 6-10 weeks old, were given an intra-peritoneal, i.p., injection of 50 µg (25 mg/kg) LPS (Escherichia Coli O55:H5, Sigma St. Louis, MO, USA) dissolved in 200 µl PBS (Mediatech Inc, Herndon, VA, USA). The mice were also given a subcutaneous injection of 500 µl PBS after the LPS-injection to restore the blood pressure, which tends to drop as a result of the LPS challenge. The mice were returned to the cage and given free access to feed and water for 24 hours. The mice then demonstrated signs of illness including marked lethargy, hair ruffling and piloerection. As controls, 3 C57/BL6 mice were given 200 µl of only PBS and otherwise treated in the same way as the LPS-injected mice.

### 2. Plasma Generation

Blood was taken from the non-treated and LPS-treated mice by retro-orbital plexus bleeding using heparinized capillary tubes. The mice were first anesthetized with an i.p. injection of 350-500 µl Avertin 2.5% (2,2,2-tribromoethanol (Fluka, Buchs, Switzerland) dissolved in tertiary amyl alcohol 100% and subsequently dissolved in sterile PBS). 1 ml of blood was collected in a tube with 100 µl of the anticoagulant Aster Jandyl (85 mM sodium citrate, 69 mM citric acid and 20mg/ml glucose at pH 4.6). The mice were euthanized by cervical dislocation prior to recovery from anesthesia. The blood was centrifuged at 100 X g for 8 minutes and the supernatant was collected. The supernatant was centrifuged at 1200 x g for 5 minutes and the plasma was collected, allocated into new tubes and frozen in 20 µl aliquots at -80°C for later use.

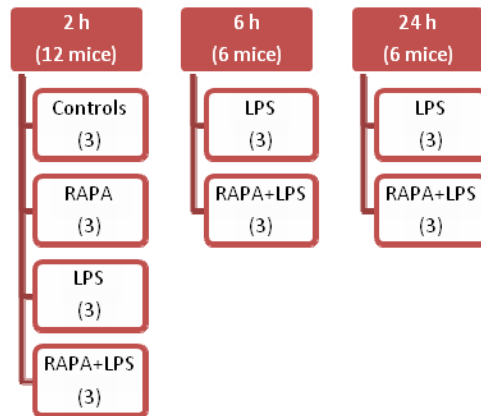
### 3. Platelet isolation

Blood was taken from the mice as described above. The blood was centrifuged at 100 x g for 8 minutes, which produced three distinct layers; the top layer being plasma, the middle layer white blood cells and the bottom layer red blood cells. 500 µl from the top and middle layer was collected. 500 µl of washing buffer (140 mM NaCl, 5 mM KCl, 12 mM trisodium citrate, 10mM glucose and 12.5 mM sucrose at pH6) was added to both tubes and the tubes were lightly agitated and then centrifuged at 100 x g for 6 minutes. Two distinct layers accrued and the top layer from both tubes were combined in a new tube and centrifuged at 100 X g for 6 minutes. The supernatant contained platelet in wash buffer and it was taken to a new tube and centrifuged at 1200 x g for 6 minutes. The pellet was resuspended in 1000 µl Washing Buffer and again centrifuged at 1200 x g for 6 minutes. The pellet was again resuspended, this time in 200 µl Resuspension Buffer (10 mM Hepes, 140 mM NaCl, 3 mM KCl, 0.5 mM MgCl<sub>2</sub>, 5 mM NaHCO<sub>3</sub>, 10 mM glucose at pH 7.4) and the platelet concentration was counted using a known concentration of Sphero™ Rainbow Fluorescent particles 5.5 µm (Spherotech Inc., Libertyville, IL, USA) in a FACScan flowcytometer (Becton Dickinson). The platelet concentration was adjusted to 4 \*10<sup>8</sup> platelets per ml, using Resuspension Buffer.

### 4. Collecting plasma samples for cytokine quantification

24 male C57/BL6 mice, 6-10 weeks old, from Charles River Laboratories (Wilmington, MA, USA), were divided into four treatment groups; Controls, LPS, RAPA and LPS+RAPA. Half of the mice

were given Rapamycin (Lc laboratories, Woburn, MA, USA) and the other half were given only the vehicle containing 5% PEG40 (Sigma-Aldrich Corp., St Louis, MO, USA) and 5% Tween80 (Sigma-Aldrich Corp., St Louis, MO, USA) in sterile water i.p. 30 minutes before the LPS treatment. To induce sepsis, the mice were given 50 µg (25 mg/kg) LPS i.p. The mice were also given a subcutaneous injection of 500 µl PBS, to help restore the blood pressure in the mice that received LPS-treatment. The mice were returned to their cages with free access to water for 2, 6, and 24 hours, respectively. After this, plasma samples were collected as described above. The plasma was frozen in aliquots at -80°C until used in experiments.



## 5. Cytokine quantification

The plasma samples were analyzed using Quantikine® ELISA kit for TNF-α (R&D Systems, Minneapolis, MN, USA), as a pilot study. The experiment was conducted according to the kit manual, with one exception; the plasma was first diluted in a 1:1 ratio in PBS. The manual is enclosed, as appendix 1. All samples were sent to Millipore (St. Charles, MO, USA) for analysis of the cytokine level in the plasma. The samples were analyzed for IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IFN-γ, and GM-CSF.

## 6. Platelet count in whole blood

Blood from control, LPS, and/or rapamycin treated mice, was collected as described above and diluted 1:10 in HBSS. 5 µl of the dilution was added together with Sphero™ Rainbow Fluorescent particles to 300 µl HBSS and the platelet number was determined using a FACScan flowcytometer. The number of platelets was related to the total blood volume and the concentration of platelets per ml was calculated.

## 7. Platelet Activation

### 7.1. Optimizing Platelet Activation

To optimize the activation, normal platelets were collected from one C57/BL6 male mouse, and processed as described above. The activation time and the concentration of the activator were varied to find the right time and concentration. Platelets were diluted by Resuspension Buffer to  $2 \times 10^8$  platelets per ml and activated in 37°C water bath using three different activators; Collagen Related Peptide - CRP (P2415, Sigma St. Louis, MO, USA) 3, 10, and 30 µg/ml, Thrombin (Sigma St. Louis, MO, USA) 1, 10, and 100 u/ml and TRAP (AYPGKF-peptide, synthesized by Tufts University Core Facility, Boston, MA, USA) 500, 750, and 1000 µM. The platelets were activated for 1, 2, 5, and 10 minutes and then 10 µl were transferred to prepared tubes with FITC labeled antibodies against CD62 - P-selectin (BD Biosciences

Pharmingen, San Diego, CA, USA), diluted 1:100 in HBSS (Mediatech Inc, Herndon, VA, USA). The antibodies were left to bind to the platelets during 30 minutes incubation. The platelet-antibody solution was diluted with 200  $\mu$ l HBSS and then analyzed in a FACScan flow cytometer using CellQuest software, where 20000 events were registered from each sample. The data analysis was done using the FlowJo software, where the activated platelets were defined as cells marked with labeled p-selectin. The readings were compared to resting platelets incubated with antibodies and percentage of activated cells and the mean fluorescence were recorded.

#### 7.2. Optimizing platelet activation in the presence of plasma

To optimize the platelet activation in the presence of plasma, normal plasma and platelets were collected from one male C57/BL6 mouse and processed as described above. The concentration of the activators was varied to find the most efficient concentration. The platelets was diluted to a final concentration of  $2 \times 10^8$  platelets per ml in plasma and activated in 37°C water bath using the three activators; CRP 10, 100, and 1000  $\mu$ g/ml, Thrombin 10 and 100 u/ml and TRAP 1, 10, and 1000 mM. The platelets were activated for 2 and 10 minutes and then analyzed as described above.

#### 7.3. Activating platelets from Control, RAPA-, LPS- and LPS+RAPA-treated mice

From the optimization, one concentration of activator and two activation times were chosen. Platelets from either non-treated and LPS-, RAPA-, or LPS/RAPA-treated mice, from the cytokine experiment mentioned above, were activated for 2 and 10 minutes using the following concentrations of the activators; CRP 10  $\mu$ g/ml, Thrombin 10 u/ml, and TRAP 1000  $\mu$ M.

#### 7.4. Platelet Activation with Plasma

From the optimization, one concentration of activator was chosen. Normal platelets from one male C57/BL6 mouse were activated in the presence of plasma, from either non-treated mice or LPS-treated mice mentioned under point 1, or resuspension buffer for controls. Platelets and plasma or resuspension buffer were added together in 1:1 ratios, 0 minutes and 60 minutes before the activation, and activated in the same way as described above.

#### 7.5. Platelet Activation with LPS and Rapamycin in vitro

Platelets from one male C57/BL6 mouse were incubated with LPS in 37°C water bath in the following concentrations; 100 ng/ml, 1  $\mu$ g/ml, and 10  $\mu$ g/ml, for 5 minutes and the activated as described above.

Platelets from 2 male C57/BL6 mice were incubated with rapamycin in 37°C water bath for 30 minutes in the following concentrations; 2 nM, 20 nM, and 200 nM and subsequently activated. The activation with rapamycin in vitro was also conducted in a narrower concentration range; 0.5 nM, 1 nM, 2 nM, 5 nM, and 10 nM, using only TRAP as an activator.

### 8. Platelet lysates

Platelets, from the experiment above, were centrifuged at 1200 x g for 5 minutes, the supernatants were removed and the pellets were re-suspended in lyses buffer, consisting of RIPA Buffer (Boston Bio Prod inc., MA, USA) 1 ml, Protease Inhibitor (Sigma-Aldrich Corp., St Louis, MO, USA) 40  $\mu$ l, and Phosphatase Inhibitor (Sigma-Aldrich Corp., St Louis, MO, USA) 15  $\mu$ l, to a final concentration of  $8 \times 10^8$  platelets per ml. The samples were centrifuged at 14000 RPM at 4° C for 10 min. The supernatants were collected and frozen at -80° C for later use.

9. Lysates from activated platelets

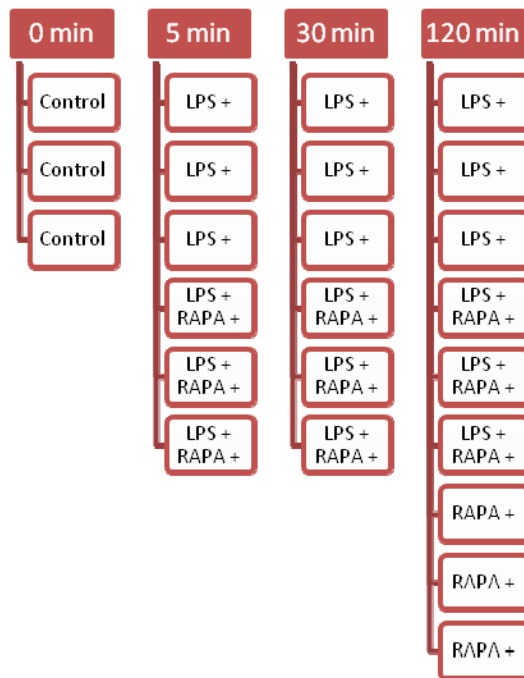
Platelets, from one male C57/BL6 mouse, in a concentration of  $8 \times 10^8$  platelets per ml, were activated for 1, 2, and 10 min using HBSS for controls, CRP, Thrombin, and TRAP and subsequently lysed in lyses buffer in a 1:1 volume ratio. The lysates were centrifuged, to get rid of the cell debris, and frozen in  $-80^\circ\text{C}$ .

