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IMMUNOMODULATION OF INFLAMMATION IN A MURINE PNEUMOCOCCAL SEPSIS MODEL

By

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Submitted in Fulfilment of the Requirements for the Award of the Degree of Doctor of Philosophy in the subject of Microbiology at the University of Venda

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Declaration

I, **Mbulaiseni Edgar Musie**, hereby declare that this thesis for the award of a Doctor of Philosophy degree in Microbiology at the University of Venda, submitted by me, has not previously been submitted for a degree at this or any other university, and that it is my own work in design and execution and that all the references materials contained therein have been duly acknowledged

Signature: ..... **Date:** 05 MAY 2013

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DEDICATION

This thesis is dedicated to my late son, **Ntanganedzeni Musie**, may our God bless your soul, my wife, **Magreth**, son **Lushaka**, and my beautiful daughters, **Phophi and Nditshilidzi** for their unconditional support

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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[illegible] *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 immu... 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 considered 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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National and international conferences attended and presentations:

Edgar Musie, Edward Martin, W Michael Scheld. Etanercept as Adjunctive Therapy Provides Survival Enhancement in a Clinically Relevant Murine Model of Lethal Systemic *Streptococcus pneumoniae.* IDSA 46th Annual Meeting, October, 2008, Washington DC.

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Edgar Musie, Christopher Moore, Edward Martin, Pascal Bessong, Michael W Scheld. Adenosine A_{2A} receptor activation reduces pneumococcal inflammation by inhibiting NK cell activation in a murine pneumococcal sepsis model.

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ABREVIATIONS

µg	microgram
µl	microliter
^0C	Degree Celsius
A_{2A} AR	Adenosine A_{2A} receptor
A_{2A}KO	Adenosine 2A receptor knockout
A_{2A}R	Adenosine 2A receptor
ACIP	Advisory committee on Immunization Practices
AIDS	Acquired immunodeficiency syndrome
ALT	Alanine aminotransferase
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
ATL	4-{3-(6-amino-9-(5-cycloproprylcarbamoyl-3,4-dihydroxytetrahydrofuran-2-yl)-9H-purin-2-yl)prop-2-ynyl}piperidine-1-carboxylic acid methyl ester)
ATP	Adenosine Triphosphate
ATTC	American Type culture collection
BHI	Brain Heart infusion
CAP	Community acquired pneumonia
CD	Cluster of differentiation
CDC	Centre for Disease Control and Prevention
CEF	Ceftriaxone
CFU	Colony forming unit
CLP	Cecal ligation puncture
CO2	Carbon dioxide
COPD	Chronic obstructive pulmonary disease

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CpG	deoxy-cytidylate-phosphate-deoxy-guanylate
CPS	Capsular polysaccharide
CSF	Cerebral spinal fluid
DCCs	Daycare Centers
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
DOC	Deoxycholate
Fig	Figure
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
H_2O_2	Hydrogen peroxide
HIV	Human immunodeficiency syndrome
Hr	hour
i.p.	Intraperitoneal
I.v.	Intravenous
IFN-	interferon
IgG	Immunoglobulin G
IL-1	Interleukin-1
IL-10 KO	Interleukin-10 knockout
IPD	Invasive pneumococcal disease
IRAK	Interleukin-I Receptor-associated kinase
KC	Keratinocyte-derived chemokine
Kd	kilodalton
Kg	kilogram
LPS	Lipopolysaccharide
LT	Lymphotoxin
LTA	Lipoteichoic acid
LytA	Autolysin

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MAP kinase	Mitogen-activated protein kinase
MCP	monocyte chemoattractant protein
Mg	milligram
MHC	Major Histocompatibility complex
MIP	Macrophage inhibitory proteins
MLA	Monophosphoryl lipid A
Mm^3	cubic millimeters
MyD88	Myeloid differentiation primary response 88
NF-κB	Nuclear factor kappa B
NK	Natural killer
NKT	Natural killer T cells
PAMPs	Pathogen associated molecular patterns
PBP	Penicillin binding protein
PBS	Phosphate buffered saline
PCV	Pneumococcal conjugated Vacine
PG	Peptidoglycan
Pg/ml	picon gram per milliliter
PMNs	Polymorphonuclear leukocytes
PRRs	Pathogen recognition receptors
Psp A	Pneumococcal surface protein A
ReLPS	repurified Lipopolysaccaharide
RNA	Ribonucleic acid
S. pneumoniae	*Streptococcus pneumoniae*
S. pyogenes	*Streptococcus pyogenes*
SCID	Severe combined immunodeficiency syndrome
SIgA	Secretary Immunoglobulin A
SIRS	Systemic inflammatory response syndrome

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SSC	Surviving sepsis campaign
STDEV	Standard deviations
TA	Teichoic acid
TCR	T cell receptor
TGF	Transforming growth factor
TLR	Toll like receptor
TNFR	Tumor necrosis factor Receptor
TNF-α	Tumour necrosis factor alpha
TRAF-6	Tumor-necrosis-factor- receptor- associated factor-6
TSA	Tryptic soy agar
USA	United States of America
WHO	World Health Organization
z-VAD-fmk	benzyloxycarbonyl- Val-Ala-Asp-fluoromethyl ketone
ZM-241385	4-(2-(7-amino-2-(furan-2-yl-)-[1,2,4]triazolo[1,5-a]triazin-5-ylamino)ethyl) phenol

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GENERAL INTRODUCTION AND LITERAURE REVIEW

1.1 INTRODUCTION

Streptococcus pneumoniae is an important human pathogen and causes serious complications in patients with underlying diseases. *S. pneumoniae* is the principal cause of bacterial pneumonia, in children and adults, in economically developed and developing countries. It is also a major cause of otitis media, bacterial meningitis and septicemia (Gonzalez et al., 2006). It is the most common cause of community acquired bacterial meningitis in children older than 1 year in developed and developing countries. The initiation of antibiotic treatment on suspicion of bacterial meningitis is important, but it is not enough to improve the prognosis for patients, especially those with pneumococcal meningitis. Mortality and morbidity due to pneumococcal meningitis are still devastating. Despite significant advances in antibiotic therapy and critical care in the past few decades, the mortality rate of acute bacterial meningitis is substantial, ranging between 10% and 30%. Patients may die several days after bacteria are eradicated from lungs by antibiotic therapy, and pneumococcal cell walls can generate exaggerated inflammation and tissue damage in pneumococcal infections (Cundell et al., 1995). A poor outcome is characteristic of severe pneumococcal pneumonia, partly because of overwhelming inflammation elicited by bacterial components.

Streptococcus pneumoniae are gram-positive, α-hemolytic, lancet-shaped, facultative anaerobes with a polysaccharide capsule which normally occur in pairs (diplococci) or in long chains. *S. pneumoniae* was first isolated in 1881 (Davis et al., 1968). At least 90 different serotypes of *S. pneumoniae* exist which are classified based on the composition of the oligosaccharides which make up their capsular polysaccharides (Van Dam et al.,

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1990). Each of these serotypes is able to colonize the nasopharynx and establish a carrier state, although some serotypes are able to colonize more readily (Casal and Tarrago, 2003). The carriage rates of *S. pneumoniae* vary from 5-30% in healthy adults and 20-50% in healthy children (Musher et al., 2000; Lexau et al., 2006). Of the 90 serotypes of *S. pneumoniae*, approximately 20 serotypes are able to cause disease in other areas of the body, such as the lungs, blood, or brain. Importantly, 10 of the 90 *S. pneumoniae* serotypes cause 62% of invasive pneumococcal disease cases worldwide (Casal and Tarrago, 2003). Young children and the elderly are not able to mount an effective immune response against capsular polysaccharides and are therefore more susceptible to *S. pneumoniae* infection than healthy adults. Pneumococcal pneumonia remains a common cause of death for young children and protection from *S. pneumoniae* disease relies on both the innate and the adaptive immune responses (Casal and Tarrago, 2003).

1.1.1 BACKGROUND AND HISTORICAL PERSPECTIVE OF *S. pneumoniae*

Streptococcus pneumoniae is a species of the genus Streptococcus of the family Streptococcaceae (Lund and Henrichsen, 1978). The genus Streptococcus comprises species of Gram – positive spherical or oval cocci that tend to be arranged in chains. Streptococci form a significant portion of the indigenous microflora of humans and animals. Most of these species are found in the oral cavity and nasopharynx, but some inhabit the intestinal tract. Although most species rarely cause disease, the genus includes three of the most important pathogens of humans: *Streptococcus pyogenes*, *Streptococcus agalactiae* and *Streptococcus pneumoniae*. *S. pyogenes*, the group A streptococcus causes a variety of acute infections and can stimulate non-suppurative post streptococcal sequel of rheumatic fever and acute glomerulonephritis. *S.*

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agalactiae, the group B streptococcus is the most important cause of neonatal sepsis and meningeal infection (Ryan and Falkow, 1994).

Streptococcus pneumoniae, originaly called *Diplococcus pneumoniae*, is commonly known simply as pneumococcus. The first isolate was found in the end of the 18th century. In 1881, the organism was first isolated and identified from human saliva concurrently in the old and new worlds, by Pasteur in France, who named it Microbe septicemique du salive and by Sternberg in the United States, who named it Micrococcus pasteuri. By the late 1880's, the term pneumococcus was generally used because of lobar pneumonia (Davis et al., 1968). The name *Diplococcus pneumoniae* was assigned in 1926 because of its appearance in gram-stained sputum and in 1974, the organism was renamed once again, this time as *Streptococcus pneumoniae* because of its morphology during growth in liquid medium (Musher, 2000). Ever since, the bacterium has been a major cause of mortality and morbidity worldwide. *S. pneumoniae* are surrounded by a polysaccharide capsule. Depending on differences of the chemical structures of the capsule, they are divided into different serotypes (Van Dam et al., 1990). Thus, 48 different serogroups have been recogniosed, further subgrouped into a total of 90 serotypes (Henrichsen, 1995).

1.1.2 CULTIVATION AND MORPHOLOGY OF *S. pneumoniae*

Streptococcus pneumoniae is fastidious bacterium, growing best in 5% carbon dioxide. Nearly 20% of fresh clinical isolates require fully anaerobic conditions. In all cases, growth requires a source of catalase (e.g. blood) to neutralize the large amount of hydrogen peroxide produced by the bacteria. In complex media containing blood, at 37°C, the bacterium has a doubling time of 20-30 minutes. On blood agar, pneumococci grow as glistening colonies, about 1 mm in diameter. Two serotypes, types 3 and 7, are

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mucoid. Pneumococci spontaneously undergo a genetically determined, phase variation from opaque to transparent colonies at a rate of 1 in 10^5. The transparent colony type is adapted to colonization of the nasopharynx, whereas the opaque variant is suited for survival in blood. The chemical basis for the difference in colony appearance is not known, but significant difference in surface protein expression between the two types has been shown (Bruyn et al., 1992).

Streptococcus pneumoniae is a fermentative aerotolerant anaerobe. It is usually cultured in media that contain blood. On blood agar, colonies characteristically produce a zone of alpha (green) hemolysis, which differentiates *S. pneumoniae* from the group A (beta hemolytic) streptococcus, but not from commensal alpha hemolytic (viridans) streptococci which are co-inhabitants of the upper respiratory tract. Special tests such as inulin fermentation, bile solubility, and optochin (an antibiotic) sensitivity must be routinely employed to differentiate the pneumococcus from *Streptococcus viridians* (Lund and Henrichsen, 1978). They are catalase negative, but they generate hydrogen peroxide (H_20_2) via a flavo enzyme system and therefore grow better in the presence of a source of catalase such as red blood cells. Pneumococci grow diffusely in serum broth and give rise to smooth colonies surrounded by narrow zones of α-haemolysis on blood agar. Under optimal conditions *in vivo*, pneumococci undergo optimal elaboration of their capsules; in contrast, the organisms may lose their capsules with repeated passage in vitro, especially under adverse conditions. These unencapsulated strains give rise to rough colonies (Bruyn et al., 1992).

Streptococcus pneumoniae is a very fragile bacterium and contains within itself the enzymatic ability to disrupt and to disintegrate the cells. The enzyme is called an autolysin. The physiological role of this autolysin is to cause the culture to undergo a characteristic autolysis that kills the entire culture when grown to stationary phase.

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Virtually all clinical isolates of pneumococci harbor this autolysin and undergo lysis usually beginning between 18-24 hours after initiation of growth under optimal conditions. Autolysis is consistent with changes in colony morphology. Colonies initially appear with plateau-type morphology, and then start to collapse in the centers when autolysis begins.

Pneumococci have a remarkable capacity (termed competence) to internalize DNA from other pneumococci or from other bacterial species. Because of this property, pneumococci are able to acquire new phenotypic traits, a process called transformation. Transformation of capsular types occurs under experimental conditions but more importantly also occurs in nature. Careful study of clinical isolates with fingerprinting techniques has shown that pneumococci can acquire a cassette of DNA that encodes the production of a serotypically different capsule in other words pneumococci can switch capsules (Nesin et al., 1998; Musher, 2000).

Pneumococci are capsulated lanceloate diplococci often arranged in short, straight chains (Davis *et al.*, 1968). Nearly every clinical isolate of *S. pneumoniae* contains an external capsule with the exception of unencapsulated isolates, which have mainly been implicated in outbreaks of conjunctivitis (Ertugrul et al., 1997). The pneumococcal outer surface consists of the capsule, cell wall and plasma membrane. Capsules are made up of repeating oligosaccharide (di- to penta-) units that are linked to one another by phosphodiester bonds and are synthesized within the cytoplasm, polymerized and transported to the bacterial surface by cell membrane transferases (Musher et al., 2000). Some of the polysaccharides that compose the capsule have a high proportion of such constituents as cellobiuronic acid, hexuronic acid and pyruvic acid. Pneumococcal polysaccharides may include sugars such as glucose, galactose, rhamnose and fucose). Most pneumococcal polysaccharides are linear polymers although some including those

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of types 7, 11, 12, 17, and 18 are branched (Bruyn et al., 1992). The antigenicity of the capsular carbohydrate is attributable to an oligosaccharide epitope consisting of no more than six or seven sugar residues (Kabat, 1966). Constituents of pneumococcal polysaccharides are common among various bacteria, thus cross reactions occur among different types of pneumococcal polysaccharides and with the polysaccharides of *Escherichia coli*, *Klebsiella species* and other *Streptococcus species* and plants (Bruyn et al., 1992). Ninety serotypes of *S. pneumoniae* have been identified based on antigen differences in their capsular polysaccharides (Lund & Henrichsen, 1978, Henrichsen 1995).

The capsular polysaccharide is the antigen that primarily elicits the B-cell response (Jawetz et al., 1991). These polysaccharides are covalently bound to peptidoglycan and cell wall C polysaccharide, which explains the difficulty of separating capsular from cell wall polysaccharide (Musher, 2000). The principal constituents of the pneumococcal cell wall are peptidoglycan and teichoic acid. Peptidoglycan consists of long chains of alternating N-acetyl –D- glucosamine and N- acetylmuramic acid from which extend chains of four to six amino acids called stem peptides. Stem peptides are cross-linked by pentaglycine bridges, which provide substantial strength to the cell wall. Teichoic acid, a carbohydrate polymer that contains phosphorylcholine, is covalently linked to the peptidoglycan on the outermost surface of the bacterial wall and protrudes into the capsule. This teichoic acid together with tightly adherent fragments of peptidoglycan make up the C- polysaccharide, a substance that is present in all pneumococci, and is otherwise detected only in a few species of viridans streptococci. This region is responsible for the reaction between pneumococci and proteins that appear in the blood stream in certain illnesses or that occur in some cases of multiple myeloma (Tomasz 1981; Musher 2000). Also expressed on the cell surface are proteins that are thought to play a role in pathogenicity such as pneumococcal surface protein A, choline binding

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proteins and proteins involved in competence. Type-specific proteins called M-proteins are proteins associated with the cell wall but do not play a role in the determination of the type, nor do they contribute to the virulence of pneumococci (Davis et al., 1968).

The plasma membrane is composed of lipoteichoic acid, lipid and protein. It also contains the Forssman antigen, which is composed of lipoteichoic acid. This antigen is common to all pneumococci and functions as a powerful and highly specific inhibitor of pneumococcal autolysin, which comprises a group of enzymes that cause rapid degradation of the cell wall under certain circumstances (Sorensen et al., 1988).

1.1.3. EPIDEMIOLOGY OF *S. pneumoniae*

Humans are the only known reservoir for *S. pneumoniae* which is spread by droplets from infected individuals. The pneumococcus colonizes the nasopharynx in 5-10% of healthy adults and 20-40% of healthy children. For reasons that remain unclear, the rate of colonization is seasonal with an increase in the midwinter period (Musher et al., 2000). The first pneumococcus to colonize an infant is generally acquired at 6 months of age and can be detected for a mean period of about 4 months (Gray et al., 1980; Sluijter et al., 1998).

Transmission of pneumococci from one individual to another occurs because of extensive close contact. Day care centers are very likely to be places for spread in toddlers (Henderson et al., 1988; Rauch et al., 1990). In adults, close crowded living conditions such as occur in military camps, prisons (Hoge et al., 1994), shelters for the homeless (Mercat et al., 1991) and nursing homes (Nuorti et al., 1998) are associated with epidemics but contact in schools or in the work place is generally not.

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Pneumococcal infections appear at all ages, but peak incidences are seen in young children and the elderly (Burman et al., 1985). In the development of illness, predisposing factors (Table 1.1) are more important than exposure to infectious agents and the healthy carrier is more important in disseminating pneumococci than the sick patient (Jawetz et al., 1991).

The overall rate of invasive pneumococcal disease, defined as the isolation of *S. pneumoniae* from a normally sterile site such as blood, pleural fluid or cerebrospinal fluid, is about 15 per 100 000 persons per year. The incidence may be increased in certain populations (Davidson et al., 1994) due to possible genetic or environmental factors. Invasive pneumococcal disease is common in newborns and infants up to two years of age and much less so, in children and young adults, again increasing in adults older than 65 years (Burman et al., 1985). Pneumococcal pneumonia accounts for 60 – 80 % of all bacterial pneumonias.

In South Carolina, the incidence of pneumococcal bacteremia was shown to be 162 per 100 000 in infants less than two years of age and 53 per 100 000 in adults more than 65 years of age (Breiman et al., 1990). In Finland, Eskola et al. (1992), reviewed the incidence of lobar pneumonia with bacteremia or meningitis in children. They determined the incidence of invasive pneumococcal infections as 9 per 100 000 children less than 16 years of age and 45 per 100 000 children less than 2 years. According to Baer *et al.* (1995) in 1993, the incidence rate in children less than 16 years rose to 20 per 100 000 and in 1994 to 30 per 100 000. In children under 20 years, the incidence rate for 1993 was 70 per 100 000 and in 1994, 110 per 100 000. Some studies showed that the peak incidence of meningitis occurs among infants three to five months of age, that of otitis media occurs among infants six to twelve months of age and that of hospitalization for pneumonia occurs among infants 13 – 18 months of age (Klein, 1981).

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In a study from Ghana the mortality rate of invasive pneumococcal disease (IPD) was as high as 47% (Holliman et al., 2006). In France a mortality rate was 20% among IPD patients was found, whereas a Swedish study reported a corresponding rate of 14% (Sjostrom et al., 2006). Like carriage, the pattern of invasive disease flunctuates with season. In a surveilance study spanning three years, it was shown that the rate of IPD increased during winter and early spring each year (Dowell et al., 2003). Moreover, annual flunctuations in serotype distribution among IPD cases have been observed (Henriques Normark et al., 2001). The minimum annual incidence for children younger than five years of age increased from 61 per 100000 (179 per 100000 for those <12 months old) in 1987 to 130 per 100000 (349 per 100000 for those < 12 months) in 1999 as a result of the impact of the HIV epidemic (Karstaedt et al., 2000).

1.1.4. PATHOGENESIS OF *S. pneumoniae*

Pneumococci are normal inhabitants of the upper respiratory tract. The mucosa of the lower respiratory tract has extraordinarily efficient defense barriers. These include: the epiglottal reflex, which prevents gross aspiration of infected secretions, the sticky mucus to which airborne organisms adhere on the epithelial lining of the bronchial tree, the cilia of the respiratory epithelium, which keep the infected mucus moving upward into the pharynx where much of it is swallowed, the cough reflex which aids the cilia in propelling accumulated mucus from the lower respiratory tract, the lymphatics draining the terminal bronchi and bronchioles and the mononuclear "dust cells" which patrol the normal alveoli (Davis et al., 1968).

If the natural immunity/defense barriers are overcome by pneumococci, disease ensues. Pneumococci produce disease through their ability to multiply in the tissues. The

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virulence of the organism is a function of the capsule that prevents or delays ingestion of encapsulated cells by phagocytosis. Risk factors lower the natural resistance and thus predispose to pneumococcal infection (Jawetz et al., 1991).

1.1.4.1 Virulence factors

Many Streptococcus pneumoniae virulence factors are known today. Some are better understood and more deeply explored than others, like the capsular polysaccharide and the cytotoxic pneumolysin for which the knowledge is substantial. The pneumococcal capsule is the major virulence factor present in pneumococci. Several other constituents, including pneumolysin, surface proteins and autolysin also contribute to the pathogenesis of pneumococcal disease (Mitchell, 2000). Genetically engineered mutants that lack the ability to produce one or more of these substances have generally been showed to have diminished virulence, and immunization with the purified substance has stimulated production of antibodies that confer protection in experimental animals. It has to be mentioned, however, that no antibody to any of these substances has been shown to be protective in humans (Musher, 2000).

1.1.4.1.1 The capsular polysaccharide

The capsule prevents or delays ingestion of pneumococci by phagocytes (Kingsbury & Wagner, 1990). The antiphagocytic effect of the capsule has long been attributed to its physical size and electrostatic charge, but recent studies suggest that it has the ability to prevent antibody independent opsonization via the alternate pathway that allows organisms to survive. The capsular polysaccharide either interferes with C3b deposition directly or covers C3 fragments that are deposited on the cell wall. In either case, the

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complement fragments recognised by phagocyte receptors are not available. When antibody binds to the capsular polysaccharide, C3b generated by the classical pathway binds and opsonophagocytosis proceeds efficiently (Ryan & Falkow, 1994; Hostetter, 1986). The capsular polysaccharide (CPS) needs to be attached to the pneumococcal cell wall in order for the bacterium to cause IPD. CPS mutants are unable to enter the blood stream due to the loss of the regulation of CPS production and decreased attachment to the cell wall (Morona et al., 2006). As mentioned previously, the capsule is composed of different structures of polysaccharide, which are distinct for each of the 90 serotypes recognized. Independent studies have shown that different capsular types have different abilities to cause invasive disease or carriage (Brueggemann et al., 2003; Martens et al., 2004; Sandgren et al., 2004; Sjostrom et al., 2006). For example, serotype 1 is more commonly found among invasive isolates and type 19F is more commonly found among carriers (Brueggemann et al., 2003; Sandgren et al., 2004). However, not only the capsular type decides the outcome of disease. A study by Mizrah Nebenzahl et al. showed that the role of the capsule for disease outcome is minor and that clonal types are more important (Mizrachi et al., 2004)

1.1.4.1.2 Cell wall

The cell wall is mainly built up by peptidoglycan (PG), teichoic acid (TA), and lipoteichoic acid (LTA). It seems like the LTA is anchored to the underlying cell membrane and that TA might participate in adhesion to the host cells (Nau et al., 2002). TA and PG mediate inflammatory responses in the host with induction of TNF- alpha and IL-6 production (Heumann et al., 1994). LTA apprears to bind CD14, Toll like receptors, and phospholipids. This activates host cells of different types and mediates the release of cytokines and a number of tissue damaging agents, such as superoxide and lysosomal enzymes including hydrolases (Ginsburg, 2002). It has been suggested that LTA and TA

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do not influence disease outcome. A german study compared clinical isolates causing invasive disease with nasopharyngeal isolates and did not observe any difference in release of TA and LTA (Spreer et al., 2004)

1.1.4.1.3 Pneumolysin

Pneumolysin is a thiol-activated, haemolytic toxin that is a member of a family of toxins produced by diverse genera of Gram - positive bacteria and is produced by all clinical isolates. Unlike other sulfhdryl- activated toxins, it is not secreted by the bacterium but is released during autolysis (Steinfort et al., 1989, Huo et al., 2004). These toxins interact with their target cell membrane possibly through cholesterol followed by oligomerization of toxin molecules to form transmembrane pores resulting in cell lysis (Gilbert et al., 1999).

Pneumolysin is toxic for pulmonary endothelial cells, a property that might contribute to disruption of the endothelial barrier and the spread of pneumococcus from the alveoli into the blood stream. Pneumolysin can slow ciliary beating and disrupt the surface integrity of cultured human respiratory epithelium (Steinfort et al., 1989). The toxic effect itself and the inhibition of phagocyte and immune cell function lead to suppression of the host inflammatory and immune response (Jedrzejas, 2001). Pneumolysin has been shown to be of importance for the development of invasive disease like meningitis, bacteremia, and pneumonia (Hirst et al., 2004).

According to a study by Benton et al., pneumolysin seems to play a major role in the beginning of an infection, determining whether pneumocci will cause bacteremia or sepsis. In a mouse study a pnemolysin-negative mutant developed a bacteremia in the beginning of the infection and stoppedgrowing after that, in contrast to a pneumolysin –

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positive starin that reached a higher level of bacteria in the blood, causing a sepsis with lethal outcome (Benton et al., 1995). Hearing loss in association with meningitis and otitis media is another result of this protein, due to the hair cells in the cochlea being highly sensitive to the cytotoxic effects (Winter et al., 1997). Most patients with pneumococcal disease exhibit an antibody response to pneumolysin and pneumolysin immunization is protective in animals (Ryan & Falkow 1994; Davis et al., 1968) and has suggested pneumolysin as vaccine candidate (Huo et al., 2004).

1.1.4.1.4. Autolysin

Autolysin, also called LytA amidase, is a choline binding protein located in the cell wall. It is responsible for the release of pneumolysin as well as cell wall components. The gene encoding autolysin is, just as the pneumolysin-encoding gene, highlyconserved but since anti-LytA antibodies have no direct effect on the adherence or clearance of invading pneumococci, LytA has been questioned asa potential vaccine candidate (Tai, 2006). The cell wall, consisting of peptidoglycan fragments, is degraded naturally by autolysin during cell division, leading to cell lysis (Jedrzejas, 2001). This bacterial cell lysis also occurs during inactivated biosynthesis, caused by penicillin treatment or nutrient starvation (Mitchell, 2000). Two other autolysins have been described, LytB and LytC and both are shown to play a role in colonization of the nasopharynx (Gosink et al., 2000). In addition, LytB is suggested to be important for cell separation of newly formed daughter cells (Garcia et al., 1999). In general, autolysins act mainly as indirect virulence factors allowing other virulence factors to be released

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1.1.4.1.5 Pneumococcal surface protein A (PspA)

Psp A is highly variable both immunologically and in molecular mass (Crain et al., 1990). In 1988, Briles *et al.*, inactivated the *Psp A* genes in three virulent encapsulated strains of pneumococci. All three strains showed a reduction in virulence and two became totally avirulent. Passive immunization with monoclonal antibodies raised against Psp A can protect mice against intravenous challenge with certain pneumococcal strains (McDaniel et al., 1984; 1987). From these studies, it has been concluded that Psp A is a major virulence determinant in pneumococci.

1.1.4.1.6 Neuraminidase

Pneumococci also produce a neuraminidase that is released upon autolysis. Neuraminidase cleaves terminal N- acetyl muramic acid from glycoproteins of the host cell membrane and may account for neurotoxicity seen in pneumococcal meningitis (Kingsbury and Wagner, 1990; Berry, 1989).

1.1.4.1.7 Secretary immunoglobulin A (sIgA) protease

Pneumococci produce a sIgA protease, a property shared with other species causing pneumonia and meningitis. This enzyme could play a part in the establishment of the microorganism in the nasopharynx, but no definite role of this enzyme in virulence has been proven (Ryan and Falkow, 1994).

1.1.4.1.8 Peptidoglycan and Teichoic Acid

Another feature of pneumococcal infection is the heightened acute inflammatory response that may itself be destructive to the host. Peptidoglycan and teichoic acid components of the cell wall are released into the surrounding medium during treatment

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with β-lactam antibiotics. Thus, it conceivable that bacterial death, when accompanied by lysis and release of the cell wall components could aggravate cerebrospinal fluid (CSF) inflammation and affect the clinical outcome of disease deleteriously (Toumanen et al., 1985a; Toumanen et al., 1985b; Riesenfield- Orn et al., 1989).

1.1.4.2 Factors that predispose to pneumococcal disease

A number of different risk factors for acquiring pneumococcal infections have been identified. The incidence differs between the different factors. A representative schema of conditions that have an impact on the immunologic capacity of the host and predispose to pneumococcal infection is shown in Table 1.1.

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Table 1.1: General schema of conditions that predispose to pneumococcal infection (Musher, 2000)

- Defective antibody formation
  - Primary
    - Congenital agammaglobulinemia
    - Common variable (acquired) hypogammaglobulinemia
    - Selective IgG subclass deficiency
  - Secondary
    - Multiple myeloma
    - Chronic lymphocytic leukemia
    - Lymphoma
    - HIV infection
- Defective complement (primary or secondary)
  - Decreased or absent C1, C2, C3, C4
- Insufficient numbers of Polymorphonuclear leukocytes (PMNs)
  - Primary
    - Cyclic neutropenia
  - Secondary
    - Drug-induced neutropenia
    - Aplastic anemia
- Poorly functioning Polymorphonuclear leukocytesPMNs
  - Alcoholism
  - Cirrhosis of the liver
  - Glucocorticosteroid treatment
  - Renal insufficiency
  - poorly avid receptors for FcγII (R131 allele)
- Defective clearance of pneumococcal bacteremia
  - Primary
    - Congenital asplenia, hyposplenia
  - Secondary
    - Splenectomy
    - Sickle cell disease (autosplenectomy)
- Multifactorial
  - Infancy and aging

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- Glucocorticosteroid treatment
- Malnutrition
- Cirrhosis of the liver
- Renal insufficiency
- Diabetes mellitus
- Alcoholism
- Chronic disease
- Fatigue
- Stress
- Cold exposure

Excess likelihood of exposure

- Daycare centers
- Military training camps
- Prisons
- Shelters for the homeless

Prior respiratory infection

- Influenza
- Other

Inflammatory condition

- Cigarette smoking
- Asthma
- Chronic obstructive pulmonary disease (COPD)

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1.1.4.2.1 Age

Age is a major risk factor for acquiring a pneumococcal disease. It is more common that elderly individuals and children under 5 years acquire an invasive disease. Among children, the risk decreases with increasing age. An under-developed immune system is likely the reason for the increased incidence in children and elderly. Factors such as exposure and lack of breastfeeding are also of importance, exemplified in a case-control study from North America where day-care centers attendance and lack of breastfeeding was strongly associated with obtaining an IPD (Levine et al., 1999). Furthermore, pre-term birth and low birth weight have been shown to increase the risk for IPD (Shinefield et al., 2002).

1.1.4.2.2 The socio-economic status and the status of the surroundings

This may as well constitute risk factors in the development of pneumococcal disease. Atmospheric pollution badly ventilated and crowded milieus might increase the risk. Treatments are expensive and healthcare sometimes inadequate and these are problems especially associated with developing countries. The source of information may also be vague, limiting the ability to know how to handle a situation when someone is infected and can spread the infection.

1.1.4.2.3 Immunocompromised patients and/or people with underlying disease/condition

People with underlying disease/condition display an increased risk for obtaining a pneumococcal disease, in some cases with a fatal outcome. Examples are HIV, cancer, heart and lung failure and asplenia (Kyaw et al., 2005; Barry et al., 2006). In a mouse experiment by coil et al. a higher mortality rate was shown in splenectomised mice

compared to non-splenectomised upon exposure to an aerosolized suspension of pneumococci (Coil et al., 1978). Diabetes, asthma, and alcohol abuse also constitute an increased risk for acquiring an infection (Kyaw et al., 2005; Talbot et al., 2005). Additionally, cigarette smoking, with its degenerative effect on lung tissue has been described as a strong independent risk factor for IPD (Nuorti et al., 2000). The importance of non-smoking programs and non-smoking areas may therefore be vast for the reduction of IPD incidence.

