

**Twenty-Seventh Annual Conference on Shock
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Sepsis constitutes the main cause of death at intensive care units worldwide. Although an ample variety of physiopathology-based experimental therapies has been tested in the last 20 years only activated protein C (drotrecogin alfa) has shown to improve outcome for selected patients. Therefore, the first line of treatment remains to be antibiotic therapy along with hemodynamic, ventilatory and metabolic support, but mortality is still high [1-3]. That explains the huge amount of work that is being carried out to find novel efficacious drugs, and to reach deeper into the pathophysiologic mechanisms of sepsis.

A significant contribution to this quest is offered by The Annual Conference on Shock, a scientific meeting held yearly by The Shock Society to debate and encourage experimental and clinical investigations on etiologic, pathogenic and therapeutic aspects of injury-associated pathologic conditions and responses, including sepsis, inflammation, shock, trauma, and ischemia.

This year's Twenty-Seventh edition included a plenary session, two workshops, three symposia, four minisymposia and three poster sessions.

The plenary session focused on Immunomodulation and highlighted interesting findings such as the importance of high mobility group box 1 protein (HMGB1) as both sepsis mediator and therapeutic target, as demonstrated by the significant increase in survival of septic mice subjected to either cecal ligation and puncture (CLP) or endotoxemia, obtained, respectively, with monoclonal antibodies against HMGB1 and fetuin, a negative acute phase protein capable of inhibiting endotoxin-induced HMGB1 released from murine peritoneal macrophages and reducing circulating HMGB1 levels in endotoxemic mice.

The workshop "Transferring discoveries from the lab to the patient" remarked the rational use of agents like interleukin 1 receptor antagonist and anti-tumor necrosis factor (anti-TNF) monoclonal antibody, initially designed as anti-sepsis therapeutics, for the treatment of other inflammatory diseases such as Rheumatoid Arthritis, as well as the need for addressing different sepsis mediators as HMGB1 for succeeding in sepsis treatment. The recommendations of the Shock Society/ISF Symposium for reducing the gap between pre-clinical models of shock and sepsis and the clinical context, were also reported.

Disease modeling was assessed in the first symposium. There, it was shown that mathematical modeling may help predict the dynamics of acute inflammation and organ dysfunction in experimental endotoxemia, making it an important tool for evaluating the efficacy of potential therapies. Also the relevance was noticed for data generated from gene microarray analyses for predicting time-dependent markers or therapeutic targets of sepsis.

As part of the mini-symposium "Hemorrhagic shock and ischemia" it was pointed out that diversity is higher than commonality in the mammalian response to endotoxin challenge, trauma-hemorrhagic shock or burn injury, through the analysis of gene expression by gene microarray in three representative mouse models. In addition, it was shown that the combination of hypoxia and lipopolysaccharide (LPS) caused a ten-fold increase in the metalloproteinase inhibitor TIMP-1 after 48 hours in RAW macrophages, as assessed by Western blot analysis.

Some of the results revealed at the mini-symposium "Endotoxin, Sepsis and Inflammation" included the correlation of reduction in C5a receptors (C5aR and C5L2) on blood neutrophils from CLP rats and septic shock patients, with both the loss of neutrophil H₂O₂ production and a poor outcome, the significant upregulation of macrophage scavenger receptors (MSR-AI/II and MARCO) on circulating mononuclear cells from children with septic shock by soluble and plasma-transferable factors, and the dependence of mouse bone marrow-derived-dendritic cell maturation and toll-like receptor (TLR) expression on the particular pathogen associated molecular pattern (PAMP) to which they are exposed.

Other relevant findings exposed at this mini-symposium comprised a putative role of macrophage migration inhibitory factor (MIF) and HMGB1 in acute lung injury and myocardial depression during sepsis as demonstrated by a significant increase in levels of both cytokines in the bronchoalveolar lavage fluid of rats 30 hours after CLP, along with this fluid induction of cardiac contractility depression and morphological changes in isolated myocytes, as well as a sepsis-related impairment of leucine-induced phosphorylation of translational control molecules with the consequent blockade of protein synthesis activation in the skeletal muscle of CLP rats.