10. Cell signaling in macrophage-like cells

The macrophage-like cell lines RAW and J774 (ATCC, Manassas, VA, USA) were used for in vitro studies of the influence LPS and rapamycin on protein expression. The cells were quickly thawed from  $-80^\circ\text{C}$  in a  $37^\circ\text{C}$  water bath and then seeded under sterile conditions on Petri dishes in pre-warmed GIBCO® DMEM Dulbecco's Modified Eagle's medium (Invitrogen Corporation, Carlsbad, CA, USA), with glucose, L-glutamine, and pyroxide hydrochloride but without sodium pyruvate and supplemented with 10% FBS and 1% penicillin-streptomycin. The cells were allowed to grow in an incubator at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ , until confluent.

As a pilot study, the cells were then split and seeded on 6-well plates at a concentration of  $200 \times 10^4$  cells/ml. The next day, the media was changed to Minimum Essential Alpha Medium (Invitrogen Corporation, Carlsbad, CA, USA) with L-glutamine, supplemented with 10% FBS and 1% penicillin-streptomycin, but without phenol red. This type of media makes the cells go into a "starvation-mode" and the cell machinery stops. After 24 hours of starvation, LPS was added to the wells at the following concentrations; 100 ng/ml, 1  $\mu\text{g}/\text{ml}$ , and 10  $\mu\text{g}/\text{ml}$ . The cells were incubated with LPS for 5 or 30 minutes. 500  $\mu\text{l}$  of the media was collected and the cells were lysed using 100  $\mu\text{l}$  lyses buffer. The lysates were centrifuged at 14000 RPM at  $4^\circ\text{C}$  for 10 min. The supernatants were collected and frozen at  $-80^\circ\text{C}$  for later use.

From the pilot study, the concentration of LPS for the following experiments was chosen. The cells were seeded on 24-well plates and the following day the media was changed to Minimum Essential Alpha Medium. The cells were starved for 24 hours and rapamycin and LPS was added to the plates to a concentration of 20nM and 1  $\mu\text{g}/\text{ml}$ , respectively. The cells were lysed, using 50  $\mu\text{l}$  of lyses buffer, at the time point 0, 5, 30, and 120 minutes after the substances were added and the lysates, as well as, the cell media was collected and stored at  $-80^\circ\text{C}$  for later use.



### 11. Cell signaling in mouse peritoneal macrophages

Macrophages were collected by lavage from two C57/BL6 mice, after injection of thioglycollate in to the peritoneal cavity three days earlier. The thioglycollate initially attracts neutrophils to the peritoneum and macrophages become the predominant inflammatory cells in the peritoneum after a few days.

The cells were centrifuged and re-suspended in GIBCO® DMEM Dulbecco's Modified Eagle's medium and seeded in two petri-dishes. The media was changed the second, third, and fourth day and then the cells were seeded in four 6-well plates, i.e. 24 wells in total, at a concentration of  $500 \times 10^4$  cells/ml and after 24 hours, treated with LPS, 1 µg/ml, for 5 minutes, 30 minutes, and 2 hours, with or without rapamycin, 20 nM, i.e. the same treatment groups as for the macrophage-like cells in point 10. The lysates and media was collected and stored in -80°C for later use.

### 12. Normalizing protein contineence

The protein contineence in the cell lysates was normalized so that they all had the same total protein level using BioRad 5X (BioRad Laboratories Inc. Hercules, CA, USA). 1 µl of the samples were added to 1 ml of BioRad and the absorbance was read in an Ultraspec®III UV/Visible spectrophotometer (Pharmacia LKB Biotechnology). The absorbance was related to the protein concentration using samples with known protein concentrations in creating a standard curve. The samples were diluted with lysates buffer to the same protein concentration.

### 13. Western Blot

The cell lysates were analyzed using Western Blot. The samples used were the above mentioned cell and platelets lysates. Reducing, or Non-reducing when appropriate, Nupage® LDS Sample Buffer 4X (Invitrogen Corporation, Carlsbad, CA, USA), was added to the samples in a 1:4 ratio and the samples were heated at 70° C for 10 minutes. The samples were then subsequently loaded on a Nupage® 12% Bis-Tris gel, 1.0 mm x 15 wells (Invitrogen Corporation, Carlsbad, CA, USA). Magic Mark™ XP Western Standard (Invitrogen Corporation, Carlsbad, CA, USA) and See

Blue® Plus 2 pre-stained Standard (Invitrogen Corporation, Carlsbad, CA, USA) were also loaded on the gel for detection of the molecular weight. MOPS Running Buffer 10X (Boston Bio Prod inc., MA, USA) was prepared by dilution by milliQ-water in a 1:10 ratio and 200 ml of the buffer with 500 µl of Nupage® antioxidant (Invitrogen Corporation, Carlsbad, CA, USA) added was poured into the XCell Sure lock™ Novex Mini-cell module (Invitrogen Corporation, Carlsbad, CA, USA). The gels were run for 50 minutes at 200 V. The western transfer was done in an iBlot™ Gel Transfer using iBlot™ Gel Transfer Stacks Mini (Invitrogen Corporation, Carlsbad, CA, USA). The membranes were blocked in 5 % non-fat dry milk (Nestlé) in TBS-T, TBS (Boston Bio Prod inc., MA, USA) with 0,05% Tween20, for 45 minutes on a shaker at room-temperature. The membranes were washed in TBS-T 3x5 minutes, the primary antibody was added and the membranes were incubated over night at 4°C. The antibodies used on platelets lysates were monoclonal rat anti-mouse antibodies against GPVI (Emfret Analytics, Eibelstadt, Germany), monoclonal rabbit anti-mouse antibodies against P-selectin (BioVision Research Products, Mountain View, CA, USA), and monoclonal goat anti-mouse antibodies against PAR4 (Santa Cruz Biotechnology INC, Santa Cruz, CA, USA). Also, the platelets lysates as well as the activated platelets lysates were blotted against p-S6 (S240/245 and S235/236) and p-P70S6K (T421) (Cell signaling Technology, Beverly, MA, USA). Whereas the lysates from the RAW and J774 macrophage-like cells and the primary mouse macrophages were blotted against monoclonal rabbit anti-mouse antibodies against p-S6 (S240/245 and S235/236), p-P70S6K (T421), p-IKKα/β and IκBα (Cell Signaling Technology INC, Danvers, MA, USA). The primary antibodies were all diluted in 5% bovine serum albumin (Serological Proteins Inc., Kankakee, IL, USA), BSA, in TBS-T. The membranes were washed 3x10 minutes in TBS-T on a shaker at room-temperature and the secondary antibody was added. The secondary antibodies were horseradish peroxidase, HRP, conjugated anti-rabbit (Santa Cruz Biotechnology, CA, USA) at a dilution of 1:2000, in all cases except for GPVI-antibody where the secondary antibody was HRP-conjugated anti-rat (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and PAR-4 where the secondary antibody was HRP-conjugated anti-goat (Santa Cruz Biotechnology, CA, USA). The secondary antibody was left to bind for 1 hour and after this, the membranes were washed 3x10 minutes in TBS-T. For detection of the HRP, the membranes were incubated for 30 seconds in SuperSignal® West Pico Chemiluminescent substrate (Pierce Protein Research Products, Rockford, IL, USA) and then placed in a light protective cassette (Fisher Biotech, Pittsburg, PA, USA). HyBlot CL™ Autoradiography films (Denville Scientific Inc., Metuchen, NJ, USA) were then exposed to the membranes from 30 seconds to 60 minutes, depending on the signal strength, in a dark room, and the film was developed in a M35A X-OMAT Processor from Kodak. The membranes were then stripped using Membrane Stripping Buffer (Tris Base 250mM, glycine 1.92 M and SDS 8%) and re-probed, with primary antibodies for β-tubulin (Abcam, Cambridge, MA, USA) as a reference for the concentration of protein.

#### 14. TNF-α quantification in cell media

The media from the cell signaling experiments were analyzed using Quantikine® ELISA kit for TNF-α (R&D Systems, Minneapolis, MN, USA). The experiment was conducted according to the kit manual; the manual is enclosed, as appendix 1.

#### 15. Statistical analysis

All experiments were done with n=3 samples or animals. The data is presented as mean±SD. The

statistical analysis was done using StatView software, where the differences between treated and non-treated animals were tested in pair wise comparisons using Mann-Whitney-Wilcoxon rank test. A probability value of less than 0.05 in the test was regarded as significant ( $p < 0.05$ ).

## Results

### Sepsis associated platelet dysfunction

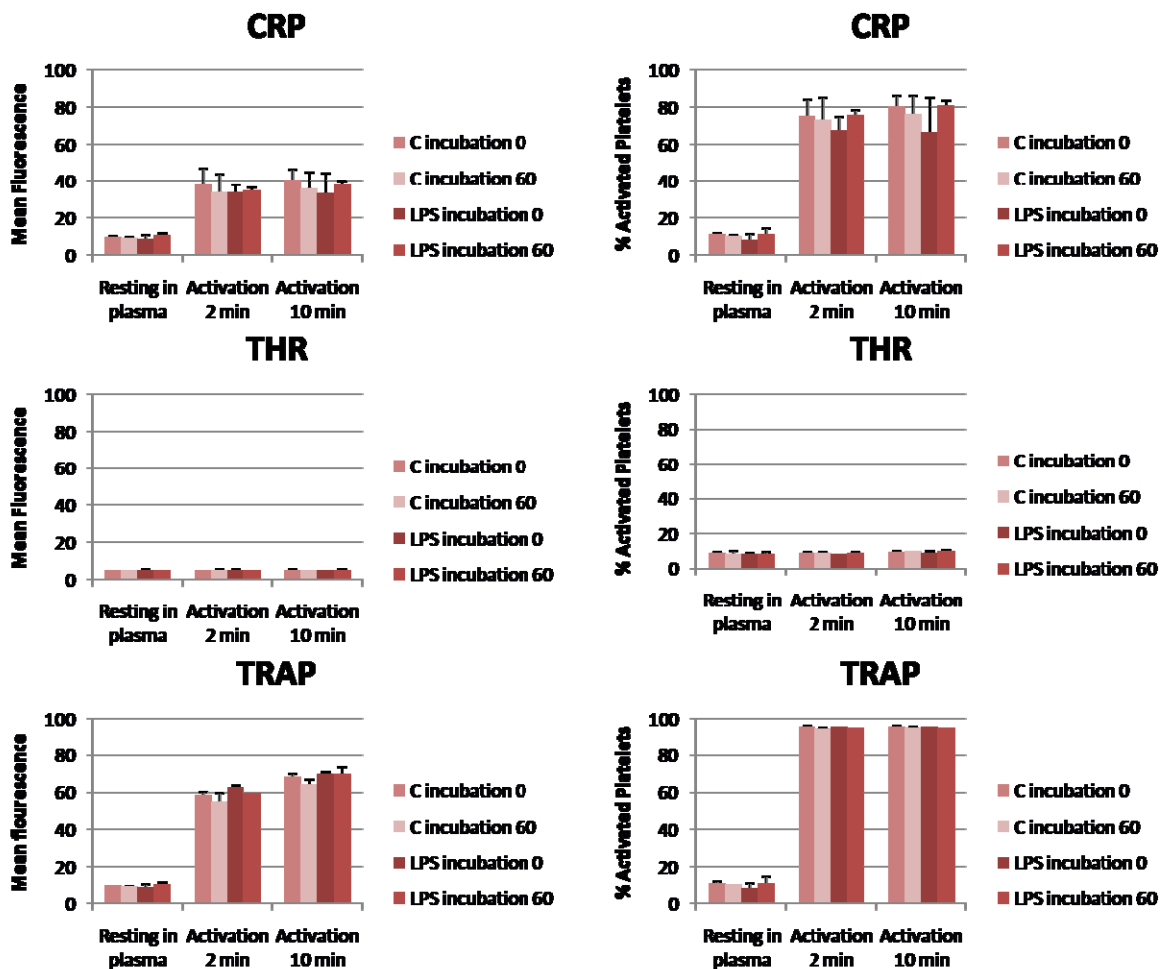
#### Platelet activation

From the result of the optimization of the platelet activation the following concentrations and times were chosen for the subsequent experiments;

Activation of platelets in resuspension buffer: 2 and 10 minutes using 10 µg/ml of CRP, 10 u/ml of Thrombin, or 1 M of TRAP as a final concentration of the activator.