Bacterial infections have emerged as an important cause of morbidity and mortality in individuals with the human immunodeficiency virus (HIV). Persons with HIV-1 infection are more susceptible to bacterial infections because of defects in both cell-mediated and humoral immunity (Mao et al., 1996). HIV-1 seroposistive individuals are particularly susceptible to infections with encapsulated bacteria, such as *Streptococcus pneumoniae*. In fact, pneumococcus is one of the most common bacterial pathogens affecting both HIV-1 infected children and adults (Janoff et al., 1992). Among female sex workers in Nairobi, Kenya, *S. pneumoniae* caused more disease than *Mycobacterium tuberculosis* or non-typhi Salmonellae (Gilks et al., 1996).

HIV-1 infection is responsible for immunologic defects not only in T lymphocytes, but also in B lymphocytes, macrophages, polymorphonuclear cells, cytokine production. Although the primary immunologic defect is in cell-mediated immunity, defects in the humoral immune system also occur and are early immunologic sequelae of HIV infection (Terpstra et al., 1989). The numerous abnormalities in the humoral immune system and phagocytic cells increase the susceptibility of the HIV-1 infected person to bacterial pathogens or infections, particularly those caused by encapsulated organisms (Terpstra et al., 1989). In the setting of HIV-1 infection, the predisposition to pneumococcal infection is probably due to dysfunctional host defenses rather than to increased colonization. Rates of

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pharyngeal carriage with *S. pneumoniae* in ambulatory adults in Denver were similar among HIV-1 infected patients (14% colonized) and HIV-seronegative controls (9%) (Janoff et al., 1993). Rodriquez-Barrades et al. (1997) revealed that the frequency of colonization with *S. pneumoniae* among participants of a vaccine study was 7% in subjects with <200 cells/mm^3, 20% in subjects with at least 200 cells/mm^3, and 10% in controls.

Defects in mucosal immunity could play a role in the susceptibility to pneumococcal infections because mucosal IgA may prevent bacterial adherence to mucosal surfaces and colonization with *S. pneumoniae* (Janoff et al., 1992). Other defects in mucosal cellular responses and systemic immunity are likely to play a more important role in predisposing HIV-1 infected patients to invasive pneumococcal disease (Opstad et al., 1995).

As in general population, the treatment of HIV-1 related pneumococcal disease depends on the site of infection and whether or not the organism is susceptible to penicillin. HIV-1 seropositive patients with moderate to severe pneumococcal pneumonia should be hospitalized and treated with paranteral antimicrobial agents. Despite the presence of underlying immunodeficiency and a high frequency of bacteremia, HIV-1 infected patients generally respond rapidly to antimicrobial therapy and no extended therapy is needed (Garcia et al., 1992). Patients with pneumococcal pneumonia who fail to respond to appropriate antimicrobial therapy or whose condition worsens after initial improvement may have a coexistent opportunistic infection. Despite the good response to therapy, recurrent bacterial disease is more common in patients with HIV-1 infection. Recurrent pneumococcal infection has been reported in 8% to 25% of HIV infected patients compared with 7% of controls (Polsky et al., 1986). Using serotyping, ribotyping, and DNA typing, Jordens et al. (1995) have demonstrated that recurrence can occur because of

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relapse with the same strain or reinfection with another strain of *S. pneumoniae* and recurrent bacterial pneumonia is a reporting criteria for AIDS (Centres for Disease and control prevention, 1993).

The increased incidence of pneumococcal infections and the high rate of bacteremia and recurrence make prevention of invasive pneumococcal disease an important aspect of the care of HIV-1 infected patients. Possible preventative strategies include pneumococcal vaccination, intravenous immunoglobulin, and oral antibiotics. The advisory committee on Immunization Practices of the CDC recommends that all HIV-1 infected patients over 2 years of age receive the 23-valent pneumococcal polysaccharide vaccine (Centers for Disease and control prevention, 1997). The CDC also recommended that all HIV-1 infected persons receive a single revaccination 5 years or more after receipt of the first dose. A 1992 report indicated that oral TMP-SMX significantly decreased the risk of bacterial infection in adults with AIDS. Oral TMP-SMX used as PCP prophylaxis has been associated with a reduction in the episodes of bacterial pneumoniae and a lower risk of community-acquired bacteremia but given the rising resistance of *S. pneumoniae* to TMP-SMX and penicillin, this approach cannot be recommended routinely (Hardy et al., 1992)

1.1.4.3 Host factors

The immune response can be categorized into two groups, innate and adaptive immunity. Innate immunity is a non-specific immune response present from birth and does not require gene recombination or clonal selection to be functional. Innate immunity acts directly when an organism enters the tissue and targets certain concerved molecular motif s specific for different organisms. This immune system has no lifelong or organism-specific memory. The complement system is an important part of the innate immune system with many effector mechanisms which

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lead to membrane pore-formation and bacterial lysis. Bacteria with a polysaccharide capsule, such asa pneumococci, can eliminate the C3b deposition on the surface and prevent opsonization (Salyers et al., 2002).

Streptococcus pneumoniae induces an adaptive immune response with the production of both anti-capsular and anti-surface protein antibodies and protection is due primarily to antibody-complement dependent phagocytosis (Casal and Taraggo, 2003). The adaptive immune system has an acquired and a specific immunity where the memory is organism –specific. When the organism is engulfed by macrophages, bacterial peptides are digested and presented by the major histocompatibility complex type II (MHC- II) on antigen presenting cells, activating the T-helper cells to stimulate B-cells, which produce capsular specific antibodies. The B-cells become memory cells and anti-capsular antibodies and then opsonise bacteria that enter the body, leading to phagocytosis. Antibody-dependent clearance of *S. pneumoniae* that results from prior exposure to *S. pneumoniae* is the basis for the development of the current *S. pneumoniae* vaccines (McCullers et al., 2001)

1.1.5. PNEUMOCOCCAL DISEASES

S. pneumoniae colonizes the upper respiratory tract and cause respiratory diseases such as pneumonia, sinusitis, and otitis media and invasive infections such as meningitis and bacteremia (Mitchell et al., 1997).

1.1.5.1 Pneumonia

If potentially protective mechanisms fail to prevent both the access of pneumococci to the alveoli and their subsequent replication, pneumonia results. Bacteria proliferate in

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alveolar spaces, and they are carried along the alveolar septa where they activate complement and generate vasoactive substances (Musher, 2000). Exudate fluid and white blood cells accumulate in the septa and alveoli. The inflammatory exudate also moves onto uninvolved areas through the pores of Kohn. This filling of alveoli with microorganisms and inflammatory exudate defines the presence of pneumonia and a clinical diagnosis is made when fluid accumulation has been great enough to allow it to be seen radiographically as a non lucent or "consolidated" area (Musher, 2000).

Symptoms of pneumonia include cough, fatigue, fever, chills, sweats, and shortness of breath and sputum production (Murphy and Fine, 1984). These are all more prominent in younger patients than in older patients. Elderly patients may have only a slight temperature elevation or be febrile but are more likely to have an increased respiratory rate. The absence of fever in young or middle aged adults is associated with increased morbidity and mortality, especially hypothermia (Murphy and Fine, 1984). A quarter to a third of cases of pneumococcal pneumonia is accompanied by bacteremia (Austrian & Gold, 1964). Bacteremic pneumococcal pneumonia has been shown to cause high mortality among adults (Mufson et al., 1974). By contrast, pneumococcal bacteremia in children seems to be associated with little risk of death (Breiman et al., 1990).

1.1.5.2 Meningitis

With *Neisseria meningitidis* and *Haemophilus influenzae*, *S. pneumoniae* is one of the three leading causes of bacterial meningitis. Acute purulent meningitis may follow pneumococcal pneumonia, or appear with no antecedent infection. It may also appear after trauma involving the skull (Ryan and Falkow, 1994). Progressive infections of the mastoid or respiratory sinuses sometimes extend directly to the subarachnoid space to cause pneumococcal meningitis (Davis et al., 1968). Haematogenous spread is yet

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another route in which pneumococci can gain access to the meninges (Boulnois, 1992). All ages are affected, however in later life pneumococcal meningitis is the most common form of the disease. The mortality and frequency of sequelae are slightly higher with pneumococcal meningitis than with other forms of pyogenic meningitis. This is generally attributed to the inflammatory response (Ryan and Falkow, 1994).

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1.1.5.3 Bacteremia

Bacteremia is frequently associated with pneumonia in adults or otitis media in children (Breiman et al., 1990), but it can also occur in the absence of a primary focus. The site at which pneumococci gain access to the blood stream and the mechanism by which this occurs is not clear although several routes are possible. In some studies, pneumococci were found in the blood only a few minutes after intranasal challenge of mice and might have invaded via the nasopharyngeal mucosa (Breiman et al., 1990). Invasion from the lower respiratory tract involving transport in the lymphatics via the hilar nodes is also possible since pneumococci were also found in the alveoli shortly after challenge. Similar experiments in rabbits indicated that bacteremia was preceded by the appearance of pneumococci in the cervical lymphatics. In intravenously challenged rabbits, pneumococci can cross both vascular and lymphatic endothelium and the lymphatics can serve as a resevoir of replicating pneumococci even in the presence of anti-pneumococcal antibody. Additionally pneumococci in the subarachnoid space may also gain access to the cerebral venous system (Boulnois, 1992).

1.1.5.4 Otitis media

Streptococcus pneumoniae has been shown to be the most common causative organism for otitis media or second only to non-typable *Haemophilus influenzae* (Dowel

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et al., 1999). Prior infection by a respiratory virus is thought to play a major role by causing congestion of the opening to the eustachian tube. Some studies (Gray et al., 1980) have shown that when infection occurs, in most cases it follows fairly closely after colonization by a new serotype, although of course, most instances of colonization occur without disease. Results from the study of Salazar et al., (1997), demonstrated that low maternally derived serum immunoglobulin G (IgG) antibodies to pneumococcal capsular polysaccharides combined with the inability of infants to produce anti-capsular polysaccharide antibody are the reasons for the onset of otitis media in the first six months of life. Faden et al., (1997) demonstrated a direct relationship between frequency of colonization and episodes of otitis media in infants less than 1 year. They also stated that early colonization might be a risk factor for first infection and recurrent disease as well. Otitis media does not only affect infants and children but it also affects adults who ultimately may suffer complications such as mastoiditis, meningitis or brain abscess (Schwartz and Brown, 1992).

1.1.5.5 Sinusitis

Important in the pathogenesis of this infection is congestion of the mucosal membranes caused by allergy or viral infection. The resulting obstruction prevents clearance of bacteria. The accumulation of fluid in the para-nasal sinus cavities, even during colds (Gwaltney et al., 1994) provides a medium for bacterial proliferation and subsequent acute sinus infection.

1.1.5.6 Other pneumococcal infections

Pneumococcal peritonitis, resulting from transient bacteremia following a primary respiratory infection, occurs most commonly in children with pre-existing ascites due to nephrosis (Gorensek et al., 1988). Endocarditis is an important complication of

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pneumococcal disease. Cure can be achieved with early recognition and appropriate antibiotic treatment but unrecognized or untreated pneumococcal endocarditis remains a rapidly lethal infection (Powderly et al., 1986). Pneumococci have also been implicated as a cause of cellulitis (Thirumoorthi et al., 1978). Bone and joint infections may also be caused by pneumococci (Burman et al., 1985).

1.1.6 IMMUNITY TO *S. pneumoniae*

Immunity to infection with pneumococci is type specific and depends on antibodies to capsular polysaccharide and on intact phagocytic function. Vaccines can induce production of antibodies to capsular polysaccharide. The Advisory Committee on Immunization Practices (ACIP) of the United States of American recommends that the vaccine be used more extensively and administered to all persons in the following groups: a) persons aged > 65 years, b) immunocompetent persons aged ≥ 2 years who are at increased risk for illness and death associated with pneumococcal disease because of chronic illness, c) persons aged ≥ 2 years with functional or anatomic asplenia, d) persons aged ≥ 2 years living in environments in which the risk for disease is high and e) immunocompromised persons aged ≥ 2 years who are at high risk of infection (Centers for Disease Control and Prevention, 1997).

With increasing emergence of penicillin-resistant pneumococci, it seems reasonable to consider improving conditions under which the vaccine is administered. For example, adults older than 60 years could be vaccinated and then re-vaccinated every 5 – 7 years. Persons whose lifestyle places them at risk of acquiring HIV infection could be vaccinated whenever they first have contact with the health care system (Musher, 2000).

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1.1.7. TREATMENT OF PNEUMOCCAL INFECTION

Before penicillin became widely available, mortality and morbidity estimates for pneumococcal disease were extremely high. The sulfonamides were the only antimicrobial agents that could be used at the time but sulfonamide-resistant strains were known as early as 1943 (McGee, 1998).

Pneumococci used to be highly susceptible to penicillin and other beta-lactam agents, which made this group of antimicrobials the drugs of choice. However, in recent years pneumococci with decreased susceptibility to beta lactams have emerged. Resistance to penicillin is now wide spread and is rapidly increasing all over the world (Appelbaum, 1992; Krinstinsson et al., 1992).

Tetracycline-resistant pneumococci were not reported until 1962. Erythromycin-resistant pneumococci were recognized in 1967 and chloramphenicol-resistant strains were reported in 1970 (Mc Gee, 1998). Pneumococci resistant to penicillin appeared for the first time in South Africa, in Durban in 1977. These strains were also resistant to chloramphenicol (Appelbaum et al., 1977). These findings prompted an increased surveillance in Johannesburg where the presence of resistant pneumococci with at least five different patterns of resistance was uncovered: resistance to penicillin and tetracycline, to penicillin, tetracycline and chloramphenicol and multiple antibiotics (Jacobs et al., 1978). The problem of penicillin resistance or multiple resistances still continues in South Africa and is rapidly increasing all over the world (Appelbaum, 1992, Kristinson et al., 1992).

Antimicrobial resistance in pneumococci involves either modification of chromosomal genes often by horizontal gene transfer (penicillin and probably co-trimoxazole

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resistance) or chromosomally located transposons (tetracycline, kanamycin, erythromycin and chloramphenicol resistance) (Hall, 1998). A combination of vancomycin with a broad spectrum cephalosporin (ceftriazone or cefotaxime) is recommended for treatment of meningitis caused by resistant strains (Quagliarello & Scheld, 1997). These cephalosporins are also used for the treatment of pneumonia caused by pneumococci that are susceptible or intermediately resistant to these drugs.

1.1.8 PREVENTION OF PNEUMOCOCCAL INFECTION

The history of attempts to prevent pneumococcal infection by prophylactic vaccination extends over 65 years, dating from the original efforts of Wright and his associates to prevent pneumococcal pneumonia in South African gold miners using heat-killed pneumococci. Their studies were carried out without knowledge about the variety of pneumococcal types with antigenically different polysaccharide capsules; therefore they were unable to demonstrate with certainty that their vaccination could prevent pneumococcal pneumonia (Lund and Henrichsen, 1978; Austrian, 1977).

By the end of 1977 licence was given in the USA to a vaccine composed of 14 highly purified pneumococcal polysaccharides produced from types 1, 2, 3, 4, 6A, 7F, 8, 9, 12F, 14, 18C, 19F, 23F and 25. One dose consisted of 50 μg of each polysaccharide. A number of studies showed that the vaccine was safe, antigenic and 80 – 90% effective in providing protection against type specific pneumonia (Austrian, 1977). However, in children under 2 years of age (Sell et al., 1981; Makela et al., 1981), and in splenectomised patients/individuals, the pneumococcal vaccine has greatly reduced immunogenicity (Hosea et al., 1981). In certain groups, persons are at risk of acquisition of pneumococcal infection throughout their lives. Such groups comprise patients with sickle cell anemia, Hodgkin's diseases, immune deficiencies or chronic disorders. The

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14-valent vaccine induces antibody production in some but not all of these individuals. However, even if the antibody response is reduced, it is worthwhile to offer vaccination to these defined risk groups (Burman et al., 1985).

The duration of protection is not yet known, but the increased level of type specific antibodies appears to persist for at least five years and may even be longer. Re-vaccination does not lead to a booster-effect and apparently should not be performed at less than three-year interval (Lund and Henrichsen, 1978; Hilleman et al., 1981). Robbins et al. (1983) proposed a second and expanded formulation for the pneumococcal vaccine which was to include types 1, 2 3 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F and 33F. This vaccine is currently in use and is recommended for individuals particularly susceptible to pneumococcal infection because of age, underlying disease or immune status. Several researchers have studied the efficacy of this vaccine (Christenson et al., 2001). Since this vaccine has greatly reduced immunogenicity in children less than 2 years of age, several clinical trials of immunogenicity and impact on carriage of various global conjugate vaccine formulations have been done (Obaro et al., 1996; Mbelle et al., 1999).

In the study by Byington et al., in which IPD isolates from children were compared, the introduction of PCV 7 vaccine decreased the incidence of IPD with 27%. However, an increase in IPD caused by non-PCV7 serotypes was observed (Byington et al., 2005). A decrease of IPD was observed among infants in the USA during the study period 1997-2004, where PCV was introduced year 2000 (Poehling et al., 2006). The efficacy of PCV 9 in rural Africa was shown to be high. In this trial, the vaccine covered 65% of all identified serotypes in the placebo group (Cutts et al., 2005). In conclusion, several studies have shown a general decrease in IPD after the vaccine introduction.

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1.2 IMMUNOMODULATION OF INFLAMMATION

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The limited efficacy of the available antimicrobial agents in immunocompromised individuals, combined with the increasing resistance to these agents, has led to an urgent need for new therapies for infectious diseases (Pirofski and Casavedall, 2006). Immunomodulation represents a novel approach to antimicrobial therapy that depends on bolstering host immunity, rather than direct antimicrobial activity. Immunomodulation refers to the action undertaken by the medication on autoregulating processes that steer the immunological defense system (Casadevall, 2006). Immunomodulators can be divided into those that are specific to pathogens (pathogen specific) and those that are not specific to pathogens (non-specific). Pathogen -specific immunomodulators include antibody reagents and vaccines and non- specific immunomodulators include cytokines, antimicrobial peptides, certain antimicrobial drugs and microbes such as probiotics (Casadevall and Pirofski, 2003).

The inflammatory process is one of these responses and involves a set of localized events designed to restrict pathogen spread and reduce the amount of tissue damage that occurs. Consequently, the ability of an organism to mount and successfully resolve effective inflammatory responses is imperative to subsequent health of the host. However, damage to host tissues as a side effect of the inflammatory response is more prominent (Strieter et al., 2003). This unregulated runaway inflammation can lead to organ failure and death. Inflammation is the hallmark of the majority of bacterial infections and therefore, managing inflammation, by administering or influencing cytokines, is an attractive adjuvant to targeted antibiotic therapy in severe infections (Barton, 2008).

Inflammation is a general term used to describe the cascade of events that occurs in response to infection or tissue damage. The study of this process predates the modern

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era of molecular biology, and consequently many of the hallmarks of inflammation involve qualitative measures of cellular responses. However, the last several decades have seen an enormous increase in the understanding of molecular pathways controlling inflammation and the early response to microbial infection. In particular, Toll-like receptors (TLRs) and other pathogen recognition receptors (PRRs) play a crucial role in the induction of early signals that establish the inflammatory setting (Medzhitov, 2007). The inflammatory response to infection has traditionally been classified as 4 distinct phases: recognition of infection, recruitment of cells to the site of infection, elimination of the microbe, and resolution of inflammation and return to homeostasis (Kumar et al., 2007). Layered on this local inflammatory response is the induction of adaptive immunity, which occurs as DCs and antigen drain from the site of inflammation to regional lymph nodes (Mellman and Steinman, 2001).

1.2.1. IMMUNOMODULATION: A THERAPEUTIC APPROACH TO INFECTIONS

Systemic inflammation and the ensuing organ damage are a major cause of mortality for bacterial infection. Defining the signals that orchestrate coordinated inflammatory response is a clinical relevant problem with implications for our overall understanding of how the immune system senses stress in the form of infection and tissue damage (Kumar et al., 2007).

Soon after the first adequate dose of an antimicrobial drug to treat a wide spectrum of infectious disease such as syphilis, relapsing fever, etc, a complex clinical reaction may occur, often characterized by sudden fever and hypotension. This phenomenon was first described by Jarisch and Herxheimer during therapy of secondary syphilis using mercury and is well known as the Jarisch –Herxheimer reaction. Typically, this systemic reaction occurs 1 to 2 h after the initial treatment of syphilis with effective antibiotics, especially

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penicillin. It consists of the abrupt onset of fever, chills, myalgias, headache, hyperventilation, vasodilation with flushing, and mild hypotension (Herxheimer and Krause, 1992; Jarisch, 1895). In the early days of antibiotic therapy, lethal vasomotor collapsed after the first dose of chloramphenicol was noted. A rapid and strong release of endotoxin was suspected as the cause of adverse effects associated with the initiation of treatment. This led to the advice to start with a low dose of antibiotic. Worsening of clinical symptoms after initiation of antibiotic treatment during sepsis or meningitis has also frequently been observed, leading to the concept of immunosuppresion by corticosteroids and other agents concomitant or prior to the first antibiotic dose.

A therapeutic approach in meningitis and sepsis, which avoids immunosuppression, is to influence the liberation of bacterial proinflammatory cell wall products. Compared to continuing replication and autolysis, all bacteriostatic antibiotic regimens eventually reduce the release of bacterial components. Whereas cell wall–active antibacterials can temporarily enhance the liberation of toxic or proinflammatoty bacterial compounds, bactericidal antibiotics acting by inhibition of RNA or protein synthesis or DNA replication (macrolides, rifamycins, clindamycin) delay or even circumvent bacterial lysis (Bhakdi et al., 1991). They are promising with respect to reducing toxic or proinflammatory bacterial compounds when they possess bactericidal in vivo activity against Gram-positive and gram-negative bacteria causing sepsis and meningitis (Bhakdi et al., 1991; Timmerman et al., 1993).

Gram-positive as well as gram-negative microorganisms can cause septic shock. The cell walls of gram positive organisms contain various components, mainly LTA, TA, and peptidoglycan, which elicit the host's inflammatory response (Bhakdi et al., 1991; Timmerman et al., 1993). LTAs exhibit many biological activities mediated by the induction of cytokines like IL-1,IL-6, IL-8, IL-12, and other molecules activating

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inflammation and peptidoglycan induce the release of TNF alpha, IL-1 beta, and IL-6 from cultured macrophages/ monocytes (Bhakdi et al., 1991).

Bacterial products stimulate monocytes, macrophages, and endothelial and glial cells and can exert direct cytotoxicity. Initiation of treatment by cell wall-active antibiotics causes a rapid release of proinflammatory or toxic compounds from both gram-negative and gram-positive bacteria. This phenomenon is less pronounced with PBP-2 than with PBP-3 specific beta-lactam antibiotics and can be absent when antibiotics inhibiting bacterial protein synthesis are employed. High antibiotic concentrations release smaller amounts of bacterial compounds than doses close to the MIC. Quantitative differences in the release of proinflammatory bacterial compounds correlate with antibiotic –initiated morphological changes in the bacteria (Bone et al., 1992).

1.2.1.1. Neutralization of proinflammatory bacterial products

Neutralization of proinflammatory bacterial products is a logical strategy to prevent harmful effects. However in practice the potential of this approach is limited because no single agent neutralizes both Gram-negative and Gram-positive bacterial products. Preliminary studies evaluating antibody neutralization of the gram-positive pneumococcal cell wall have not been followed by successful clinical studies (Toumanen, 1988). Neutralization of the Lipid A moiety in gram-negative bacterial meningitis by intrathecal administration of neutralizing antibodies, polymyxin B has been studied in several animal models of bacterial meningitis and had only limited efficacy when administered at clinical relevant time-point in live infection models (Tauber et al., 1987). Therefore, this type of adjuvant treatment is likely to fail in clinical practice, most probably because the treatment is given too late following the unleashing of the inflammatory cascade. This is

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supported by the disappointing results of clinical trials evaluating LPS neutralization in gram-negative sepsis (Ziegler, 1991)

1.2.1.2 Neutralization of cytokines and inhibition of cytokine production

Cytokines are key regulators of the immune response, switching on and off whole cascades of events. These cytokines are released by macrophages, microglia, etc. Four major avenues exist to intervene in the inflammatory response at the cytokine level: inhibition of transcription of the cytokine genes, blockage of the cytokine itself or by cleavage to activate the procytokine, and antagonism of the cytokine receptor and its downstream signaling pathway. Administration of immunomodulatory or anti-inflammatory cytokines has been investigated as an alternative approach to modulate or inhibit pro-inflammatory cytokine production. Modulation of the cytokine cascade in sepsis has focused primarily upon those agents which sit at the "head" of the pathway, TNF-alpha and IL-1 (Saukkonen et al., 1990).

An alternative target for cytokine inhibition may be found in enzymes involved in the activation of procytokines. TNF- alpha is produced as membrane associated pro-TNF-alpha and cleaved to its active soluble form by TNF-alpha-converting enzyme, a metalloproteinase closely related to matrix metalloproteinases. Experiments in the neonatal rat model of bacterial meningitis showed significantly downregulated CSF levels of TNF by combined inhibition of matrix metalloproteinases and TNF-alpha converting enzyme with a broad spectrum hydroxamic acid-based metalloproteinase inhibitor (BB-1101) and the incidence of seizures, mortality, and neural injury was significantly decreased (Leib et al., 2001). Current inhibitors of TNF-alpha-converting enzyme have a low potency, necessitating the use of high doses; however, it is expected that modifications will result in improved agents (Barlaam et al., 1999). Similarly, IL-1 beta is

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formed by the cleavage of its precursor pro-IL-1beta by caspase-1. Treatments of rabbits and rats with experimental pneumococcal meningitis with the caspase-1 inhibitor z-VAD-fmk (benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone) resulted in a reduced inflammatory response with lower fever and less neuronal apoptosis (Koedel et al., 2002).

Intervention at the transcriptional level regulates the expression of many cytokines at once. NK-kappa B is an important transcriptional factor, first recognized for its role in the immune system. Various anti-inflammatory drugs including corticosteroids and anti-inflammatory cytokines such as IL-10 inhibit the NF-kappa B pathway (Koedel et al., 2000). In resting cells, the NF- kappa B heterodimer remains in the cytoplasm, where it is associated with inhibitory I-kappaB proteins. Stimuli from outside the cell activate I-kappaB via phosphorylation and induce its proteolytic degradation, leading to translocation of NF-kappa B to the nucleus, which results in a concerted activation of target genes. Pharmacological manipulation of NF-kappaB activation was investigated in experimental meningitis –associated Central nervous system complications and clinical symptoms and NF-kappaB inhibitor improved clinical status (Koedel et al., 2000). Given the broad involvement of NF-kappa B in cell regulation, it is expected that systemic inhibition will also affect nonimmune cells. The challenge is to examine components of the NF-kappa B signaling pathway that can be inhibited without causing unacceptable side effects (Koedel et al., 2000).

1.2.2 ENDOTOXIN

The incidence of Gram-positive sepsis has increased dramatically over the past decade making it almost as common as Gram-negative sepsis (Bone, 1991). The manifestations of Gram-positive sepsis and Gram-negative sepsis share some common clinical features suggesting common pathways of activation. Macrophages and monocytes are thought to

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play a major role in the systemic inflammatory response in Gram-negative and Gram-positive sepsis (Sweet and Hume, 1996; Fenton and Golenbock, 1998). Bacterial cell wall components of Gram-positive and Gram-negative bacteria activate monocytes and macrophages to produce several inflammatory cytokines such as TNF-alpha and IL-6 and also induce a symptom like endotoxin shock in experimental animals. These include bacterial lipopeptides, peptidoglycan, muramyl dipeptide from Gram-positive bacteria, LPS from Gram-negative bacteria and bacterial DNA containing unmethylated CpG motif. These bacterial components have also been shown to induce tolerance to the subsequent stimulation (Ziegler-Heitbrock, 1995; Zeisberger and Roth, 1998).

1.2.2.1 TLR signaling pathway

TLRs are PAMPs and signal the presence of infection and can recognize several, structurally unrelated ligands by directly binding to them. Activation of signal transduction pathways by TLRs leads to the induction of various genes that function in the host defence, including inflammatory cytokines, chemokines, major histocompatibility complex (MHC) and co-stimulatory molecules (Verstak et al., 2007). Stimulation of IL-1R or TLR by their respective ligands causes the adapter protein MyD88 to be recruited to the receptor complex, which in turn promotes its association with the IL-1R –associated kinase (IRAK) via an interaction between the respective death domains of each molecule. This is followed by the autophosphorylation of IRAK, which dissociates from the receptor complex and interacts with the TRAF-6 (tumor –necrosis –factor –receptor-associated-factor 6). From TRAF-6, two signaling pathways diverge; one ultimately leads to nuclear factor-kB (NF-kB) activation, and the other to mitogen-activated protein (MAP) kinase activation (Hemmi et al., 2000).

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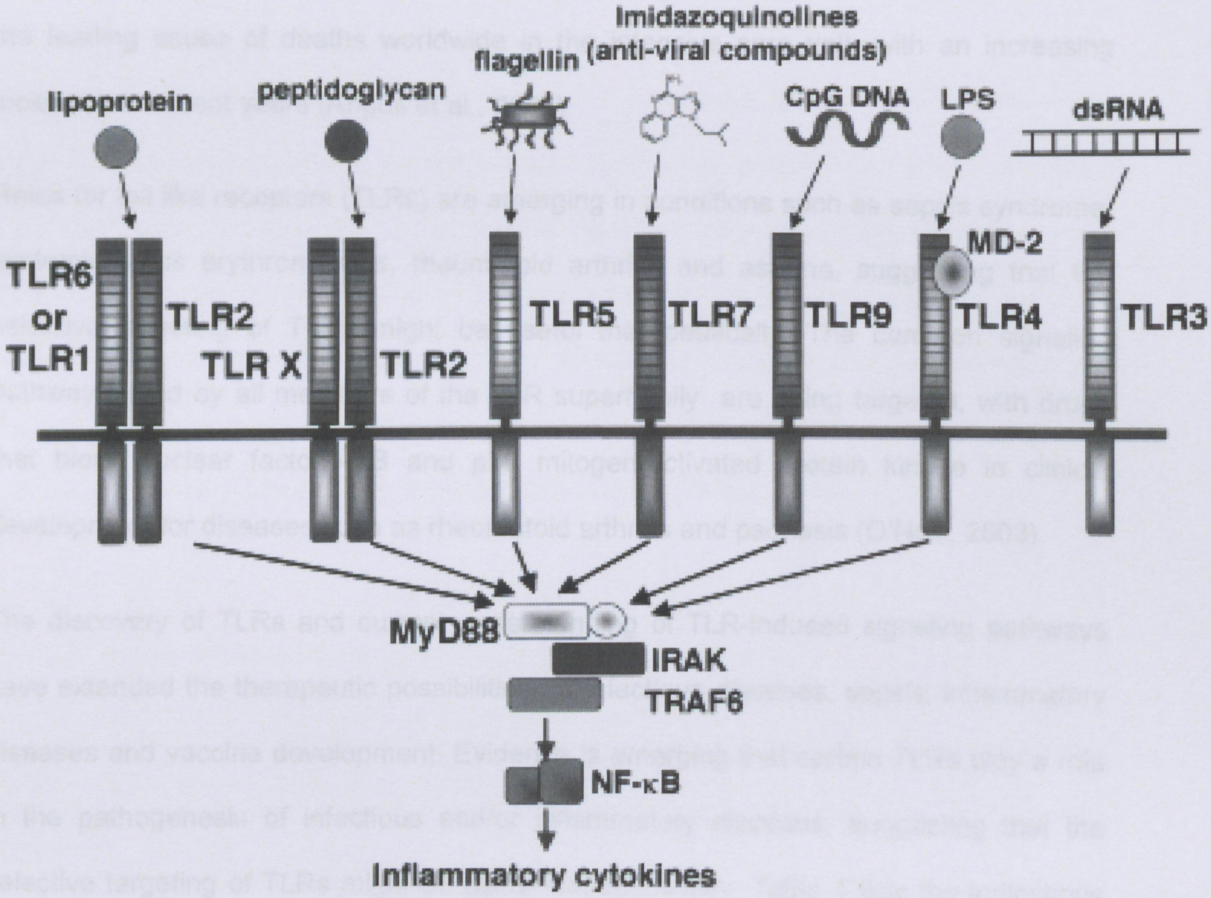


Figure 1.2: Summary of ligands recognized by TLR family (Akira et al., 2003).

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1.2.2.2 Targeting TLRs and Potential targets on the TIR domain pathway

Despite improvements in patient management, bacterial sepsis and septic shock remain the leading cause of deaths worldwide in the intensive care unit, with an increasing incidence in recent years (Angus et al., 2001).