The symposium "Allostasis and stress response", reported that manipulation of the va-

1. Dremsizov TT, Kellum JA, Angus DC. Incidence and definition of sepsis and associated organ dysfunction. *Int J Artif Organs* 2004;27(5):352-9.

2. Polderman KH, Girbes AR. Drug intervention trials in sepsis: divergent results. *Lancet* 2004;363(9422):1721-3.

3. Bochud PY, Calandra T. Pathogenesis of sepsis: new concepts and implications for future treatment *BMJ*. 2003; 326(7383):262-6.

gus nerve activates the cholinergic anti-inflammatory pathway and permits an anti-inflammatory response in sepsis as demonstrated by the significant decrease in serum TNF levels obtained after mechanical or electrical stimulation of the vagus nerve in a murine endotoxemia model.

Several current successful therapeutic approaches in sepsis such as low tidal volume mechanical ventilation, low-dosage corticosteroids and early fluid reposition were highlighted at the symposium "Clinical successes in sepsis and trauma".

Presentations at the workshop "Transforming discoveries from the lab to the patient" featured hopeful therapeutic options as bactericidal phage lytic enzymes, effective in experimental bacteremia, and ethyl pyruvate, which has been shown to have favorable effects in experimental sepsis, including an increase in survival, a reduction of HMGB1 release, the blockade of nuclear factor kappa B (NF κ B) translocation and an anti-inflammatory action on liver microcirculation.

The outstanding results discussed at the mini-symposium "Cell signaling and adhesion molecules" included the demonstration of the involvement of p38 MAPK kinase in the inflammatory LPS/TLR signaling pathway using flow cytometry of LPS-stimulated splenic macrophages from mice subjected to thermal injury, the tissue-dependent direct link of NF κ B activation to the suppressor of cytokine signaling 3 (SOCS-3) induction through TLR signaling in sepsis as demonstrated employing a murine CLP model,

NF κ B activation inhibitors and myeloid differentiation factor 88 (MyD88) deficient mice, and the role of protein kinase C delta (-PKC) as a regulator of the TNF mediated assembly of the TNF receptor 1 (TNFR-1) signaling complex in neutrophils, enhancing TNF receptor-associated factor 2 (TRAF2) and receptor interacting protein (RIP) recruitment and inhibiting TNFR-associated death domain protein (TRADD) recruitment, as revealed through the use of -PKC inhibitors and co-immunoprecipitation assays.

Noticeable findings debated at the mini-symposium "Inflammation and burn trauma" included the protective effect of leptin in a murine model of posttraumatic CLP sepsis through the reduction of serum interleukin 6 (IL-6) levels as supported by the absence of this effect in IL-6 deficient mice, and the increase of serum heat shock protein 72 (HSP72) levels produced by intravenous glutamine in both patients with pancreatitis and systemic inflammatory response syndrome (SIRS), and CLP rats which also showed an improved survival.

Poster sessions also comprised very outstanding studies in the sepsis field, some of which are abstracted at the end of this report.

In conclusion, the Twenty-Seventh Annual Conference on Shock met all the expectations in compliance with its tradition of bringing together up-to-date advances in sepsis and related conditions within a collaborative environment, that should help in man's fight against these life-taking diseases.

HMGB1 SIGNALS THROUGH TOLL-LIKE RECEPTOR 2

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High mobility group box 1 (HMGB1), a protein previously known only as a nuclear transcription factor, is now understood to be a pro-inflammatory cytokine, released from activated immune cells or necrotic cells. The late kinetics of HMGB1 during murine severe sepsis identifies this novel cytokine as an attractive therapeutic target for diseases of systemic inflammation. Although HMGB1 can bind to the receptor for advanced glycation endproducts (RAGE) to mediate cellular activation, recent observations suggest that there may be other cell surface receptor(s) that mediate the pro-inflammatory effects of HMGB1. To explore the potential contribution of Toll-Like receptors (TLRs) to HMGB1 bioactivity, we studied human embryonic kidney (HEK) 293

cells over-expressing TLR2 or TLR4; interaction of the receptors with ligand induces the release of IL-8 as a reporter. Highly purified HMGB1 from *E. coli* or mammalian cells dose-dependently induced IL-8 release from TLR-2-expressing cells, but not from cells expressing TLR-4 or the control vector. HMGB1 B box also triggered IL-8 release specifically via TLR2, suggesting that the cytokine activity of HMGB1 maps to B box. Antibodies directed against HMGB1 or TLR2 each suppressed HMGB1-induced IL-8 release by >70%. In addition, HMGB1 signaling requires MyD88, a signaling/adaptor molecule downstream of Toll-like receptors. These data indicate that HMGB1 is capable of signaling through TLR2, and present new opportunities to regulate HMGB1 activity.

