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IMMUNOMODULATION OF INFLAMMATION IN A MURINE PNEUMOCOCCAL SEPSIS MODEL

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SUMMARY OF THE STUDY

Mortality from pneumococcal infections remains high, despite the development of potent antibiotics. Antibiotic resistance among pathogens including *S. pneumoniae* calls for new therapeutics. Immunomodulation represents a novel approach to antimicrobial therapy that depends on bolstering host immunity, rather than direct antimicrobial activity. The use of innate immune stimulation to improve survival has previously been described for Gram negative pathogens.

The effect of TLR4 stimulation on survival in mice during lethal *S. pneumoniae*, serotype 2 infections was examined. C57BL/6, BALB/c, CBA/CaHN-Btk$^{xid/}$/J, A/J, Rag-1 KO, IL-10 KO , C3H/HeN and C3H/HeJ mice were inoculated intravenously with a lethal dose of 10^6 cfu of *S. pneumoniae* serotype 2 48 hours after treatment with five doses of 10ug highly purified LPS or vehicle (PBS) at 12 hours interval. Another group of LPS or PBS treated C57bl/6 received 25 mg/kg ceftriaxone at 6 hours post infection. Survival was monitored for 5 days. Blood samples were collected at different time points (6h, 12h and 24h) after bacterial challenge for bacteriological examinations, serum cytokine measurements, and biochemical assays for liver function. Spleens were harvested for flow cytometric analysis of splenic lymphocytes or NK activation.

Innate stimulation with LPS reduced systemic bacteremia by at least four logs in LPS- pretreated C57BL/6 (10^4 v.s 10^8 cfu/ml) mice compared with controls during the recorded course of infection. Death in experimental controls occurred within 48 hours. Reduced bacteremia corresponded with improved survival in all 3 strains. Survival for LPS-treated C57bl/6, Balb/c and C3H/HeN was 90% (N=29; p=0.001), 50% (N=14; p = 0.017) and 60% (N=8; p=0.009), respectively, and mortality for controls was 100% for all the strains. Mortality for ceftriaxone-treated C57bl/6 was

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75% (N=12, p=0.0018). Survival for LPS-treated and non-treated mice was 80% (n=10) and 0 % (n= 6) in CBA/CaHN-Btk$^{xid/}$/J mice and 85% (n=14) and 0 % (n=9) in A/J mice which suggests that deficiency in complement and antibodies did not ablate survival benefits. Lack of B and T cells ablated survival benefits of LPS pretreatment in RAG-1 KO mice even when mice received a high dose of LPS. These results suggest that T cells are responsible for protection and B cells are partially involved in tolerance. The level of TNF-a, IFN-g, IL-12 (p40/70), IL-6, IL-1a/b, IL-10, Eotaxin, MCP-1, and MIP-1a/b were attenuated in C57BL/6 LPS-treated mice 12 hours after infection compared to untreated group. Pretreatment with a low dose of LPS also prevented hypoglycemia (glucose level was 149 vs. 45) and liver failure (reduced the AST [139 vs. 445, $p<0.025$] and ALT [27 vs. 129, $p<0.03$] to near baseline) induced by *S. pneumoniae* infection. LPS pretreatment restored the splenic NK population and decrease their activation demonstrated by lower mean percentage of CD69 expression of 35.3 vs 97.3 ($p<0.05$) than of control infected mice.

This study demonstrates a survival benefit from TLR4 stimulation prior to infection with *S. pneumoniae.* It provides evidence that induction of profound LPS tolerance, despite reducing cytokine production, improves host defense against infection with virulent strain of *S. pneumoniae.* TLR4 agonist activity can be potentially exploited to provide short-term resistance to infectious challenge such as might occur in the setting of exposure to bio-threat agents or epidemic infections. These observations have implications for prophylactic treatment after an index case is identified or as adjunctive therapies with antibiotics. It is plausible that compounds capable of stimulating early host defense and microbial clearance, but not the latter phases of inflammatory tissue injury associated with sepsis, may be advantageous.

The effect of LPS postinfection, as adjunctive therapy, in a lethal murine model of systemic *Streptococcus pneumoniae* was investigated. Mice were inoculated

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intravenously with a lethal dose of 10^7 cfu of *S. pneumoniae* serotype 2; and treated with a combination of 25 mg/kg Ceftriaxone (i.p) and 10ug of LPS (i.v) at 6 hours post infection or ceftriaxone alone. The survival of mice treated with the combination of ceftrioxone and LPS was 80% in C57BL/c (n=20), compared to 40% of mice receiving ceftrioxone only (n=20) and 0% of vehicle controls (n=10) (p=0.0001). Treatment with a non-lethal dose of LPS beginning at 3 hours after infection reduced mortality by 50% in C57BL/6 mice (n=7) (p=0.03) and no survival benefits (100 % mortality) were observed when LPS alone was administered 6h after bacterial challenge (n=5) (p=0.07). Administration of LPS in combination with ceftriaxone resulted in a significant reduction of bacteremia at 7h (2.5 ± 1.6 vs 6.6 ± 1.6 log10 CFU/ml, p=0.002 and 12h (0.5 ± 1.0 vs 4.9 ±1.6 log10 CFU/ml, p=0001) after infection, compared with animals treated with ceftriaxone alone and also decreased significantly the level of TNF-alpha, IFN-gamma, IL-12p70, MIP-1 alpha, IL-1 beta, and RANTES 12h after bacterial challenge and elevated levels of IL-2, IL-3, IL4, and KC were elevated 12h after bacterial challenge compared to those treated with ceftriaxone alone ($p<0.05$).