Roles for toll like receptors (TLRs) are emerging in conditions such as sepsis syndrome, systemic lupus erythromatosis, rheumatoid arthritis and asthma, suggesting that the selective targeting of TLRs might be useful therapeutically. The common signaling pathways used by all members of the TLR superfamily are being targeted, with drugs that block nuclear factor –kB and p38 mitogen-activated protein kinase in clinical development for diseases such as rheumatoid arthritis and psoriasis (O'Neill, 2003).

The discovery of TLRs and current understanding of TLR-induced signaling pathways have extended the therapeutic possibilities for infectious diseases, sepsis, inflammatory diseases and vaccine development. Evidence is emerging that certain TLRs play a role in the pathogenesis of infectious and/or inflammatory diseases, suggesting that the selective targeting of TLRs might be useful therapeutically. Table 1 lists the indications where manipulation of TLRs might have therapeutic potential. The most studied TLRs in this regard are TLR4, anti-viral TLRs and TLR9.

TLRs are potential therapeutic targets in septic shock. In sepsis, bacteria and bacterial products activate an uncontrolled network of host-derived mediators, such as pro-inflammatory cytokines, leading to multi-organ failure, cardiovascularcolapse and death. Inappropriate TLR response to bacterial products closely associates with the pathogenesis of septic shock (O'Neill, 2003). Most TLR pathways once stimulated ultimately lead to the activation of the transcription factor NFkB and the MAP kinase family. There are already clinical trials underway to try and block NFkB and p38 MAP

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kinase; however, there is no drug that has been found to be clinically efficacious in the treatment of inflammatory conditions (Khor et al., 2007).

Antagonists for TLR proteins might, therefore, be useful to inhibit the harmful pro-inflammatory response that is associated with systemic microbial infections. There might be three basic strategies that could reduce signal transduction through TLRs in septic shock: (1) soluble TLRs that bind and neutralize the microbial ligands, (2) antibodies or small molecules that block the binding of ligands or protein-ligand complexes to the receptors, (3) specific small-molecular-mass inhibitors of the common signaling pathways that are induced by ligand-TLR interaction (O'Neill, 2003).

1.2.2.3 Endotoxin tolerance

A unique property of endotoxin is that it can modulate in mammals a transient state of either hypersensitivity to itself, seen in animals with bacterial infections, or low responsiveness induced by single or repeated injections of low LPS amounts in human volunteers and experimental animals (Johnston and Greisman, 1985). The later phenomenon, referred as endotoxin tolerance, and its molecular mechanism(s) has been the subject of extensive studies, in large part due to its potential application for correction and/or prevention of the pathophysiologic sequelae associated with endotoxemia and Gram-negative sepsis (Freudenberg et al., 1996; 1998).

Endotoxin tolerance is defined as a reduced responsiveness to a lipopolysaccharide (LPS) challenge following a first encounter with endotoxin. Endotoxin tolerance has been also named deactivation, adaptation, desensitization, anergy, refractoriness, or reprogrammation. In many aspects it resembles the

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immunodepression/immunosupression/immunoparalysis which has been reported in patients with sepsis or non-infectious systemic inflammatory response (SIRS) such as trauma, surgery, cardiopulmonary bypass, cardiac arrest and resuscitation and hemorrhagic shock. It is an attenuated inflammatory and physiologic response to an endotoxin challenge that may be induced experimentally by prior exposure of an animal to nonlethal doses of endotoxin or other molecules that cause substantial activation of an inflammatory response (Murphey et al., 2007).

1.2.2.4 Endotoxin prophylactic protection

Some studies suggested that the induction of endotoxin tolerance might impair resistance to infectious processes. For example, a reduced level of interferon was reported in animals pretreated with LPS and further challenged with Newcastle disease virus (Youngner and Stinebring, 1965). In addition, a reduced Leishmanicidal activity was observed after pre-exposure of macrophages to LPS (Severn et al., 1993), and an impaired lung clearance of *Pseudomonas aeruginosa* was shown after LPS exposure (Mason et al., 1997).

However, administration of purified LPS has been found to confer prophylactic protection from subsequent bacterial or viral challenge in various animal models, presumably via stimulation of innate immunity (Berger, 1967). Recently, the analysis of infectious models using either *Cryptococcus neoformans* (Rayhane et al., 2000), or *Salmonella enteriditis* (Lehner et al., 2001) established that mice rendered tolerant to LPS had an increased resistance to fungal and bacterial infections, associated with a reduced burden of pathogens within the tissues. The intrauterine administration of LPS in cattle was shown to facilitate clearance of chronic intrauterine infections associated with infertility (Singh et al., 2000). However, despite these potentially beneficial effects, the pharmacologic use of

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purified LPS (or lipid A) is precluded by its extreme toxicity; LPS is highly pyrogenic and promotes systemic inflammatory response syndrome (Johnson, 1994). In an effort to uncouple the immunomodulatory effects of lipid A from its toxicity, Masihi et al. (1986) developed monophosphoryl lipid A (MLA); MLA comprises the lipid A portion of LPS from which the (*R*)-3-hydroxytetradecanoyl group and the 1-phosphate have been removed by successive acid and base hydrolysis. LPS and MLA induce similar cytokine profiles, but MLA is at least 100-fold less toxic (Myers et al., 1990). MLA, the active ingredient of MPL adjuvant, has been administered in over 273,000 doses in human clinical studies of the next generation of vaccine adjuvants. MLA alone or in combination with other molecules elicits protective responses when administered prior to challenge with pathogenic viruses (influenza virus), bacteria (*Listeria monocytogenes* and *Salmonella enterica* serovar Enteritidis), or parasites (*Toxoplasma gondii*) (Masihi et al., 1986; Persing et al., 2002).

The mechanisms by which endototxin tolerance develops are still not completely clear and over the last decades several pathophysiological pathways have been proprosed. The anti-inflammatory hypothesis is based on the finding that endotoxin tolerance does not attenuate the release of different cytokines similarly, resulting in attenuated levels of proinflammatory cytokines with concomitant increased levels of anti-inflammatory cytokines, leading to the observed tolerance (Sly et al., 2004)

1.2.3 ADENOSINE AS ANTIINFLAMMATORY

Inflammatory processes are crucial for defense against pathogens, but inappropriate and or prolonged inflammation contributes to the pathogenesis of major diseases (Tarkowski and Wagner, 1998). The demonstration that the extracellular adenosine A_{2A} receptor participates in an endogenous immunosuppressive loop that serves to down-regulate

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inflammation in vivo by a non-redundant mechanisms (Ohta and Sitkovsky, 2001) heightened the interest in naturally occurring purines in the regulation of inflammation and tissue protection. It is known that adenosine possesses anti-inflammatory properties and that adenosine analogs can protect mice from a variety of inflammatory diseases such as septic shock by inhibiting the release of free radicals and pro-inflammatory cytokines from different immune cells (Hasko et al., 1998). The attractiveness of an adenosine-mediated anti-inflammatory pathway has been enhanced by the realization that widely used anti-inflammatory drugs, such as methotrexate and sulfasalazine; seem to exert their effects by inducing the release of endogenous adenosine, which then signals through adenosine receptor. Thus, the triggering of a natural anti-inflammatory pathway by ligands of adenosine receptors represents an attractive immunomodulation strategy for the treatment of inflammatory disease (Cronstein, 1995).

1.2.3.1 Adenosine and adenosine receptors

Adenosine, an endogenous purine nucleoside, is a biological active extracellular signaling molecule that is formed at the site of infection or metabolic stress associated with hypoxia, ischemia, trauma, or inflammation and is one of the best-characterized endogenous immunomodulator molecules (Ohta and Sitkovsky, 2001). This environment triggers immunosuppressive cAMP accumulation in immune cells via signaling through A_{2A} adenosine receptors. The A_{2A} adenosine receptor signaling suppresses inflammation in a delayed, negative feedback manner because of inhibition by cyclic AMP of molecular intermediates of pro-inflammatory signaling pathways (Ohta and Sitkovsky, 2001). Appropriate A_{2A} adenosine receptor agonists and antagonists have promise for

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the engineering of inflammatory processes in vivo by selectively utilizing or disengaging the natural, endogenous anti-inflammatory pathway.

Adenosine is an extracellular messenger with a large spectrum of biological activities, including a protective role in acute inflammation and the promotion of wound healing (Montesinos et al., 1997). Adenosine mediates many of its effects through one or more of four different types of G-coupled cell surface molecules or receptors, the adenosine A_1, A_{2A}, A_{2B}, and A_3 receptors (Linden, 1991; Linden, 1994, Ongini and Fredholm, 1996). These receptors are seven membrane-spanning proteins that couple to heterotrimeric G proteins to trigger a variety of intracellular signaling pathways (Linden, 1994, Ongini and Fredholm, 1996). Cells of the monocytes/macrophages lineage have been documented to express all four adenosine receptor (Khoa et al., 2001).

Ligation of these receptors located on immune cells has important effects on a variety of immune cell functions including antigen presentation (Xaus et al., 1999), phagocytosis (Salmon et al., 1993), negative selection (Resta et al., 1997), or target cell killing (Priebe et al., 1990). An important feature of the regulation of immune processes by adenosine is that adenosine alters immune cell production of soluble mediators such as cytokines (Xaus et al., 1999), free radicals (Hasko et al., 1996). Stimulation of adenosine receptor has been shown to result in an anti-inflammatory, deactivated macrophage phenotype (Khoa et al., 2001). Macrophage deactivation by adenosine also suppresses antibacterial defense mechanisms indirectly. Adenosine decreases the production of proinflammatory cytokines that orchestrate inflammatory/immune functions of other cell types and act as autoregulators of macrophage function. The adenosine receptor subtypes mediating the inhibitory effect of adenosine on TNF-alpha production have been extensively studied (Hasko and Szabo, 1998). In LPS-stimulated

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monocytes/macrophages, adenosine receptor activation attenuates the production of several proinflammatory cytokines, including TNF-alpha, (Reinstein et al., 1994) and IL-12 (Khoa et al., 2001). In contrast to the suppressive effect of adenosine on the production of these proinflammatory mediators, adenosine up-regulates IL-10 production by LPS –stimulated monocytes/macrophages (Le Moine et al., 1996). Thus, adenosine acts as an anti-inflammatory agent, suppressing expression of inflammatory cytokines. Adenosine helps maintain tissue integrity by modulating immune system function (Hasko et al., 2002). Although the immune response to tissue injury has an essential role in preventing tissue homeostasis, uncontrolled inflammation or immune activation can inflict further damage on the affected tissue. It appears that the release of adenosine followed by its binding to adenosine receptor on immune cells represents a potent endogenous immunosuppressive pathway that regulates the exuberant immune response to harmful external insults.

1.2.3.2 Role of adenosine as an immune modulator

Recent in vitro and in vivo studies clearly confirm the beneficial role of adenosine as an immune modulator. First adenosine is released in the vicinity of immune cells in tissues subjected to various forms of injurious stimuli, including inflammation. Second, in the majority of experimental systems, adenosine is immunosuppressive as a result of adenosine receptor occupancy on various immune cell types. Third, removal of endogenous adenosine signaling exacerbates immune activation and consequently aggravates tissue dysfunction following injurious stimuli.

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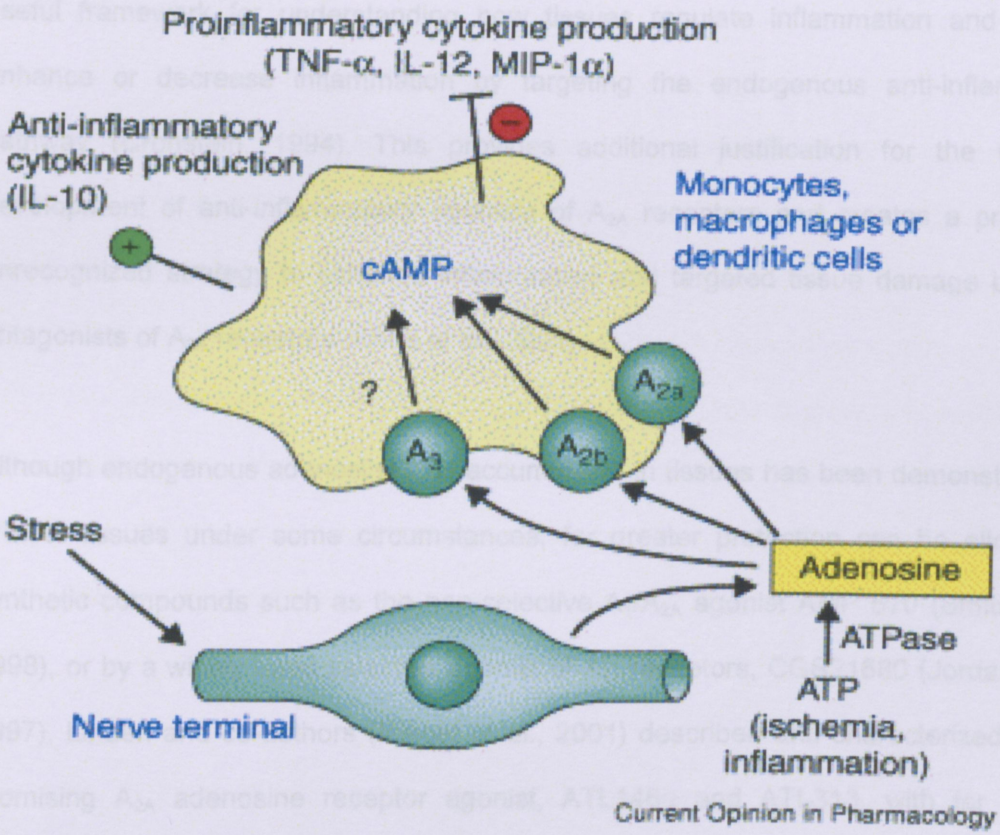


Figure 1.3. Adenosine is released from a variety of sources during multiple organ failure. Stress can cause the release of adenosine from sympathetic nerve terminals and ischemia and inflammation can generate adenosine via the release of ATP. The released adenosine can bind to A_{2a}, A_{2b}, and A_3 receptors that are present on immune cells (monocytes, macrophages or dendritic cells). Adenosine receptor stimulation suppresses production of proinflammatory cytokines (e.g. TNF-α, IL-12 and macrophage inflammatory protein-1α [MIP-1α]) and increases production of anti-inflammatory cytokines (e.g. IL-10) (Hasko et al., 2002).

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Identification of A_{2A} adenosine receptors as "natural" brakes of inflammation provided a useful framework for understanding how tissues regulate inflammation and how to enhance or decrease inflammation by targeting the endogenous anti-inflammatory pathway (Cronstein, 1994). This provides additional justification for the vigorous development of anti-inflammatory agonists of A_{2A} receptors and creates a previously unrecognized strategy to enhance inflammation and targeted tissue damage by using antagonists of A_{2A} receptors (Khoa et al., 2001).

Although endogenous adenosine that accumulates in tissues has been demonstrated to protect tissues under some circumstances, far greater protection can be elicited by synthetic compounds such as the non-selective A_1/A_{2A} agonist AMP 570 (Smits et al., 1998), or by a widely used selective agonist of A_{2A} receptors, CGS21680 (Jordan et al., 1997). Linden and co-authors (Rieger et al., 2001) described and characterized a very promising A_{2A} adenosine receptor agonist, ATL146e and ATL313, with far greater potency and selectivity than CGS21680. This agonist has been shown to markedly reduce ischemia-reperfusion or traumatic injury and neutrophil infiltration in many tissues. Selective A_{2A} receptor agonists may be effective than endogenous adenosine as inhibitors of inflammation for two reasons. First, adenosine and its breakdown product, inosine, may activate pro-inflammatory pathways that are mediated by A_1 (Crostein et al., 1990) or A3 receptor (Jin et al., 1997). Second, optimal effectiveness of A2A receptor activation probably requires the triggering of receptors on circulating leucocytes, lymphocytes, platelets, and /or vascular endothelium. In this sense, synthetic compounds are much more effective at activating receptors in blood than adenosine, which is rapidly metabolized in blood (Moser et al., 1989). Thus, results of preliminary studies point to the promising uses of A_{2A} receptor agonists as therapeutic agents to treat ischemic, infection-associated, and trauma-associated inflammation provided that

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possible side effects are taken care of by designing more tissue-specific treatments (Jacobson et al., 2001).

The demonstration of a crucial role of extracellular adenosine A2A receptors in the down-regulation of inflammation in vivo represents the first example that physiological, metabolic changes in the inflamed local tissue environment can serve as a "reporter" of excessive tissue damage that can provide a signal to terminate inflammatory processes. This immunosuppressive mechanism functions by taking advantage of very fundamental cellular processes where physiologically abundant and ubiquitous molecules of adenosine can be produced by virtually all cells, resulting in a negative feedback loop to inhibit inflammation-producing immune cells and protect surrounding tissues (Sitkovsky, 2003).

Adenosine, whether endogenously released or exogenously administered, is a potent anti-inflammatory agent and is a potent inhibitor of stimulated neutrophil function (Cronstein, 1994). Endogenously released and exogenously added adenosine analogs can reduce neutrophil-mediated cellular injury (Cronstein et al., 1986) and block selectin- and Beta2-integrin-mediated neutrophil adhesion to endothelial cells in vitro (Cronstein et al., 1992). Adenosine, acting at its receptor, inhibits the production of a variety of inflammatory mediators by stimulated monocytes (Lappin and Whaley, 1984). Adenosine may also be mildly immunnosuppresive, since occupancy of adenosine receptors inhibits some lymphocyte functions as well (Kammer et al., 1983). Development of agents that enhance extracellular adenosine concentrations is a useful approach to the discovery of potent and possibly less toxic anti-inflammatory agents for the treatment of a wide variety of inflammatory conditions. Manipulation of inflammatory processes may include not only efforts to inhibit inflammation, but also the development of approaches to

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enhance local inflammatory processes. This is illustrated by the desirability to develop better adjuvants for vaccine development (Schijns, 2001) and stronger proinflammatory local tissue damage during immunotherapies of cancerous tumors (Chambers et al., 2001).

Accordingly, a current goal of pharmacological research is to develop both pro-inflammatory and anti-inflammatory drugs. This should be done with the understanding that the pharmacological can be a useful anti-inflammatory drug even if it targets a pathway that is not an important part of a physiological mechanism, but the rational development of valuable proinflammatory agents will require an improved understanding of natural inhibitors of inflammation. A rather obvious option is to enhance inflammatory processes simply by increasing the dose of inflammatory stimuli, but this may be accompanied by unacceptable side-effects. It appears that the least dangerous approach to enhancing inflammation may lie in antagonizing the physiological mechanisms that attenuate it in vivo, but this has not been possible. Thus, the efforts to reveal the endogenous mechanisms that terminate inflammation and thereby protect tissues from excessive damage may be richly rewarded by identification of better targets for immunomodulation and by providing options of enhancing inflammation through the use of novel drugs that antagonize natural anti-inflammatory processes and result in persistence of pro-inflammatory molecules in targeted local tissue environments. In addition, the identification of endogenous inhibitors of inflammation is expected to improve the design and development of novel inflammation-inhibiting drugs that will be able to recruit the natural pathway.

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1.2.4 ETANERCEPT

Etanercept (Enbrel, Immunex, Seattle, WA), an injectable tumor necrosis factor (TNF) receptor fusion protein that binds and inactivate human TNF, is used in active rheumatoid arthritis (www.centocor.com). Etanercept consists of the extracellular ligand-binding portion of the human p75 TNF receptor linked to the Fc portion of the human IgG1. Because it does not contain nonhuman material, it is theoretically less immunogenic. It is a genetically engineered TNF alpha receptor fusion protein, which is able to bind TNF alpha and reduce its bioavailability (www.immunex.com)

1.2.4.1 The pathological activities of TNF-α

The pathological activities of TNF-α have attracted much attention. For instance, although TNF-α causes necrosis of some types of tumors, it promotes the growth of other types of tumor cells. High levels of TNF-α correlate with increased risk of mortality (Rink and Kirchner, 1996). TNF-α participates in both inflammatory disorders of inflammatory and non inflammatory origin (Strieter et al., 1993). Originally sepsis was believed to result directly from the invading bacteria itself, but it was later recognized that host system proteins, such as TNF-α induced sepsis in response. Exogenous and endogenous factors from bacteria, viruses, and parasites stimulate production of TNF-α and other cytokine (Tracey and Cerami, 1994). Lipopolysaccharide from from bacteria cell walls is an especially potent stimulus for TNF-α synthesis. When cytokine production increases to such an extent that it escapes the local infection, or when infection enters the bloodstream, sepsis ensues. Systematic edema results in low blood volume, hypoproteinanemia, neutropenia and then neutrophilia and body organs fail and death may result. Victims of septic shock experience fever, falling blood pressure,

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myocardial suppression, dehydration, acute renal failure and then respiratory arrest (Janeway et al., 1999).

TNF-α seems to serve as a mediator in various pathologies. A few such examples include: Septic shock, Cancer, AIDS, Transplantation rejection, Multiple Sclerosis, Diabetes, Rheumatoid arthritis, Trauma, Malaria, Meningitis, Ischemia-Reperfusion Injury, and Adult respiratory distress syndrome. Since TNF-α plays a role in several diseases, a substantial amount of research has been conducted concerning TNF-α therapies and anti- TNF-α therapies. Because TNF-α exhibits anti tumor activity, research has been conducted to determine the protein's effectiveness against certain forms of cancers. Utilizing TNF-α tumoricidal activities has proved problematic, especially due to the cytotoxin's systematic toxicity. While higher doses of TNF-α may exhibit higher cytotoxicity, high doses also lead to systematic toxicity (National Cancer Institute, 1995). Some studies involving TNFR-75 and TNFR-55 mutants have suggested that the TNFR-75 receptor plays a role in systematic toxicity, while TNFR-75 mutants will exhibit cytotoxicity but not systematic toxicity (Van Ostade et al., 1993). Additionally, a mutant form of TNF-α which exists only in the transmembrane form acts only by cell-to-cell contact and may result only in cytotoxicity, suggesting that mutant forms of TNF-α might effectively be used therapeutically as against specific types of cancers (Perez et al.,1990).

Other research has focused upon inhibiting the effects of TNF-α in such diseases as Rheumatoid Arthritis, Crohn's Disease, AIDS, bacterial septic shock (caused by certain gram negative bacteria), and bacterial toxic shock (caused by superantigens) as well as in prevention of alloreactivity and graft rejection (Janeway et al. 1999). Mutant mice that lack TNF-α are resistant to gram-negative bacteria induced sepsis, and anti-TNF monoclonal antibodies have been used to effectively reduce or inhibit TNF-α activity

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(Beutler et al., 1985b). One hypothetical advantage of treatment with anti- TNF-α antibodies results from its role in multiple types of inflammation. It is often difficult to determine that inflammation in burn and trauma victims are of infectious etiology and warrant treatment with antibiotics; therefore another treatment strategy might involve anti- TNF-α therapy (Strieter, et al., 1993).

Strategies for preventing TNF-α activity include neutralization of the cytokine via either anti-TNF antibodies, soluble receptors, or receptor fusion proteins; supression of TNF-α synthesis via drugs such as cyclosporine A, glucocorticoides, or cytokine IL-10; reduction of responsiveness to TNF-α via repeated low dose stimulation; and lastly, by inhibition of secondary mediators such as IL-1, IL-6, or nitric oxide (Tracey et al., 1994). Pharmaceutical companies such as Peptech limited have developed different antibodies to TNF-α, some of which inhibit various TNF-α functions and others which do not affect protein activity. For instance, Remicade (TM) is a chimeric Igk monoclonal anti-TNF antibody manufactured by Centocor which has been used to treat Crohn's disease--a chronic inflammatory disease of the intestines (www.centocor.com). Soluble TNF-R will also neutralize TNF-α before it can bind to its target cell receptor. Another drug, Enbrel (TM), developed by Imunex Corporation is a fusion of two soluble TNF receptors and a human immunoglobulin (www.centocor.com). It has been approved for treatment of rheumatoid arthritis.

However, the efficacy of preventing septic shock has been questioned as a result of recent research which suggests that, in the absence of TNF-α, other cytokines will eventually initiate the inflammatory response. The authors of this study speculate that TNF-α production may instead play a key kinetic role by amplifying release of cytokines IL-A, IL-B, and IL-6 and thereby affecting the severity of a response to LPS (Amiot et al., 1997). Additionally, eliminating the stimulatory effects of TNF-α in disease such as AIDS

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presents problems because inactivation of TNF-α leaves the host at even greater risk for bacterial infections normally countered by TNF-α activity (Amiot et al., 1997).

Induction of TNF production along with other cytokines such as IL-1 and IL-6 during the early phase of bacterial infection is an example of the adverse effects following the excessive release of some cytokines. These cytokines are released in access during the acute host response to bacterial infection, cause worsening of the inflammation, and increase the severity of sequelae of the infection (Saez-Llorens et al., 1990). The production of TNF is induced by bacterial products during the initial phase of the lysis of bacteria that follows interaction with antimicrobial agents. Dexamethasone, which inhibits the production of TNF and IL-1 by the monocytes in vitro (Szefler et al., 1989), has been found to have a beneficial effect on the clinical outcome of bacterial meningitis caused by *H. influenza* in children. Other agents such as antibodies to TNF and antimicrobial agents that kill but do not lyse the bacteria are also promising (Odio et al., 1991).

Given the key role that TNF has in the pathogenesis of certain infections and in the modulation of the host defenses against them, it may have an important role in the prevention of certain infectious diseases, particularly with the use of antagonists that attenuate the adverse clinical consequences it induces.

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1.3. RATIONALE OF THE STUDY

Streptococcus pneumoniae is a major human pathogen and causes serious complications in patients with underlying diseases. The bacterium is the principal cause of bacterial pneumonia, in children and adults, in economically developed and developing countries. It is also a major cause of otitis media, bacterial meningitis and septicemia. Mortality from pneumococcal disease is particularly high in developing countries, where pneumococcal poneumonia has been estimated to account for 20-25% of all deaths in children under the age of 5 years (Gonzalez et al., 2006). Although major advances have been made in pneumococcal vaccines, they cannot be considered a complete solution because of the serotype variability and genomic plasticity of the pneumococcus. In addition, access to the vaccine is limited in in developing countries. Furthermore, antibiotic treatments are hampered by an ever-increasing frequency of resistant strains and even if treated with appropriate antibiotics *S. pneumoniae* septicemia has a mortality of 20-25%. The treatment of pneumococcal infections, such as meningitis, pneumonia, and bacteremia, with beta-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 (Nau and Eiffert, 2002; Orman and English, 2000).

The use of innate immune stimulation to improve survival has previously been described for Gram negative bacteria (*Salmonella typhimurium*) and Gram-positive bacteria (*Staphylococcus aureus, Listeria monocytogenes*. The current study determined if the enhancement of the innate immune response by exposure to sublethal doses of LPS would extend to a bacterial species that does not have an LPS component. Stimulation of innate immune response can be potentially exploited to provide short-term resistance

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to pneumococcal infections such as might occur in the setting of exposure to bio-threat agents or epidemic infections. These have implications for prophylactic treatment after an index case is identified or as adjunctive therapies with antibiotics in developing countries and may suggest that interventions designed to improve innate immunity to infection could have therapeutic benefits. 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.

Antibiotics can kill bacteria, but most do not strongly modulate the inflammatory response (Casadevall and Pirofski, 2003). Therefore, the addition of host response modulators to antibiotics might be beneficial for the treatment of severe pneumococcal pneumonia. Immunomodulation represents a novel approach to antimicrobial therapy that depends on bolstering host immunity, rather than direct antimicrobial activity. At present, clinical experience with non-specific immunomodulators as antimicrobial tools has been predominantly limited to cytokines. The rationale for the use of cytokines as adjunctive immunomodulators for infectious diseases is based on the concept that replacement or augmentation of natural mediators of host defense should enhance the antimicrobial effect of host immune mechanisms and/or antimicrobial agents (Casadevall and Pirofski, 2003).

The future use of adjunctive immunomodulators for infectious diseases requires a better understanding of microbial pathogenesis and the relative need for immune activation versus immune modulation in the context of the immune response of the affected individual (Casadevall and Pirofski, 2003). Knowledge of the effects of timing during the course of disease and the optimal dose agents often seems to be crucial to reaching a

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beneficial effect. Mediators may be beneficial early on in the disease process or in higher or low doses but may have detrimental effects later or in higher doses.

To our knowledge, investigations examining the effect that treatment with anti-inflammatory agents (Adenosine agonist ATL313, Eternecept), as an adjunctive therapy, has during either clinical or experimental pneumonia have not been performed. Identification of endogenous molecules such as adenosine, using adenosine agonist ATL313, that are capable of inhibiting activated immune cells is important in order to understand the physiologic mechanisms that regulate inflammation and to develop novel targets for anti- inflammatory drugs. The analysis of NK cell activation during the septic process may yield insights into importance of NK cells and their interactions that occur between the monocytes or macrophages compartment during the course of severe bacterial infections. 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. Studying the effect of ATL313 on NK cell activation will permit clarity on whether organ damage corresponds to areas where NK cells traffic and activation. 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.

In patients with sepsis, the success of treatment with anti-TNF is limited, in spite of an abundance of experimental data indicating that, during overwhelming sepsis in animals, elimination of endogenous TNF activity exerts strong protective effects (Van der Poll, 1995). These studies focused on the role that TNF plays in the pathogenesis of pneumococcal infection and do not explain how treatment with anti-TNF (treatment administered to animals with already -ongoing pneumococcal infection) influences the course of pneumococcal infection in the context of treatment with antibiotics.

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Consequently, it is important to determine the effect Etanercept (tumor necrosis factor (TNF) receptor fusion protein), therapeutically administered together with antibiotic ceftriazone, has in mice with ongoing systemic pneumococcal infection. 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. The main aim of the study was to study how immunomodulation of inflammation would affect progression of pneumococcal infection, making use of a lethal murine model of systemic *S. pneumoniae* infection.

1.4 INVESTIGATION PRESENTED IN THIS THESIS

The next successive chapters explore specific aims which this study set out to investigate. In Chapter 2 the study aimed at determining if enhancing the innate immune system, through TLR4, would initiate a stronger immune response, leading to faster elimination of bacteria and modulation of certain components of the host response and therefore improve the outcome of pneumococcal infection. The study determined if the enhancement of the innate immune response by exposure to sublethal doses of LPS would extend to a bacterial species that does not have an LPS component. LPS was administered before bacterial challenge and the response of mice to a subsequent challenge with the live *Streptococcus pneumoniae* was studied.

Chapter 3 comprises an investigation on the effect of innate stimulation with highly purified LPS after antibiotic treatment, on the progression of pneumococcal infection in a lethal murine model of systemic *Streptococcus pneumoniae* sepsis. The study

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determined the effect LPS treatment as an adjunctive therapy to antibiotic ceftriaxone on survival, bacterial load and cytokine production after pneumococcal challenge.

Chapter 4 explored whether anti-inflammatory agent A_{2A} receptor agonist (ATL313) as an adjunctive therapy, can increase the survival of mice exposed to live *Streptococcus pneumoniae* in a sepsis model. To test whether the effect of ATL313 was through functional A_{2A} adenosine receptors and also determine if hematopoietic cells are important in the protective effect of A_{2A}AR.

Chapter 5 sought to characterize the effects of A_{2A} R activation on the NK cell activity in pneumococcal sepsis model and determine the molecular and functional mechanisms of NK cell impact on the pathophysiology of pneumococcal sepsis.

Chapter 6 sought to determine the effect of etarnecept (tumor necrosis factor (TNF) receptor fusion protein) in mice with ongoing systemic pneumococcal infection when therapeutically administered together with antibiotic ceftriazone. We tested the hypothesis that eternecept could be a promising adjunctive agent to antibiotic ceftriaxone for the treatment of severe pneumococcal sepsis.

Each of the respective chapters consists of an abstract, introduction, objectives, experimental procedures, results, discussion and conclusion, and references. Lastly, a general overview of the entire study is presented where the main findings with recommendations are highlighted.

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1.5 REFERENCES

Akira, S. 2003. Mammalian Toll-like receptors. *Curr Opin Immunol. 15:5-11.*

Akira, S., and M. Yamamoto, and Takeda. K. 2003. Role of adapter in Toll-like receptors. *Biochem Society Trans* 31:637-642

Amiot, F., Fitting, C., Tracey, K. 1997. Lipopolysaccharide-Induced Cytokine Cascade and Lethality in LT-A/TNF-A Deficient Mice. *Molecular Med* 3 (12): 864-875.

Angus, D.C., Linde-Zwirble, W.T., Lidicker, J., Clermont, G., Carcillo, J., Pinsky, M.R. 2001. Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. *Crit Care Med* 29: 1303-1310.