Activation of platelets in plasma: 2 and 10 minutes using 1000 µg/ml of CRP, 100 u/ml of Thrombin, or 10 M of TRAP as a final concentration of the activator.

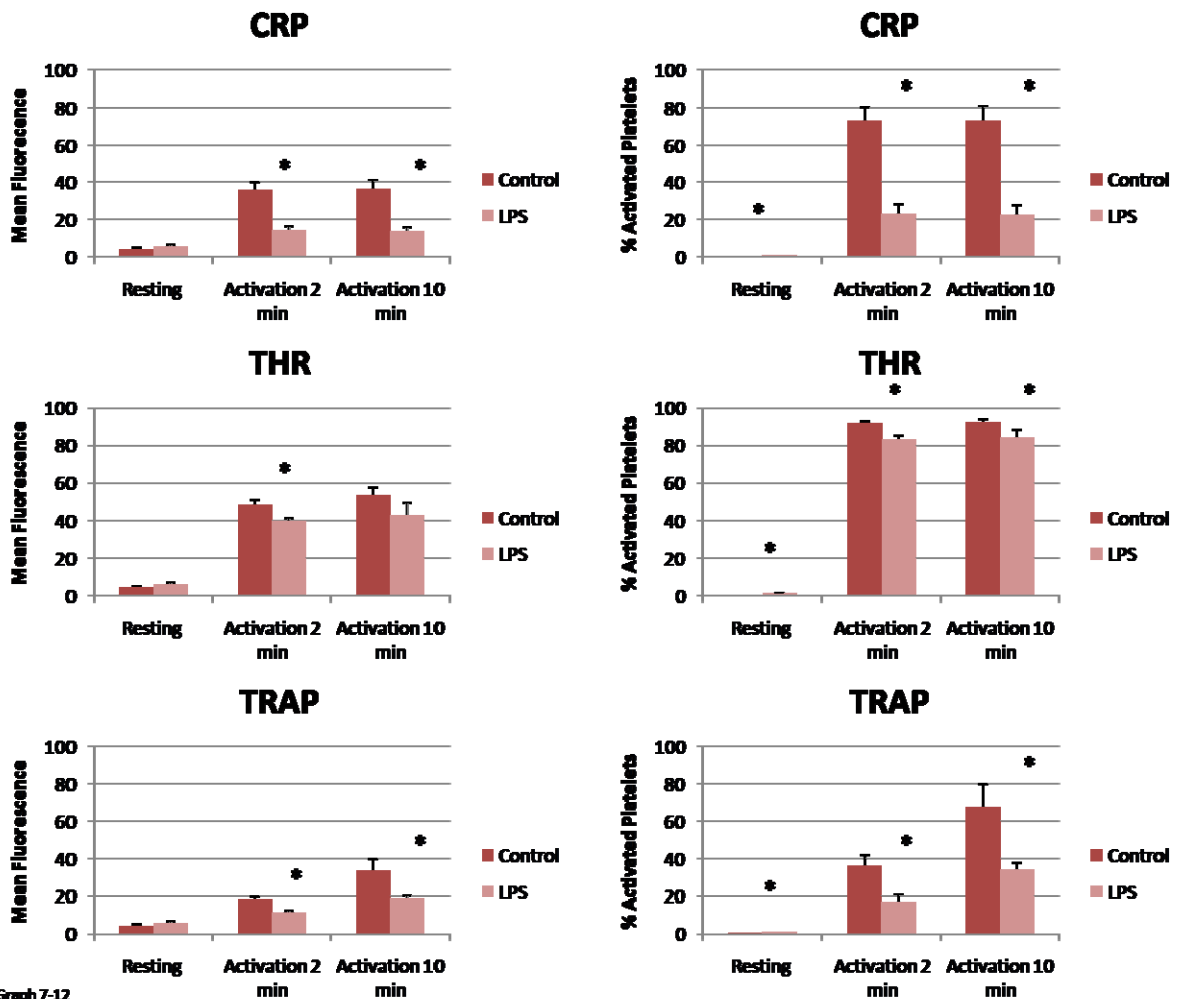
Normal platelets incubated in plasma from LPS-challenged mice did not activate differently from normal platelets incubated in plasma from unchallenged mice. Thrombin at a concentration of 10 u/ml did not activate platelets in the presence of plasma. The data are presented in graph 1-6 as mean value and SD as error bars.



Graph 1.6 Platelet activation on normal platelets incubated in plasma from control (C) and LPS-challenged mice.

Platelet activation in LPS-challenged mice was compared to non-challenged mice and the result shows that LPS-treatment significantly decreases platelet ability to activate and secrete P-selectin. The data are presented in graph 7-12 as mean value and SD as error bars and in table 1 as mean±SD and p-values.

The resting platelets from the both groups had different P-selectin expression on the surface, where the normal platelets had a lower level of P-selectin than the platelets from the LPS-challenged mice.

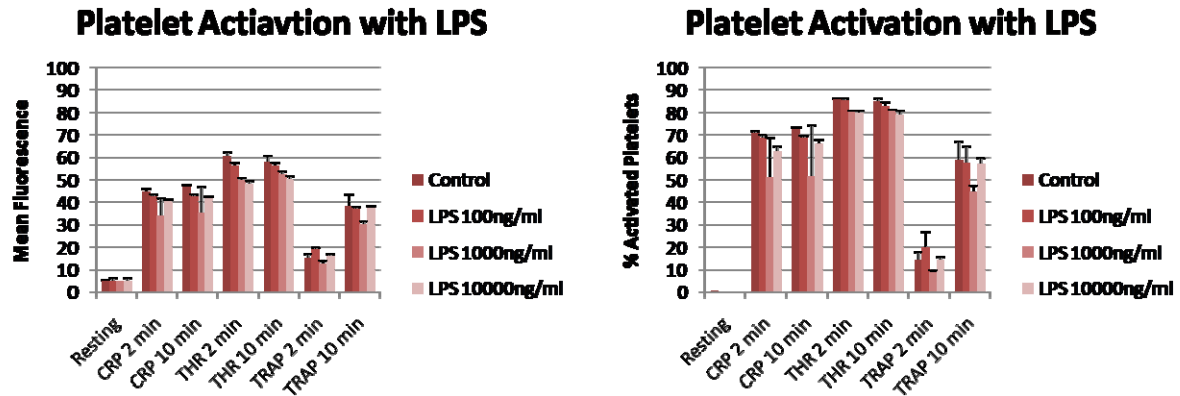


Graph 7-12  
Platelet activation on platelets from control and LPS-challenged mice.  
\* = p<0.05

**Table 1: Platelet Function as Mean Fluorescence and % Activated platelets after activation of platelets from control and LPS-challenged mice.**

	Mean Fluorescence			% Activated Platelets			
	Control	LPS	p-value	Control	LPS	p-value	
Resting	4.3±0.7	5.9±1	0.0809	Resting	0.3±1	0.9±0.2	<0.05
CRP 2 min	36±4	14±2	<0.05	CRP 2 min	73±7	23±5	<0.05
CRP 10 min	37±4	14±2	<0.05	CRP 10 min	73±8	23±5	<0.05
THR 2 min	49±2	40±1	<0.05	THR 2 min	92±1	83±2	<0.05
THR 10 min	54±4	43±6	0.1266	THR 10 min	92±2	84±4	<0.05
TRAP 2 min	18±2	11±1	<0.05	TRAP 2 min	36±5	17±4	<0.05
TRAP 10 min	34±6	18±2	<0.05	TRAP 10 min	68±12	34±3	<0.05

As platelets from LPS-challenged mice have shown to be hypo-reactive, the effect of LPS on platelets was studied in vitro. Platelet activation with LPS in vitro, in the concentration range 100ng/ml – 10µg/ml, showed that LPS does not affect platelet activation in vitro. The data are presented in graph 13 and 14 as mean values and SD as error bars.



Graph 13 and 14  
The result from the platelet activation with LPS in vitro presented as mean fluorescence and % activated platelets.

To assure that the observed differences in platelet activation did not depend on different amounts of the receptors for CRP – GPVI – and for thrombin and TRAP – PAR-4 – or on the amount of P-selectin in the granules, the amount of the proteins was analyzed using Western Blot. There was no difference in concentration of either of the proteins, as shown in figure 9-11.



Fig 9  
Western Blot: GPVI on platelet lysates from control and LPS-Challenged mice with or without rosoydnin pre-treatment.



Fig 10  
Western Blot: PAR-4 on platelet lysates from Control and LPS-Challenged mice with or without rosoydnin pre-treatment.

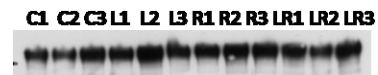
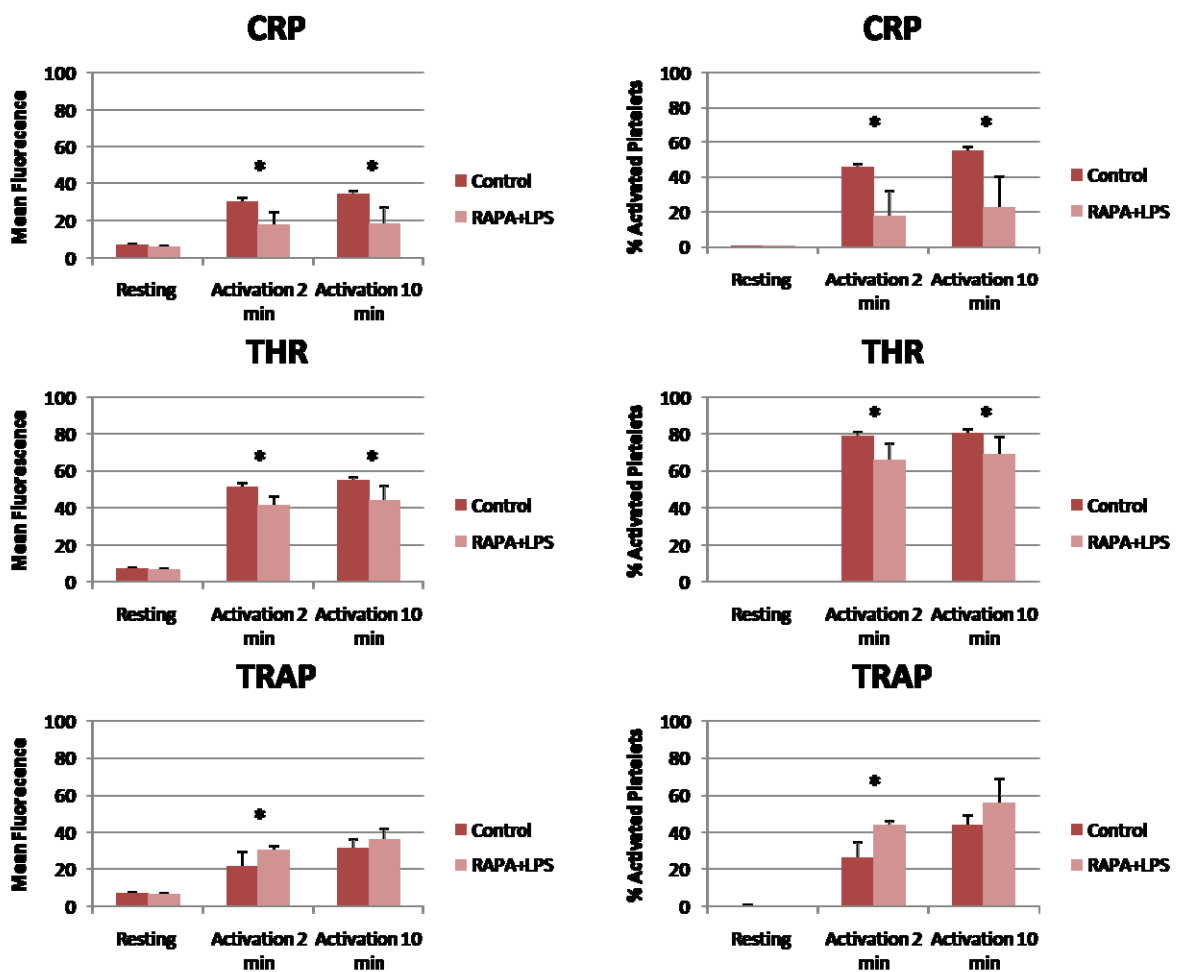


Fig 11  
Western Blot: on platelet lysates from control and LPS-Challenged mice with or without rosoydnin pre-treatment.

