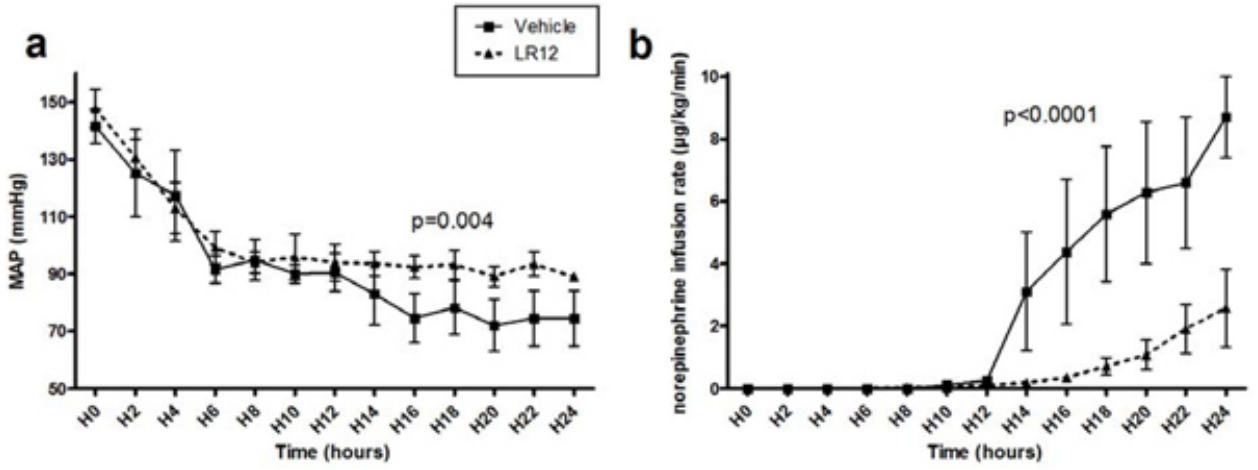

Figure 1(abstract P48) LR12 protects from sepsis-induced hypotension. Evolution of mean arterial pressure **(a)** and norepinephrine requirements **(b)** during the 24-hour study period. MAP was constantly higher and norepinephrine dose lower in LR12-treated animals than controls.

P49

Role of TREM-1 in endothelial dysfunction during experimental sepsis

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Background: Triggering receptor expressed on myeloid cells-1 (TREM-1) is a receptor of the immunoglobulin superfamily expressed on the surface of neutrophils and monocytes/macrophages. It plays an important role during sepsis by amplifying the inflammatory response. Modulation of TREM-1 through the administration of a short synthetic peptide (LR12) increases survival during experimental sepsis. This study aimed to explore the mechanisms by which LR12 prevented sepsis-induced cardiovascular dysfunction.
Methods: We studied the effect of TREM-1 modulation by a synthetic peptide LR12 (3 mg/kg) on MAP and blood lactate during experimental sepsis (CLP). Aortic and mesenteric arterial vessels of these animals were collected to study the vasoreactivity to phenylephrine (Phe) and acetylcholine (Ach) *ex vivo* (Myograph). Alternatively, vasoreactivity was studied in the vessels of healthy animals (with and without endothelial lawyer) stimulated with LPS or with a specific agonist of TREM-1 (αTREM-1, 10 µg/ml), with or without LR12 (20 mg/ml). The effect of LR12 on arterial vessels was also studied through western blotting (eNOS, iNOS, Akt, COX-1, COX-2) and qRT-PCR. Mouse lung microvascular endothelial cells (LUMECs; $CD146^+$) were analyzed by flow cytometry, qRT-PCR, and ELISA to decipher the effect of LR12 on LPS-induced endothelial activation.
Results: CLP induced MAP decrease and lactate elevation were prevented by the administration of LR12. Arterial vessels from septic animals treated with LR12 showed better response to Phe and Ach compared with controls. The reactivity of aortic and mesenteric vessels (contraction and relaxation) stimulated *in vitro* with LPS or by αTREM-1 was altered: this phenomenon was reversed by LR12. LR12 restored the phosphorylation of Akt and eNOS while it reduced the activation of inducible pathways (iNOS, COX-2). FACS analysis showed that TREM-1 is constitutively expressed by LUMECs ($CD146^+$/$VEGFR2^+$). *In vivo*, the expression of *Trem-1* was increased in septic animals and was inducible *in vitro* upon stimulation with LPS. *Trem-1*, *Tnf-α* and *Il-6* expression was upregulated by LPS; once again LR12 attenuated this upregulation. Finally, the production of several cytokines by LPS-stimulated LUMECs was decreased by LR12.
Conclusion: Here we show that TREM-1 is expressed on endothelial cells from the aorta, mesenteric artery, and microvascular endothelial cells, and that TREM-1 might be directly involved in endothelial dysfunction during experimental sepsis.