Appelbaum, P.C., Bhamjee, A., Scragg, J.N., Harlett, A.F., Bowen, A.J., Cooper, R.C. 1977. *Streptococcus pneumoniae* resistant to penicillin and chloramphenicol. *Lancet* 2: 995-997.

Appelbaum, P.C. 1992. Antimicrobial resistance in *Streptococcus pneumoniae*: An overview. *Clin Infect Dis* 15:77-83

Austrian R., and Gold J. 1964. Pneumococcal bacteremia with special reference to bacteremic pneumococcal pneumonia. *Annals Intern Med* 60: 759–776.

Austrian R. 1977. Prevention of pneumococcal infection by immunization with capsular polysaccharides of *Streptococcus pneumoniae*: Current status of polyvalent vaccines. *J Infect Dis* 136 (supplement): 38 – 42

Baer, M., Vuento, R., Vesikari T. 1995. Increase in bacteremic pneumococcal infections in children. *Lancet* 345:661

Barlaam, B., Bird, T.G., Lambert-Van Der Brempt, C., Campell, D., Foster, S.J., and Maciewic Z. 1999. New alpha-substituted succinate-based hydoxamic acids as TNF alpha convertase inhibitors. *J Med Chem* 42: 4890-4908.

© University of Venda

University of Venda
Creating Future Leaders

Barry, P.M., Zetola, N., Keruly, J.C., Moore, R.D., Gebo, K. A., and Lucas, G.M. 2006. Invasive pneumococcal disease in a cohort of HIV –infected adults: incidence and risk factors, 1990-2003. *AIDS* 20 (3): 437-44.

Barton, G.M. 2008. A calculated response: control of inflammation by the innate immune system. *J Clin Invest* 118: 413-420

Benton, K.A., Everson, M.P, and Briles, D.E. 1995. A pneumolysin-negative mutant of Streptococcus pneumoniae causes chronic bacteremia rather than acute sepsis in mice. *Infect Immun* 63 (2): 448-55.

Berger, F. M. 1967. The effect of endotoxin on resistance to infection and disease. *Adv Pharmacol 5:19-46.*

Berry, A.M., Lock, R.A., Hansman, D., Paton, J.C. 1989. Contribution of autolysin to virulence of *Streptococcus pneumoniae. Infect Immun* 57: 2324 -2330

Beutler, B., Greenwald, D., Hulmes, J., Chang, M., Pan, Y., Mathison, J., Ulevitch, R., Cerami, A. 1985a. Identity of Tumour Necrosis Factor and the Macrophage-Secreted Factor Cachectin. *Nature.* 316 (8): 552-554.

Beutler, B., Milsark, L., Cerami, A. 1985b. Passive Immunization Against Cachectin/Tumor Necrosis Factor Protects Mice from Lethal Effects of Endotoxin. *Science* 229: 867-871.

Bhakdi, S., Klonisch, T., Nuber, P., and Fischer, W. 1991. Stimulation of monokine production by lipoteichoic acids. *Infect Immunol* 59:4614-4620.

Bone, R.C., Balk, R.A., Cerra, F.B., Dellinger, R.P.,Fein, A.M., Knaus, W.A., Schein, R.M., Sibbald, W.J. 1992. Definations for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus conference committee. American College of Chest Physicians/Society of Critical Care Medicine. *Chest* 101:1644-1655.

Bone, R.C.1991. The pathogenesis of sepsis. *Ann Inter Med 115:457-469.*

© University of Venda

University of Venda
Creating Future Leaders

Boulnois, G.J. 1992. Pneumococcal proteins and the pathogenesis of disease caused by *Streptococcus pneumoniae*. *J Gen Microbiol* 138: 249–259

Breiman, R.F., Spika, J.S., Nava, R.R.O., Darden, P.M., Darby, C.P. 1990. Pneumococcal bacteremia in Charleston County, South Carolina. *Arch Intern Med* 150: 1401-1405.

Briles, D.E., Yother, J., McDaniel, L.S. 1988. Role of pneumococcal surface protein A in the virulence of *Streptococcus pneumoniae*. *Rev Infect Dis* 10 (supplement): 372-374.

Brueggemann, A.B., Griffiths, D.T., Meats, E., Peto, T., Crook, D.W. and Spratt, B.G. (2003). Clonal relationships between invasive and carriage *Streptococcus pneumoniae* and serotype- and clone-specific differences in invasive disease potential. *J Infect Dis 187* (9): 1424-32.

Bruyn, G.A.W., Zegers, B.J.M., van Furth, R. 1992. Mechanisms of host defense against infection with *Streptococcus pneumoniae*. *Clin Infect Dis* 14: 251-262.

Burman, L.A, Norrby R, Trollfors B. 1985. Invasive pneumoccoccal infections: incidence, predisposing factors and prognosis. *Rev Infect Dis* 7: 133-142.

Byington, C.L., Samore, M.H., Stoddard, G.J., Barlow, S., Daly, J., Korgenski, K., Firth, S., Glover, D., Jensen, J., Mason, E.O., Shutt, C.K. and Pavia, A.T. 2005. Temporal trends of invasive disease due to Streptococcus pneumoniae among children in the intermountain west: emergence of novaccine serogroups. *Clin infect Dis 41(1): 21-9*

Casadevall, A. 2006. The third age of antimicrobial therapy. *Clin Infect Dis* 42:1414-1416.

Casadevall, A., and Pirofski, L. 2003. The Damage-response framework of microbial pathogenesis. *Nat Rev Microbiol* 1: 17-24.

© University of Venda

University of Venda
Creating Future Leaders

Casal, J. and T.G. Tarrago. 2003. *Immunity to Streptococcus pneumoniae: Factors affecting production and efficacy. Curr Opin Infect Dis* 16(3): 219-24.

Center for Disease Control and Prevention. 1993. Revised classification system for HIV-1 infection and expanded surveillance case definition for AIDS among adolescences. *MMWR* 41:1-19.

Center for Disease Control and Prevention. 1997. Prevention of pneumococcal disease. Recommendations of the advisory committee on immunization practices (ACIP). *MMWR* 46:1-24

Chambers, C.A., Kuhns, M.S., Engen, J.G., Allison, J.P. 2001. CTLA-4-mediated inhibition in regulation of T cell responses:mechanisms and manipulation in tumor therapy. *Annu Rev Immunol* 19:565-94

Christenson, B., Lundbergh, P., Hedlund, J., Ortqvist, A. 2001. Effects of a large – scale intervention with influenza and 23-valent pneumococcal vaccines in adults aged 65 years or older: a prospective study. *Lancet* 357: 1008-11.

Coil, J.A., Dickerman, J.D. and Boulton, E. 1978. Increased susceptibility of splenectomized mice to infection after exposure to an aerosolized suspension of type III Streptococcus pneumoniae. *Infect Immun* 21(2):412-6

Crain MJ, Waltman WD, Turner S, Yother J, Talkington DF, McDaniel LS, Gray BM, Briles DE. 1990. Pneumococcal surface protein A (PspA) is serologically highly variable and is expressed by all clinically important capsular serotypes of *Streptococcus pneumoniae*. *Infect Immun* 58: 3293-3299.

Cronstein B.N., Levin, R.N., Phillips, M.R., Hirschhorn, R., Abramson, S.B., Weissmann, G. 1992. Neutrophil adherence to endothelium is enhanced via adenosine A1 receptors and inhibited via adenosine A2 receptors. *J Immunol* 148: 2201-2206.

Cronstein, B. 1995. The antirheumatic agents sulphasalazine and methaotrexate share an anti-inflammatory mechanism. *Br J Rheumatol* 34:30-32.

© University of Venda

University of Venda
Creating Future Leaders

Cronstein, B.N. 1994. Adenosine, an endogenous anti-inflammatory agent. *J Appl Physiol* 76:5-13.

Cronstein, B.N., Daguma, L., Nichols, D., Hutchison, A.J., Williams, M. 1990. The adenosine/neutrophil paradox resolved: human neutrophils posses both A1 and A2 receptors that promote chemotaxis and inhibit O2 generation, respectively. *J Clin Invest* 85: 1150-7.

Cronstein, B.N., Levin, R.N., Belanoff, J., Weissmann, G., Hirschhorn, R. 1986. Adenosine: an endogenous inhibitor of neutrophil-mediated injury to endothelial cells. *J Clin Invest* 78:760-770.

Cutts, F.T., Zaman, S.M., Enwere, G., Jaffar, S., Levine, O.S., Okoko, J.B., Oluwalana, C., Vaughan, A., Obara, S.K., Leach, A., MacAdam, K.P., Biney, E., Saaka, M., Onwuchekwa, U., Yallop, F., Pierce, N.F., Greenwood, B.M. and Adegbola, R.A. 2005. Efficiency of nine-valent pneumococcal conjugate vaccine against pneumonia and invasive pneumococcal disease in the Gambia: randomized, double-blind, placebo-controilled trial. *Lancet* 365 (9465): 1139-46.

Davidson, M., Parkinson, A.J., Bulkow, L.R., Fitzgerald, M.A., Peters, H.V., Parks, D.J. 1994. The epidemiology of invasive pneumococcal disease in Alaska, 1986-1990-ethnic difference and opportunities for prevention. *J Infect Dis* 170: 368-376.

Davis, B.D., Dulbecco, R., Eisen, H.N., Wood, W.B. 1968. Pneumococci. *In* Davis BD, Dulbecco R, Eisen HN, Wood WB eds. Microbiology: A text emphasizing molecular and genetic aspects of microbiology and immunology and the relations of bacteria, fungi and viruses to human disease USA. Harper and Row Publishers, 684 -700.

Dinarello, C.A. 1997. Proinflammatory and anti-inflammatory cytokines as mediators in the pathogenesis of septic shock. *Chest* 112:321S-329S.

Dowell, S.F., Butler, J.C., Giebink, G.S., Jacobs, M.R., Jenigan, D., Musher, D.M. 1999. Otitis media management and surveillance in the era of pneumococcal

© University of Venda

University of Venda
Creating Future Leaders

resistance: A report from the Drug – Resistant *Streptococcus pneumoniae* Therapeutic Working Group. *Pediatr Infect Dis J* 18: 1-9.

Dowell, S.F., Whitney, C.G., Wright, C., Rose, C.E., Jr., and Schuchat, A. 2003. Seosonal patterns of invasive pneumococcal disease. Emerg Infect Dis 9 (5): 573-9

Ertugrul, N., Rotriguez- Barradam, M.C., Musher, D.M., Ryan, M.A.K., Agin, C.S., Murphy, S.J., Shayegani, M., Watson, D. 1997. BOX-polymerase chain reaction-based DNA analysis of nonserotypeable *Streptococcus pneumoniae* implicated in outbreaks of conjunctivitis. J *Infect Dis* 176: 1401-1405

Eskola, J., Takala, A.K., Kela, E., Pekkanen, E., Kalliokoski, R., Maija, L. 1992. Epidemiology of invasive pneumococcal infections in children in Finland. *JAMA* 268: 3323-3327.

Faden, H., Duffy, L., Wasielewski, R., Wolf, J., Krystofik, D., Tung, Y. 1997. Relationship between nasopharyngeal colonization and the development of otitis media in children. *J Infect Dis* 175: 1440-1445.

Fenton, M.J., Golenbock, D.T. 1998. LPS-binding proteins and receptors. *J Leukoc Biol 64:25-32.*

Freudenberg, M. A., C. Galanos. 1988. Induction of tolerance to lipopolysaccharide (LPS)-D-galactosamine lethality by pre-treatment with LPS is mediated by macrophages. *Infect Immun 56:1352.*

Freudenberg, M. A., Y. Yaegashi, P. Nielsen, Y. Kumazawa, M. Aguet, C. Galanos. 1996. Mechanism of sensitization to endotoxin by bacteria. E. Faist, and A. E. Baue, and F. W. Schildberg, eds. *The Immune Consequences of Trauma, Shock and Sepsis: Mechanisms and Therapeutic Approaches 804-815.* Pabst Science Publishers, Lengerich.

Friendland, I.R., McCracken, G.H. 1994. Management of infections caused by antibiotic –resistant *Streptococcus pneumoniae*. *N Eng J Med* 331: 377-382

© University of Venda

University of Venda
Creating Future Leaders

Garcia, P., Gonzalez, M.P., Garcia, E., Lopez, R., and Garcia, G.L. 1999. LytB, a novel pneumococcal murein hydrolase essential for cell separration. *Mol Microbiol* 31(4) : 1275-81.

Garcia-Leoni, M.E., Moreno, S., Rodeno, P. 1992. Pneumococcal pneumonia in adult hospitalized patients infected with the human immunodeficiency virus. *Arch Intern Med* 152: 1808-1812.

Gilbert, R.J., Jimenez, J.L., Chen, S., Tickle, I.J., Rossjohn, J., Parker, M., Andrew, P.W. and Saibil, H.R. 1999. Two structural transitions in membrane pore formation by pneumolysin, the pore-forming toxin of Streptococcus pneumoniae. *Cell* 97(5) : 647-55

Gilks, C..F, Ojoo, S.A., Ojoo, J.C., Brindle, R.J., Paul, J., Batchelor, B.I., Kimari, J.N., Newnham, R., Bwayo, J., Plummer, F.A. 1996. Invasive pneumococcal disease in a cohort of predominantly HIV-1infected female sex-workers in Nairobi, Kenya. *Lancet* 347:718-723

Ginsburg, I. 2002. Role of lipoteichoic acid in infection and inflammation. *Lancet infect Dis* 2 (3):171-9.

Gonzalez, B.E., Hulten, K.G., Lamberth, L., Kaplan, S.L., and Mason, E.O.,Jr. 2006. *Streptococcus pneumoniae* serogroups 15 and 33 : an increasing cause of pneumococcal infections in children in the United States after the introduction of the pneumococcal 7-valent conjugate vaccine. *Pediatr Infect Dis J* 25(4) : 301-5

Gorensek, M.J., Lebi, M.H., Nelson, J.D. 1988. Peritonitis in children with nephrotic syndrome. *Pediatrics* 81: 849-856.

Gosink, K.K., Mann, E.R., Guglielmo, C., Tuomanen, E.I., and Masure, H.R. 2000. Role of novel choline binding proteins in virulence of *Streptococcus pneumoniae*. *Infect Immun* 68(10): 5690-5.

© University of Venda

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Creating Future Leaders

Gray, B.M., Converse, G.M., Dillon, H.C. 1980. Epidemiologic studies of *Streptococcus pneumoniae* in infants: Acquisition, carriage and infection during the first 24 months of life. *J Infect Dis* 142: 923-933.

Gwaltney, J.M., Phillips, C.D., Miller, R.D., Riker, D.K. 1994. Computed tomographic study of the common cold. *N Engl J Med* 330: 25-30.

Hall, L.M.C. 1998. Application of molecular typing to the epidemiology of *Streptococcus pneumoniae*. *J Clin Pathol* 51: 270-274.

Hardy, D.W., Feinberg, J., Finkelstein, D.M, et al.1992. A controlled trial of Trimethoprim-sulfamethoxazole or aerosolized pentamidine for secondary prophylaxis of pneumocystis carinni pneumonia in patients with the acquired immunodeficiency syndrome. *N Engl J Med* 327:1842-8.

Hasko, G., Deitch, E.A., Szabo, C., Nemeth, Z.H. 2002. Adenosine: a potential mediator of immunosuppression in multiple organ failure. *Curr Opin Pharmacol* 2:440-444.

Hasko, G., Nemeth, Z.H., Vizi, E.S., Salzman, A.L., Szabo, C. 1998. An agonist of adenosine A3 receptors decreases interleukin-12 and interferon-gamma production and prevents lethality in endotoxemic mice. *Eur J Pharmacol* 358:261-268.

Hasko, G., and Szabo, C. 1998. Regulation of cytokine and chemokine production by transmitters and co-transmitters of the autonomic nervous system. *Biochem Pharmacol* 56:1079-1087.

Hasko, G., Szabo, C., Nemeth, S.H., Kvetan, V., Pastores, S.M., and Vizi, E.S. 1996. Adenosine receptor agonists differentially regulate IL-10, TNF-alpha, and nitric oxide production in RAW264.7 macrophages and in endotoxemic mice. *J. Immunol.* 157: 4634-4640.

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H.I. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature* 408:740-745.

© University of Venda

University of Venda
Creating Future Leaders

Henderson, F.W., Gilligan, P.H., Wait, K., Goff, D.A. 1988. Nasopharyngeal carriage of antibiotic – resistant pneumococci by children in group day care. *J Infect Dis* 152: 256-263.

Henrichsen, J. 1995. Six newly recognized types of *Streptococcus pneumoniae*. *J Clin Microbiol* 33: 2795-2762

Henricson, B. E., W. R. Benjamin, S. N. Vogel. 1990. Differential cytokine induction by doses of lipopolysaccharide and monophosphoryl lipid A that result in equivalent early endotoxin tolerance. *Infect Immun 58:2429*.

Henriques Normark, B., Novak, R., Ortqvist, A., Kallenius, G., Tuomanen, E. and Normark, S. (2001). Clinical isolates of *Streptococcus pneumoniae* that exhibittolerance of vancomycin. *Clin Infect Dis* 32(4) :552-8

Herxheimer, K., and Krause, K. 1902. Ueber eine bei Syphilitischen vorkommende Quecksilberreakton. Dtsch. *Med.Wochenschr* 28:895-897.

Heumann, D., Barras, C., Severin, A., Glauser, M.F., and Tomasz, A. 1994. Gram-positive cell walls stimulate synthesis of tumor necrosis factor alpha and interleukin-6 by human monocytes. *Infect Immun* 62(7) : 2715-21

Hilleman, M.R., Carlson, A.J., McLean, A.A., Vella, P.P., Weibel, R.E., Woodhour, A.F. 1981. *Streptococcus pneumoniae* polysaccharide vaccine: age and dose responses, safety, persistence of antibody, revaccination and simultaneous administration of pneumococcal and influenza vaccines. *Rev Infect Dis* 3 supplement: S31-S42.

Hirst, R.A., Kadioglu, A., O'Callaghan, C. and Andrew, P.W. 2004. The role of pneumolysin in pneumococcal pneumonia and meningitis. *Clin Exp Immunol* 138 (2): 195-201.

Hoge, C.W., Reichler, M.R., Dominguez, E.A., Bremer, J.C., Mastro, T.D, Hendricks, K.A., Musher, D.M., Elliott, J.A., Facklam, R.R., Breiman, R.F. 1994. An epidemic of

© University of Venda

University of Venda
Creating Future Leaders

pneumococcal disease in an overcrowded inadequately ventilated jail. *N Engl J Med* 331: 643- 648.

Holliman, R.E., Liddy, H., Johnson, J.D., and Adjei, O. 2006. Epidemiology of invasive pneumococcal disease in Kumasi, Ghana. *Trans R Soc Trop Med Hyg*

Hosea, S.W., Brown, E.J., Burch, C.G., Berg, R.A., Frank, M.M. 1981. Impaired immune response of splenectomized patients to polyvalent pneumococcal vaccine. *Lancet* 2: 804 -807.

Hostetter, M.K. 1986. Serotypic variations among virulent pneumococci in deposition and degradation of covalently bound C3b: implications for phagocytosis and antibody production. *J. Infect Dis* 153: 682-693.

Huo, Z., Spencer, O., Miles, J., Johnson, J., Holliman, R., Sheldon, J. and Riches, P. 2004. Antibody response to pneumolysin and to pneumococcal capsular polysaccharide in healthy individuals and Streptococcus pneumoniae infected patients. *Vaccine* 22(9-10) : 1157-61

Jacobs, M.R., Koornhof, H.J., Robins–Brown, R.M. 1978. Emergence of multiply resistant pneumococci. *N Eng J Med* 299: 735-740.

Jacobson, K.A., Gao, Z-G, Chen, A., Barak, D., Kim, S.A, Lee, K., Link, A., Rompaey, P.V., van Calenbergh, S., Liang, B.T. 2001. Neoceptor concept based on molecular complementarity in GPCRs: a mutant adenosine A3 receptor with selectively enhanced affinity for amine-modified nucleosides. *J Med Chem* 44:4125-36.

Janeway, C., Travers, P., Walport, M., Capra, D. 1999. Immunobiology: the Immune System in Health and Disease. 5th edition. New York: Garland Science. 13-14.

Janoff, E.N., Breiman, R.F., Daley, C.L, Hopewell, P.C. 1992. Pneumococcal disease during HIV infection: Epidemiologic, clinical, and immunologic prospective. *Ann Inter Med* 117:314-324.

© University of Venda

University of Venda
Creating Future Leaders

Jarisch, A. 1895. Therapeutische Versuche bei Syphilis. Wien Med Wochenschr 45: 721-724.

Jawetz, E., Melnick, J.L., Adelborg, E.A. 1991. Medical Microbiology. San Mated California: Appleton and Lange 200-211.

Jedrzejas, M.J. 2001. Pneumococcal virulence factors: structure and function. *Microbiol Mol Biol Rev* 65 (2):187-207.

Jin, X., Shepherd, R.K., Duling, B.R., Linden, J. 1997. Inosine binds to A3 adenosine receptors and stimulates mast cell degranulation. *J Clin Invest* 100:2849-57.

Johnson, A. G. 1994. Molecular adjuvants and immunomodulators: new approaches to immunization. *Clin. Microbiol. Rev.* 7:277-289.

Johnston, C. A., S. E. Greisman. 1985. Mechanism of endotoxin tolerance. L. B. Hinshaw, ed. In *Handbook of EndotoxinVol. 2:359.-391.* Elsevier, Amsterdam, New York, and Oxford.

Jones, E., Stuart, D., Walker, N. 1989. Structure of Tumour Necrosis Factor. *Nature.* 338 : 225-228.

Jordan, J.E., Zhao, Z-Q, Sato, H., Taft, S., Vinten-Johansen, J. 1997. Adenosine A2 receptor activation attenuates reperfusion injury by inhibiting neutrophil accumulation, superoxide generation and coronary endothelial adherence. *J Pharmacol Exp Ther* 280:301-9.

Jordens, J.Z., Paul, J., Bates, J., Beaumont, C., Kimari, J., Gilks, C. 1995. Characterization of *Streptococcus pneumoniae* from human immunodeficiency virus-seropositive patients with acute and recurrent pneumonia. *J Infect Dis* 172:983-987.

Karstaedt, A.S., Khoosal, M., Crewe-Brown, H.H. 2000. Pneumococcal bacteremia during a decade in children in Soweto, South Africa. *Pediatr Infect Dis J* 19 (5): 454-7

© University of Venda

University of Venda
Creating Future Leaders

Kammer, G.M., Birch, R.E., Polmar, S.H. 1983. Impaired immunoregulation in systemic lupus erythematosus: defective adenosine –induced suppressor T lymphocyte generation. *J Immunol* 130:1706-1712.

Khoa, N.D., Montesinos, M.C., Reiss, A.B., Delano, D., Awadallah, N., Cronstein, B.N. 2001. Inflammatory cytokines regulate function and expression of adenosine A2Ar receptors in human monocytic THP-1 cells. *J Immunol* 167:4026-4032.

Khor, C.C., Chapman, S.J., Vannberg, F.O., Dunne, A., Murphy, C., Ling, E.Y. 2007. A Mal functional variant is associated with protection against invasive pneumococcal disease, bacteremia, malaria and tuberculosis. *Nat Genet* 39:523-528.

Kim, P.E., Musher, D.M., Glezen, W.P., Rotriguez- Barradas, M.C., Nahm, W.K., Wright, C.E. 1996. Association of invasive pneumococcal disease with season, atmospheric conditions, air pollution and the isolation of respiratory viruses. *Clin infect Dis* 22: 100-106.

Kingsbury, D.T., and Wagner GE. 1990. Microbiology 2nd ed. Pennyslavania: Harwal Publishing Company, 91-93

Klein, J.O. 1981. The epidemiology of pneumococcal disease in infants and children. *Rev Infect Dis* 3: 246-253.

Koedel, U., Bayerlein, I., Paul, R., Sporer, B., and Pfister, H.W. 2000. Pharmacologic interference with NF-kappa B activation attenuates central nervous system complications in experimental pneumococcal meningitis. *J Infect Dis* 182:1437-1445.

Koedel, U., Winkler, F., Angele, A., Fontana, A., Flavell, R.A., and Pfister, H.W. 2002. Pathogenesis and pathophysiology of pneumococcal meningitis. *Lancet Infect Dis* 2:721-736.

Kristinsson, K.G., Hjalmarsdottir, M.A., Steingrimsson, O. 1992. Increasing penicillin resistance in pneumococci in Iceland. *Lancet* 339: 1606-1607.

© University of Venda

University of Venda
Creating Future Leaders

Kumar, V., Abbas, A.K., Fausto, N., and Mitchell, M. 2007. Robbins basic pathology. W.B. Saunders. Philadelphia, Pensylvania, USA. Pp 960.

Kyaw, M.H., Lynfield, R., Schaffner, W., Craig, A.S., Hadler, J., Reingold, A., Thomas, A.R., Harrison, L.H., Bennett, N.M., Farley, M.M., Facklam, R.R., Jorgensen, J.H., Labro, M.T. 2000. Interference of antibacterial agents with phagocyte functions: immunomodulation or "immuno-fairy" tales? *Clin Microbiol Rev* 3:615-650.

Lappin, D., and K. Whaley. 1984. Adenosine A2 receptor s on human monocytes modulates C2production. *Clin Exp Immunol* 57:454-460.

Lehner, M.D., Morath, S., Michelsen, K.S., Schumann, R.R., Hartung, T. 2001. Induction of cross-tolerance by lipopolysaccharide and highy purified lipoteichoic acid via different Toll-like receptors independent of pancrine mediators. *J Immunol 166:5161-5167.*

Leib, S.L., Clements, J.M., Lindberg, R.L., Heimgartner, C., Loeffler, J.M., Pfister, L.A., Tauber, M.G., and Lebppert, D. 2001. Inhibition of matrix metalloproteinases and tumor necrosis factor alpha converting enzyme as adjuvant therapy in pneumococcal meningitis. *Brain* 124:1734-1742.

LeMoine, O., Stordeur, P., Schandene, L., Marchant, A., de Groote, D., Goldman, M., Deviere, J. 1996. Adenosine enhances IL-10 secretion by human monocytes. *J Immunol* 156:4408-4418.

Levine, O.S., Farley, M., Harrison, L.H., Lefkowitz, L., McGeer, A. and Schwartz, B. 1999. Risk factors for invasive pneumococcal disease in children : a population-based case –control study in North America. *Pediatrics* 103(3) :E28

Linden, J. 1991. Structure and function of A1 adenosine receptors. FASEB J 5:2668-2676.

Linden, J. 1994. Cloned adenosine A3 receptors: pharmacological properties, species differences and receptor functions. *Trends Pharmacol Sci* 15:298-306.

© University of Venda

University of Venda
Creating Future Leaders

Lund E., and J. Henrichsen. 1978. Laboratory diagnosis, serology and epidemiology of *Streptococcus pneumoniae*. *In*: Bergan T, Norris JR, eds. Methods in Microbiology (Vol 12). New York: Academic Press 241-242.

Makela, P.H., Leinonen, M., Pukander, J., Karma, P. 1981. A study of the pneumococcal vaccine in prevention of clinically acute attacks of recurrent otitis media. *Rev Infect Dis* 3 (supplement): S124 -S130.

Mao, C., Harper, M., McIntosh, K. 1996. Invasive pneumococcal infection in human deficiency virus-infected children. *J Infect Dis* 173:870-876

Martens, P., Worm, S.W., Lundgren, B., Konradsen, H.B. and Benfield, T. 2004. Serotype –specific mortality from invasive pneumoniae disease revisited. *BMC Infect Dis* 4:21.

Masihi, K. N., W. Lange, W. Brehmer, and E. Ribi. 1986. Immunobiological activities of nontoxic lipid A: enhancement of nonspecific resistance in combination with trehalose dimycolate against viral infection and adjuvant effects. *Int. J. Immunopharmacol. 8:339-345.*

Mason, C.M., Dobard, E., Summer, W.R., Nelson, S. 1997. Intraportallipopolysaccharide suppresses pulmonary antibacterial defense mechanism. *J Infect Dis 176:1293-1302.*

Mbelle, N., Wasas, A., Huebner, R., Wasas, A.D., Kimura, A., Chang, I., Klugman, K.P. 1999. Immunogenicity and impact on nasopharyngeal carriage of a 9-valent pneumococcal congugate vaccine. *J Infect Dis* 180: 1171-1176

McCullers, J.A. and E.I. Tuomanen. 2001. *Molecular pathogenesis of pneumococcal pneumonia. Front Biosci* 6: D877-89.

McDaniel, L.S., Scott, G., Keayney, J.F., Briles, D.E. 1984. Monoclonal antibodies against protease sensitive pneumococcal antigens can protect mice from fatal infection with *Streptococcus pneumoniae*. *J Exp Med* 160: 386-397.

© University of Venda

University of Venda
Creating Future Leaders

McDaniel, L.S., Yother, J., Vitayakumar, M., McGarry, L., Guild, W.R., Briles, D.E. 1987. Use of insertional activation to facilitate studies of biological properties of pneumococcal surface protein A (PspA). *J Exp Med* 165: 381-394.

McGee, L., McDougal, J., Zhou, BG., Spratt, F.C., Tenover, R., Geroge, R. 1998. Emergence and spread of antibiotic- resistant *S. pneumoniae*. *South Afr J Epidemiol Infect* 14: 117-120.

Medzhitov, R. 2007. Recognition of microorganisms and activation of the immune response. *Nature.* 449:819-826.

Mellman, I., and Steinman, R.M. 2001. Dendritic cells: specialized and regulated antigen processing machines. *Cell* 106:255-258.

Mercat, A., Nguyen, J., Dautzenberg, B. 1991. An outbreak of pneumococcal pneumonia in two men's shelters. *Chest* 99: 147-151.

Mitchell, T.J. 2000. Virulence factors and the pathogenesis of disease caused by Streptococcus pneumoniae. *Res Microbiol* 151 (6): 413-9

Mitchell, T. J., Alexander, J. E., Morgan, P. J., Andrew, P. W. 1997. Molecular analysis of virulence factors of *Streptococcus pneumoniae. Soc Appl Bacteriol Symp Ser* 26: p. 62S-71S.

Mizrachi Nebenzahl, Y., Porat, N., Lifshitz, S., Novick, S., Levi, A., Ling, E., Liron, O., Mordechai, S., Sahu, R.K., and Dagan, R. (2004). Virulence of *Streptococcus pneumoniae* may be determined independently of capsular polysaccharide. *FEMS Microbiol Lett* 233 (1): 147-52.

Montesinos, M.C., Gadangi, P., Longaker, M., Sung, J., Levine, J., Nilsen, D., Reibman, J., Li, M., Jiang, C.K., Hirschhorn, R.I. 1997. Wound healing is accelerated by agonists of adenosine A2 receptor. *J Exp Med* 186:1615-1620.

© University of Venda

University of Venda
Creating Future Leaders

Morana, J.K., Morona, R., and Paton, J.C. 2006. Attachment of capsular polysaccharide to the cell wall of Streptococcus pneumoniae type 2 is required for invasive disease. *Proc Natl Acad Sci USA* 103 (22): 8505-10.

Moser, G.H., Schrader, J., Deussen, A. 1989. Turnover of adenosine in plasma of human and dog blood. *Am J Physiol* 256: C799-806.

Mufson MA, Kruss DM, Wasil RE, Metzger WI. 1974. Capsular types and outcome of bacteremic pneumococcal disease in the antibiotic era. *Arch Intern Med* 134: 505-510.

Murphey, E.D., Fang, G., Varma, T.K., Sherwood, E.R. 2007. Improved bacterial clearance and decreased mortality can be induced by LPS tolerance and is not dependent upon IFN-gamma. *Shock 27 (3):289-295.*

Murphy, T.F., and Fine, B.C. 1984. Bacteremic pneumococcal pneumonia in the elderly. *Am J Med Sci* 288: 114 -118.

Musher, D.M. 2000. *Streptococcus pneumoniae. In*: Mandell GC, Bennett JE, Dolin R eds. Mandell, Principles and practice of infectious disease, 4th ed. Philadelphia, Pennyslavia, *Churchhill Livingstone* 2128-2147.

Myers, K. R., A. T. Truchot, J. Ward, Y. Hudson, and J. T. Ulrich. 1990. A critical determinant of lipid A endotoxic activity, p. 145-156. *In* A. Nowotny, J. J. Spitzer, and E. J. Ziegler (ed.), Cellular and molecular aspects of endotoxin reactions. Elsevier Sciences Publishers B.V., Amsterdam, The Netherlands.

National Cancer Institute. 1995. Biological Therapies: Using the Immunse System to Treat Cancer.