Rapamycin has, in previous studies, increased the survival of mice challenged with LPS. Therefore the effect of rapamycin pre-treatment on platelets was studied. Platelet activation in LPS-challenged mice pre-treated with rapamycin was compared to non-treated mice and the result shows that, as seen earlier, LPS treatment decreased platelet ability to activate and secrete P-selectin. Rapamycin pre-treatment did not affect the decreased activation ability, except with TRAP as an activator; where the activation ability was restored to the same, or even higher, level as platelets from non-treated mice. The data are presented in graph 15-20 as mean value and SD as error bars and in table 2 as mean±SD and p-values.



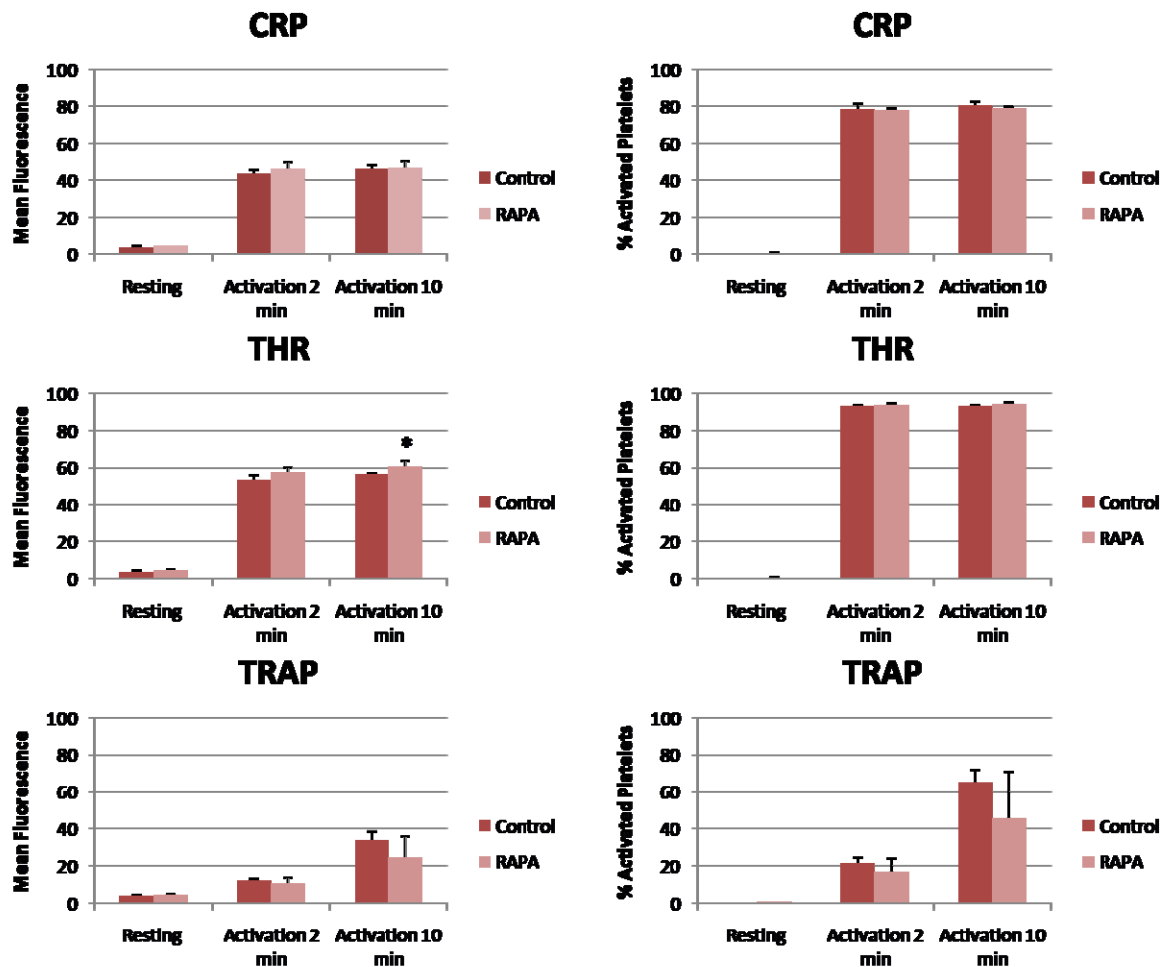
Graph 15-20  
Platelet activation on platelets from control and LPS challenged mice pre-treated with RAPA.  
\* = p<0.05

**Table 2: Platelet Function as Mean Fluorescence and % Activated platelets after activation of platelets from control and Rapamycin-treated, LPS-challenged mice.**

	Mean Fluorescence			% Activated Platelets			
	Control	RAPA+LPS	p-value	Control	RAPA+LPS	p-value	
Resting	7.2±0.5	6.3±0.5	0.1266	Resting	0.42±0.0	0.49±0.0	0.1904
CRP 2 min	31±2	18±7	<0.05	CRP 2 min	46±1	18±14	<0.05
CRP 10 min	35±2	18±9	<0.05	CRP 10 min	55±2	23±18	<0.05
THR 2 min	51±2	41±5	<0.05	THR 2 min	79±2	66±8	<0.05
THR 10 min	55±1	44±8	<0.05	THR 10 min	80±2	69±9	<0.05
TRAP 2 min	21±8	30±2	<0.05	TRAP 2 min	26±16	44±2	<0.05

TRAP 10 min	31±5	36±6	0.0765	TRAP 10 min	44±11	56±13	0.1266
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To assess the intrinsic effect of rapamycin on platelets, platelets from mice that received only rapamycin treatment was studied. Platelet activation in rapamycin treated mice was compared to non-treated mice and the result shows that rapamycin-treatment did not affect platelet ability to activate and secrete P-selectin. The data are presented in graph 21-26 as mean value and SD as error bars and in table 3 as mean±SD and p-values.

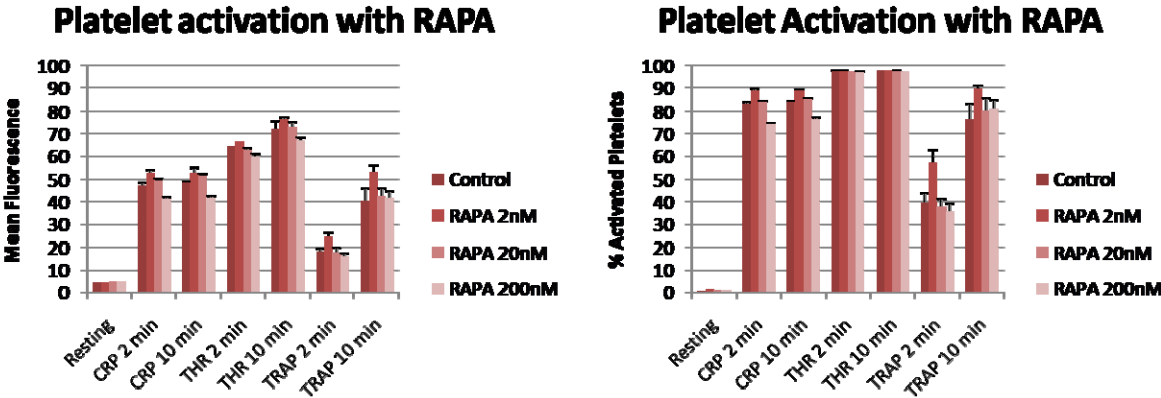


Graph 21-26  
Platelet activation on platelets from control and RAPA-treated mice.  
\* = p<0.05

**Table 3: Platelet Function as Mean Fluorescence and % Activated platelets after activation of platelets from control and Rapamycin-treated mice.**

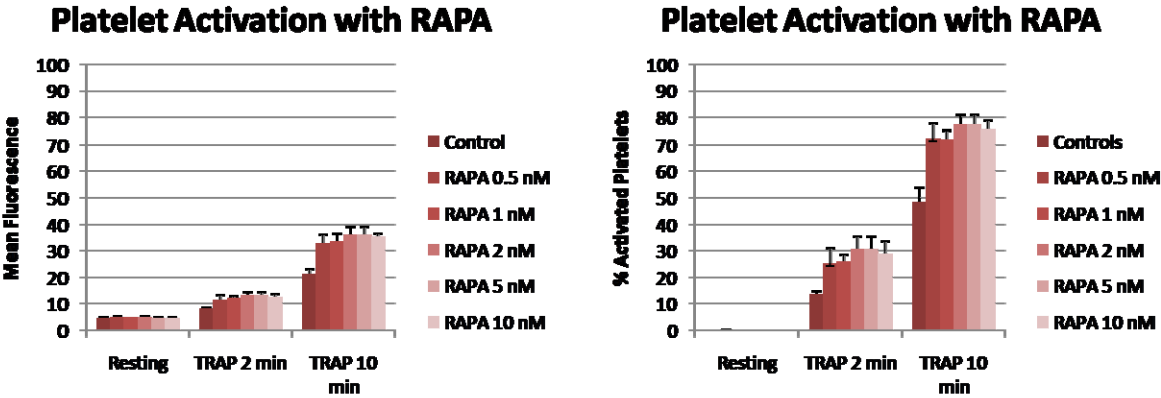
	Mean Fluorescence			% Activated Platelets			
	Control	RAPA	p-value	Control	RAPA	p-value	
Resting	3.7±0.8	4.6±0.2	0.1266	Resting	0.26±0.2	0.58±0.1	0.1266
CRP 2 min	44±2	46±4	0.8273	CRP 2 min	78±3	78±1	0.2752
CRP 10 min	46±2	47±3	0.8273	CRP 10 min	81±2	79±1	0.1266
THR 2 min	53±2	57±3	0.1266	THR 2 min	93±1	94±1	0.3827
THR 10 min	57±0	60±3	<0.05	THR 10 min	93±0	94±1	0.5127
TRAP 2 min	12±1	17±3	0.3827	TRAP 2 min	22±3	17±7	0.2752
TRAP 10 min	34±4	46±11	0.1266	TRAP 10 min	65±7	46±25	0.1266

To further assess the influence of rapamycin on platelet function, rapamycin was added to washed platelets in vitro. Platelet activation with rapamycin in vitro, in the concentration range 2nM – 200nM, showed that rapamycin in vivo affects platelet activation with TRAP as an activator at the concentration of 2nM rapamycin. Rapamycin, in the concentration range 2nM – 200nM, does not significantly influence the activation of platelets using CRP or Thrombin as activators. The data are presented in graph 27 and 28 as mean values and SD as error bars.