DIFFERENTIAL MECHANISM OF IMMUNE SUPPRESSION IN PATIENTS WITH SEPTIC AND NON-SEPTIC SURGICAL STRESS

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There were no detailed studies concerning the immune suppression after surgical stress. To investigate the different immune response in patients with septic and non-septic surgical stress, we studied IFN production by peripheral blood mononuclear cells (PBMCs), and IL-18 R (receptor) expression on NK or NKT cells in such patients. Patients and Methods: Twenty patients with alimentary tract carcinoma who underwent elective surgery (OP), and 26 patients with sepsis (SP) were enrolled in this study. Ten healthy volunteers served as controls. Blood was collected on the 5th postoperative day (POD 5) in OP and immediately after admission in SP. PBMCs were incubated ex vivo either with IL-12 and IL-2 stimulation (cy) or with cy and additional IL-18 stimulation, and the culture supernatants were assayed for IFN production by ELISA. IL-8 R expression on NK or NKT cells were evaluated by flow cytometry. Results: IFN production by

PBMCs with cy in both OP and SP were significantly decreased compared with those of the healthy control. In contrast, IFN production by PBMCs with cy and IL-18 stimulation in OP was significantly increased compared with that without IL-18 stimulation, but for those in SP it was not increased. IL-18 R expression on NK cells in SP was significantly decreased compared with that of healthy controls (0.57 0.05 vs 0.80 0.04), but that of OP is not decreased. IL-18 R expression on NKT cells in SP was not decreased. Conclusions: IFN production by PBMCs with IL-12 and IL-2 stimulation is suppressed in both patients with sepsis and elective surgery on POD 5. However, IL-18 R expression on NK cells is different between patients with septic and non-septic surgical stress. This may explain the different IFN response by additional IL-18 stimulation in patients with septic and non-septic surgical stress.

MULTIPLEXING APPROACH TO DETECT NOVEL CYTOKINES NOT ABROGATED IN SEPSIS AND SEPTIC SHOCK

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Non responsiveness of NF κ B inducible cytokines such as TNF- and IL-6 is a well known phenomenon in patients with trauma-induced reaction and subsequent immune deficiency related to hyperinflammation and endotoxemia. Using a multiplexing determination approach by anticytokine-coupled beads we addressed the question whether other cytokines or chemokines may also play a major role in sepsis and septic shock. Multi cytokine standards as well as beads and antibodies were specific for IL-2, IL-4, IL-12 (p70), IL-15, IFN-, MCP-1, RANTES, IP-10, IL-1, IL-1, IL-8, eotaxin, GM-CSF, IL-6 and IL-10. In addition, TNF-, IL-6, IL-8 and IL-10 were analyzed by standard ImmunliteTM, chemiluminescence assays. In more than 200 samples derived from about 30 patients with severe sepsis and at transition to septic shock we confirmed anergy insofar as serum levels of IL-6 and TNF- but also IL-1, were mostly undetectable. However, the release of the IFN- inducible chemokines IP-10 but

also MCP-1 and MIP-1a was increased. Amongst the Th1 cytokines we found IFN- as high as 300 pg/ml and IL-2 at 120 pg/ml but also classical Th2 cytokines such as eotaxin, IL-4, IL-10, and IL-13 were elevated to about identical protein levels. Most remarkably, the plasma concentrations of IL-12 p40 but also the biologically active p70 heterodimer ranged between 10 and 50 pg/ml. These results suggest that immunological non-responsiveness in septic shock is restricted to the classical macrophage cytokines TNF- IL-1 and IL-6, whereas cytokines driven by the stimulation of dendritic cells appear to be elevated and guide the immune function in these critically ill patients. Current studies are under way testing the cytokine release in patients before and shortly after major surgical trauma to learn more about the soluble mediators responsible for the initiation of dendritic cell and T-cell activation patterns by systemic inflammatory responses (SIRS).