Nau, R. and Eiffert, H. 2002. Modulation of release of proinflammatory bacterial compounds by antibacterials: potential impact on course of inflammation and outcome in sepsis and meningitis. *Clin Microbiol Rev* 15(1): 95-110.

© University of Venda

Nesin, M., Ramirez, M., Tomasz, A. 1998. Capsular transformation of a multidrug – resistant *Sreptococcus pneumoniae* in vivo. *J Infect Dis* **177**: 707-713.

Nuorti, J.P., Butler, J.C., Crutcher, J.M., Guevara, R., Welch, D., Holder, P., Elliot, J.A. 1998. An outbreak of multidrug-resistant pneumococcal pneumonia and bacteremia among unvaccinated nursing home residents. *N Engl J Med* 338: 1861-1868.

Nuorti, J.P., Butler, J.C., Farley, M.M., Harrison, L.H., McGeer, A., Kolczak, M.S., and Breiman, R.F. 2000. Cigarette smoking and invasive pneumococcal disease. Active Bacterial Core Surveillance Team. *N Engl J Med* 342(10):681-9

O'Neill, L.A. 2003. Therapeutic targeting of Toll-like receptors for inflammatory and infectious diseases. *Curr Opin Pharmacol* 3:396-403

Obaro, S.K., Adegbola, R.A., Banya, W.A.S, Greenwood, B.M. 1996. Carriage of pneumococci after pneumococcal vaccine. *Lancet* 348: 271-272.

Obaro, S. and Adegbola, R. 2002. The pneumococcus: carriage, disease and conjugate vaccines. *J Med Microbiol* 51(2):98-104

Odio, C.M., Faingezicht, I., Paris, M. 1991. The beneficial effects of early dexamethasone administration in infants and children with bacterial meningitis. *N. Engl J Med* 324: 1525-31.

Ohta, A., and Sitkovsky, M. 2001. Role of G-coupled adenosine receptor s in down regulation of inflammation and protection from tissue damage. *Nature* 414:916-920

Ongini, E., and Fredholm, B.B. 1996. Pharmacology of adenosine A2A receptors. *Trends Pharmacol Sci* 17: 364-372

Opal, S.M., and DePalo, V.A. 2000. Anti-inflammatory cytokines. *Chest* 117:1162-1172.

Opstad, N.L., Daley, C.L., Thurn, J.R., Rubins, J.B., Merrifield, C., Hopewell, P.C, Janoff, E.N. 1995. Impact of *Streptococcus pneumoniae* bacteremia and human immunodeficiency virus type 1 on oral mucosal immunity. *J Infect Dis* 172:566-570

© University of Venda

University of Venda
Creating Future Leaders

Perez, C., Albert, I., DeFay, K., Zachariades, N., Gooding, L., Michael, K. 1990. A Nonsecretable Cell Surface Mutant of Tumor Necrosis Factor (TNF) Kills by Cell-to Cell Contact. *Cell* 63: 251-258.

Persing, D. H., Coler, R. N., Lacy, M.J., Johnson, D.A., Baldridge, J.R., Hershberg, R.M. and Reed, S.G. 2002. Taking toll: lipid A mimetics as adjuvants and immunomodulators. *Trends Microbiol. 10:S32-S37.*

Pirofski, L. and Casadevall, A. 2006. Immunomodulators as an antimicrobial tool. *Curr Opinion Microbiol* 9:489-495.

Poehling, K.A., Talbot, T.R., Griffin, M.R., Craig, A.S., Whitney, C.G., Zell, E., Lexau, C.A., Thomas, A.R., Harrison, L.H., Reingold, A.L., Hadler, J.L., Farley, M.M., Anderson, B.J., and Schaffner, W. 2006. Invasive pneumococcal disease among infants before and after introduction of pneumococcal conjugate vaccine. *JAMA* 295(14):1668-74.

Polsky, B., Gold, J.W., Whimbey, E. 1986. Bacterial pneumonia in patients with the acquired immune deficiency sundrome. *Ann Intern Med* 104:38-41

Powderly WG, Stanley SL, Medoff G. 1986. Pneumococcal endocarditis: Report of a series and review of the literature. *Rev Infect Dis* 8: 786-791.

Priebe, T., Platsoucas, C.D., and Nelson, J.A. 1990. Adenosine receptors and modulation of natural killer cell activity by purine nucleosides. *Cancer. Res* 50:4328-4331

Quagliarello VJ, Scheld WM. 1997. Treatment of bacterial meningitis. *N Engl J Med* 336: 708-716.

Rauch, A.M., O'Ryan, M., Van, R., Pickering, L.K. 1990. Invasive disease due to multiply resistant *Streptococcus pneumoniae* in a Houston, Tex, day-care center. *AJDC* 144: 923- 927.

© University of Venda

University of Venda
Creating Future Leaders

Reinstein, L.J., Lichtman, S.N., Currin, R.T., Wang, J., Thurman, R.G., Lemasters, J.J. 1994. Suppression of lipopolysaccharide-stimulated release of necrosis factor by adenosine: evidence for A2 receptors on rat Kupffer cells. *Hepatology* 19:1445-1452.

Resta, R., Hooker, S.W., Laurent, A.B., Jamshedur Rahman, S.M., Franklin, M., Knudsen, T.B., Nadon, N.L., and Thompson, L.F. 1997. Insights into thymic purine metabolismand adenosine deaminase deficiency revealed by transgenic mice overexpressing ecto-5'-nucleotidase (CD73). *J Clin Invest* 99:676-683.

Rieger, J.M., Brown, M.L., Sullivan, G.W., Linden, J., Macdonald, T.L. 2001. Design, synthesis, and evaluation of novel A2A adenosine receptor agonist. *J Med Chem* 44:531-9.

Riensenfeld – Orn I, Wolpe S, Garcia – Bustos JF, Hoffmann MK, Toumanen E. 1989. Production of interleukin – 1 but not tumor necrosis factor by human monocytes stimulated with pneumococcal cell surface components. *Infect Immun* 57: 1890-1893.

Rink, L., Kirchner, H. 1996. Recent Progress in the Tumor Necrosis Factor-Alpha Field. *Int. Arc Allergy and Immunol.* 111 (3): 199-209.

Robbins, J.B., Austrian, R., Lee, C.J., Rastogi, S.C., Schiffman, G., Henrichsen, J., Makela, P.H., Broome, C.V., Facklam, R.R., Tiesjema, R.H., Parke, J.C. 1983. Considerations for formulating the second - generation pneumococcal capsular polysaccharide vaccine with emphasis on the cross-reactive types within groups. *J Infect Dis* 148: 1136-1158.

Rodriguez-Barradas, M.C., Tharapel, R.A., Groover, J.E. 1997. Colonization by *Streptococcus pneumoniae* among human immunodeficiency virus-infected adults: Prevalence of antibiotic resistance, impact of immunization, and characterization of by polymerase chain reaction with BOX primers of isolates from persistent *S. pneumoniae* carriers. *J Infect Dis* 175: 590-597.

Ryan K.J., and Falkow S. 1994. *Streptococcus* and *Enterococci. In*: Ryan KJ, Champoux JJ, Falkow S, Plorde JJ, Ray CG, Drew WL, Neidhardt FC eds. Medical

© University of Venda

University of Venda
Creating Future Leaders

Microbiology: An introduction to infectious diseases. Appleton and Lange Norwalk Connecticut, 265 -282.

Saez-Llorens, X., Ramilo, O., Mustafa, M.M., Mertsola, J., McCracken, G.H. Jr. 1990. Molecular pathophysiology of bacterial meningitis: current concepts and therapeutic implications. *J. Pediatr* 116:671-84.

Salazar JC, Daly KA, Giebink GS, Lindgrew BR, Liebeler CL, Meland M, Le CT. 1997. Low cord blood pneumococcal immunoglobin G (lgG) antibodies predict early onset acute otitis media in infancy. *Am J Epidemiol* 145: 1048-1056.

Salmon, J.E., Brogle, N., Brownlie, C., Edberg, J.C., Kimberly, R.P.,Chen, B.N., and Erlanger, B.F. 1993. Human mononuclear phagocytes express adenosine A1 receptors. *J Immunol* 151:2775-2785.

Salyers, A.A., and Whitt, D.D. 2002. Bacterial pathogenesis: A molecular approach. ASM Press.Washington DC, p 567-574

Sandgren, A., Sjostrom, K., Olsson-Liljequist, B., Christensson, B., Samuelsson, A., Kronvall, G. and Henriques Normark, B. 2004. Effect of clonal and serotype-specific properties on the invasive capacity of Streptococcus pneumoniae. *J Infect Dis* 189 (5):785-96.

Saukkonen, K., Sande, S., Cioffe, C., Wolpe, S., Sherry, B., Cerami, A., and Tuomanen, E. 1990. The role of cytokines in the generation of inflammation and tissue damage in experimental gram-positive meningitis. *J Exp Med* 171:439-448.

Schinjns, V.E. 2001. Induction and direction of immune responses by vaccine adjuvants. *Crit Rev Immunol.* 21:75-85.

Schwartz L.E., and Brown R.B. 1992. Purulent otitis media in adults. *Arch Intern Med* 152: 2301-2304.

© University of Venda

University of Venda
Creating Future Leaders

Sell, S.H., Wright, P.F., Vaughn, W.K., Thompson, J., Schiffman, G. 1981. Clinical studies of pneumococcal vaccines in infants. Reactogenicity and immunogenicity of two polyvalent polysaccharide vaccines. *Rev Infect Dis* 3 (supplement): S97-S107.

Severn, A., Xu, D., Doyle, J., Leal, L.M., O'Donnell, C.A., Brett, S.J., Moss, D.W., Liew, F.Y. 1993. Pre-exposure of murine macrophages to lipopolysaccharide inhibits the induction of nitric oxide synthase and reduces leishmanicidal activity. *Eur J Immunol 23: 1711-1714.*

Shinefield, H., Black, S., Ray, P., Fireman, B., Schwalbe, J., and Lewis E. 2002. Efficacy, immunogenicity and safety of heptavalent pneumococcal conjugate vaccine in low birth weight and preterm infants. *Pediatr Infect Dis* 21(3):182-6.

Singh, J., S. S. Sidhu, G. S. Dhaliwal, G. R. Pangaonkar, A. S. Nanda, and A. S. Grewal. 2000. Effectiveness of lipopolysaccharide as an intrauterine immunomodulator in curing bacterial endometritis in repeat breeding cross-bred cows. *Anim Reprod Sci 59:159-166.*

Sitkovsky, M.V. 2003. Use of the A2A adenosine receptor as aphysiological immunosuppressor and to engineer inflammation *in vivo*. *Biochem Pharmacol* 65:493-501.

Sjostrom, K., Spindler, C., Ortqvist, A., Kalin, M., Sandgren, A., Kuhlmann-Berenzon, S., and Henriques –Normark, B. 2006. Clonal and capsular types decide whether pneumococci will act as a primary or opportunistic pathogen. *Clin Infect Dis* 42(4):451-9.

Sluijter, M., Faden, H., de Groot, R., Lemmens, N., Goessens, W.H.F., van Belkum, A., Hermans, P.W.M. 1998. Molecular characterization of pneumococcal nasopharynx isolates collected from children during their first 2 years of life. *J Clin Microbiol* 36: 2248 -2253.

Sly, L.M., Rauh, M.J., Kalesnikoff, J., Song, C.H., Krystal, G. 2004. LPS-induced upregulation of SHIP is essential for endotoxin tolerance. *Immunity 21: 227-239.*

© University of Venda

University of Venda
Creating Future Leaders

Smits, G.J., McVey, M., Cox, B.F., Perrone, M.H., Clark, K.L. 1998. Cardioprotective effects of the novel adenosine A1/A2 receptor against AMP 579 in a porcine model of myocardial infarction. *J Pharmacol Exp Ther* 286:611-8.

Sorensen UFS, Blom J, Birch – Anderson A, Henrichsen J. 1988. Ultrasturctural localization of capsules, cell wall polysaccharide, cell wall proteins and F antigen in pneumococci. *Infect Immun* 56: 1890-1896.

Spreer, A., Lis, A., Gerber, J., Reinert, R.R., Eiffert, H. and Nau, R. 2004. Differences in clinical manifestation of Streptococcus pneumoniae infection are not correlated with in vitro production and release of the virulence factors pneumolysin and lipoteichoic and teichoic acids. *J Clin Microbiol* 42(7):3342-5.

Steinfort, C., Wilson, R., Mitchell, T., Feldman, C., Rutman, A., Todd, H., Sykes, D., Walker, J., Saunders, K., Andrew, P.W., Boulnois, G.J., Cole, P.J. 1989. Effect of *Streptococcus pneumoniae* on human respiratory epithelium *in vitro*. *Infect Immun* 57: 2006-2013.

Strieter, R., Kunkel, S., Bone, R. 1993. Role of Tumor Necrosis Factor-Alpha in Disease States and Inflammation. *Crit Care Med.* 21 (10 Supplement): S447-S463.

Strieter, R.M., Belperio, J.A., Keane, M.P. 2003. Host innate defenses in the lung: the role of cytokines. *Curr Opin Infect Dis* 16:193-198

Sweet, M.J., Hume, D.A. 1996. Endotoxin signal transduction in macrophages. *J Leukoc Biol 60:8-26.*

Szefler, S.J., Norton, C.E., Ball, B., Gross, J.M., Aida, Y., Pabst, M.J. 1989. IFN-gamma and LPS overcome glucocorticoid inhibition of priming for superoxide release in human monocytes. Evidence that secretion of IL-1 and tumor necrosis factor –alpha is not essential for monocyte priming. *J Immunol* 142:3985-92

Tai, S.S. 2006. *Streptococcus pneumoniae* protein vaccine candidates: properties, activities and animal studies. *Crit Rev Microbiol* 32(3):139-53

© University of Venda

University of Venda
Creating Future Leaders

Talbot, T.R., Hartert, T.V., Mitchel, E., Halasa, N.B., Arbogast, P.G., Poehling, K.A., Schaffner, W., Craig, A.S. and Griffin, M.R. 2005. Asthma as a risk factor for invasive pneumococcal disease. *N Engl J Med* 352(20): 2082-90.

Tarkowski, A., Wagner, H. 1998. Arthritis and sepsis caused by Staphylococcus aureus: can the tissue injury be reduced by modulating the host's immune system? *Mol Med Today* 4:15-18.

Tauber, M.G., Shibl, A.M., Harckbarth, C.J., Larrick, J.W., Sande, M. 1987. Antibiotic therapy, endotoxin concentration in cerebrospinal fluid, and brain edema in experimental Escherichia coli meningitis in rabbits. *J Infect Dis* 156:456-462.

Terpstra, F.G., Ai, B.J., Roos, M.T. 1989. Longitudinal study of leukocyte functions in homosexual men seroconverted for HIV: Rapid and persistent loss of B cell function after HIV infection. *Eur J Immunol* 19:667-673

Thirumoorthi, M.C., Asmar, B.I., Dajani, A.S. 1978. Violaceous discoloration in pneumococcal cellulitis. *Pediatrics* 62: 492-493.

Timmerman, C.P., Mattsson, E., Martinez-Martinez, L., De Graaf, L., Van Strijp, J.A., Verbrugh, H.A., Verhoef, J., and Fleer, A.1993. Induction of release of tumor necrosis factor from human monocytes by staphylococci and staphyloccal peptidoglycans. *Infect Immunol* 61:4167-4172

Tomasz, A. 1981. Surface components of *Streptococcus pneumoniae*. *Rev Infect Dis* 3: 190-211.

Toumanen, E., Liu, H., Hengstler, B., Zak, O., Tomasz, A. 1985. The induction of meningeal inflammation by components of the pneumococcal cell wall. *J. Infect Dis* 151: 859-868.

© University of Venda

University of Venda
Creating Future Leaders

Toumanen, E., Tomasz, A., Hengstler, B., Zak, O. 1985. The relative role of bacterial cell wall and capsule in the induction of inflammation in pneumococcal meningitis *J Infect Dis* 151: 535-540.

Toumanen, E. 1988. Partner drugs: a new outlook for bacterial meningitis. *Ann. Intern Med* 109:690-692.

Tracey, K., and Cerami, A. 1994. Tumor Necrosis Factor: A Pleiotropic Cytokine and Therapeutic Target. *Ann Rev Med.* 45: 491-503.

Van Dam, J.E., Fleer, A. and Snippe, H. 1990. Immunogenecity and immunochemistry of *Streptococcus pneumoniae* capsular polysaccharides. Antonie van Leuwenhoek 58(1):1-47.

Van Ostade, X., Vandenabeele, P., Everaerdt, B., Loetscher, H., Gentz, R., Brockhaus, M., Lesslauer, W., Tavernier, J., Brouckaert, P., Fiers, W. 1993. Human TNF Mutants with Selective Activity on the P55 Receptor. *Nature.* 361: 266-269.

Verstak, B., P. Hertzog, and A. Mansell. 2007. Toll-like receptor signaling and the clinical benefits that lie within. *Inflamm Res* 56: 1-10

Winter, A.J., Comis, S.D., Osborne, M.P., Tarlow, M.J., Stephen, J., Andrew, P.W., Hill, J. and Mitchell, T.J. 1997. A role for pneumolysin but not neuraminidase in the hearing loss and cochlear damage induced by experimental pneumococcal meningitis in guinea pigs. *Infect Immun* 65(11): 4411-8

Xaus, J., Mirabet, M., Lloberas, J., Soler, C., Lluis, C., Franco, R., and Celeda, A. 1999. IFN-gamma upregulate the A2B adenosine receptor expression in macrophages: a mechanism of macrophage deactivation. *J Immunol* 162:3607-3614.

Youngner, J.S., Stinebring, W.R. 1965. Interferon appearance stimulated by endotoxin bacteria or viruses in mice pre-treated with Escherichia coli endotoxin or infected with Mycobacterium tuberculosis. *Nature 208: 456-458.*

Zeisberger, E., and Roth, J. 1998. Tolerance to pyrogens. *Ann NY Acad Sci 856: 116.*

© University of Venda

Ziegler, E.J., Fisher, Jr, C.J., Sprung, C.L., Straube, R.C., Sadoff, J.C., Foulke, G.E., Wortel, C.H., Fink, M.P., Dellinger, R.P., and Teng, N.N. 1991. Treatment of gram-negative bacteremia and septic shock with HA-1A human monoclonal antibody against endotoxin. A randomized, double-blind, placebo-controlled trial. The HA-1A Sepsis study Group. *N Engl J Med* 324:429-436

Ziegler-Heitbrock, H.W. 1995. Molecular mechanism in tolerance to lipopolysaccharide. *J Inflamm 45: 13.*

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CHAPTER TWO

Toll-like Receptor 4 (TLR4) Stimulation Provides Survival Enhancement in a lethal Murine Model of Systemic *Streptococcus pneumoniae* sepsis

Abstract

Background: Antibiotic resistance among pathogens including *S. pneumoniae* calls for new therapeutics. 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 a lethal *S. pneumoniae*, serotype 2 infections was examined.

Methods: 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 at 12 hours interval. Controls for all strains received PBS. 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 and spleens were harvested for flow cytometric analysis of splenic lymphocytes or NK activation.

Results: Systemic bacteremia was reduced 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-stimulated 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

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for ceftriaxone-treated C57bl/6 was 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 deficient 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. High levels of TNF-a, IL-6, IL-10, IL-1a, Rantes, IL-12 (p70), G-CSF, KC, and MIP-1a were detected in plasma of LPS-treated mice compared to naïve control mice. 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 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

Conclusions: This study demonstrates a survival benefit from TLR4 stimulation prior to infection with *S. pneumoniae.* This suggests that interventions designed to improve innate immunity to infection could have therapeutic benefits.

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2.1 INTRODUCTION

Administration of purified LPS (endotoxin) has been found to confer prophylactic protection from subsequent bacterial or viral challenge in various animal models, presumably via stimulation of innate immunity (Berger, 1967; Neter, 1969). Endotoxin, a predominant glycolipid of the cell membrane of Gram-negative bacteria, is one of the most potent known stimulators of immune responses. The immune system responds to LPS with a systemic production of proinflammatory cytokines and chemokines which recruit and activate immune cells to eliminate invading pathogens (Nakano et al., 1992; Mastoeni et al., 1999). Although these cytokines are indispensible for the efficient control of growth and dissemination of the pathogen (Mastroeni, et al., 1999; Beutler et al., 1985; Galanos et al., 1993), an overshooting inflammatory response is potentially autodestructive and may lead to microcirculatory dysfunction causing tissue damage, shock and eventually death (Beutler et al., 1985; Galanos et al., 1993). "Endotoxin tolerance" describes the observation that pretreatment of experimental animals with an initial low dose of LPS confers protection against the detrimental consequences of a subsequent high dose of LPS (Greisman et al., 1966).

The innate immune system first detects invading pathogens by recognizing conserved motifs found in the microorganisms, but not in the vertebrate host. One of the central components of the innate immune system is the family of Toll like receptors (TLRs). These pattern recognition receptors (PRR) recognize evolutionarily conserved pathogen-associated molecular patterns (PAMPs) present on most types of microorganisms (Iwasaki and Medzhitov, 2004). Once TLRs are activated, they signal "danger" to the host and trigger signaling cascades, leading to antimicrobial and inflammatory responses involving innate and adaptive immunity (Iwasaki and Medzhitov, 2004). To date, 10 TLRs

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have been identified in humans, and they each recognize different microbial structures. They have been emerging as the key regulators of innate immune responses to infection in mammals in recent years (Basset et al., 2003, Medzhitov and Janeway, 2000). TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are located on the cell surface and mainly recognize bacterial products unique to the invading organism.

TLR2 recognizes bacterial lipoproteins (Hirschfeld et al., 1999), peptidoglycan, and lipoteichoic acids (Schwandner et al., 1999); TLR4 recognizes lipopolysaccharide (LPS) (Poltorak et al., 1998); and TLR5 detects bacterial flagellin (Hayashi et al., 2001). In contrast, TLR3, -7, and -8, which together with TLR9, are located within endosomal compartments, are specialized primarily in viral detection and more generally, in recognition of RNA (Mogensen and Paludan, 2005). Double-stranded RNA is recognized by TLR3 (Alexopoulou et al., 2001) and single-stranded RNA by TLR7 and -8 [Diebold et al., 2004, Heil et al., 2004]. TLR9 recognizes unmethylated CpG DNA (Hemmi et al., 2000), which is a component of viruses and bacteria, implying that this TLR may play a role in a broader range of bacterial and viral infections (Hemmi et al., 2000). TLRs are expressed by most cell types, although clear differences between cell populations are apparent. However, the most important TLR-expressing cell types are believed to be dendritic cells (DC), macrophages, and B lymphocytes (Iwasaki and Medzhitov, 2004).

An interesting recent development has been the demonstration of involvement of TLRs in pneumococcal infection. Cell wall components (peptidoglycan and lipoteichoic acid) of the pneumococcus are recognized by TLR2 (Khan et al., 2002) and recent preliminary evidence also suggests that the inflammatory response of murine macrophages to the pneumococcal toxin pneumolysin depends on TLR4, and that mutant mice that lack TLR4 are significantly more susceptible to invasive disease and death following infection

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with pneumococci, compared to wild-type mice (Yoshimura et al., 1999). These findings, which suggest that the innate immune response to pneumococci is mediated in part by TLR4, challenges the generally accepted model whereby TLR4 is a receptor associated with ligands that are products of Gram-negative bacteria only. The suggestion that TLR4 and TLR2 are involved in the host innate immune response to pneumococcal infection is of great interest and hints at the important protective role these receptors might have in the host response to infection.

TLR4 activation leads to the initiation of the MyD88-dependent intracellular signaling pathway, which results in the activation of NF-κB, a transcription factor responsible for promoting, among other things, the production of multiple inflammatory cytokines (e.g., interleukin-6 [IL-6], IL-8, and IL-12). LPS exposure also results in the production of defensins, which comprise several distinct families of antibacterial, antifungal, and antiviral peptides (Ayabe et al., 2000). In addition to activating MyD88-dependent signaling, TLR3 and TLR4 agonists activate the TRIF-dependent pathway (also known as the MyD88-independent pathway), which results in the expression of interferon regulatory factor 3 and production of alpha/beta interferons (particularly beta interferon) (Toshchakov et al., 2002), as well as the activation of mitogen-activated protein kinases and NF-κB (Kawai et al., 1999), production of inducible nitric oxide synthase, and functional maturation of dendritic cells (Akira, 2003). Induction of beta interferon appears to be crucial for the antiviral effects associated with activation of TLR3 and TLR4, since mice deficient in TRIF are highly susceptible to viral infection (Hoebe et al., 2003). Thus, TLR4 activation results in short-term antimicrobial effects, which can be observed within minutes to hours of exposure to microbial components, as well as long-term effects on the adaptive response as dictated by the tenor of the cytokine environment in which antigens are presented.

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TLR2 plays a crucial role in the host defense against Gram-positive bacteria and their cell wall components (Echchannaoui et al., 2002; Branger et al., 2004). Therefore, it seems that TLR2 and TLR4 are activated primarily by different PAMPs to initiate the host response to Gram-positive and Gram-negative bacterial infections, respectively. How the host responds to different bacterial ligands and how prior exposures may influence responses to infection are basic questions that may ultimately provide insight into the pathogenesis of sepsis (Malley et al., 2003). Stimulation of the innate immune system causes the release of cytokines and other mediators that can drive the adaptive immune system in a specific direction. The use of innate immune stimulation to improve survival has previously been described for other pathogens. Studies in animal models have shown that beneficial effects can be achieved by immunomodulation of certain components of the host response to improve the outcome of disease (Medvedev et al., 2001).

In this study it is hypothesized that enhancing the innate immune system, through TLR4, would initiate a stronger immune response, leading to faster elimination of bacteria and modulation of certain components of the host response and therefore improve the outcome of the disease. The study determined if the enhancement of the innate immune response by exposure to sublethal doses of LPS would extend to a bacterial species that does not have an LPS component. LPS was administered before bacterial challenge and studied the response of the mice to a subsequent challenge with the live *Streptococcus pneumoniae*. It was observed that endotoxin pretreatment improves bacterial clearance and decreases mortality in mice challenged with *Streptococcus pneumoniae*.

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2.2. Objectives of the study

The main objective of the study was to determine if enhancing the innate immune system, through TLR4, would initiate a stronger immune response, leading to faster elimination of bacteria and modulation of certain components of the host response and therefore improve the outcome of pneumococcal infection.

2.2.1. The specific objectives of the study were:

2.2.1.1. To determine the effect of innate stimulation through TLR4 on the survival of mice after bacterial challenge

2.2.1.2 To determine the effect of innate stimulation on bacterial load in blood and lungs of mice after bacterial challenge in order to examine whether survival benefits resulted from improved bacterial clearance or from managing high numbers of bacteria.

2.2.1.3. To determine serum cytokine concentration from control and LPS pre-treated mice

2.2.1.4. To determine the effect of pretreatment with low dose of LPS on blood chemistry and determine if excessive liver injury may have contributed to increased mortality

2.2.1.5 To determine the activation status of specific lymphocyte populations from splenocytes from infected and LPS treated mice, and analyze for upregulation of CD69 and NK1.1 expression as an indication of cellular activation.

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2. 3. MATERIALS AND METHODS

2.3.1 Experimental animals

Female C57BL/6, BALB/c, CBA/CaHN-Btk^{xid}/J, A/J (complement deficient mice), Rag-1 Knock-out, IL-10 Knock-out (Jackson laboratories, Bar Harbor), C3H/HeN and C3H/HeJ (Charles River) mice, 8-10 weeks of age, weighing approximately 20 grams each, were kept in a sterile environment prior to start of experiments. The mice were maintained on standard lab chow and water *ad libitum* with a 12-hr light/dark cycle. The protocol used in the present study was approved by the Animal Care and Use Committee, University of Virginia, USA.

2.3.2 Reagents

Ultrapure Escherichia coli 0111:B4 LPS was purchased from InvivoGen (San Diego, USA).

2.3.3 Bacteria

Streptococcus pneumoniae ATCC 6302 ™ from the American Type Culture Collection (Manassas, VA, USA) were grown as described by Restrepo *et al.* (2005). Briefly, bacterial stock stored at -70^{0}C was thawed and resuscitated by two consecutive passes on 5% sheep blood Tryptic soy agar (TSA) supplemented with 0.5% yeast extract and incubated for 15 hours under 5% CO_2 atmosphere. Colonies from the second plate were inoculated into 10 ml of supplemented Brain Heart Infusion broth, followed by 3 successive 1:10 dilutions into identical liquid media. All tubes were incubated for 12 hours under 5% CO_2 atmosphere. 1 ml of bacterial suspension taken from the most diluted tube with visible growth was inoculated into 9 ml of supplemented Brain Heart Infusion broth and incubated for 3 hours at 37^{0}C under 5% CO_2 atmosphere. Optical

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density was measured to obtain the desired concentration (Vitek 15 = appr. 10^8 CFU/ml) and serial dilutions were plated on 5% sheep blood TSA to ensure final concentration of approximately 10^8 CFU/ml.

2.3.4 Infection and survival

Mice were infected intravenously with a lethal dose ($5x10^6$-$5 x10^7$ cfu) of *S. pneumoniae* suspension 48 hours after intravenous treatment with five injections of 10ug highly purified LPS or vehicle intravenously (i.v) or intraperitoneally (i.p) at 12 hours interval. Controls for all strains received PBS. Survival was monitored for 5 days after bacterial challenge.

2.3.5 Bacteriological examinations

Mice were anaesthetized with Isothesia-isoflurane™ and blood was collected from mice by cardiac puncture under anesthesia and then killed by cervical dislocation. Lungs were dissected carefully and excised, and then separately homogenized in 10 ml of saline by teasing with a stainless mesh at room temperature. Blood (collected at t=6h,12h and 24h after bacterial challenge) and lung homogenates (collected at t=6h) were serially diluted 10-fold with sterile physiological saline or PBS, and 0.2ml samples of the various dilutions were inoculated onto 5% sheep blood TSA. The plates were incubated at 37^0C 5% CO_2 overnight. Colonies were enumerated and bacterial counts were expressed as the log number of CFU per milliliter of blood.

2.3.6 Cytokine measurements

Blood samples from untreated control, LPS-treated C57Bl/6, C3H/HeN and C3H/HeJ mice were collected at different time points after bacterial infection. Individual sera were separated from blood by centrifugation and stored at -70^0C until cytokine assays were

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performed. Serum cytokines were determined by using protein-based multiplex immunoassay system (Bio-Rad Laboratories, Hercules, CA) in duplicates per sample.

2.3.7 Biochemical assays for liver function

Level of blood glucose and activities of liver enzymes; levels of aspartate aminotransferase(AST), alanine aminotransferase (ALT) in serum, as an indication of hepatic cellular injury, were determined in blood samples collected 6 and 12 hours after *S. pneumoniae* inoculation. Heparinized blood samples were spun at 10, 000 x g for 15 minutes; then serum samples were collected and frozen at -70^{0}C until blood chemistry activities were analyzed. Biochemical assays for liver function were analyzed by the Clinical Pathology Laboratory at the University of Virginia Medical Center by use of an automated spectrophotometric assay (Abbott Architect C8000).

2.3.8 Multiparameter flow cytometric analysis of splenic lymphocytes

Mice were anaesthetized with Isothesia-isoflurane™ and then killed by cervical dislocation. Spleens from uninfected, infected and LPS treated mice were harvested for analysis. Single-cell suspensions were prepared or isolated. Spleens were minced and serially passed through 100-and 40μm cell strainers (BD Biosciences). Spleenocytes were resuspended at 10^6 cells/ml in 100μl staining buffer (1% bovine serum albumin [BSA], 0.1% sodium azide in phosphate-buffered saline [PBS]). Cells were Fcγ receptor-blocked and stained with cell surface markers. A flexible live/dead stain trypsin blue (Invitrogen) was used to assess viability. All staining reagents were purchased from eBioscience. Nonspecific antibody binding was blocked by incubation with an anti-CD16/32 antibody prior to specific antigen staining. The activation status of lymphocytes subsets were determined by incubation with an anti-CD69-biotin antibody (H1.2F3) detected with streptavidin–allophycocyanin. Specific lymphocytes subsets were detected

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by using the following directly conjugated (with fluorescein isothiocyanate, phycoerythrin, or PerCP-Cy5.5) antibodies: anti-CD3 (145-2C11) PE, and anti-CD161 (anti-NK1.1; clone PK136) PE, anti-CD49b (DX5) PE. Flow cytometric acquisition was performed on a FACSCalibur benchtop analyser (Becton Dickinson/Cytek) at the flow cytometry core facilities at the University of Virginia, USA. At least 10,000 events were collected for each analysis. Data analyses were performed using the Cellquest (Becton Dickinson) software package.