Graph 27 and 28  
The result from the platelet activation with RAPA in vitro presented as mean fluorescence and % activated platelets.

Due to the observations above, a narrower concentration range around 2nM was chosen. Rapamycin enhances platelet activation at even very low concentrations. The response does not follow a dose-response curve and this may be due to that the maximum response has already been reached at the lowest concentration or that the effect is non-specific. Rapamycin has been seen to affect cells at concentrations around 1 nM. The results from the activation experiment are shown in graph 29 and 30 as mean values and SD as error bars.

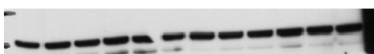


Graph 29 and 30  
The result from the platelet activation with rapamycin in vitro, using TRAP as an activator.

## Cell signaling in platelets

To evaluate if the mTOR pathway is active in platelets and supporting that rapamycin can directly affect platelet function, platelets lysates from non-challenged and LPS-challenged mice, with or without rapamycin treatment was analyzed using western blot for p-P70S6K and p-S6 to see if the rapamycin treatment had influenced the expression of the proteins. The results are showed in figure 12-14. Rapamycin treatment blocks, as expected, mTOR which results in decreased levels of p-S6 in the cells. The expression of p-P70S6K is not altered by rapamycin treatment using this antibody, which is specific for the T421 phosphorylation. This phosphorylation site is known to be under the control of other cell signaling pathways in addition to mTOR, explaining the observation.

C1 C2 C3 L1 L2 L3 R1 R2 R3 LR1 LR2 LR3



**Fig 12**  
Western blot: p-P70S6K (T421) on platelet lysates from control and LPS-challenged mice with or without rapamycin pre-treatment.

C1 C2 C3 L1 L2 L3 R1 R2 R3 LR1 LR2 LR3



**Fig 13**  
Western blot: p-S6 (235/236) on platelet lysates from control and LPS-challenged mice with or without rapamycin pre-treatment.

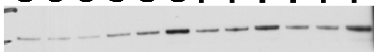
C1 C2 C3 L1 L2 L3 R1 R2 R3 LR1 LR2 LR3



**Fig 14**  
Western blot: p-S6 (240) on platelet lysates from control and LPS-challenged mice with or without rapamycin pre-treatment.

To further assess the function of the mTOR pathway in platelet response to agonists platelets from a normal mouse were activated using HBSS as control and the three activators CRP, thrombin, and TRAP for 1, 2, and 10 minutes, respectively, and subsequently tested using western blot for p-P70S6K and p-S6. The result shows that activation of platelets results in an increase in both p-P70S6K and p-S6 after 10 minutes of activation. The result is shown in figure 15-17.

Control 1 min  
Control 2 min  
Control 10 min  
CRP 1 min  
CRP 2 min  
CRP 10 min  
THR 1 min  
THR 2 min  
THR 10 min  
TRAP 1 min  
TRAP 2 min  
TRAP 10 min



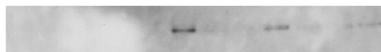
**Fig 15**  
Western blot: p-P70S6K (T421) on platelet lysates from activated platelets, using HBSS for control, CRP, THR and TRAP as activators 1, 2 and 10 minutes.

Control 1 min  
Control 2 min  
Control 10 min  
CRP 1 min  
CRP 2 min  
CRP 10 min  
THR 1 min  
THR 2 min  
THR 10 min  
TRAP 1 min  
TRAP 2 min  
TRAP 10 min



**Fig 16**  
Western blot: p-S6 (235/236) on platelet lysates from activated platelets, using HBSS for control, CRP, THR and TRAP as activators 1, 2 and 10 minutes.

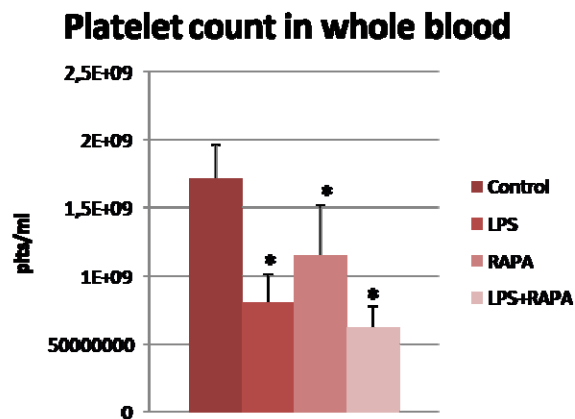
Control 1 min  
Control 2 min  
Control 10 min  
CRP 1 min  
CRP 2 min  
CRP 10 min  
THR 1 min  
THR 2 min  
THR 10 min  
TRAP 1 min  
TRAP 2 min  
TRAP 10 min



**Fig 17**  
Western blot: p-S6 (240) on platelet lysates from activated platelets, using HBSS for control, CRP, THR and TRAP as activators 1, 2 and 10 minutes.

### Platelet count in whole blood

As thrombocytopenia is a common feature of sepsis, circulating platelets after LPS-challenge with or without rapamycin pre-treatment was quantified. Platelet concentration in whole blood after LPS treatment shows that mice treated with LPS has significantly lower platelet concentration than control animals. The data are presented in graph 31 as mean values and SD as error bars. The number of platelets per ml in non-treated mice was  $1.7 \cdot 10^9 \pm 2.4 \cdot 10^8$  platelets/ml and in LPS treated mice the number was significantly lower;  $8.1 \cdot 10^8 \pm 1.9 \cdot 10^8$  platelets/ml ( $p < 0.05$ ). Rapamycin treatment alone also seem to decrease the platelet count, the animals treated with only rapamycin had a mean value of  $1.2 \cdot 10^9 \pm 3.5 \cdot 10^8$  platelets/ml, which is significantly lower than the non-treated mice ( $p < 0.05$ ). The mice that received both LPS and rapamycin had the lowest platelet counts, only  $6.2 \cdot 10^8 \pm 1.6 \cdot 10^8$  platelets/ml, which is significantly lower than non-treated mice ( $p < 0.05$ ).

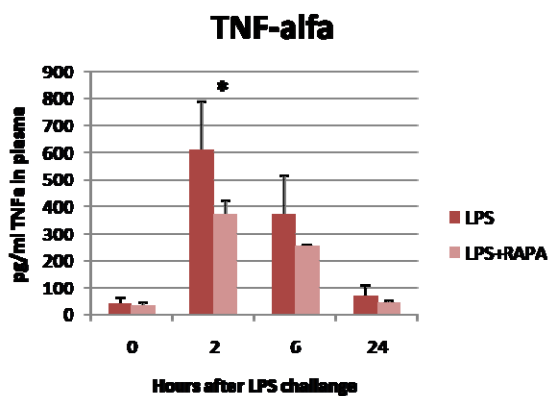


Graph 31  
Platelet count in whole blood after LPS and/or rapamycin treatment.  
\* =  $p < 0.05$  compared to WT.

## Rapamycin sensitive pathway of LPS signaling

### Cytokine expression in septic mice

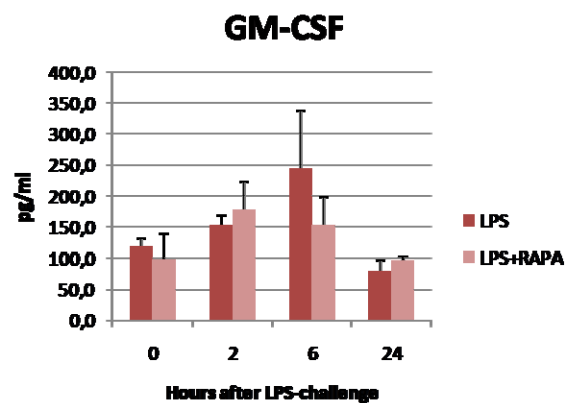
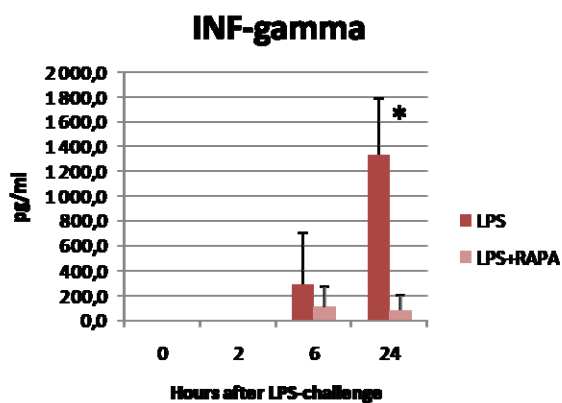
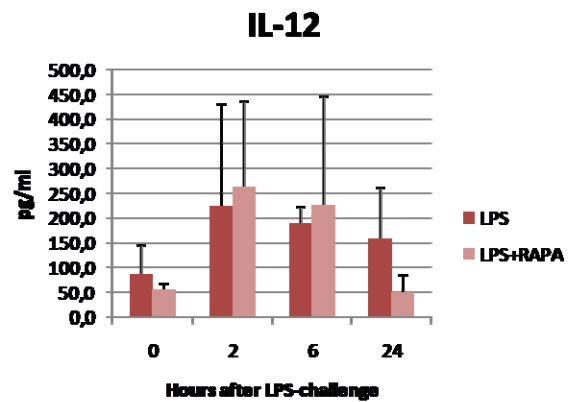
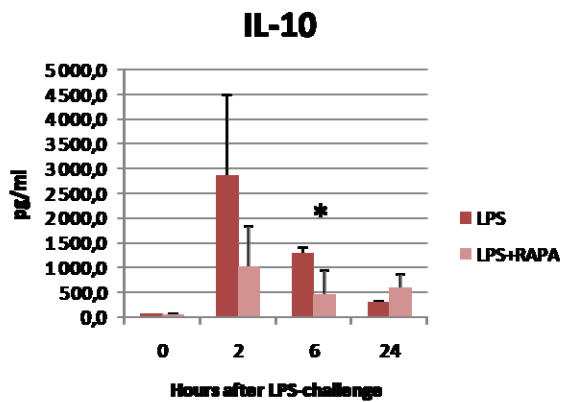
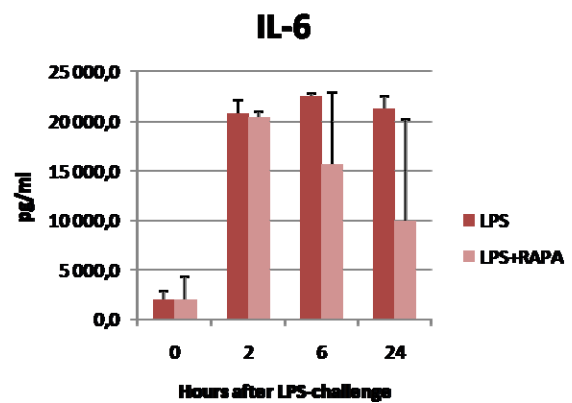
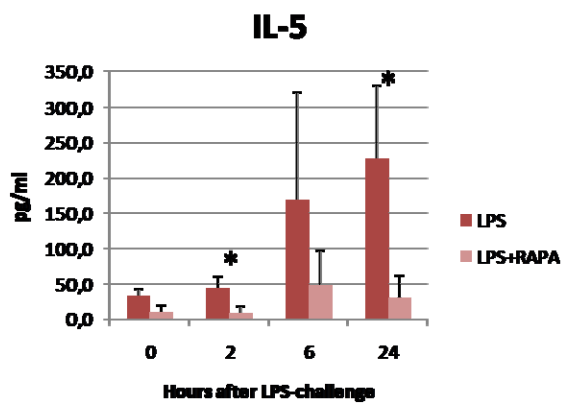
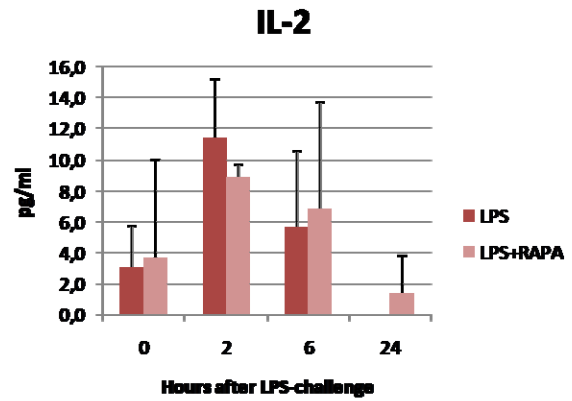
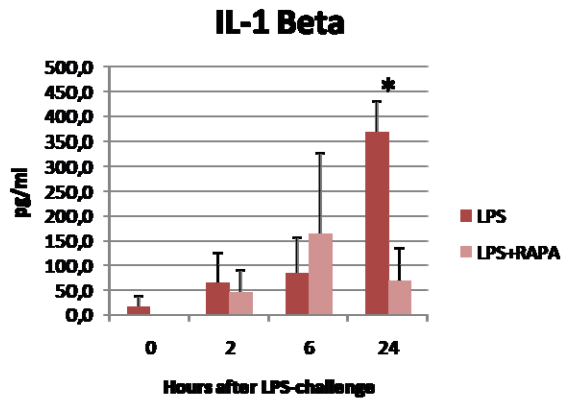
The pilot test measuring TNF- $\alpha$  using ELISA showed that pre-treatment with rapamycin decreased the pro-inflammatory cytokine TNF- $\alpha$  in plasma. At time-point zero, the levels of TNF- $\alpha$  were low in both groups;  $38 \pm 20$  pg/ml in the group that did not get rapamycin pre-treatment and  $33 \pm 9$  pg/ml in the rapamycin pre-treatment group ( $p = n.s.$ ). At the 2-hour time-point, the TNF- $\alpha$  levels were high in both groups but lower in the rapamycin pre-treated animals;  $610 \pm 180$  pg/ml and  $370 \pm 51$  pg/ml ( $p < 0.05$ ), respectively. At 6 hours the TNF- $\alpha$  level had gone down, but still, the rapamycin treated group had lower levels;  $250 \pm 5.0$  pg/ml as compared to the non-rapamycin treated group where the concentration was  $370 \pm 150$  pg/ml ( $p = n.s.$ ). 24 hours after LPS-challenge, the TNF- $\alpha$  level was almost back to normal with the values of  $67 \pm 42$  pg/ml and  $41 \pm 9.3$  pg/ml, respectively ( $p = n.s.$ ). The result is shown in graph 32 as mean values and SD as error bars.