In this mouse model, stimulation of TLR4 by highly purified LPS as an additional therapy to the antibiotic Ceftrioxone achieved a survival benefit in the reduction of mortality in severe *S. pneumoniae* infections. These results show the potential for innate immune stimulation as adjunctive therapy in the treatment of pneumococcal infections and these results warrant further studies.

The treatment of pneumococcal infections- such as meningitis, pneumonia, and bacteremia- with β-lactam antibiotics can result in the paradoxical enhancement of inflammation as a result of the release of proinflammatory bacterial cell components, such as lipoteichoic acid, peptidoglycan, and bacterial toxin pneumolysin. Adenosine has strong immunosuppressive and anti-inflammatory effects mediated by A_{2A}

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receptor ($A_{2A}R$) expressed on immune cells. We investigated the effect of $A_{2A}R$ agonist, ATL313, as adjunctive therapy in a lethal C57bl/6 mouse model of systemic *Streptococcus pneumoniae*.

Female C57bl/6 mice were inoculated intravenously with 10^7 colony forming units (CFU) of *S. pneumoniae* and treated with the A_{2A} AR agonist ATL313 (5ug/kg or 25ug/kg), or PBS, at t=1, 6hrs and then every 24 hours after bacterial challenging spanning 48 hours. To test whether the effect of ATL313 was through functional A_{2A} adenosine receptors and if hematopoietic cells are important in the protective effect of $A_{2A}AR$, C57BL/6 A_{2A}-receptor-deficient mice, and chimera were used and groups of C57BL/6 mice were co-injected intraperitoneally with ATL313 and ZM243185 (antagonist ligand selective for the adenosine A_{2A} receptor). Survival was monitored for 7 days. Blood samples were collected at 7h and 12h after bacterial challenge for bacterial counts and cytokine measurements.

ATL 313 (2.5- 25ug/kg), had no survival benefits (100% mortality) when administered without antibiotic ceftriaxone. But when administered in combination with antibiotic ceftriaxone at 6h after infection, 25ug/kg ATL313 increased survival (89%, n=26) of mice compared to ceftriaxone alone treated mice (23%, n=31). Survival benefit was reversed after treatment with ZM241385 (25%; n=8/group) and after ATL313 treatment in A_{2A} AR KO and chimeric mice (17%; N=6/group) compared to ATL 313 treated C57BL/6 mice (89%; $p<0.001$). Treatment with ATL313 plus ceftriaxone reduced bacteremia by 2.1 log10 and 3.3 log10 fewer bacteria at t=12h and 24h, respectively, than those of mice treated with ceftriaxone alone ($p<0.05$); and also decreased level of TNF-alpha ($p=0.010$), IL-6 ($p=0.027$) , IFN-gamma ($p=0.001$), IL-12p70 ($p=0.031$), MIP-1 alpha/ beta ($p=0.005$), IL-10 ($p=0.022$), IL-1 beta ($p=0.0071$), IL-5 ($p=0.014$), and Eotaxin ($p=0.023$) at 12h after bacterial challenge compared to those treated with ceftriaxone alone ($p<0.05$).

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In this study, ATL313 in association with ceftriaxone reduced both the magnitude and the duration of inflammation and increased survival in a lethal murine model of systemic *Streptococcus pneumoniae*. The anti –inflammatory effects of A_{2A} AR agonist ATL313 may be particularly useful for the treatment in bacterial diseases when antibiotics have been administered. Further investigations are warranted to assess the therapeutic benefit of A_{2A} receptor agonists as an adjunctive agent to antibiotics in the treatment of pneumococcal infection and also to further understand the mechanism of protection involved in the ATL 313 protection.

The mechanism of protection was investigated by characterizing the effects of A_{2A} R activation on the NK cell activity in pneumococcal sepsis model. Natural killer (NK) cells are potent mediators of the innate immune response and their effect is attributed to both direct cytotoxiciy and indirect stimulation of macrophages by cytokine signaling. A_{2A} adenosine receptor signaling has been implicated in adenosine-mediated inhibition of cytokine production and cytotoxic activity by activated natural killer (NK) cells, although the process of NK cell granule exocytosis is apparently suppressed via a distinct and as yet uncharacterized adenosine receptor. There is evidence that NK cells can be activated during septic events and may contribute to the pathogenesis of this condition via the secretion of IFN-γ, which acts primarily to augment macrophage function. Whether modulation of NK activity during pneumococcal sepsis could improve pneumococcal sepsis outcomes and the ability of an A_{2A} AR agonist to modulate the NK response to improve sepsis has not been tested or available data is scanty and controversial.