2.3.9 Statistical analysis

Culture data from each time point were expressed as mean log CFU ± SD for each group. Statistical comparisons of cytokine values were done by a 2 tailed Student's t-Test (Microsoft Excel software, Microsoft Corporation, Redmond, WA). Survival data were plotted and statistical analysis for survival studies was performed using the log-rank test with GraphPad Prism, version 5.0 for windows (GraphPad PRISM Software, San Diego, CA). Data are displayed as means ± SEM unless otherwise stated. Differences were considered statistically significant when $p<0.05$.

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2.4. RESULTS

2.4.1 Survival

C57BL/6 and Balb/c mice were pretreated with PBS or LPS as described above and infected with *S. pneumoniae* 2h after the last LPS injection. Mice were inoculated intravenously with a lethal dose $2x10^6$ CFU of *S. pneumoniae* suspension 48 hours after intravenous treatment with five injections of 10ug highly purified LPS or vehicle at 12 hours interval. LPS –treated mice survived significantly longer than untreated control mice. Survival for LPS-treated C57bl/6 and Balb/c was 90% (N=29; $p<0.001$), and 50% (N=14; $p = 0.0056$), respectively and mortality for untreated controls was 100% (Fig. 2.1). The onset of clinical symptoms such as piloerection and inactivity were observed earlier by 6-12h in the control group, whereas LPS-treated group showed no symptoms of disease during the first 24 hours. Median survival times for control and LPS-tolerant C57BL/6 mice were 48 hours and 140 hours, respectively and 72 hours and 120 hours for control and LPS-tolerant Balb/c mice, respectively. The significance of the Kaplan-Meier survival curves was assessed by the Prism log rank test.

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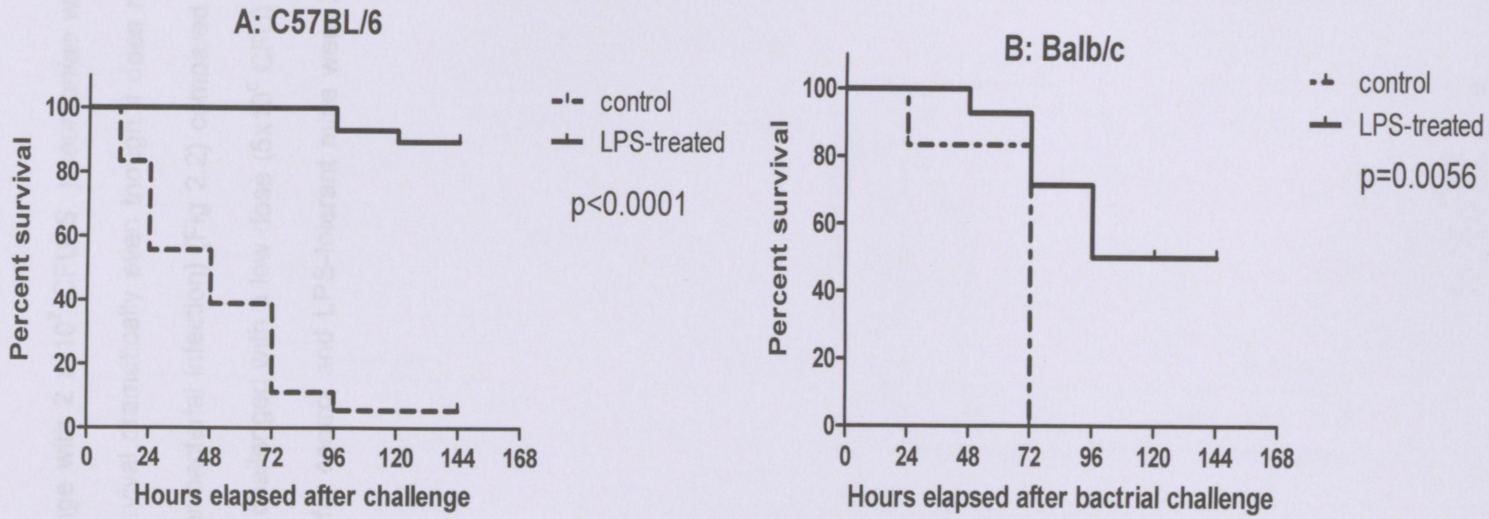


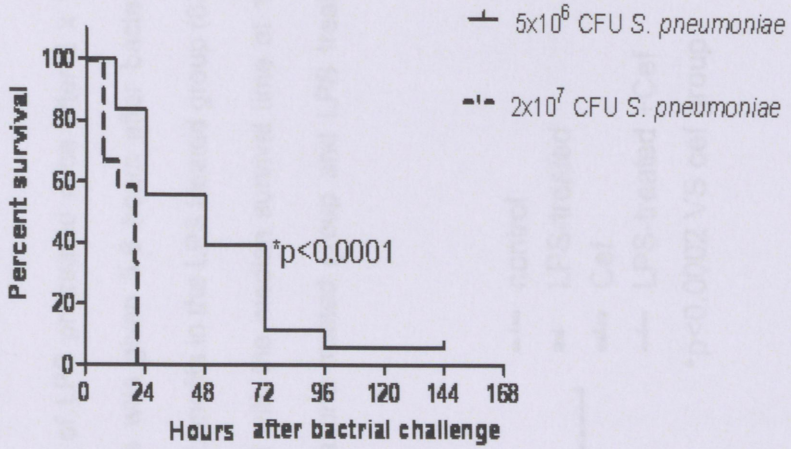
Figure 2.1: Survival curves for control and LPS –pretreated mice following intravenous bacterial challenge with 2 x 10^6 CFU of *S. pneumoniae* culture. Median survival times for control (n=18) and LPS-pretreated (n=29) C57BL/6 mice (**A**) were 48 hours and 140 hours, respectively and for control (n=6) and LPS-pretreated (n=14) Balb/c mice (**B**) were 72 hours and 120 hours, respectively. The significance of the Kaplan-Meier survival curves was assessed by the Prism log rank test ($p<0.05$ for statistical significant difference).

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Survival rate for C57BL/6 mice after challenge with 2 x 10^7 CFU *S. pneumoniae* was reduced and LPS pretreatment extended survival dramatically even though it does not reduce mortality (100% mortality 5 days after bacterial infection) (Fig 2.2) compared to 90% survival in the LPS treated group when challenged with a low dose ($5x10^6$ CFU) of *S. pneumoniae*. The median survival times for control and LPS-tolerant mice were 20 hours and 70 hours, respectively ($p<0.001$).

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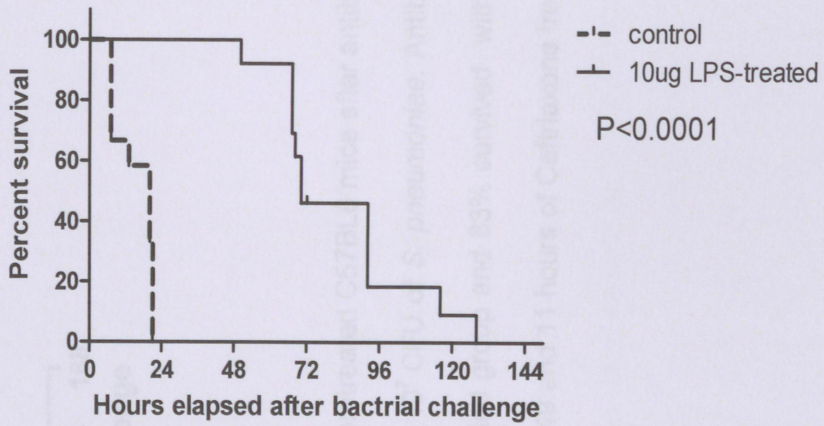


Figure 2.2. Survival curves for C57BL/6 mice after challenge with 5 x 10^6 (n=18) and 2 x 10^7 CFU of *S. pneumoniae (n=12)*. Median survival times for low dose and high dose challenged mice were 48 hours and 20 hours, respectively. Median survival times for control (n=12) and LPS-pretreated (n=13) mice challenged with high dose of 2 x 10^7 *CFU S. pneumoniae* were 20 hours and 70 hours, respectively (p<0.001).

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The effect of antibiotic treatment on the survival of LPS pretreated mice after 2 x 10^7 CFU *S. pneumoniae* was examined. Ceftriaxone was given 5-6 hours after bacterial challenge. Antibiotic treatment enhanced survival benefits in the LPS treated group (83% vs 12% and 0% cef and LPS treated group) and with the median survival time of 168 hours compared with 49 and 11 hours of ceftriaxone treated group and LPS treated group, respectively (Fig. 2.3).

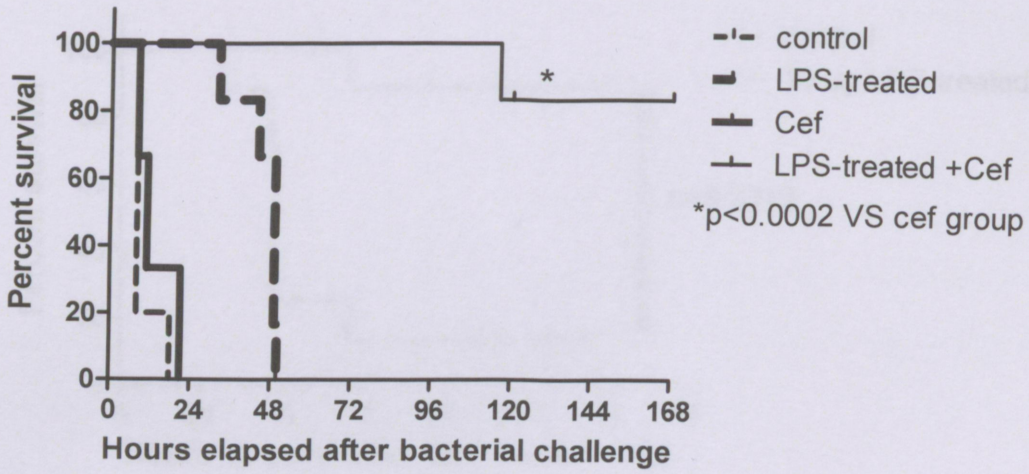


Figure 2.3. Survival curves for control and LPS –pretreated C57BL6 mice after antibiotic following intravenous bacterial challenge with 2 x 10^7 CFU of *S. pneumoniae*. Antibiotic treatment enhanced survival benefits in LPS treated group and 83% survived with the median survival time of 168 hours compared with 49 and 11 hours of Ceftriaxone treated group and LPS treated group, respectively.

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LPS pretreatment protected mice from intranasal pneumococcal infection and 87 % of LPS treated mice survived and median survival time of 144h compared with 12% survival and median survival time of 46 hours in the control group ($p<0.0013$) (Fig. 2.4). Experimental induction of tolerance did not cause increased susceptibility of mice to *S. pneumoniae* as observed in innately LPS-unresponsive mice (C3H/HeJ), but instead improved or extended survival.

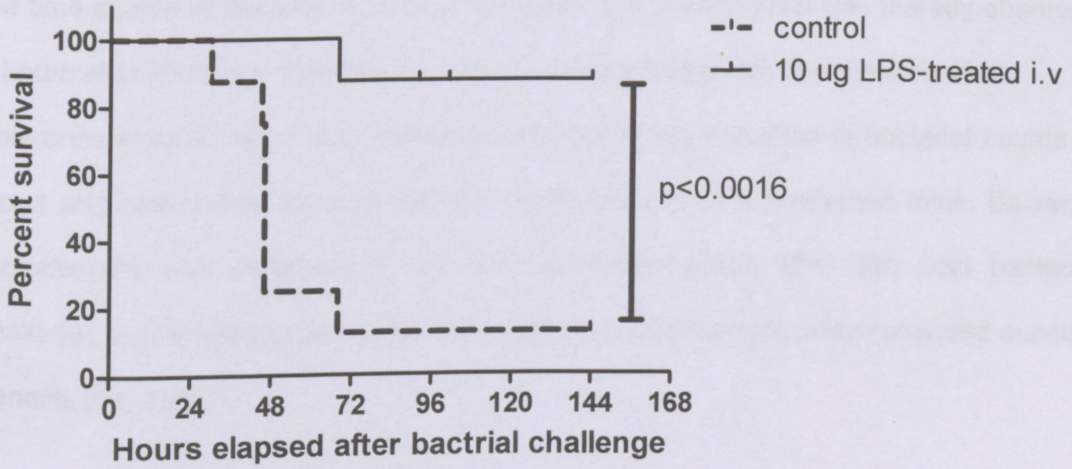


Figure 2. 4. Survival curves for control and LPS –pretreated C57BL6 mice following intranasal bacterial challenge with 2 x 10^7 CFU/ml. The data expressed as percent surviving mice, and are cumulative results of different experiments. LPS pretreatment protected mice from intranasal pneumococcal infection. 87 % survival and median survival time of 144h compared to 12% survival and median survival time of 46 hours ($p<0.0016$).

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2.4.2 Bacteriological analysis from blood

The study examined whether survival benefits resulted from improved bacterial clearance or from managing high numbers of bacteria. We studied bacterial load in blood and lung homogenates of control and LPS-treated mice collected at different time points (t=3h, 6h, 12h, 24h, 72h) after infection with *S. pneumoniae*. Systemic bacteremia (blood and lung) was significantly reduced by at least four logs in LPS-pretreated mice compared to the control untreated group (Fig. 2.5a). A comparison of the time course of bacterial load in LPS-treated and control mice was already observed 6 hours after infection. Reduced bacteremia corresponded with improved survival.

Tolerance induced by LPS pretreatment resulted in the reduction in bacterial counts in blood and ceftriaxone reduced the level of bacteria in LPS pretreated mice. Bacterial recrudescent was observed in the LPS pretreated group after 24h post bacterial challenge and antibiotic eliminated and prevented recrudescent, which provided survival benefits (Fig 2.5b).

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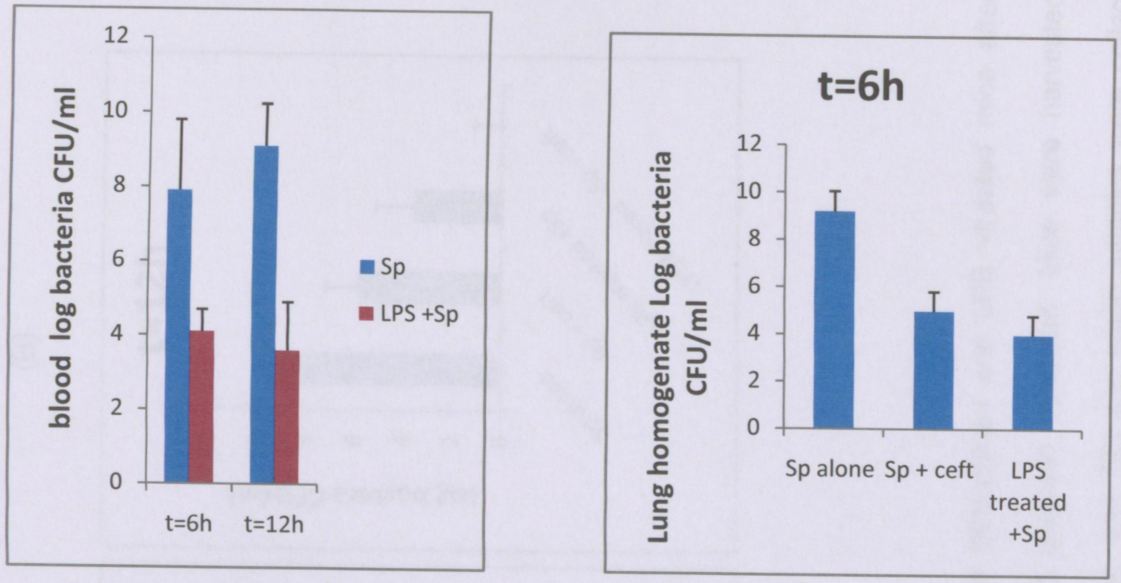


Figure 2. 5a. Bacterial clearance of LPS -pretreated and PBS-primed mice after *S. pneumoniae* challenge. LPS tolerance induced via the intravenous route was associated with a significant reduction in bacterial colonies in blood and lung tissues when compared with PBS-pretreated control groups (n=5-8 per group, lung homogenate n=3). The results are expressed as the median, and standard deviations are indicated by error bars. ($p<0.05$ vs respective control group based on t-Test).

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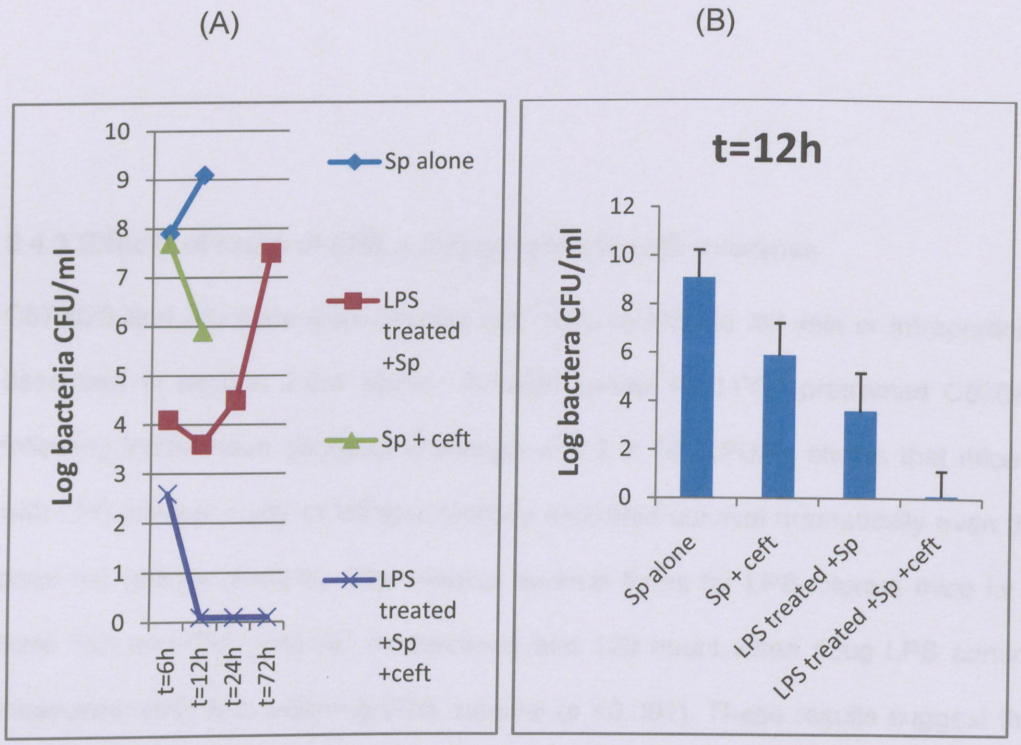


Figure 2. 5b. Bacterial clearance of PBS-primed and LPS –treated mice after *S. pneumoniae* bacterial challenge and antibiotic treatment. Mice were harvested at different time points at t=6h, 12h, 24, and 72h and blood samples were collected. Bacterial CFUs were counted to assess bacterial burden. Antibiotic ceftriaxone was given 5-6 hours after bacterial challenge. Tolerance induced by LPS pretreatment resulted in the reduction in bacterial counts in blood, and antibiotic ceftriaxone reduced the level of bacteria in LPS pretreated mice ($p<0.05$ vs respective control group based on t-Test). Data from 4 to 6 mice per group are presented as Mean/Median, with the standard deviation indicated by error bars.

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2.4.3 Effects of route of LPS administration in LPS tolerance

C57BL/6 and A/J mice were treated with 10ug reLPS via tail vein or intraperitonially as described in section 2.2.4 above. Survival curves for LPS –pretreated C57BL6 mice following intravenous bacterial challenge with 2 x 10^7 CFU/ml shows that mice treated with LPS intravenously or intraperitoneally extended survival dramatically even though it does not reduce mortality. The median survival times for LPS-tolerant mice i.v and i.p were 70h and 73h ($p>0.05$), respectively and 120 hours when 50ug LPS administered intraperitoneally also showing 60% survival ($p <0.001$). These results suggest the route of LPS administration to induce tolerance is not important. The degree of enhanced survival induced by LPS pretreatment is dependent on the dose of LPS. Greater efficacy in protection in terms of clinical symptoms was observed when LPS and bacterial challenge were injected by the same route in mice (mice were healthier than the other group) than employing heterologous inoculation routes. But there was no statistical significant difference between these groups in terms of the survivors.

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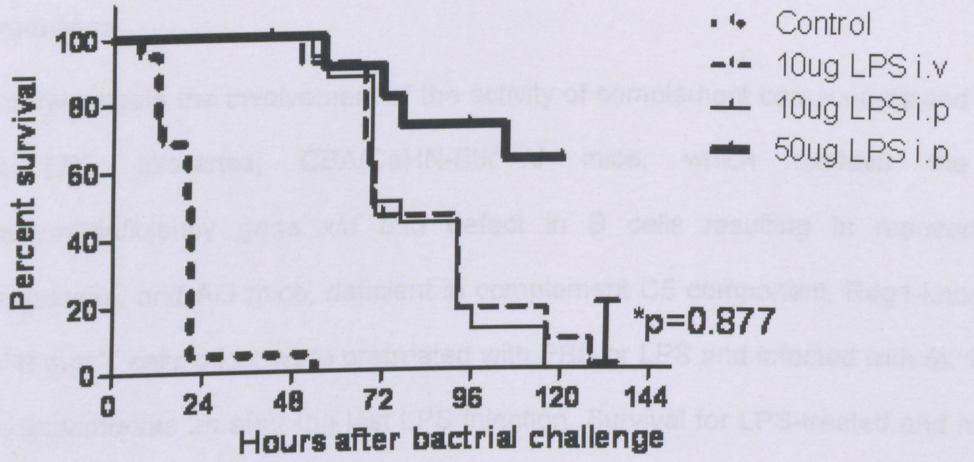


Figure 2.6. Survival curves for control and LPS –pretreated C57BL6 following intravenous bacterial challenge with 2 x 10^7 CFU of *S. pneumoniae*. The data expressed as percent surviving mice, are cumulative results of different experiments. LPS was administered intravenously or intraperitoneally before bacterial challenge and the median survival times for LPS-pretreated mice treated with 10ug LPS intravenously or intraperitoneally were 70h and 73h ($p>0.05$), respectively, and 120h when 50ug LPS administered *i.p* ($p<0.05$).

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2.4.4 Importance of the activity of the immune cells and antibodies in LPS tolerance

To investigate the involvement of the activity of complement components and antibodies in LPS tolerance, CBA/CaHN-Btk$^{xid/}$J mice, which express the X-linked immunodeficiency gene *xid* and defect in B cells resulting in reduced antibody responses, and A/J mice, deficient in complement C5 component, Rag1-knockout, lack of B and T cells mice, were pretreated with PBS or LPS and infected with 5x 10^6 CFU of *S. pneumoniae* 2h after the last LPS injection. Survival for LPS-treated and non-treated mice was 80% (n=10) and 0 % (n= 6) in CBA/CaHN-Btk$^{xid/}$J mice with median survival time 46 hours and 140 hours ($p<0.005$), respectively and 85% (n=14) and 0 % (n=9) in A/J mice, respectively and with median survival time 34 hours and 96 hours ($p<0.0001$), respectively (Fig. 2.7). These results with LPS- treated A/J and CBA/CaHN-Btk$^{xid/}$J mice also indicated that deficient in complement and antibodies did not ablate survival benefits. The fact that the A/J mice were protected indicates that opsonization not formation of membrane attack complex is involved in the elimination of bacteria LPS treated mice.

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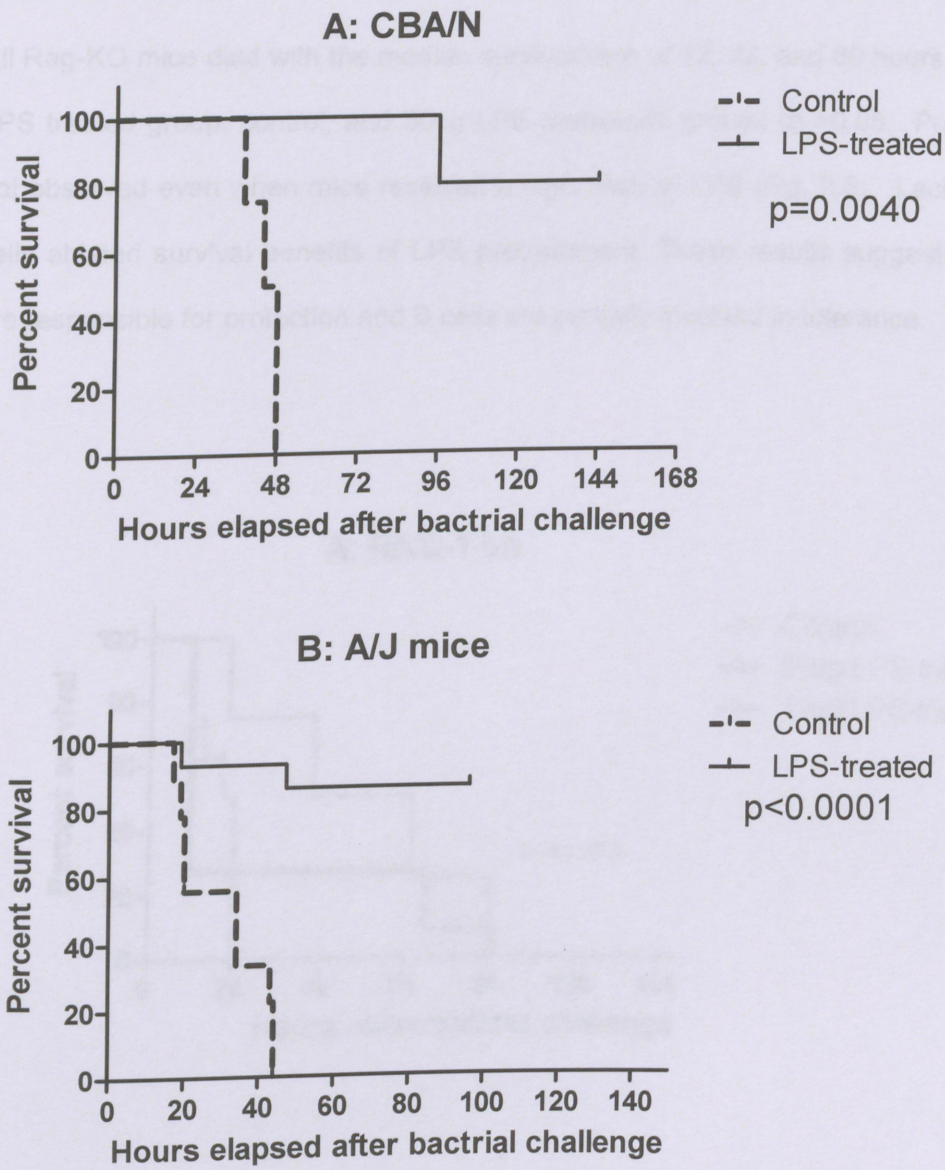


Figure 2.7. Survival curves for CBA/N and A/J mice after challenged with *S. pneumoniae*. Mice were pre-treated via intravenous (i.v) route with vehicle (PBS-Controls), five injections of 10ug reLPS 48h prior to challenge with a lethal dose of *S.pneumoniae* (10^7cfu). There was a significant difference between pretreated and untreated group (p=0.05). Median survival times for control (n=4) and LPS-tolerant (n=10) mice CBAN **(A)** were 46hours and 140 hours, respectively and for control (n=9) and LPS-tolerant (n=14) CBA/ J **(B)** mice were 34 hours and 96 hours, respectively.

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All Rag-KO mice died with the median survival time of 12, 22, and 60 hours for the 10ug LPS treated group, control, and 50ug LPS pretreated groups ($p > 0.05$. Protection was not observed even when mice received a high dose of LPS (Fig. 2.8). Lack of B and T cells ablated survival benefits of LPS pretreatment. These results suggest that T cells are responsible for protection and B cells are partially involved in tolerance.

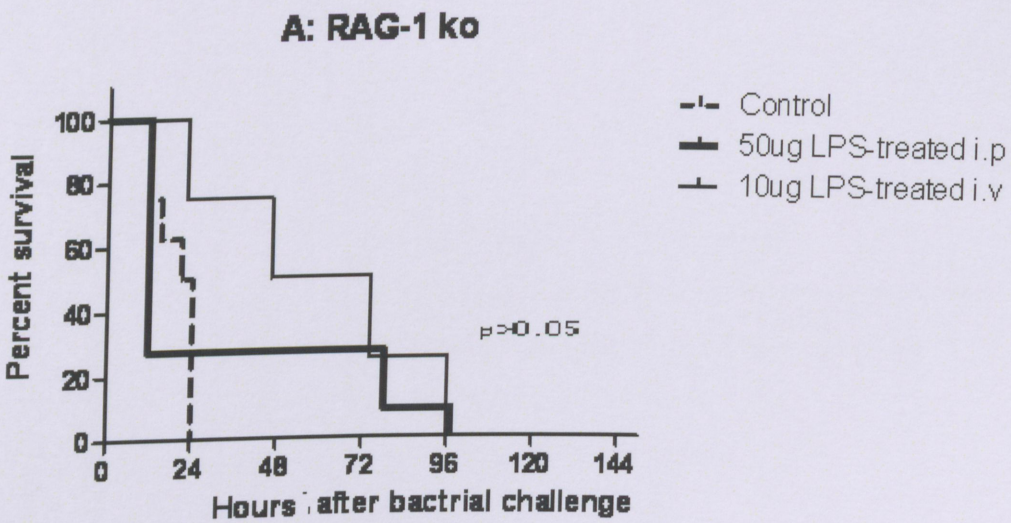


Figure 2. 8. Survival curves for control and LPS –pretreated Rag-1 KO mice following intravenous bacterial challenge with 2 x 10^7 CFU/ml of *S.pneumoniae*. The data expressed as percent surviving mice. All Rag-KO mice died with the median survival time of 12, 22, and 60 hours for the10ug LPS preteated group, control, and 50ug LPS pretreated groups (P >0.05).

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2.4.5 The effect of LPS pretreatment on cytokine production in C57Bl/6 mice

Blood was taken from LPS-treated uninfected and naïve C57BL/6 mice for determination of cytokine levels. High levels of TNF-a, IL-6, IL-10, IL-1a, Rantes, IL-12 (p70), G-CSF, KC, and MIP-1a were detected in plasma of LPS-treated mice compared to naïve control mice (Fig. 2.9a). The results indicated that inflammatory immune response is of crucial importance for the early containment of infection as indicated by high survival percentage in LPS-treated groups.