Graph 32  
TNF- $\alpha$  levels in plasma 0, 2, 6 and 24 hours after LPS-Challenge.  
\* -  $p < 0.05$

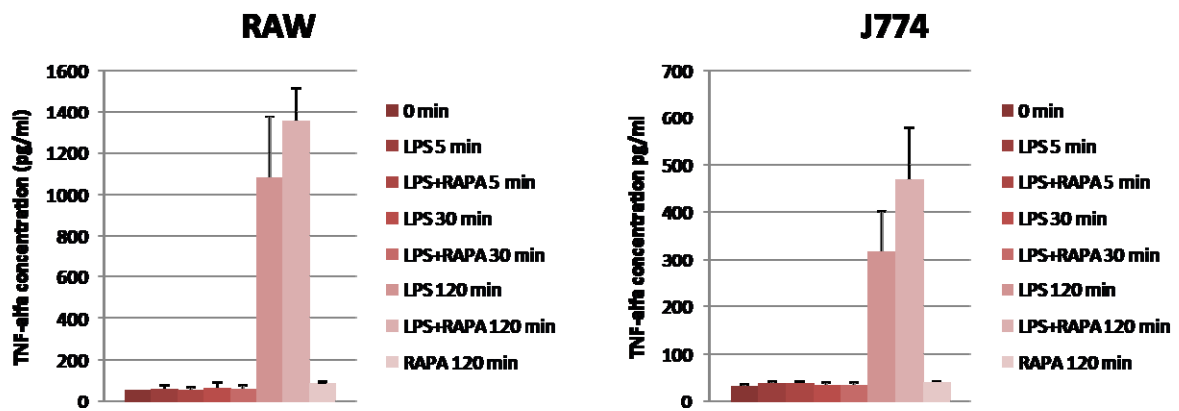
The result from the cytokine panel is shown in graph 33-40 as mean values and standard deviations as error bars.

IL-1 $\beta$  is increasing as a response to LPS-challenge and the effect of rapamycin pre-treatment is first obvious at the 24 hour time-point ( $p < 0.05$ ), at the earlier time-point the values are not significant. Rapamycin treatment does not seem to influence the release of IL-2 and none of values were significant. The level of IL-4 was undetectable in all samples. IL-5, on the other hand, seems to be affected by rapamycin pre-treatment and at the 2 and 6 hour time-points the difference was significant ( $p < 0.05$ ). IL-6, on the other hand, do not seem to be affected by rapamycin pre-treatment ( $p = n.s.$ ). The level of IL-10 is decreased by rapamycin treatment although only the 6 hour time-point reached significance ( $p < 0.05$ ). IL-12 is not affected by rapamycin treatment ( $p = n.s.$ ). Interferon  $\gamma$  gave a later response; the levels were undetectable at 0 and 2 hours. At 6 hours there is a trend against lower levels in the rapamycin treated group ( $p = n.s.$ ) and at 24 hours the level in the rapamycin treated group is clearly lower than in the non pre-treated group ( $p < 0.05$ ). The levels of the macrophage stimulatory factor GM-CSF does not seem to be affected by rapamycin treatment.



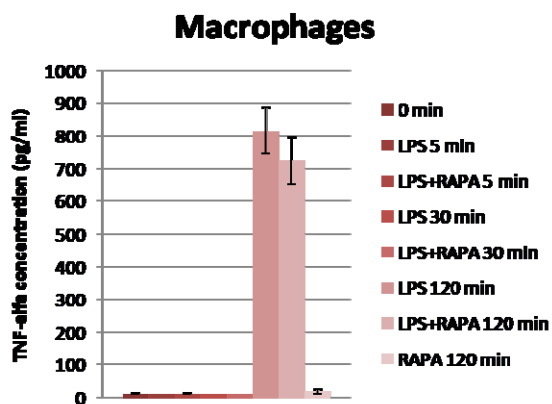
Graph 33-40  
The levels of cytokines in the plasma at 2, 6, and 24 hours post LPS-challenge in mice with and without rapamycin pre-treatment.  
\*= $p < 0.05$

The ELISA test of the TNF- $\alpha$  level in media from the RAW and J774 cells show that TNF- $\alpha$  is up-regulated first 120 minutes after LPS treatment and that rapamycin do not affect the level of TNF- $\alpha$  secretion from the macrophage-like cells ( $p=n.s.$ ). The result is shown in graph 41 and 42.



Graph 41 and 42  
The TNF- $\alpha$  levels in media from the RAW and J774 macrophage-like cells.

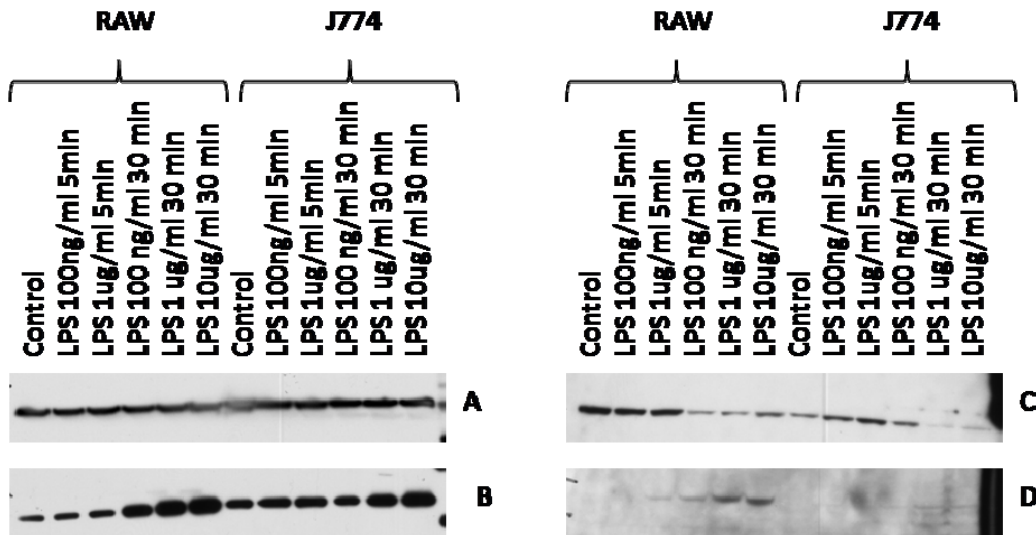
The ELISA test of the TNF- $\alpha$  level in media from the primary mouse peritoneal macrophages also show that TNF- $\alpha$  is increased first after 120 minutes after addition of LPS. Rapamycin does not affect the TNF- $\alpha$  level at the 120 minute time-point ( $p=n.s.$ ). The result is shown in graph 43.



Graph 43  
The TNF- $\alpha$  level in media from primary macrophages.

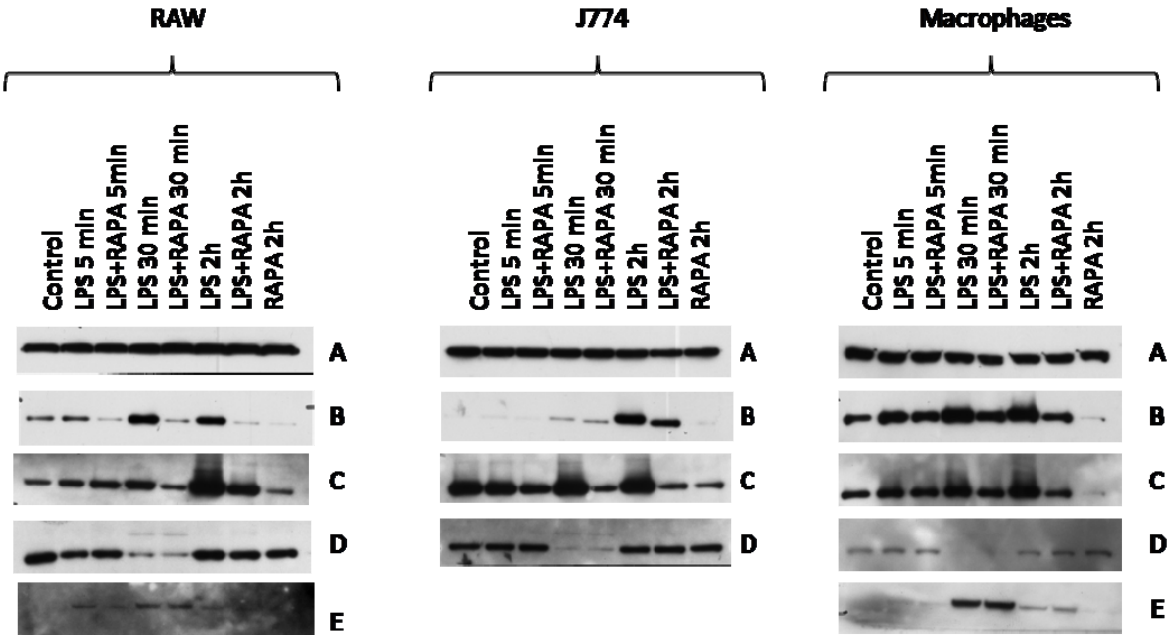
### Cell signaling in macrophage-like cell lines and primary macrophages

The pilot study of the RAW and J774 cells showed that LPS increases p-S6 after 30 minutes, and that the increase is dose dependant, with an increased expression with increased doses. IκBα is present in control sample and after 5 minutes with LPS, but goes down to the 30 minutes time-point. IKKα/β is up-regulated after 30 minutes. The results from the western blots are showed in figure 18 A-D.