IN VIVO DELIVERY OF CASPASE 8 siRNA IMPROVES THE SURVIVAL OF SEPTIC MICE

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Silencing (si) RNA is a novel technique frequently used in vitro due to a wide supply of agents available assisting the uptake of siRNA into cell lines. However, few studies have been conducted using RNA interference in the intact animal, let alone models of disease. Our lab has shown that dys-regulated apoptotic cell death is shown to be associated with the pathology of sepsis, in particular, the Fas-FasL signaling pathway in the liver, spleen, and intestine. With this in mind, the hypothesis we address here is that "hydrodynamically" injecting siRNA against a downstream molecule in the Fas pathway, Caspase 8, will improve the morbidity and mortality of mice when given after septic challenge (CLP). Experiments to denote tissue distribution by siRNA after "hydrodynamic" i.v. injection in transgenic enhanced-GFP expresser mice, illustrated a decrease in the intensity of GFP. This was seen in virtually all tissues studied 24 h after administering GFP siRNA. This supports the concept that the injection

and uptake of siRNA is systemic and functional. Subsequently, CLP (with/without siRNA to Caspase 8, to GFP [as control] or saline, given at 1 h-post) or Sham-CLP in C3H/HeN was performed. 24 h post-CLP TUNEL analyses showed a decrease in apoptosis in the liver and spleen of septic mice receiving Caspase 8 siRNA, as compared to saline or the nonsense GFP siRNA control (n=5/grp, p<0.05; Mann-Whitney U test). In addition, plasma liver enzymes ALT and AST (markers of liver damage) decreased after Caspase 8 siRNA treatment. Lastly, Caspase 8 siRNA injected mice show a 40% increase in septic survival at 5-10 days over the saline and GFP siRNA controls (n=20/grp, p<0.05; Chi-sq. test). This data coupled with that of our Fas siRNA studies shows not only that this pathway plays a major role in the pathology of sepsis, but that the in vivo application of siRNA is a novel approach to silencing these genes, as well as potentially other targets.

NEW INSIGHTS ON THE PATHOPHYSIOLOGIC STAGES OF SEVERE SEPSIS (TRANSLATION FROM PRIMATE MODELS TO VARIANTS OF SEVERE SEPSIS IN HUMANS)

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Severe sepsis is an acute inflammatory/coagulopathic disorder of the microvasculature. Depending on the nature and intensity of the insult and the status of the host at the time of challenge, this disorder can exhibit variants ranging from cardiovascular collapse and shock to multiple organ failure. The pathophysiologic conditions underlying these variants are revealed in models of sub lethal human endotoxemia and baboon *E. coli* sepsis. There are two stages of inflammatory/coagulopathic activity. The first stage is symptomatic, and is dominated by a neutrophil/endothelial interaction driven by inflammatory (cytokines) and hemostatic (thrombin) mediators. The second stage (which may be asymptomatic) is dominated by inflammatory (complement) and hemostatic (TP/VIIa) events driven by oxidative stress following ischemia reperfusion (IR). At one extreme following LD100 *E. coli*, stage 1 can dominate leading to cardiovascular collapse and

death in hours. This corresponds to 13 to 15% of severe sepsis patients, all of whom die. Following LD25 *E. coli*, stage 2 can dominate in some leading to multiple organ failure and death in days. This corresponds to ~54% of patients with severe sepsis about half of whom die. The remaining survivors of the LD25 *E. coli* exhibit a transient inflammatory/coagulation response. This corresponds to ~35% of patients with severe sepsis whose initial organ failure is non-progressive and from which all recover. Using a platelet count of 1×10^5 or below and a protime of 14.5 seconds or above, we identified a subgroup of patients admitted with organ failure that exhibited a pattern of ischemia reperfusion similar to that observed in the second stage, and that was distinct from the inflammatory overt coagulopathic responses. This observation may aid in early, more explicit classification of therapeutic targets.