P50

LPS-induced Pellino3 degradation is mediated by p62-dependent autophagy

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Background: In macrophages Toll-like receptor 4 (TLR4) is activated in response to lipopolysaccharide (LPS) and induces proinflammatory cytokine expression [1]. Therefore, mechanisms terminating proinflammatory gene expression are important. Autophagy plays a central role in controlling innate immune responses by lysosomal degradation of signaling proteins, thus contributing to the resolution of inflammation [2]. Autophagic proteins like p62 directly interact with molecules involved in the TLR4-signaling pathway, but a correlation with the IRAK E3 ligase and scaffold protein Pellino3 remains obscure [3,4]. Hence, we are interested in elucidating the function of Pellino3 to prove our hypothesis that it is a key regulator in the TLR4-signaling cascade [5].
Methods: We used the cecal ligation and puncture (CLP) mouse model causing polymicrobial sepsis to analyze Pellino3 protein and mRNA expression. Furthermore, we induced endotoxemia in RAW264.7 mouse macrophages by LPS treatment to verify *in vivo* experiments. Lentiviral Pellino3 knockdown in RAW264.7 macrophages was used for cytokine measurements at mRNA level. To analyze potential Pellino3 binding partners in TLR4-signaling by mass spectrometry (MS), we overexpressed FLAG-tagged Pellino3 in RAW264.7 macrophages, treated cells for 3, 6 and 24 hours with LPS and immunoprecipitated Pellino3 via its FLAG-tag. To consider Pellino3 degradation as a result of p62-mediated autophagy, we transiently knocked down p62 by siRNA in RAW264.7 macrophages and also pharmacologically blocked LPS-induced autophagy by Bafilomycin A1.
Results: We demonstrated Pellino3 protein degradation in primary $CD11b^+$ splenocytes after 24 hours following CLP operation and confirmed this in RAW264.7 macrophages after 24-hour LPS stimulation. Knockdown of Pellino3 attenuates proinflammatory cytokines, for example IL-6 mRNA, after 6 hours of LPS. Furthermore, we found by MS and verifying immunoprecipitation experiments that p62 is a Pellino3 binding partner, thus targeting Pellino3 for degradation. In line, both p62 knockdown and Bafilomycin A1 treatment prevent Pellino3 degradation, supporting an autophagic mechanism.
Conclusion: Our observations highlight a regulatory role of Pellino3 on TLR4 signaling. Thus, antagonism of Pellino3 in the hyperinflammatory

phase of sepsis may counteract the cytokine storm. Furthermore, stabilization of Pellino3 by inhibition of autophagy in the hypoinflammatory phase of sepsis may improve immunity. In consideration of these two conflictive sepsis phases, modulation of Pellino3 may provide a new strategy for the development of a therapy approach in sepsis.

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References

1. Zhu J, Mohan C: **Toll-like receptor signaling pathways - therapeutic opportunities.** *Mediators Inflamm* 2010, **2010**:781235.
2. Xu Y, Liu XD, Gong X, Eissa NT: **Signaling pathway of autophagy associated with innate immunity.** *Autophagy* 2008, **4**:110-112.
3. Levine B, Mizushima N, Virgin HW: **Autophagy in immunity and inflammation.** *Nature* 2011, **469**:323-335.
4. Schauvliege R, Janssens S, Beyaert R: **Pellino proteins are more than scaffold proteins in TLR/IL-1R signalling: a role as novel RING E3-ubiquitin-ligases.** *FEBS Lett* 2006, **580**:4697-4702.
5. Hennessy EJ, Parker AE, O'Neill LA: **Targeting Toll-like receptors: emerging therapeutics?** *Nat Rev Drug Discov* 2010, **9**:293-307.

P51

Attenuated NOX2 expression impairs ROS production during the hypoinflammatory phase of sepsis

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Background: The multicomponent phagocytic NADPH oxidase produces reactive oxygen species (ROS) after activation by microorganisms or inflammatory mediators [1]. In the hypoinflammatory phase of sepsis, macrophages are alternatively activated by contact with apoptotic cells or their secretion products. This inhibits NADPH oxidase and leads to attenuated ROS production [2] and furthermore contributes among others to a hyporeactive host defense. Due to this immune paralysis, sepsis patients suffer from recurrent and secondary infections [3]. We focused on the catalytic subunit of NADPH oxidase, the transmembrane protein NOX2 [4]. We assume that after induction of sepsis the expression of NOX2 is reduced and hence ROS production is decreased.
Methods: We induced polymicrobial sepsis in mice by cecal ligation and puncture. The ability of peritoneal macrophages (PMs) to produce ROS was determined by FACS via hydroethidine assay. NOX2 expression of PMs was determined by western blot and qPCR. To elucidate the mechanism causing mRNA destabilization, we performed *in vitro* experiments using J774 macrophages. To obtain an alternatively activated phenotype, macrophages were stimulated with conditioned medium from apoptotic T cells (CM). By luciferase assays we figured out a 3'UTR-dependent regulation of NOX2 mRNA stability. Assuming that a protein is involved in the mRNA degradation, we performed a RNA pulldown with biotinylated NOX2-3'UTR constructs followed by mass spectrometry. We verified the role of SYNCRIP by siRNA approach. Additionally, we overexpressed NOX2 in J774 cells and analyzed the ROS production (w/wo CM treatment) by FACS.
Results: We found an impaired expression of NOX2 at RNA and protein level along with decreased ROS production after induction of sepsis in mice as well as stimulating J774 macrophages with CM of apoptotic T cells. This is due to a time-dependent NOX2 mRNA degradation depending on SYNCRIP, a RNA-binding protein, which stabilizes NOX2 mRNA through binding to its 3'UTR under normal conditions. In line, knockdown of SYNCRIP also decreases NOX2 mRNA expression. We assume that a CM-dependent modification or degradation of SYNCRIP prevents its stabilizing function. As the overexpression of NOX2 restores ROS production of CM-treated J774 cells, we assume that NOX2 expression is crucial for maintaining NADPH activity during the hypoinflammatory phase of sepsis.
Conclusion: Our data imply a regulatory impact of SYNCRIP on NOX2 stability during the late phase of sepsis. Therefore, further understanding of the regulation of NADPH oxidase could lead to the design of a therapy to reconstitute NADPH oxidase function, finally improving immune function in sepsis patients.