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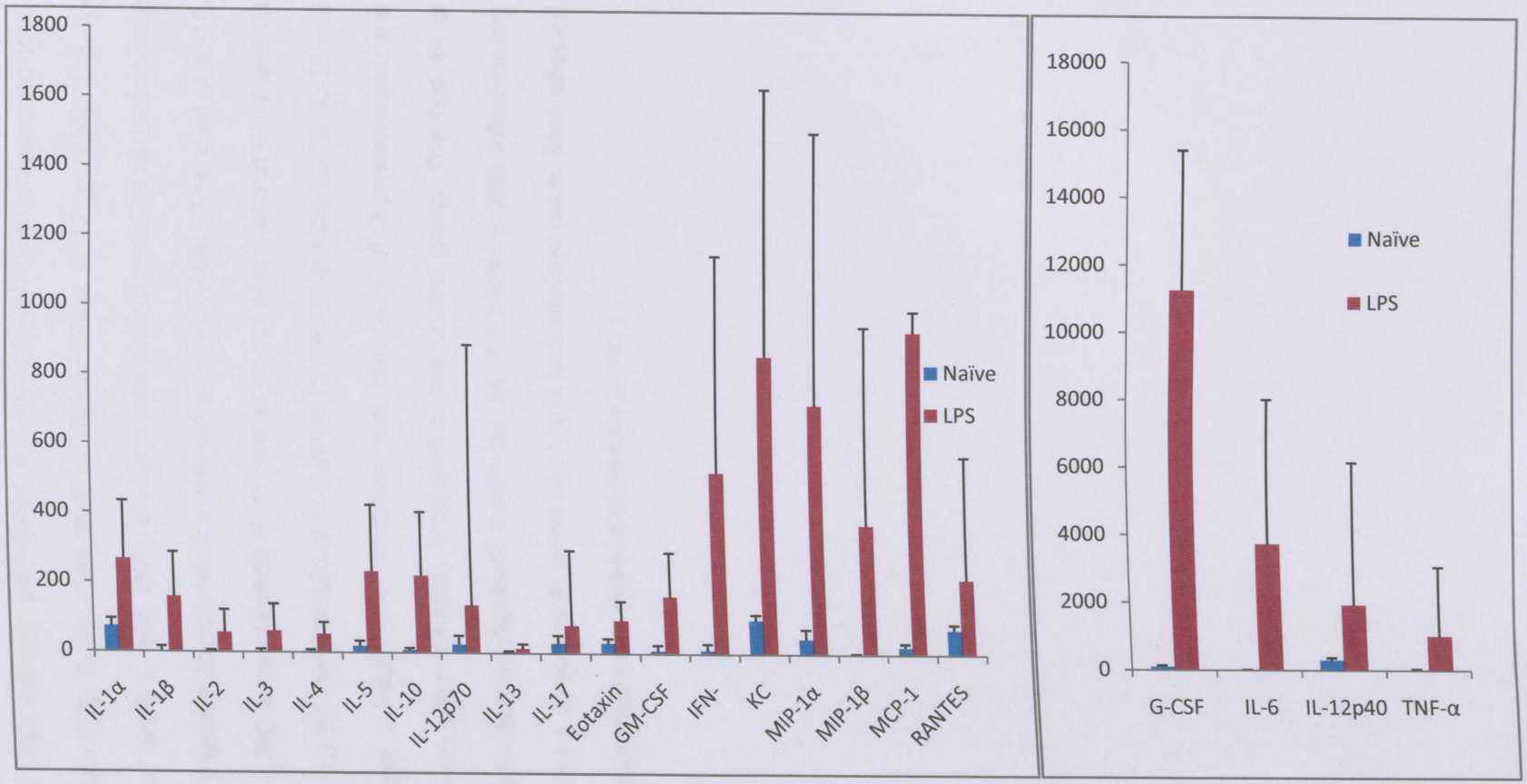


Figure 2. 9a. Levels of proinflammatory cytokines (pg/ml) in blood at the time of infection (t=0) before bacterial challenge in normal PBS-primed (naive) and LPS-pretreated (LPS) uninfected mice. The results are expressed as the means for 4-5 mice in each group and standard deviation represented by error bars. $p<0.05$ vs respective control group based on student t-Test

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Cytokine production was strongly attenuated in LPS- treated mice in response to *S. pneumoniae* challenge (Fig. 2. 9b). 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 ($p<0.05$). The level of IL-10, MCP-1, IL-5, IL-6 and KC were elevated at 3h time point but attenuated at 6, 12 and 24 hours after infection in LPS- treated group compared to control groups. IL-13, IL-17 and G-CSF were elevated 6 hours after infection with low dose of *S.pneumoniae* and remained high 12 hours after infection compared to the control group. The first 4h of infection were characterized by apparent quietness, as few bacteria, little inflammatory mediator production were observed in blood of control and infected mice. Mice showed no symptoms of infection and were active and healthy looking.

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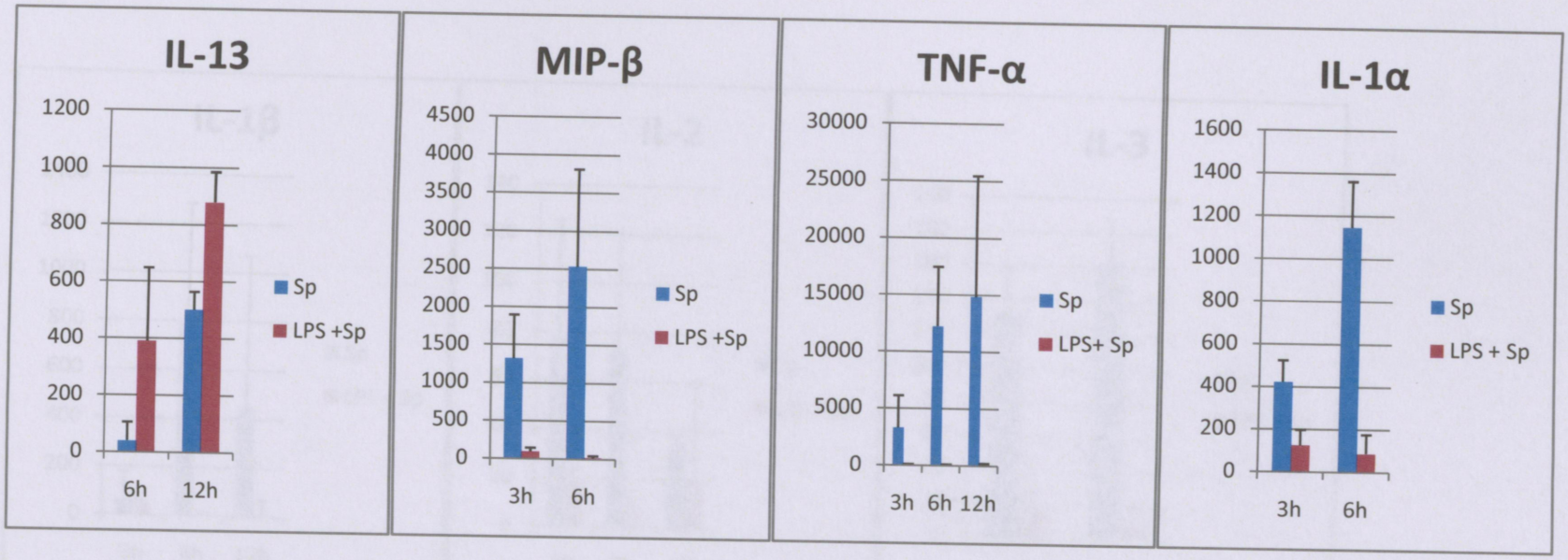


Figure 2. 9b (i). Levels of proinflammatory cytokines in blood collected 3h, 6h and 12 h after bacterial challenge in normal PBS-primed control (Sp) and LPS-pretreated (LPS) mice. The results are expressed as the means for 7-10 mice in each group and standard deviation represented by error bars. $p<0.05$ vs respective control group based on student T-test

NB. *x-axis= time in hours after bacterial challenge * y-axis = concentration in pg/ml

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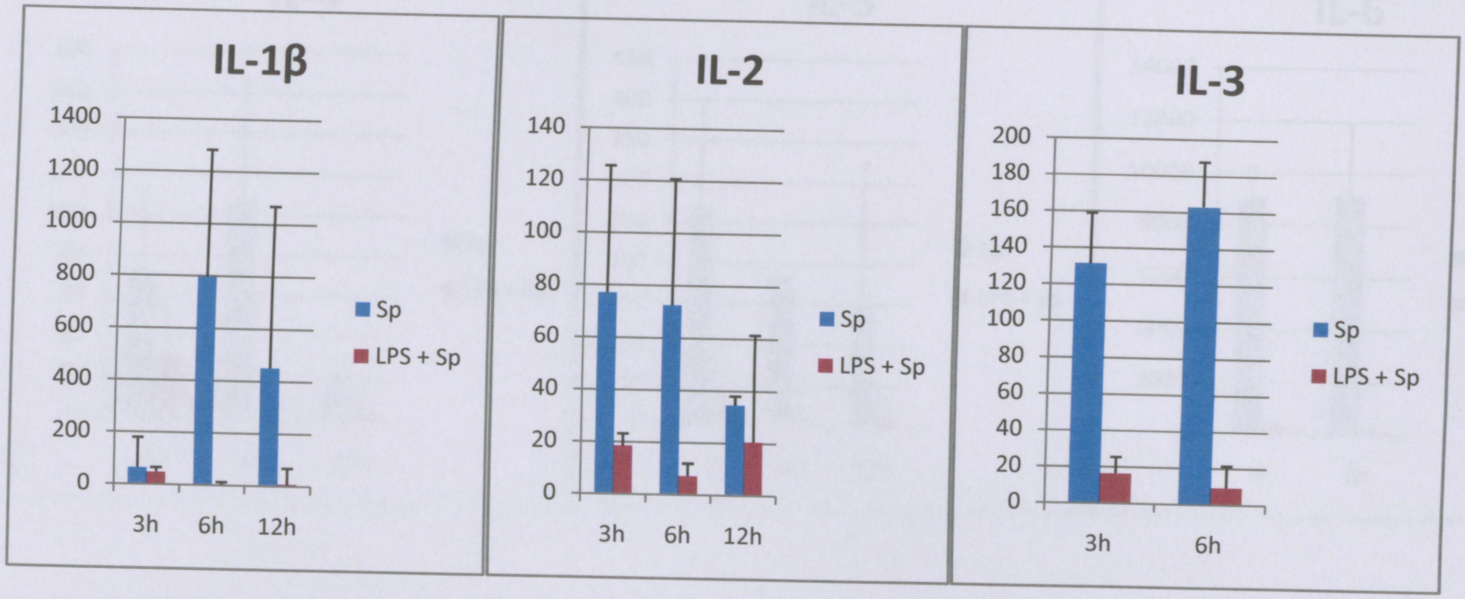


Figure 2. 9b (ii). Levels of proinflammatory cytokines in blood collected 3h, 6h and 12 h after bacterial challenge in normal PBS-primed control (Sp) and LPS-pretreated (LPS) mice. The results are expressed as the means for 7-10 mice in each group and standard deviation represented by error bars. $p<0.05$ vs respective control group based on student T-test

NB. *x-axis= time in hours after bacterial challenge * y-axis = concentration in pg/ml

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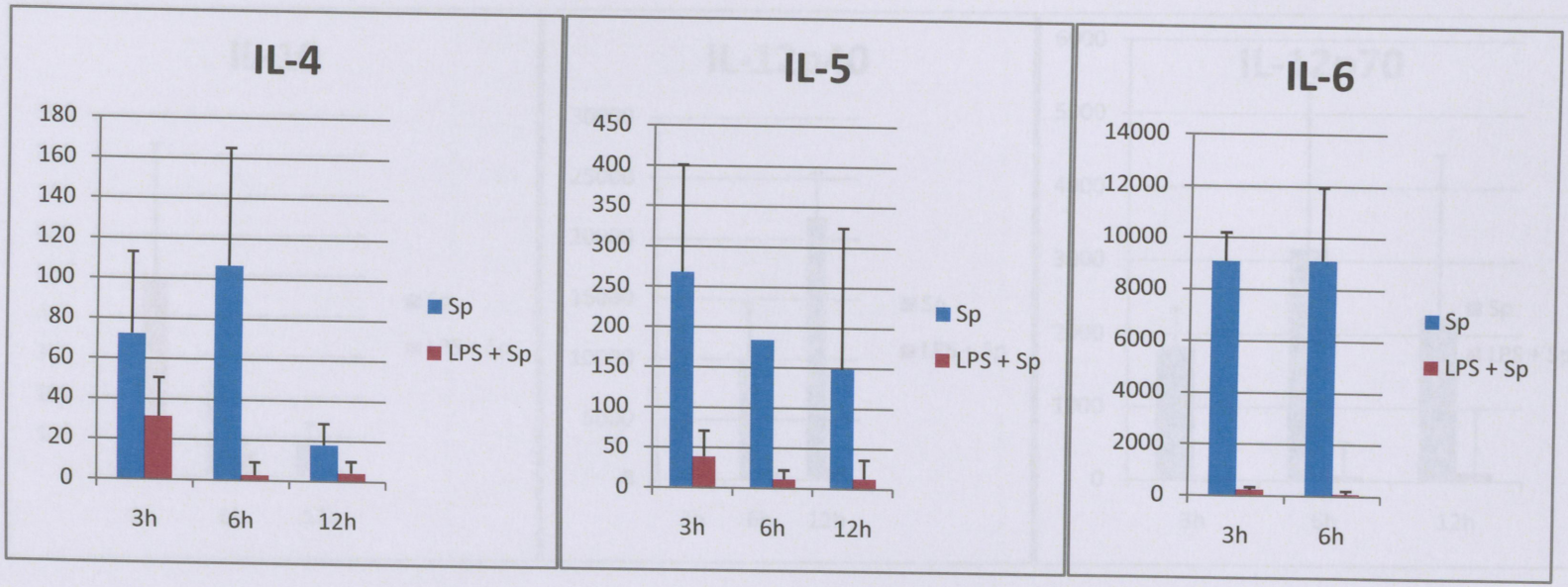


Figure 2. 9b (iii). Levels of proinflammatory cytokines in blood collected 3h, 6h and 12 h after bacterial challenge in normal PBS-primed control (Sp) and LPS-pretreated (LPS) mice. The results are expressed as the means for 7-10 mice in each group and standard deviation represented by error bars. $p<0.05$ vs respective control group based on student T-test

NB. *x-axis= time in hours after bacterial challenge * y-axis = concentration in pg/ml

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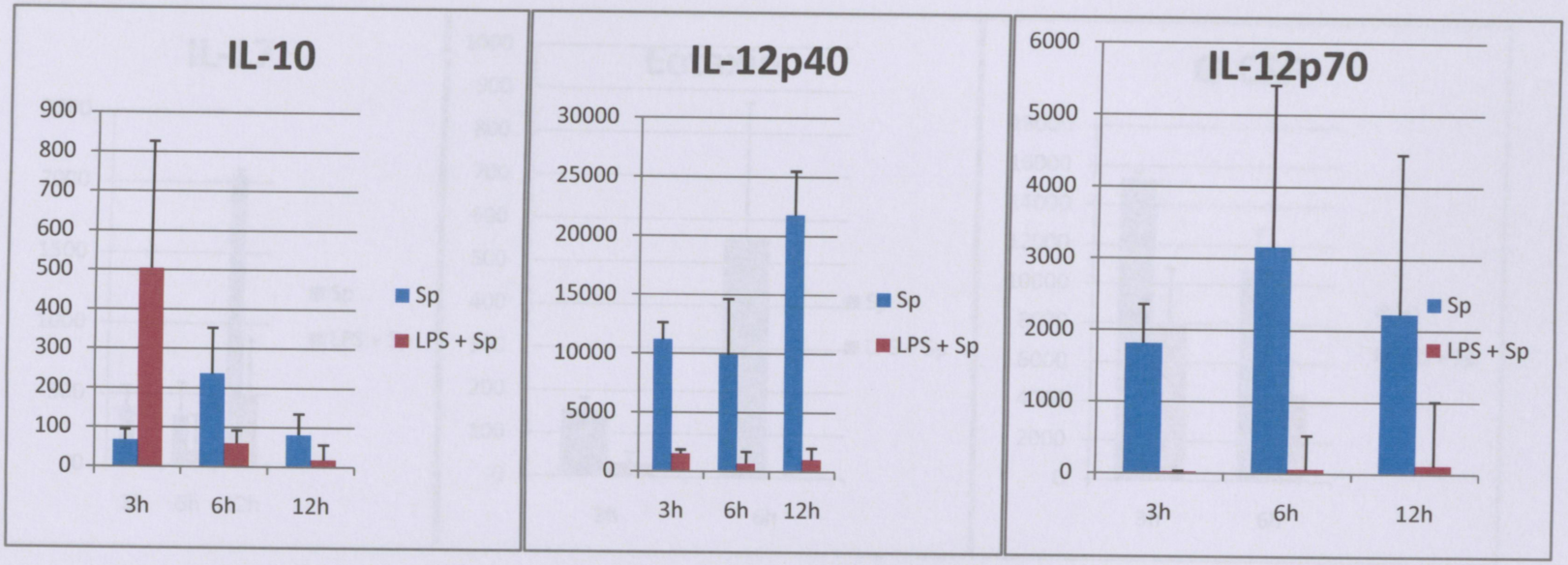


Figure 2. 9b (iv). Levels of proinflammatory cytokines in blood collected 3h, 6h and 12 h after bacterial challenge in normal PBS-primed control (Sp) and LPS-pretreated (LPS) mice. The results are expressed as the means for 7-10 mice in each group and standard deviation represented by error bars. $p<0.05$ vs respective control group based on student T-test

NB. *x-axis= time in hours after bacterial challenge * y-axis = concentration in pg/ml

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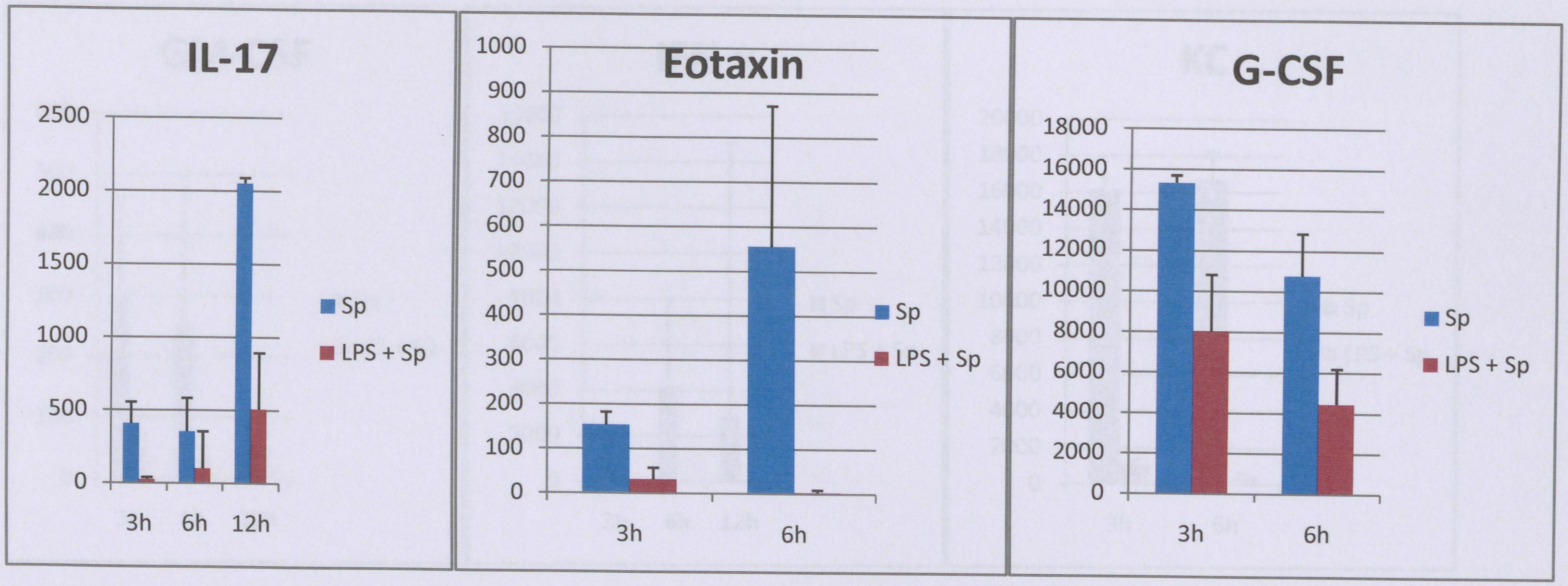


Figure 2. 9b(v). Levels of proinflammatory cytokines in blood collected 3h, 6h and 12 h after bacterial challenge in normal PBS-primed control (Sp) and LPS-pretreated (LPS) mice. The results are expressed as the means for 7-10 mice in each group and standard deviation represented by error bars. $p<0.05$ vs respective control group based on student T-test

NB. *x-axis= time in hours after bacterial challenge * y-axis = concentration in pg/ml

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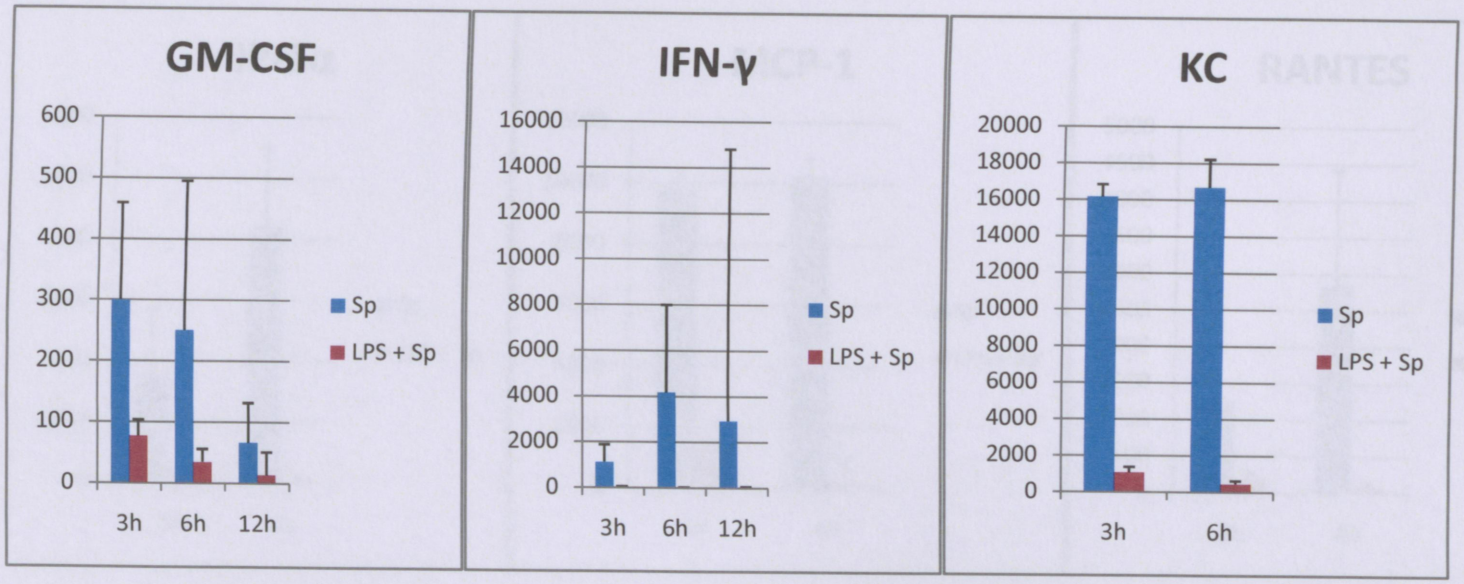


Figure 2. 9b(vi). Levels of proinflammatory cytokines in blood collected 3h, 6h and 12 h after bacterial challenge in normal PBS-primed control (Sp) and LPS-pretreated (LPS) mice. The results are expressed as the means for 7-10 mice in each group and standard deviation represented by error bars. $p<0.05$ vs respective control group based on student T-test

NB. *x-axis= time in hours after bacterial challenge * y-axis = concentration in pg/ml

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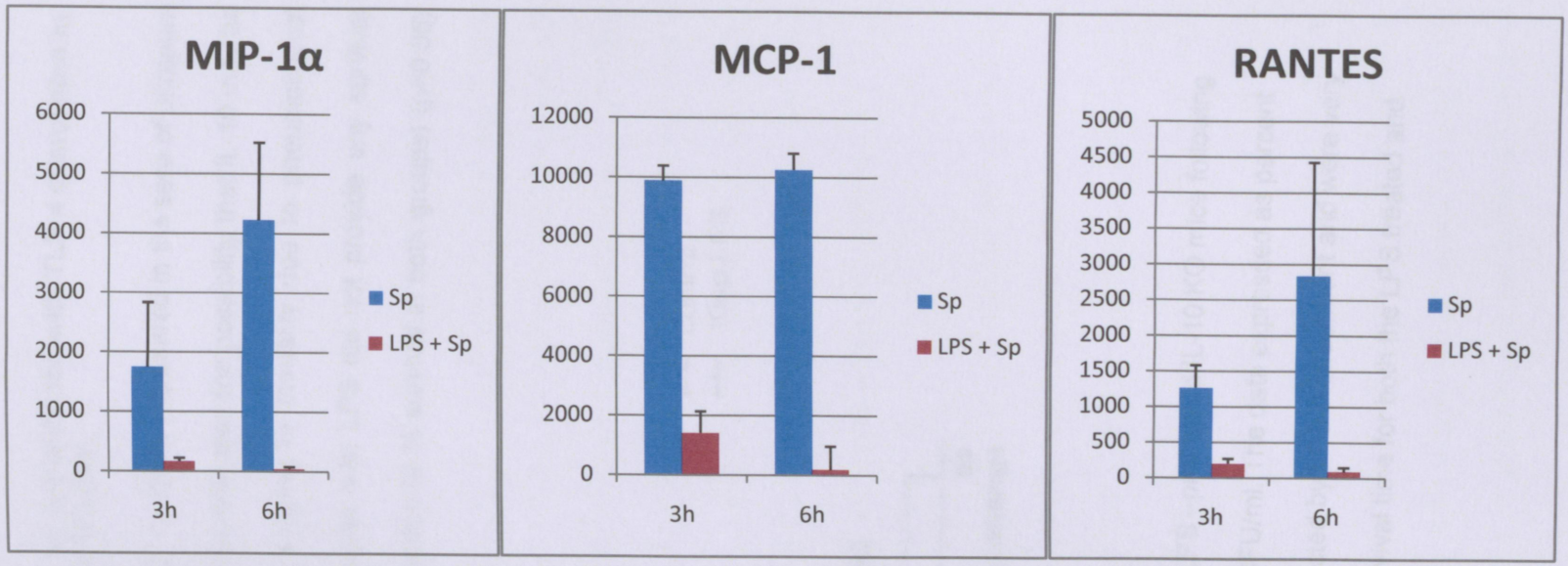


Figure 2. 9b (vii). Levels of proinflammatory cytokines in blood collected 3h, 6h and 12 h after bacterial challenge in normal PBS-primed control (Sp) and LPS-pretreated (LPS) mice. The results are expressed as the means for 7-10 mice in each group and standard deviation represented by error bars. $p<0.05$ vs respective control group based on student T-test

NB. *x-axis= time in hours after bacterial challenge * y-axis = concentration in pg/ml

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2.4.6 Role of IL-10 in the survival enhancement provided with TLR 4 stimulation in a lethal murine model of systemic S. pneumoniae

This study showed that IL-10, TNF and other cytokines appeared in the sera of ultrapure pretreated mice at the time of infection. We investigated the possibility that IL-10 may be important for directly suppressing events leading to mortality due to pneumococcal infection. Pretreatment of IL-10 knockout mice with LPS did not provide any survival benefit compared to the control untreated group (0 % survival in both groups) (p=0.35) (Figure 2.10).

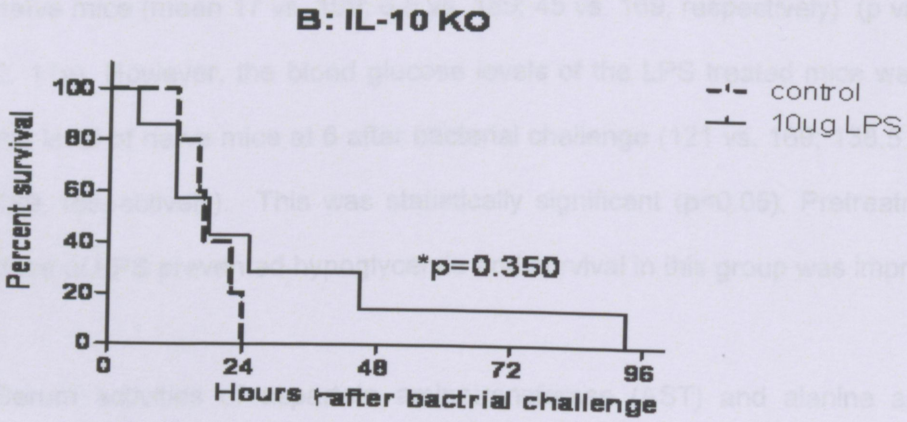


Figure 2.10. Survival curves for control and LPS –pretreated IL-10 KO mice following intravenous bacterial challenge with 2 x 10^7 CFU/ml. The data expressed as percent surviving mice. IL-10 KO mice were not protected by LPS pretreatment and were very sensitive to low dose of LPS. The median survival time for both the LPS treated and control group was 18 hours, p=0.35.

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2.4.7 The effect of pretreatment with low dose of LPS on blood chemistry in C57BL/6 mice

To determine the effect of pretreatment with low dose of LPS on blood chemistry and determine if excessive liver injury may have contributed to increased mortality, release of the hepatocyte-associated enzymes AST and ALT into the peripheral blood were examined. Fig 2.11a shows the mean serum concentration of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and glucose, in LPS treated and control mice. The results showed that the blood glucose level significantly decreased at 6 after bacterial challenge in the control infected mice when compared with naïve mice (mean 17 vs. 169; 6.5 vs. 169; 45 vs. 169, respectively) (p value <0.05) (Fig. 2. 11a). However, the blood glucose levels of the LPS treated mice were very close to the level of naïve mice at 6 after bacterial challenge (121 vs. 169; 138.5 vs. 169; 149 vs. 169, respectively). This was statistically significant ($p<0.05$). Pretreatment with a low dose of LPS prevented hypoglycemia and survival in this group was improved.

Serum activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were significantly reduced in the LPS treated mice compared with the control mice 12 hours after bacterial challenge (180 vs. 343, $p<0.006$ and 139 vs. 445, $p<0.025$ respectively) (Fig. 2.11b). Serum activities of alanine aminotransferase (ALT) were significantly reduced in the LPS treated mice compared with the control mice 12 hours after bacterial challenge (35.5 vs. 65.5, $p<0.05$ and 27 vs. 129, $p<0.03$ respectively) (Fig. 2. 11b). LPS treatment reduced the AST and ALT to near baseline in LPS treated mice. This data suggest that excessive liver injury likely explain the increased mortality in the control group and increased survival in the LPS treated mice is associated with intact or

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less liver damage. Pretreatment with low dose of LPS prevented experimental liver failure induced by *S. pneumoniae* infection.

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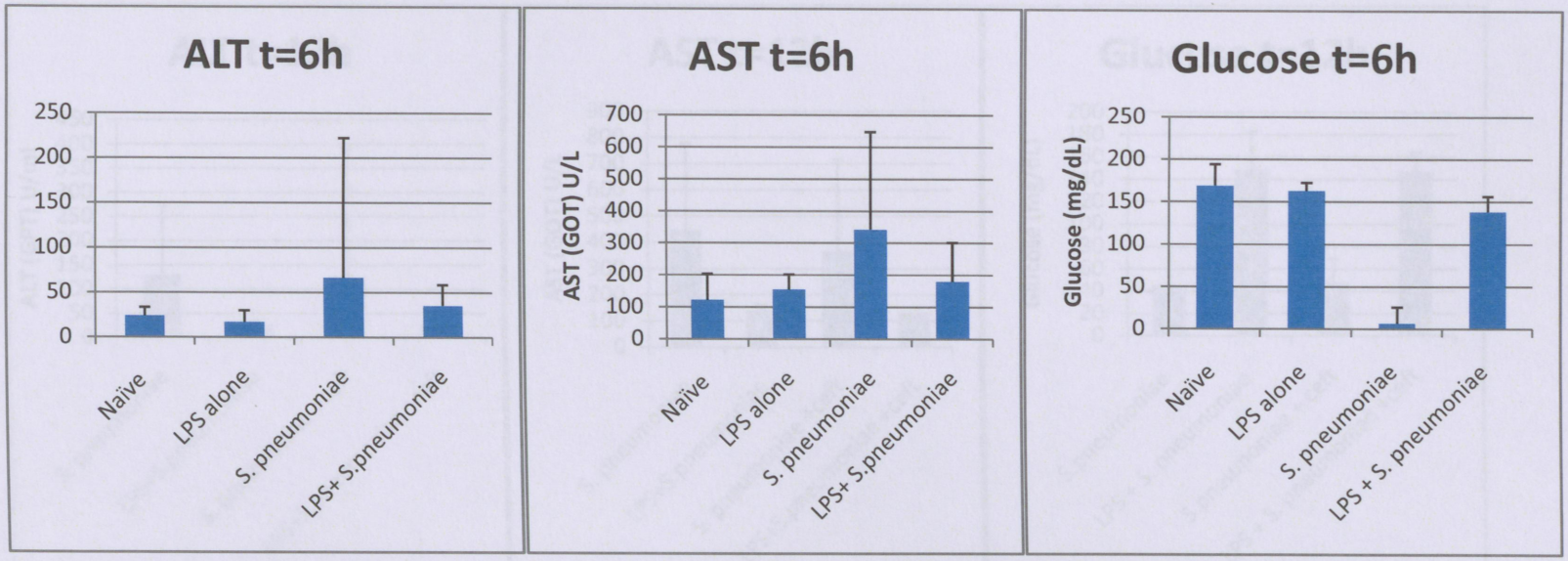


Figure 2. 11a. Serum ALT (A), AST (B) and Glucose (C) levels determined 6hours after pneumococcal bacterial challenge. Mice were sacrificed 6 hours after challenge and mouse serum samples were collected and detected as described in Materials and Methods. Data from four to 6 mice per group are presented as Mean. $p<0.05$ vs respective control group based on T-test.