PROTEOMIC ANALYSIS OF PLASMA OVER TIME IN A MURINE MODEL OF INTRAABDOMINAL SEPSIS

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We hypothesized that characterization of the plasma proteome of intra-abdominal infection would reveal novel disease patterns and markers. Methods. Male C57/BL6 mice underwent cecal ligation and puncture (CLP, 2x25 gauge, 7-day mortality 80%) or sham laparotomy (mortality =0%). Plasma was pooled from groups of 6-8 mice at each of three time points (0, 3 h, 6 h, 24 h) and depleted for immunoglobulin. 2-D differential in-gel electrophoresis (DIGE) was used to compare the protein composition of baseline, sham and CLP plasma at each time point. Differentially abundant spots were excised from the gel, digested with trypsin and characterized by mass spectrometry. Results. Approximately 1500 gel features were detected in each gel with 22 differentially abundant features at 3 h,

39 at 6 h and 91 at 24 h. The pattern of protein abundance and the identities of those proteins distinguished sham from CLP from baseline at all times. Forty-five gel features were identified from the 24 h time point. The proteins identified to date are part of the acute phase response (e.g., complement C3 and C8, serum amyloid P, fibrinogen A, B and , hemopexin, ceruloplasmin, haptoglobin, and apolipoprotein A-I). Twelve of the excised spots contained fibrinogen A indicating ongoing disseminated intravascular coagulation (DIC) in the septic mice. Conclusion. This first report of the global response to intraabdominal sepsis revealed time-dependent changes in plasma protein content constituting the canonical acute phase response with DIC.

CENTRALLY ACTING ACETYLCHOLINESTERASE INHIBITORS IMPROVE SURVIVAL IN A MURINE MODEL OF SEPSIS

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The efferent vagus nerve is an important component of the inflammatory reflex, neural loop through which the central nervous system can detect and suppress inflammation [Nature, 420:853]. The inflammatory reflex can be centrally stimulated [J. Exp. Med., 195:1] through activation of central cholinergic mechanisms [Shock Suppl., 19:65]. We have recently shown that the central administration of the acetylcholinesterase inhibitor galantamine attenuates serum TNF levels during endotoxemia. Peripheral administration of galantamine, which crosses the blood brain barrier, also causes firing of the vagus nerve. The goal of this study was to test the therapeutic efficacy of galantamine and tacrine (another centrally-acting acetylcholinesterase inhibitor) in the cecal ligation and puncture (CLP) model of sepsis.

Mice were subjected to CLP and treated intraperitoneally with drug or vehicle, twice daily, for 3 consecutive days, beginning 24 h after surgery; survival was monitored for 3 weeks. Galantamine significantly and dose-dependently increased survival from lethal sepsis (vehicle-treated survival =37%; vs. galantamine [10 g/kg] survival =58%; vs. galantamine [100 g/kg] survival =90%, $p<0.05$). Similarly, tacrine significantly protected mice from lethal sepsis (vehicle-treated survival =50%; vs. tacrine [250 g/kg] survival =100%, $p<0.05$). These results indicate that activation of central cholinergic pathways contributes to protection against sepsis. Acetylcholinesterase inhibitors may be novel anti-inflammatory therapeutics by activating the efferent part of the inflammatory reflex.

EFFECT OF RECOMBINANT HUMAN ACTIVATED PROTEIN C (DROTRECIGIN ALFA ACTIVATED-DAA) IN THE INFLAMMATORY RESPONSE OF MONOCYTES AND NEUTROPHILS IN WHOLE BLOOD OF HEALTHY VOLUNTEERS (HV)

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It has been well established that beyond its role in coagulation, DAA has fibrinolytic and anti-inflammatory properties. Objectives: To evaluate the effect of DAA in LPS-induced cellular activation in whole blood. Methods. Blood samples were obtained from HV, and diluted 1:8 in medium and kept on a shaker for 30'. DAA was added (40 ng/mL, 80 ng/mL, and 160 ng/mL), followed by 30' with the addition of LPS (100 ng/mL), and incubated for 5hs. For intracellular staining, monensin was added after the first hour. Neutrophils were identified based on FSC vs. SSC parameters and CD66b-FITC staining and monocytes on FSC vs. SSC and CD14-PerCP. Surface cell staining was performed using TLR-4-PE, CD11c-APC, and HLA-DR-PE, whereas intracellular cytokines were detected with IL-6-PE and TNF-FITC. Six to eight volunteers were studied for each condition. Results. DAA (160 ng/mL) induced a decreased expression of CD14 on