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References

1. Bedard K, Krause KH: **The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology.** *Physiol Rev* 2007, **87**:245-313.
2. Serikan BF, Gambelli F, Potapovich AI, Babu H, Di Giuseppe M, Ortiz LA, Fabisiak JP, Kagan VE: **Apototic cells quench reactive oxygen and nitrogen species and modulate TNF-alpha/TGF-beta1 balance in activated macrophages; involvement of phosphatidylserine-dependent and independent pathways.** *Cell Death Differ* 2005, **12**:1141-1144.
3. Monneret G: **How to identify systemic sepsis-induced immunoparalysis.** *Adv Sepsis* 2005, **4**:42-49.
4. Babior BM, Lambeth JD, Nauseef W: **The neutrophil NADPH oxidase.** *Arch Biochem Biophys* 2002, **397**:342-344.

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Kinetic characterization of selective peroxisome-proliferator-activated receptor gamma modulators *in vitro*

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Background: The ligand-activated transcription factor, peroxisome-proliferator-activated receptor gamma (PPARγ), has been shown to play an essential role in immunosuppression during sepsis. PPARγ is upregulated in T cells of septic patients, sensitizing these cells to PPARγ-dependent apoptosis and thus contributing to T-cell depletion [1,2]. In the polymicrobial cecum ligation and puncture (CLP) sepsis model in mice, both T-cell-specific gene knockout (Lck-Cre PPARγ$^{fl/fl}$) and systemic pharmacological PPARγ antagonism by GW9662 improved survival [3]. Because GW9662 was only effective when applied 3 hours after CLP, we were interested to extend this time frame. For this reason we characterized the kinetics of SPPARγMs when administered before or in combination with the agonist thiazolidinedione, rosiglitazone.
Methods: A PPARγ-dependent transactivation assay was used in HEK293T cells. It is based on the vector pFA-PPARγ-LBD-GAL4-DBD encoding the hybrid protein PPARγ-LBD-GAL4-DBD and the reporter vector pFR-Luc, carrying a GAL4-responsive element in front of the *Firefly* luciferase gene. These two vectors were co-transfected, in combination with a control vector encoding *Renilla* luciferase (pRL-CMV) to normalize *Firefly* luciferase activity for transfection efficiency. Following transfection, cells were incubated with the SPPARγMs F-MOC and MCC-555 and the PPARγ antagonist GW9662 for different times (2 to 48 hours) and at increasing doses (0.01 to 10 μM), with or without rosiglitazone (0.01 to 10 μM). Transactivation was analyzed using a 96-well plate format.
Results: Rosiglitazone transactivated PPARγ in a time-dependent and dose-dependent manner, the response gradually increasing to a maximum at 48 hours with 10 μM. Low concentrations (0.01 to 0.1 μM) of SPPARγMs F-MOC and MCC-555 and the PPARγ antagonist GW9662 all exerted dose-independent antagonistic effects at an early incubation time point (2 hours). From 10 hours onwards, MCC-555 and GW9662, given alone, both exerted PPARγ agonistic effects, MCC-555 in parallel to responses to rosiglitazone, but GW9662 with characteristics of partial antagonism. F-MOC showed no dose-dependent effect at any concentration at later time points. Only GW9662 (1 to 10 μM) was able to inhibit rosiglitazone (0.1 to 1 μM)-induced PPARγ transactivation after 10 hours.
Conclusion: Our kinetic analysis reveals clear differences in the modulatory characteristics of PPARγ inhibitors, with previously unreported early inhibitory effects and late agonistic or partial agonistic activity. New SPPARγMs with extended inhibitory activity may prove useful in the therapy of sepsis.

References

1. Soller M, Tautenhahn A, Brüne B, Zacharowski K, John S, Link H, von Knethen A: **Peroxisome-proliferator-activated receptor gamma contributes to T lymphocyte apoptosis during sepsis.** *J Leukoc Biol* 2006, **79**:235-243.
2. Soller M, Dröse S, Brandt U, Brüne B, von Knethen A: **Mechanism of thiazolidinedione-dependent cell death in Jurkat T cells.** *Mol Pharmacol* 2007, **71**:1535-1544.