**Fig 18 A-D**  
The expression of (A) Tubulin, (B) p-S6 (Z35/Z36), (C) IκBα and (D) p-IKKα/β in RAW and J774 macrophage-like cells after LPS-treatment.

The RAW and J774 macrophage-like cells and the primary macrophages were treated with both LPS and rapamycin. To evaluate the influence of mTOR on the LPS signaling, the lysates were analyzed using western blot and the result is showed in figure 19 A-E, where one set of the triplicates are shown. The level of p-S6, both phosphorylation of S235/236 and S240/245, increase after 30 minutes of LPS treatment, and the increase is successfully blocked by rapamycin, which means that the increase is due to mTOR activity. The signal from p-P70S6K was too weak to be detected. This was probably due to a low protein concentration. P-IKK $\alpha/\beta$  increased rapidly after LPS treatment and is visible after 5 minutes in RAW cells and in the primary macrophages, but in J774 cells the level was too low to be detected. The level of P-IKK $\alpha/\beta$  is not affected by rapamycin treatment. The I $\kappa$ B $\alpha$  level goes down already 5 minutes after LPS treatment and is further suppressed at 30 minutes after LPS treatment. I $\kappa$ B $\alpha$  is unaffected by rapamycin treatment in RAW, J774, and peritoneal macrophages.



**Fig 19 A-E**  
 The expression of (A) Tubulin, (B) p-S6 (235/236), (C) p-S6 (240), (D) I $\kappa$ B $\alpha$  and (E) p-IKK $\alpha/\beta$  in RAW and J774 macrophage-like cells and primary macrophages, respectively.

## Discussion

### Sepsis associated platelet dysfunction

The platelet function is altered in sepsis, where platelets are hypo-reactive in response to the three activators tested and the platelet function is restored to normal with rapamycin pre-treatment, although only when using TRAP as an activator, thus bypassing several steps in the activation process used by thrombin. Platelet function does not seem to be affected by any substances in plasma from septic animals.

Flow cytometry is one of many techniques available to assess platelet function, and could be used to measure the degree of activation of platelets using monoclonal antibodies. Other techniques available are bleeding time and platelet aggregometry. In this study I used flow cytometry, which is a well-established method to assess platelet activation.

P-selectin (CD62-P) is only present on the platelet surface when the platelet is activated; therefore this protein is a good marker for platelet activation. P-selectin translocates from  $\alpha$ -granules to the cell surface within seconds after activation<sup>29</sup>. Labeled antibodies for P-selectin have been used before to differ between activated and non-activated platelets<sup>27,33,41</sup>. The western blots for P-selectin showed that platelets, from control, rapamycin treated and LPS-challenged mice with or without rapamycin pre-treatment, have the same level of P-selectin. Therefore the difference in response to activation was not due to different amount of P-selectin. Also both the receptors for CRP and thrombin, GPVI and PAR-4, respectively, seem to be present in the same amounts in the platelets lysates. Therefore that could not explain the observed hypo-responsiveness to activation. Other receptors could also affect the response to activation, for example GPV and  $\alpha_2\beta_2$  integrin, which both enhance the signal through GPVI and PAR-4. Also glycosylation of the receptors could affect their ability to convey the activation signal. It was beyond the scope of this study to evaluate the reason why the activation process is altered after LPS-challenge and further testing needs to be done to understand how and why platelets are affected during sepsis.

Platelets from LPS-challenged mice actually respond differently to the activators CRP, thrombin and TRAP compared to platelets from non-challenged mice. LPS-challenge resulted in the platelets being more resistant to activation, i.e. hypo-reactive. This is not in line with most opinions in this area; however published papers have reported conflicting data about the platelet function during sepsis. In a study performed by Jacoby et al.<sup>33</sup> on trauma patients, the platelets from trauma patients showed increased function. They tested both aggregation, determined as occlusion time in a platelet function analyzer, PFA, and activation, measured as expression of GPIIb-IIIa, P-selectin, and percentage of microparticles. The latter forms as platelets, when activated, release fragments of the cell. The result of the study on trauma patients was decreased occlusion time, which means increased function, and increased activation. The finding that inflammation can enhance platelet aggregation is further strengthened by a study conducted by Matera et al.<sup>42</sup>, where LPS enhanced human and rabbit platelet aggregation in response to ADP and thrombin. However, the opposite result of LPS has been reported by Saba et al.<sup>43</sup>, who added endotoxin to washed platelets or platelet rich plasma and found an inhibitory effect of LPS on platelet aggregation caused by interference with calcium mobilization. Similar observations were reported by Sheu et al.<sup>44</sup>; lipoteichoic acid, which is a membrane component of gram-positive bacteria, inhibit platelet aggregation after stimulation with thrombin and collagen. Cicala et al.<sup>45</sup> also demonstrated that LPS

injection *in vivo* resulted in a decrease of platelet response to ADP and collagen. Boldt et al.<sup>46</sup> found that the response of platelets from septic patients to collagen and ADP-aggregation was lower compared to platelets from trauma patients without sepsis.

The observed differences between these studies could be due to different methods to measure platelet function, i.e. secretion or aggregation, as well as the population studied, i.e. patients with sepsis, experimental animals with induced sepsis, or isolated cells treated with bacterial products. It has been argued that LPS infusion models often may not accurately mimic the changes observed during sepsis, and does not fully reflect the clinical development in human sepsis<sup>6</sup>. However, we can conclude from our data, that in this murine model of endotoxemia, platelets are hypo-responsive. The fact that rapamycin treatment restores the ability of the platelets to activate by TRAP is interesting and may be explained by several possibilities. Both a direct effect on the platelets, as we have showed that the mTOR pathway in platelets is up-regulated by activation and that rapamycin pre-treatment in platelets decreases p-S6. Rapamycin could also affect platelets in an indirect way, by lowering for example TNF- $\alpha$  level in the blood. TNF- $\alpha$ , in levels found during sepsis, seem to strongly inhibit thrombus growth in vascular injury models and results in prolonged bleeding time in mice, i.e. a function reduction of the platelets<sup>28</sup>.

In our study, plasma from LPS-challenged mice had no effect on platelet activation compared to plasma from normal mice. This is not in line with an article by Gawaz et al.<sup>47</sup>, where ADP was used to aggregate platelets in the presence of plasma. Plasma from septic patients with MODS was compared to healthy volunteers and the result showed increased aggregation using plasma from the patients compared with plasma from healthy volunteers. These results are the opposite of the findings by Cicala et al.<sup>45</sup> where normal platelets were added to plasma from LPS treated animals and the platelets were hypo-responsive. The results of these two studies, combined with the result in mine, have come to the three possible conclusions: increased platelet function, no change in platelet function, and decreased platelet function. Other studies have also showed that the coagulation cascade and platelets are activated in sepsis, using other markers for activation, such as platelet-specific proteins  $\beta$ -TG and PF<sub>4</sub> or TF levels in plasma<sup>3,24</sup>.

In this study, LPS had no effect on platelet activation when added to washed platelets *in vitro*. This finding is supported by findings by Csako et al.<sup>48</sup>, who also found that LPS did not influence washed platelets. They concluded that other blood cells were necessary to mediate the actions of LPS on platelets, as LPS added to whole blood increased aggregation. The same thing was also found by Whitworth et al.<sup>49</sup>, LPS added to whole blood enhanced aggregation but LPS added to platelet rich plasma did not affect aggregation. In a study by Camerer et al.<sup>41</sup>, the conclusion was also that endotoxin does not trigger P-selectin expression on isolated platelets, thus endotoxin acting on platelet TLR4 may contribute to platelet activation, but the major effects of endotoxin on platelets may be indirect. As mentioned in the introduction, platelets express TLR4 and should, at least in theory, be able to respond to LPS directly. Rapamycin, on the other hand, increased platelet activation when added *in vitro* to washed platelets in this study, which means that rapamycin has a direct effect on platelets.

In a study conducted by Ogura et al.<sup>27</sup>, platelets from septic patients had an increased amount of P-selectin on their surface, which would suggest that platelets from septic patients are already activated to a higher degree than platelets from healthy volunteers. In this study on mice platelets, there was also a difference in of P-selectin positive platelets between the LPS- challenged mice and the non-challenged mice, but the numbers of activated platelets was very low in both groups; therefore the conclusion would be that the difference has no clinical implication.

LPS has been shown to bind to platelets through P-selectin, and thus bind to only activated platelets. One function of LPS-binding to platelets could be to remove soluble LPS from the circulation and soluble P-selectin could provide protection against LPS-induced organ failure.<sup>8</sup> The platelet count in blood from LPS, and/or rapamycin treated mice show that all three groups had lower platelet concentration compared with normal mice. Sepsis is known to cause thrombocytopenia and the fact that rapamycin alone causes a slight decrease in platelet count has also been seen in clinical trials with rapamycin<sup>22</sup>. Thrombocytopenia has been shown to correlate with increased lethality, both in patients and in experimental animals<sup>2,31</sup>. As much as low platelet count may confer increased risk of bleeding, sepsis-associated thrombocytopenia should be recognized for what it is: a surrogate marker for the severity of the host immune response<sup>2</sup>. Previous results from this research group have shown that rapamycin pre-treatment before LPS-challenge led to increased survival. This means that the decreased lethality seen in rapamycin pre-treated animals is not due to salvaged platelet counts.

It is becoming increasingly apparent that platelets play a complex role in sepsis and interfering with platelet function may prove to be valuable in the treatment of sepsis. However, further study is needed to better define the precise mechanisms and effects of platelet function during sepsis. Many factors may, directly and indirectly, affect platelet function in sepsis including; pH, temperature, plasma coagulation abnormalities, and hormonal response<sup>46</sup>.

## Rapamycin sensitive pathway of LPS signaling

### Cytokine expression

As shown by the pilot-study, the TNF- $\alpha$  level is successfully lowered in plasma by treatment with rapamycin before LPS-challenge. The TNF- $\alpha$  levels are known to peak in one to two hours after LPS-challenge and subsequently go back to normal levels within 24 hours<sup>14</sup>. This is also seen in this study, and the same pattern is seen in the rapamycin pre-treated animals but with lower levels of TNF- $\alpha$ . High concentrations of TNF- $\alpha$  may determine pathologic abnormalities with exaggerated inflammation and TNF- $\alpha$  is thought to play a major role in the clinical manifestations of septic shock<sup>15</sup>. The lower level of TNF- $\alpha$  may be the reason why rapamycin pre-treated mice survive longer than non-treated animals after LPS-challenge.