monocytes (DAA vs. control, $p=0.028$). Although DAA had no direct effect on TLR-4 expression, it reversed the down regulation induced by LPS (LPS vs. controls, $p=0.036$) at 40 ng/mL and 80 ng/mL (DAA plus LPS vs. control, $p=0.225$ and 0.310 respectively). DAA had no direct effect but abrogated the LPS-induced expression of HLA-DR. GMFI of HLA-DR with LPS plus DAA did not differ from controls, when DAA was used at 40 ng/mL ($p=0.116$), 80 ng/mL ($p=0.176$), and 160 ng/mL ($p=0.866$). DAA had neither a direct effect on CD11c expression nor modulated the effect of LPS. The same was true with the induction of TNF- and the IL-6. Conclusions: (1) this system is suitable to study the modulating effect of DAA at a cellular level in whole blood; (2) the low doses of DAA used in our experiments (chosen based on the predicted plasma level after reposition therapy) affected CD14 and modulated HLA-DR expression.

ADJUVANT THERAPY OF MEDIASTINITIS WITH IMMUNOGLOBULINS (PENTAGLOBIN®)-A MULTI-CENTRE RANDOMIZED CONTROLLED TRIAL

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Mediastinitis is still a life-threatening complication in patients with wound infection after open heart surgery. The adjuvant application of immunoglobulins was assumed to improve the clinical situation of these patients and to reduce postoperative morbidity. Methods: A prospective double-blind randomized trial was performed in 19 European hospitals. Adult patients with wound infection after median sternotomy (mediastinitis) were included. Pentaglobin® or placebo was administered before wound revision and postoperatively until day 5 as a continuous i.v. infusion of 5 mg/kg body weight. The primary end point was the sum of daily Therapeutic Intervention Scoring System points for 28 days (cumulative TISS-28). Results: 125 patients were included, 64 received Pentaglobin, and 61 placebo. Basic data were comparable.

In each group 6 patients (10%) died before day 28. Intention-to-treat analysis showed that median cumulative TISS-28 was lower in the Pentaglobin group by 61 points (189 versus 250 points, respectively) which approximately corresponds to two days less of average intensive care. This difference was not significant ($p=0.08$; U-test, one-sided). Mean duration of intensive care (7.9 ± 9.1 vs. 9.1 ± 0.1 days) and duration of wound infection (10.4 ± 9.7 vs. 12.1 ± 10.9 days) were reduced as well in the Pentaglobin group. Adverse events were distributed equally. Conclusion: Although statistical significance was missed there is a strong trend towards a reduced morbidity in patients with adjuvant therapy with immunoglobulins, which is reflected by a reduced requirement of intensive care.

ADENOVIRAL-MEDIATED PULMONARY EXPRESSION OF HEAT SHOCK PROTEIN-70 (HSP-70) AFTER CECAL LIGATION DOUBLE PUNCTURE (2CLP) INHIBITS THE NFκB PATHWAY

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Sepsis secondary to cecal ligation and double puncture (2CLP) in rats causes acute respiratory distress syndrome (ARDS). Previous studies have shown that use of an adenoviral vector that expresses HSP-70 (AdHSP) increases intra-pulmonic HSP-70 and protects against lung injury. The mechanism by which AdHSP is protective is unknown, but HSP-70 has been reported to inhibit activation of the intracellular signaling molecule NFκB. We have previously demonstrated that AdHSP decreases inflammation by reducing NFκB activation. Activation results from phosphorylation of the NFκB inhibitor IκB. This is primarily modulated by IκB Kinase (IKK). We hypothesized that the inhibition of the NFκB pathway by HSP-70 is, in part, due to blockade of IKK. 48 hours following 2CLP (with or without AdHSP), lung tissue was har-

vested. Nuclear and cytosolic protein was isolated from both models. We evaluated fluctuations in IKK function using 35S methionine labeling and a GST-IκB kinase assay, which directly assay IKK activity. Immunoblotting demonstrated elevated levels of IKK in 2CLP rats as compared to control animals. In addition, IKK levels did not change. Both 35S methionine labeling and GST-IκB kinase assays demonstrated a decrease in the phosphorylated form of IκB in 2CLP animals treated with AdHSP as compared to other 2CLP animals. We conclude that HSP-70 modulates inflammation, in part, by decreasing NFκB activation via inhibition of IKK. Further, our data support the hypothesis that IKK is the key subunit in NFκB activation during stress. Ongoing studies will determine by which mechanism HSP-70 modulates IKK.