In this study, mice were inoculated intravenously (i.v) with a lethal dose (10^7 cfu) of *S. pneumoniae* serotype 2 and treated with a combination of ceftriaxone and 25ug/kg ATL313 or ceftriaxone alone. To assess the importance of NK cells in pneumococcal

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sepsis, C57BL/6 and Jα281 mice were treated with a single i.p. injection of 200μg anti-NK1.1 (PK136) antibodies 2 days before bacterial challenge. Survival was monitored for seven days. Spleens were harvested and processed to assess NK cell activation (expression of markers CD69 and NK1.1, perforin and intracellular interferon gamma) using flow cytometry. Treatment with the combination of ATL and ceftriaxone (n=4) showed a high level of NK cell populations (2.063 ± 0.277) compared with ceftriaxone alone treated group (1.648 ± 0.566) (p=0.535); down-regulated CD69 expression, mean percentage 32.78 ± 6.327 vs 70.03 ± 8.163) (p=0.011); reduced the release of perforin (3,4% vs 0.21%)(p<0.05); and also reduced the level of intracellular and plasma IFN-γ (0.55% compared to 12.1%) (p=0.02). Blockade of NK cell activation with the treatment with PK136 and antibiotics resulted in 80% survival compared with 40% of mice that received ceftriaxone alone (p=0.01) in pneumococcal animal sepsis model. The results of the study implicate NK cells as one of the mediator of inflammation sensitive to regulation by $A_{2A}R$ activation. Furthermore, profound protection is imparted when this early event in the inflammatory cascade is inhibited by $A_{2A}R$ activation.

Therapeutic agents that inhibit the activity of NK and NKT cells may therefore hold promise in the treatment of pneumococcal infection i.e. it is possible to reduce cytokine levels more substantially by targeting an upstream event in the cascade (NK cell activation). A further study to improve understanding on the negative impact of ATL313 on NK cell function during sepsis is needed. These results suggest that analysis of NK cell activation during the septic process may yield insights into the interactions that occur between NK cells and the monocytes or macrophages compartment during the course of severe bacterial infections. However, the precise molecular and functional mechanism by which the negative impact of NK cells occurs is not known and an improved understanding of NK cell function during sepsis is needed. To investigate the role of decreased NK cell activation by A_{2A} AR treatment

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in the improved outcomes from experimental sepsis. We need to study the impact of A_{2A} AR agonist on NK cell trafficking during sepsis i.e. study how NK cells impact the pathophysiology of sepsis and determine where NK cells traffic during experimental sepsis and where and to what extent they proliferate. This will permit clarity on whether organ damage corresponds to areas where NK cells traffic. Furthermore, the understanding gained in this process will potentially provide tools with which we may in turn control the immune system when it runs awry in sepsis.

Growing antibiotic resistance and paradoxical enhancement of lethality as a result of the release of proinflammatory cell components after antibiotic treatment prompted our study of Etanercept (a tumor necrosis factor alpha neutralizing agent) as an adjunctive therapy against systemic *S. pneumoniae*. TNF is a major therapeutic target since it appears early and is related to disease severity. Balb/c mice were inoculated intravenously with a lethal dose (10^7 cfu) of *S. pneumoniae* serotype 2 and then treated with 25mg/kg ceftriaxone with/out 100ug of Etanercept (i.v) at 4 hours after bacterial challenge. Survival was monitored for several days. Serum samples from mice were analyzed for inflammatory cytokines with bead-based multi-analyte flow cytometry at 24 hours after challenge. Etanercept as adjunctive therapy significantly improved bacterial clearance and survival (70%, n=16) compared with 25% (n=20) survival of ceftriaxone alone treated mice ($p=0.001$). Inflammatory cytokines, TNF-alpha (787 v/s 5801 pg/ml), IFN- gamma (504 v/s 2642 pg/ml), interleukin-6 (IL-6) (255 v/s 2806 pg/ml), were reduced in Etanercept treated mice compared with ceftriaxone treated mice ($p<0.05$).

In conclusion, this study indicates that early TNF-alpha is a critical component of antibacterial host defense and late neutralization of that same endogenous TNF-alpha provides survival benefits when combined with ceftriaxone. Moreover, the current wave of enthusiasm regarding the treatment of patients with anti- TNF

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antibodies or soluble receptors must be tempered by the awareness of potential infectious complications that may occur as a result of this specific therapy. The study outlines the potential usefulness of Etanercept as an adjunctive therapy for pneumococcal infection and underlines the need for further research in the field. Therefore, more investigations are warranted, to further assess the therapeutic benefit of etanercept as an adjunctive agent to antibiotics in the treatment of pneumococcal infections in a clinical set-up.

As the treatment of infections becomes increasingly complicated by aggressive immunosuppressive regimens and the emergence of resistant bacteria strains, manipulation of the immune system with cytokine, anti-cytokine strategies may play an important adjuvant role in the management of patients with severe bacterial infections. Thus, it is important to study how immunomodulation of inflammation would affect progression of pneumococcal infection, making use of a lethal murine model of systemic *S. pneumoniae* infection in order to assess therapeutic benefits of immunomodulation and antiinflammatory agents in infections.

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