The result from the cytokine panel shows that the macrophage stimulatory factor GM-CSF is not affected by rapamycin pre-treatment. Neither IL-2, IL-6, nor IL-12 seems to be affected. The levels of INF- $\gamma$  are apparent decreased by rapamycin treatment and the release of INF- $\gamma$  is delayed compared to most of the other cytokines, peaking at 24 hours or later. IL-1 $\beta$  and IL-5 also have slow kinetics compared to TNF- $\alpha$  and the levels are still high at 24 hours post LPS-challenge. Rapamycin is affecting the levels of the both cytokines, although not significantly at all time points but there is an evident trend towards decreased release. The kinetics of IL-10 resembles that of TNF- $\alpha$ , the levels peaking at 2 hours and almost returning to basal level at 24 hours post challenge, and as TNF- $\alpha$ , the IL-10 levels are influenced by rapamycin pre-treatment, and there is a fairly clear trend towards decreased release.

The cytokine response demonstrate the complexity, redundancy, and overlap of mediators, suggesting that no single agent is likely to modulate the disordered inflammatory process in a clinically significant way<sup>13</sup>. Rapamycin inhibits the release of several of the cytokines and that could contribute to the beneficial effect, seem as increased survival, in rapamycin pre-treated mice. Critics have been raised against LPS infusion as a model of sepsis<sup>6</sup>. The circulating LPS levels in the cecal ligation and puncture, CLP, sepsis model, which may more accurately reflect the dynamics of sepsis occurring in humans, were found to be very low. TNF- $\alpha$  level observed in the CLP model is generally also very low and not comparable to TNF- $\alpha$  level found after LPS infusion. The failure of anti-TNF- $\alpha$  and anti-LPS interventions in septic patients can be seen as an example of how conclusions based on animal models may not hold true in humans, or may not be applicable to human sepsis because of incorrect assumptions underlying the animal models, i.e. that LPS is a major initiator of sepsis and that LPS is present in the serum at high levels during sepsis. Currently, there is general agreement among researchers in the field that LPS injection may serve as a model for endotoxic shock but not for sepsis.

The relative contribution of the NF- $\kappa$ B and PI3K (i.e. the Akt/mTOR) pathways to the LPS-response in macrophages was analyzed by Dos Santos et al.<sup>10</sup>. The NF- $\kappa$ B and PI3K pathways exert an overlapping regulation in cells stimulated by LPS. Some genes are more specifically under the control of one of these signaling pathways. As shown by specific inhibitors of the both pathway; the NF- $\kappa$ B pathway controls pro-inflammatory genes for monocytes chemoattractant protein 1, inducible NO synthetase, and NF- $\kappa$ B itself and the PI3K pathway controls genes encoding platelet activating factor, IL-13 among others. The inhibition of PI3K results in inhibition of the phosphorylation of Akt, which suppresses the activation of NF- $\kappa$ B by attenuating the phosphorylation and degradation of I $\kappa$ B. IL-10 gene expression has also been described to be regulated by PI3K/Akt<sup>10</sup>. IL-10 is produced in response to LPS and TNF- $\alpha$ , this leads to down-regulation of other pro-inflammatory cytokines as IL-

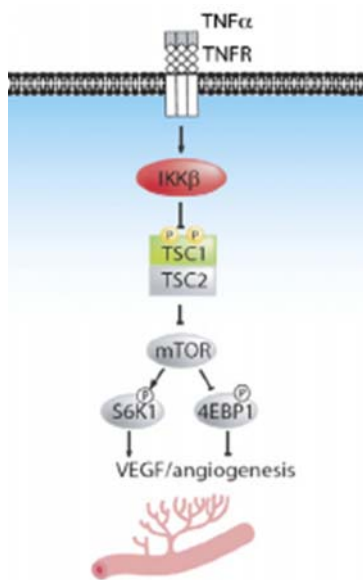
1 $\alpha$ / $\beta$ , IL-6, IL-8, and TNF- $\alpha$  <sup>15</sup>. Also IL-6 has been shown to be regulated by TNF- $\alpha$ , and is found in high levels in septic patients. IL-1, IL-6, and IL-8 have been found to correlate with clinical outcome in a study by Bozza et al. <sup>40</sup>.

The cytokine release from macrophage-like cells is increased first two hours after LPS-treatment and is not dependent on the mTOR pathway as rapamycin does not affect the release. The fact that the TNF- $\alpha$  release is affected by rapamycin treatment in animals and not in cells could be due to the difference between the whole animal system and the cell system, the later being less complicated, and also the fact that the cells used in this experiment are immortalized cell lines that could be changed compared to normal macrophages. The first wave of TNF- $\alpha$  could also be a preformed limited amount of TNF- $\alpha$ , stored in the macrophage-like cells and subsequent release could be dependent on mTOR activation. Since the same pattern was observed in primary macrophages, i.e. that rapamycin does not affect the TNF- $\alpha$  release, the second explanation to the difference between the in vivo and in vitro response; that the result was due to the characteristics of the cell lines, could be ruled out. The difference in response to rapamycin treatment observed between in vivo and in vitro could be dependent on the fact that a cell are less complicated than a live animal and that other cells except for the macrophages could be responsible for the TNF- $\alpha$  release.

### LPS signaling pathway

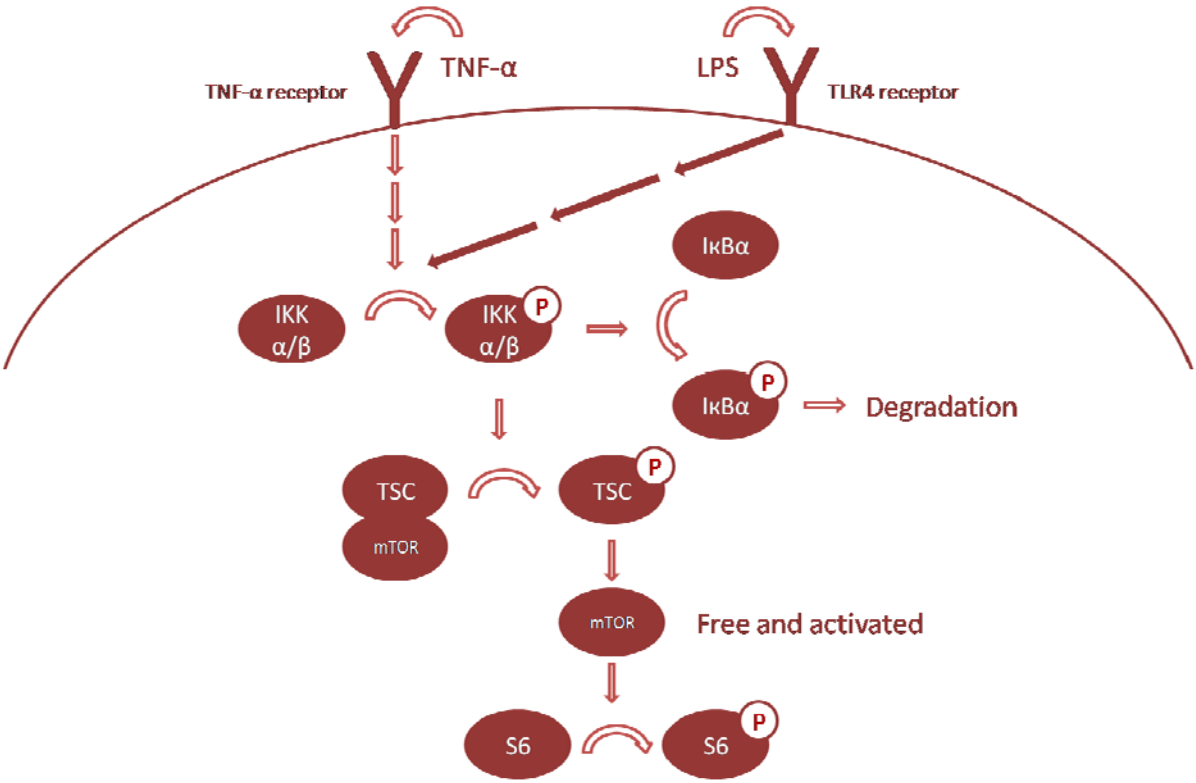
Previous studies in animals; have shown that LPS administration reproduces many of the metabolic derangements of sepsis shortly after LPS is infused<sup>14</sup>. These include elevations in temperature, heart rate, and cortical and lactate levels. Evidence suggests that when endotoxin is used in a non-lethal dose, the pathophysiological consequences are intracellular and metabolic in nature rather than hemodynamic and that those effects persists over a prolonged period of time. The metabolic effects are important for maintaining homeostasis during infection, and thus have a protective role<sup>4,50</sup>. The signaling pathway that mediates the effect of LPS on S6K1 phosphorylation, i.e. cell metabolism is not yet known<sup>21</sup>.

In a study published a few months ago by Lee et al.<sup>20</sup>, a new pathway linking inflammation to mTOR activation through TNF- $\alpha$  is proposed. IKK $\beta$ , which is major downstream kinase in the TNF- $\alpha$  signaling pathway, is shown to interact with and phosphorylate TSC1 at S487 and S511, resulting in suppression of TSC1. The IKK $\beta$ -mediated suppression subsequently activates the mTOR pathway through the inactivation of TSC1. The suggested pathway is shown in figure 20.



**Figure 20**  
The pathway in which TNF- $\alpha$  activates mTOR.<sup>19</sup>

The results from the western blots on the cell lysates shows that p-IKK is increased within 5 minutes and the IκBα is subsequently decreased in 5 to 30 minutes after LPS treatment. P-S6 is increased 30 minutes after LPS treatment and the increase is inhibited by rapamycin. The TNF-α release is increased first after 2 hours. According to the timing of the events; LPS must affect the same pathway as TNF-α, through signaling via TLR4. Of course, this needs to be confirmed by either blockade of the TNF-α receptor or by using TNF-α or the TNF-α receptor knock-out cells. Another possibility to strengthen the hypothesis is by inhibition of p-IKKβ and measurement of the p-S6 levels, so that no other pathway could be responsible for the increased levels of p-S6 than mTOR. I hereby suggest a new mechanism in which LPS affects the mTOR pathway and thereby cell metabolism. The proposed cell signaling pathway for LPS to influence p-S6 is shown in figure 20.



**Figure 21**  
LPS activates the mTOR pathway through the same pathway as TNF-α.

## Conclusions

**Sepsis is associated with platelet dysfunction and platelets are hypo-reactive in response to agonists. A new signaling pathway for LPS, through IKK to mTOR, is likely to mediate the beneficial effect of rapamycin seen during sepsis.**

During this master thesis project, I have studied two aspects of sepsis; platelet function and the influence of the mTOR signaling pathway to the pathology of the disease. The platelet function is altered in sepsis, where platelets are hypo-reactive in response to the three activators tested and the platelet function is restored to normal with rapamycin pre-treatment, although only when using TRAP as an activator, thus bypassing several steps in the activation process used by thrombin. The platelet function does not seem to be affected by any substances in plasma from septic animals. The TNF- $\alpha$  level in plasma, after LPS-challenge, is lowered with rapamycin pre-treatment and also IL-1 $\beta$ , IL-5, IL-10, and INF- $\gamma$  is affected by rapamycin pre-treatment. The lower cytokine level could be the reason why increased survival is observed in rapamycin pre-treated mice after LPS-challenge. LPS affects cell signaling through the mTOR pathway in the same manner as TNF- $\alpha$ ; as shown by time-point measurement of levels of phosphorylated proteins, TNF- $\alpha$  secretion, and by fact that the effect could be blocked by rapamycin. I propose a new signaling cascade for LPS, from TLR4 to mTOR through IKK.

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