BRUTON'S TYROSINE KINASE (BTK) INHIBITS CONSTITUTIVE NEUTROPHIL (PMN) APOPTOSIS AND PROLONGS PMN SURVIVAL IN SEPSIS

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Inflammatory stimuli can prolong PMN survival by inhibiting apoptosis. Btk, a non-receptor tyrosine kinase critical for B cell maturation and survival, is upregulated in PMN by IL-1, an anti-apoptotic cytokine. We hypothesized that Btk promotes PMN survival in experimental inflammation and clinical sepsis by inhibiting constitutive PMN apoptosis. **METHODS.** PMNs from healthy donors or septic patients were incubated for 1 hour with LFMA-13 (25-50 M), a specific inhibitor of Btk or vehicle control, then treated with LPS (1 g/ml) or TNF (50nM). Apoptosis was evaluated by flow cytometry as Annexin V fluorescence and propidium iodide uptake after 6 and 21 hours of culture respectively. Caspase-3 activity was measured using a chromogenic assay and con-

firmed by Western Blotting. Btk mRNA levels were quantified by real-time PCR using primers specific for Btk. **RESULTS.** LFMA-13 alone significantly increased PMN apoptosis, and partially abrogated the LPS-mediated delay, but had no effect on the anti-apoptotic activity of TNF. Btk mRNA levels were increased by LPS and in septic patient neutrophils (N=3) and LFMA-13 induced apoptosis in septic PMNs. Caspase-3 activity was increased in healthy donor and septic patient PMNs treated with LFMA-13. **CONCLUSION.** Btk regulates PMN survival by inhibiting constitutive PMN apoptosis. Expression is increased by LPS, and further upregulated in septic PMN, suggesting that Btk may be an attractive target for modulation of clinical inflammation.

NAD⁺ IMPROVES SURVIVAL OF ENDOTOXEMIC MICE

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We serendipitously observed that extracellular NAD⁺ preserves epithelial barrier function of human Caco-2 enterocyte-like cells following stimulation with cytomix, a mixture of IFN- (1000 U/ml), IL-1 (1 ng/ml), and TNF- (10 ng/ml). NAD⁺ decreased the activation of NFκB, iNOS mRNA accumulation, and nitric oxide (NO) production from Caco-2 cells (Han *et al.* 2003, *J Pharmacol Exp Ther* 307:443). These findings prompted us to investigate the antiinflammatory effects of extracellular NAD⁺ in the mouse myeloid RAW 264.7 cell line and in mice. The cells were exposed to graded concentrations of NAD⁺ in the absence or presence of LPS (100 ng/ml). C57Bl/6J mice were injected with LPS in the absence or presence of multiple injections of NAD⁺(137mg/kg). NAD⁺ decreased the production of NO, decreased steady-state levels of iNOS mRNA,

and decreased the activation of NF- in RAW 264.7 cells (ED50 10-100 M). NAD⁺ also decreased the release of TNF- from RAW 264.7 cells. When injected into mice that were exposed to a sublethal dose of E coli LPS (2 mg/kg), NAD⁺ decreased the accumulation of TNF-α, IL-6, and NO catabolites (NO₂-/NO₃) in serum and preserved intestinal epithelial barrier function measured ex vivo. NAD⁺(132 mg/kg at t=0 and 3 injections repeated at 12 h intervals) decreased mortality in mice injected with a lethal dose of LPS (17 mg/kg). Taken together, these studies reveal that extracellular NAD⁺ has an immunomodulatory effect on both epithelial and immune cells. The results of these studies support further investigations into the possible use of NAD⁺ for the treatment of inflammatory diseases like sepsis.