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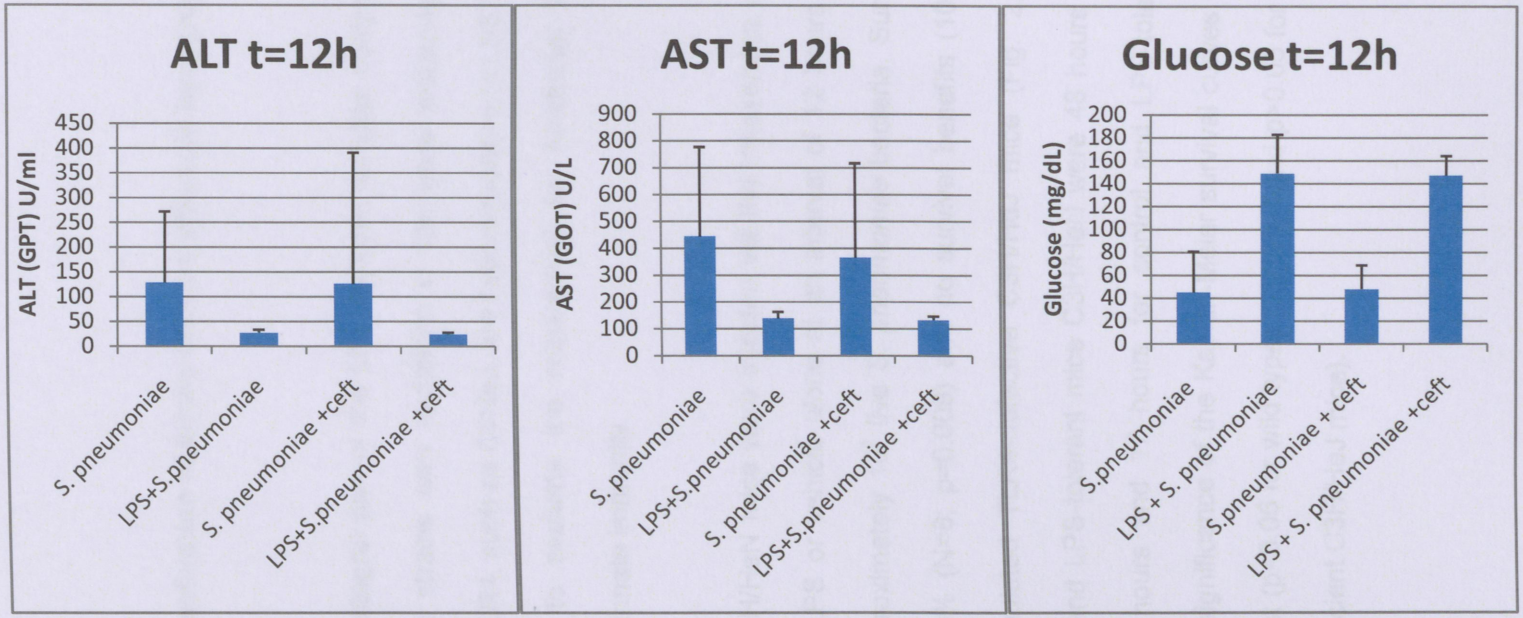


Figure 2.11b. Blood chemistry of LPS- and PBS- primed C57Bl/6 mice in response to experimental inoculation with *S. pneumoniae*. Serum ALT (A), AST (B) and Glucose (C) levels were determined 12hours after pneumococcal bacterial challenge and antibiotic ceftriaxone treatment. Antibiotic ceftriaxone was given 5-6 hours after bacterial challenge. Data from four to 6 mice per group are presented as Mean/Median, with the standard deviation indicated by error bars. $p<0.05$ vs respective control group based on T-test.

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2.4.8 Requirements of TLR4 for activation of innate immune effectors by highly purified LPS

TLR4 has been identified as an integral part of the LPS receptor complex comprising TLR4, MD-2, and CD14. Mouse strains with mutations in the gene encoding the cytoplasmic signaling domain of TLR4, such as C3HeJ, are hyporesponsive to LPS. This feature was exploited in order to evaluate the requirement for functional TLR4 expression in the enhancement of innate immunity.

Groups of C3HeJ or wild-type C3H/HeN mice were injected via the intravenous route with five doses of 10ug purified LPS or vehicle alone at an interval of 12 hours and challenged intravenously with approximately 10^7 live *S. pneumoniae* bacteria. Survival for LPS-treated C3H/HeN was 60% (N=8; p=0.009) and no survival benefits (100 % mortality) were observed in stimulated TLR4-defective C3H/HeJ mice (Fig. 2.10). Median survival times for control and LPS-tolerant mice C3H/HeN were 48 hours and 140 hours, respectively and 24 hours and 17 hours for control and LPS-tolerant C3H/HeJ were, respectively. The significance of the Kaplan-Meier survival curves was assessed by the Prism log rank test ($p<0.05$ for wild types C3H/HeJ and $p>0.05$ for toll-like receptor 4 (TLR4) complex-deficient C3H/HeJ mice).

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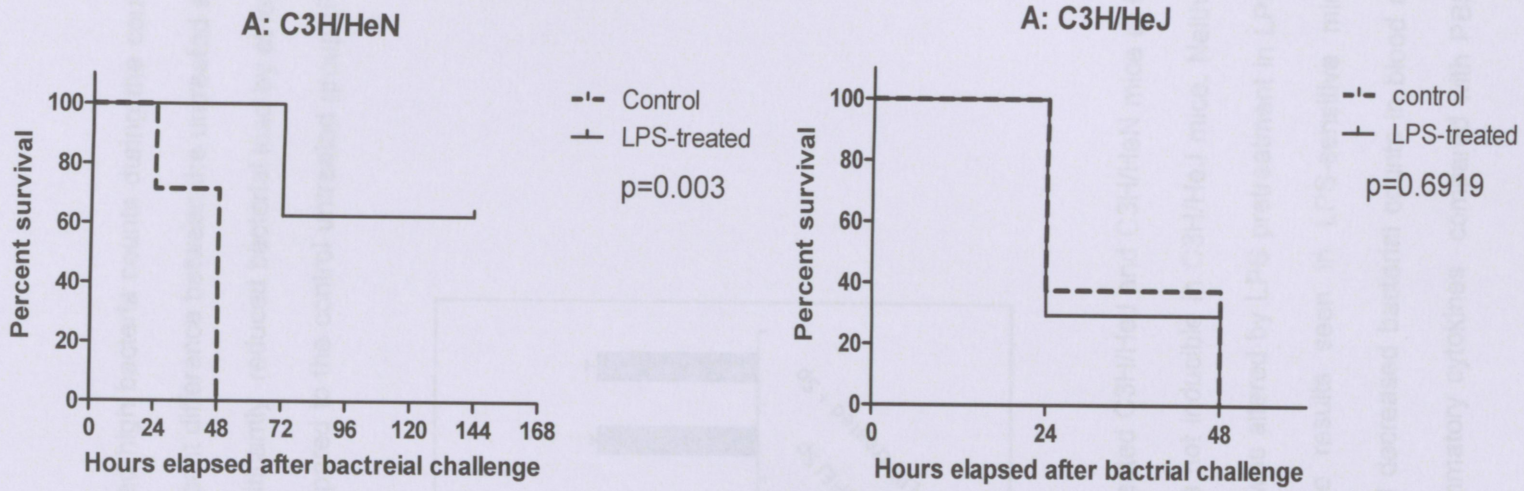


Figure 2.12. Survival curves for wild type C3H/HeN and toll-like receptor 4 (TLR4) complex-deficient C3H/HeJ mice challenged with *S. pneumoniae* after pre-treated with nonlethal doses of highly purified LPS (reLPS). Mice were pre-treated via intravenous (i.v) route with vehicle (PBS- Controls), five injections of 10ug reLPS 48h prior to challenge with a lethal dose of *S. pneumoniae* (5 X10^6 CFU). There was no significant difference between pretreated and untreated group in the toll-like receptor 4 (TLR4) complex-deficient C3H/HeJ mice. Median survival times for control (n=7) and LPS-pretreated (n=8) mice C3H/HeN (**A**) were 48h and 140h, respectively and for control (n=8) and LPS-pretreated (17) C3H/HeJ (**B**) were 24 hours and 17 hours, respectively. The significance of the Kaplan-Meier survival curves was assessed by the Prism log rank test ($p<0.05$ for wild type C3H/HeN and $p>0.05$ for toll-like receptor 4 (TLR4) complex-deficient C3H/HeJ mice).

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C3H/HeJ LPS-treated and control mice showed high bacteria counts during the course of infection and there was no statistical significant difference between the untreated and LPS treated groups but LPS pretreatment significantly reduced bacterial load by at least two logs in LPS- pretreated C3HeN mice compared to the control untreated group (Fig. 2. 13).

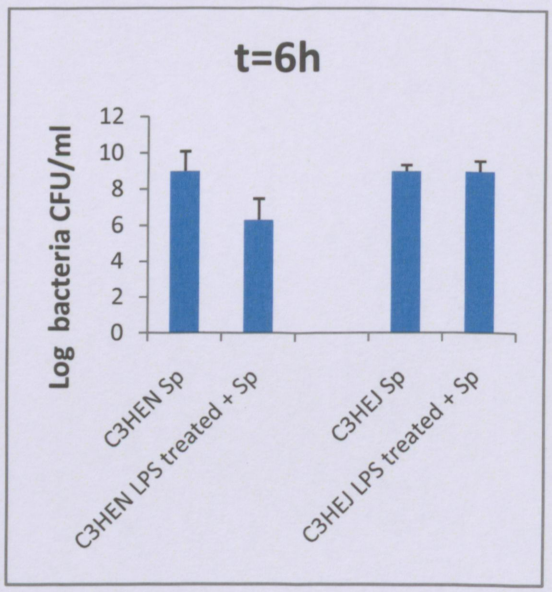


Figure 2.13. Bacteremia in control and LPS treated C3H/HeJ and C3H/HeN mice at 6h after *S. pneumoniae* challenge. Tolerance was not inducible in C3H/HeJ mice. Neither bacterial clearance nor the cytokine response was altered by LPS pretreatment in LPS-resistant mice (C3H/HeJ) in contrast to the results seen in LPS-sensitive mice (C3H/HeN). LPS –tolerant C3H/HeN mice had decreased bacterial counts in blood as well as decreased concentrations of pro-inflammatory cytokines compared with PBS-primed controls ($p<0.05$; n=5 per group).

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Cytokine production was strongly attenuated in C3H/HeN LPS- treated mice compared to untreated control mice in response to *S. pneumoniae* challenge ($p<0.05$) and there was no statistical difference in the concentration levels of inflammatory cytokines between the LPS treated and untreated C3H/HeJ mice (Fig 2.14a-e).

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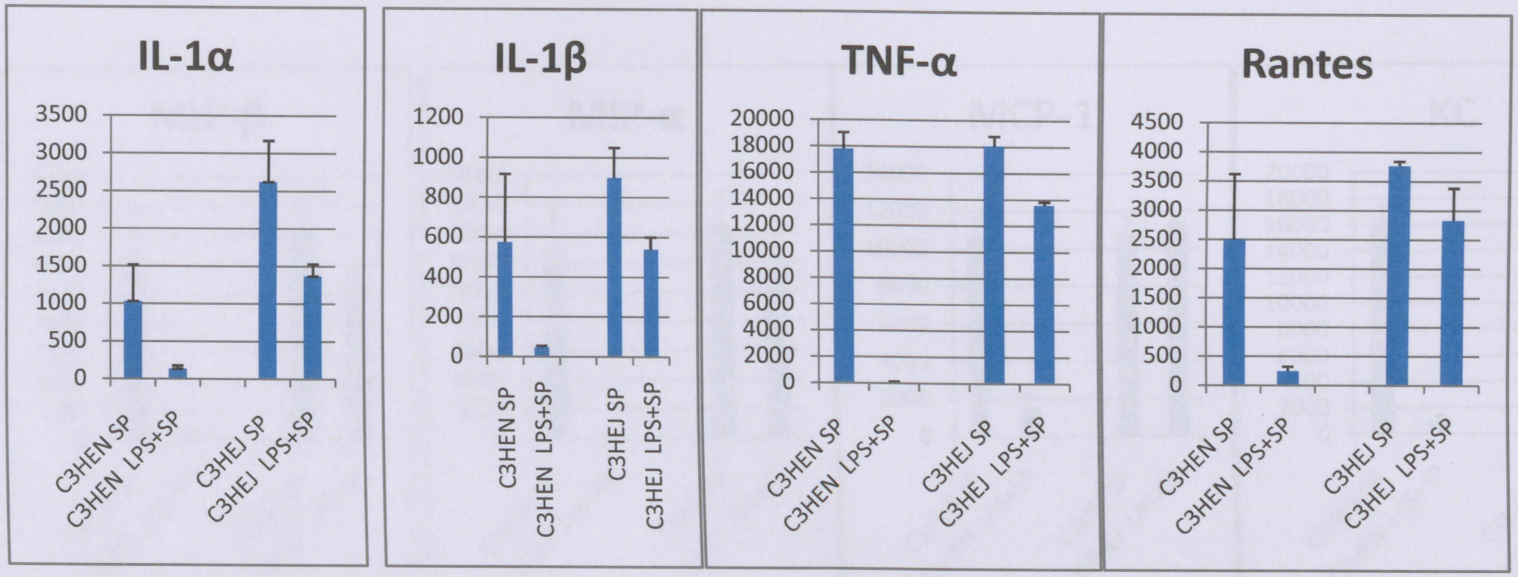


Figure 2. 14a. Levels of proinflammatory cytokines (concentration of cytokines in pg/ml) determined in plasma of C3H/HeN and C3H/HeJ control and pretreated mice 6hrs after bacterial challenge. C3H/HeN and C3H/HeJ mice were pretreated intravenously with 5 doses of PBS (Control S.p), or 10ug (in 50ul) LPS for 2 days before bacterial challenge. Data from four to 6 mice per group are presented as mean. $p<0.05$ vs respective control group based on Microsoft Excel t-Test.

Note. Y-axis = concentration in pg/ml

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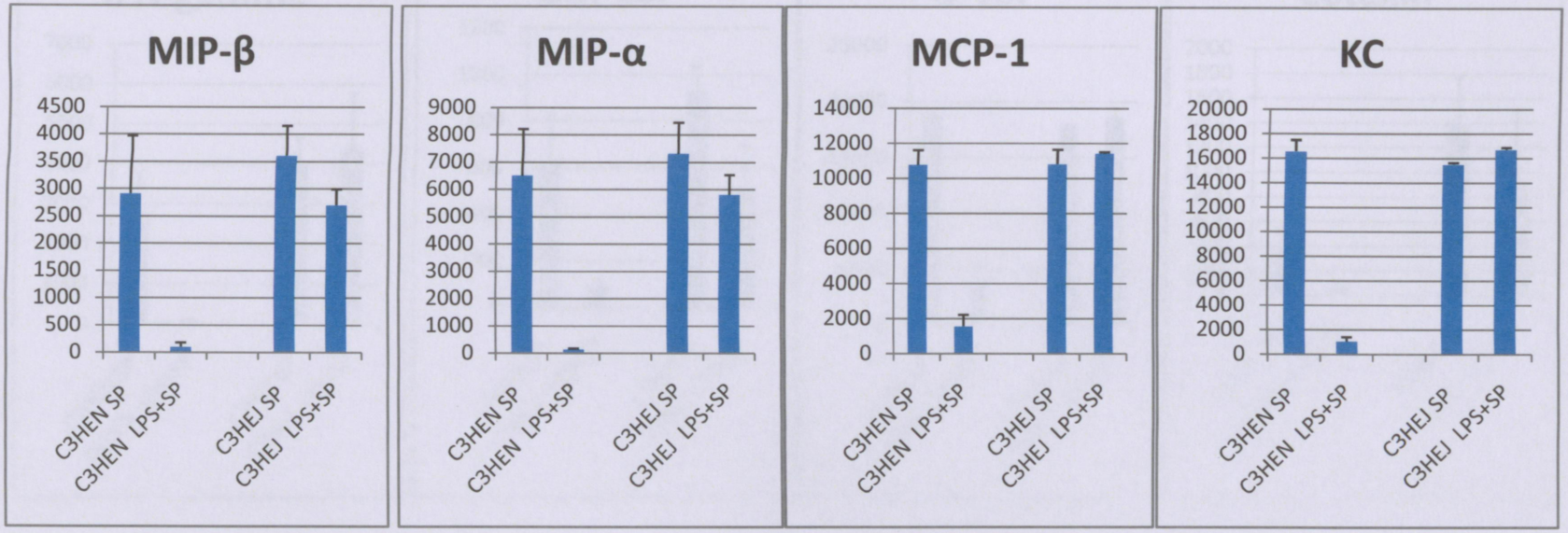


Figure 2. 14b. Levels of proinflammatory cytokines (concentration of cytokines in pg/ml) determined in plasma of C3H/HeN and C3H/HeJ control and pretreated mice 6hrs after bacterial challenge. C3H/HeN and C3H/HeJ mice were pretreated intravenously with 5 doses of PBS (Control S.p), or 10ug (in 50ul) LPS for 2 days before bacterial challenge. Data from four to 6 mice per group are presented as mean. $p<0.05$ vs respective control group based on Microsoft Excel t-Test.

Note. Y-axis = concentration in pg/ml

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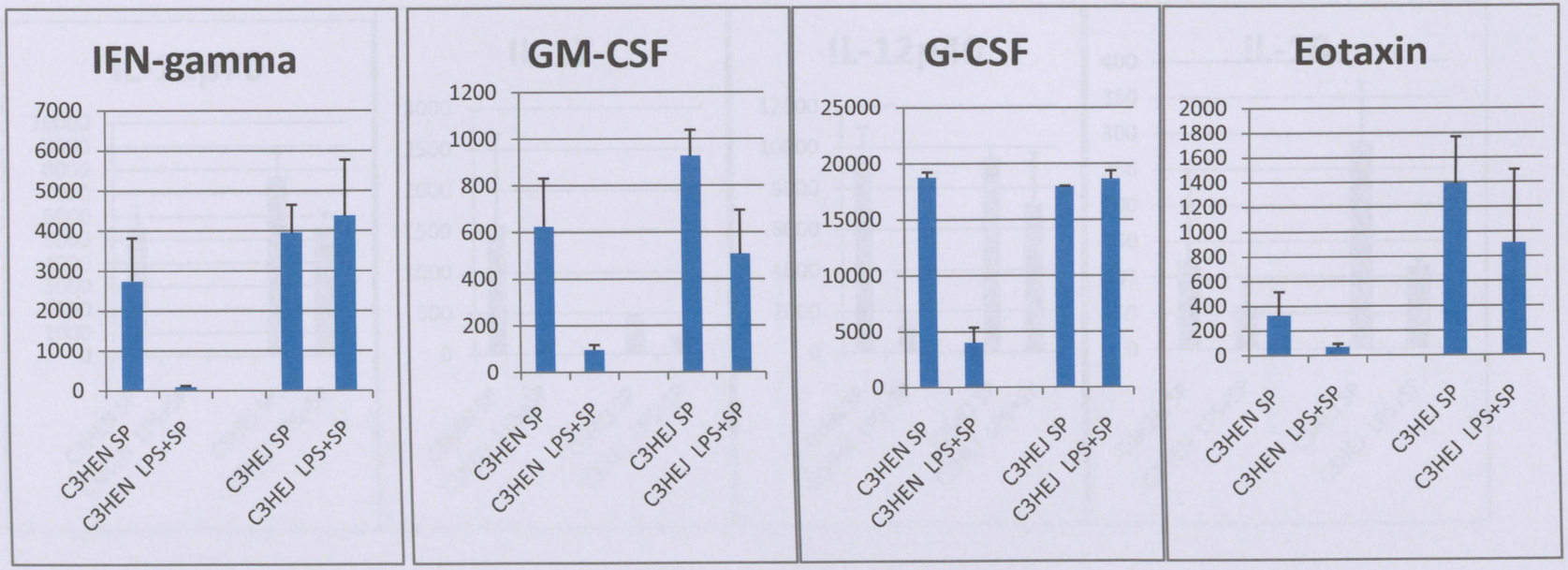


Figure 2. 14c. Levels of proinflammatory cytokines (concentration of cytokines in pg/ml) determined in plasma of C3H/HeN and C3H/HeJ control and pretreated mice 6hrs after bacterial challenge. C3H/HeN and C3H/HeJ mice were pretreated intravenously with 5 doses of PBS (Control S.p), or 10ug (in 50ul) LPS for 2 days before bacterial challenge. Data from four to 6 mice per group are presented as mean. $p<0.05$ vs respective control group based on Microsoft Excel t-Test.

Note. Y-axis = concentration in pg/ml

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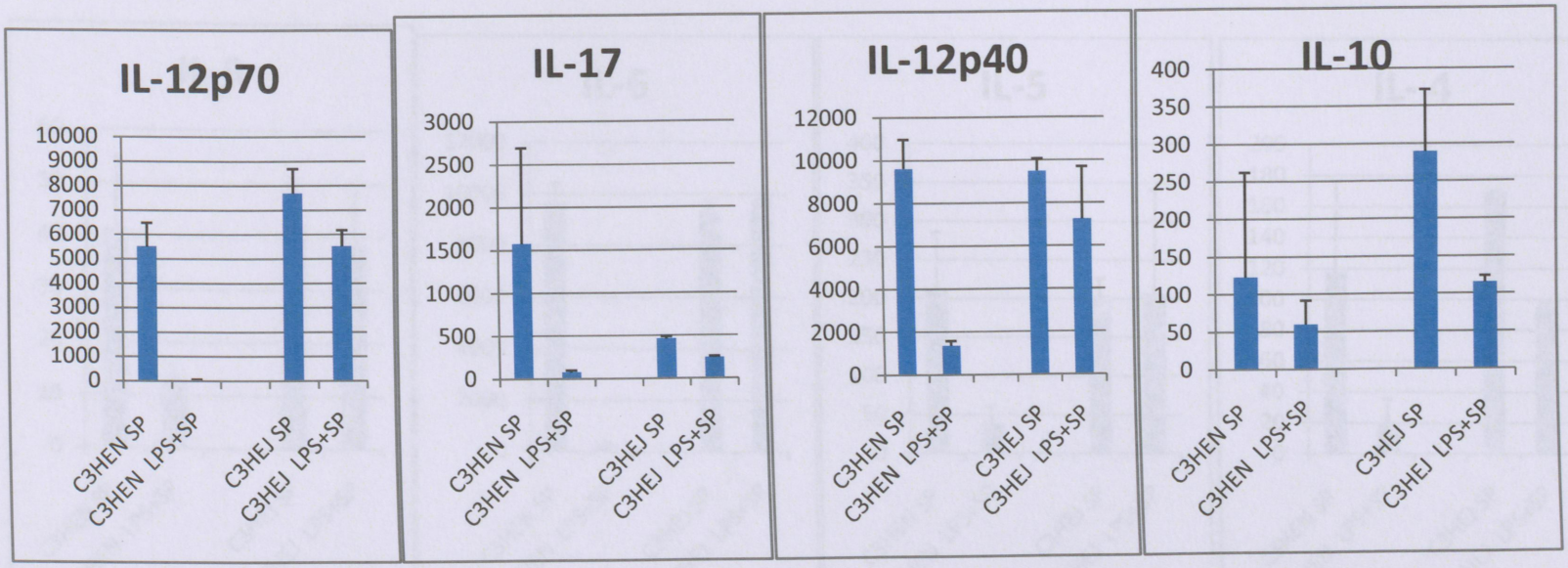


Figure 2. 14d. Levels of proinflammatory cytokines (concentration of cytokines in pg/ml) determined in plasma of C3H/HeN and C3H/HeJ control and pretreated mice 6hrs after bacterial challenge. C3H/HeN and C3H/HeJ mice were pretreated intravenously with 5 doses of PBS (Control S.p), or 10ug (in 50ul) LPS for 2 days before bacterial challenge. Data from four to 6 mice per group are presented as mean. $p<0.05$ vs respective control group based on Microsoft Excel t-Test.

Note. Y-axis = concentration in pg/ml

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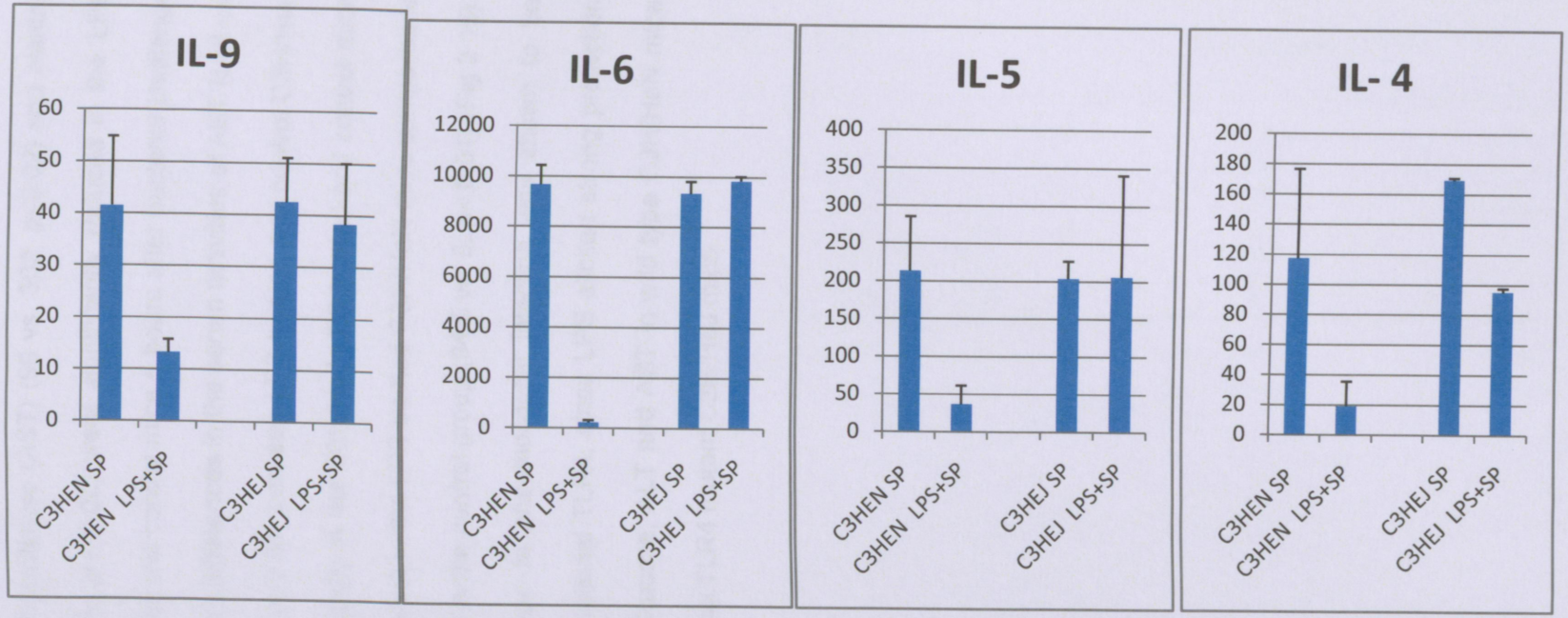


Figure 2. 14e. Levels of proinflammatory cytokines (concentration of cytokines in pg/ml) determined in plasma of C3H/HeN and C3H/HeJ control and pretreated mice 6hrs after bacterial challenge. C3H/HeN and C3H/HeJ mice were pretreated intravenously with 5 doses of PBS (Control S.p), or 10ug (in 50ul) LPS for 2 days before bacterial challenge. Data from four to 6 mice per group are presented as mean. $p<0.05$ vs respective control group based on Microsoft Excel t-Test.

Note. Y-axis = concentration in pg/ml

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Serum activities of aspartate aminotransferase (AST) (99 vs. 302; $p<0.02$) and alanine aminotransferase (ALT) (18 vs. 119; $p< 0.02$) were significantly reduced in the LPS treated C3H/HeN mice compared with the control mice 6 hours after bacterial challenge but there were no statistical significant differences in the serum activities of AST (516 vs. 379; $p< 0.78$) and ALT (115 vs. 68; $p<0.61$) between LPS treated and control C3H/HeJ mice. Glucose level dropped significantly in the C3H/HeJ LPS treated and control mice (5 vs.5.5) but remained close to baseline in the LPS treated C3H/HeN mice compared to significantly reduced level of glucose in the control group (94.5 vs. 5; $p< 0.01$) (Fig 2.15). LPS-mediated induction of resistance to pneumococcal infection was shown to be dependent on the presence of a functional TLR4, since LPS elicited strong protection and attenuation of cytokine levels, Glucose, ALT and AST in wild type C3H/HeN mice compared to control mice but not in the TLR4 mutant C3H/HeJ mice.

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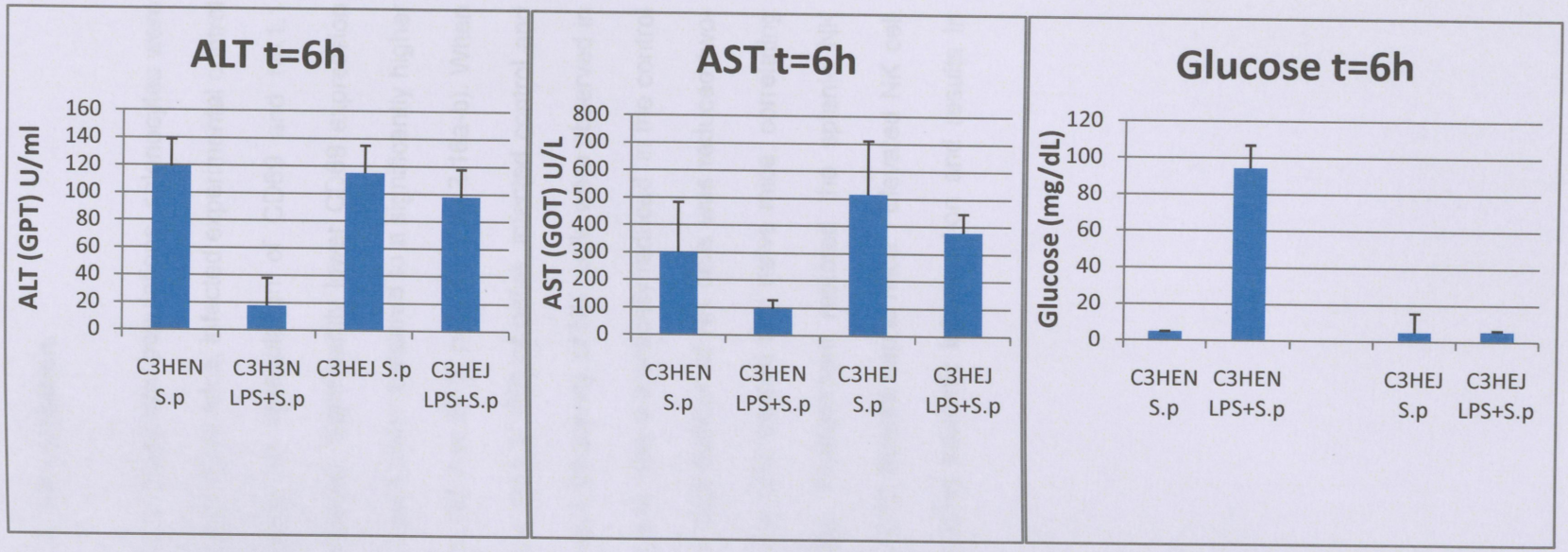


Figure 2. 15. ALT, AST and Glucose levels determined in plasma of C3H/HeN and C3H/HeJ mice at 6hrs after bacterial challenge. C3H/HeN and C3H/HeJ mice were injected intravenously with PBS (Control S.p), 10ug LPS before bacterial challenge. Data from four to 6 mice per group are presented as Mean. $p<0.05$ vs respective control group based on t-Test.

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2.4.9. Analysis of splenic lymphocytes for NK activation

To determine the activation status of specific lymphocyte populations, splenocytes were isolated 24 hours after bacterial challenge from naïve mice, infected experimental control mice, and LPS treated mice, and analysed for upregulation of CD69 and NK1.1 expression as an indication of cellular activation. Significantly lower CD69 expression was observed on spleenocytes from LPS treated mice compared to a significantly higher CD69 expression from control infected mice (35.3 vs 97.3; $p<0.05$) (Fig. 2.16a-b). When the activation status of NK cells (defined as $NK1.1^{+}$ PE) of naïve, infected control and LPS treated mice were compared, increases in frequency of NK cells were observed in LPS treated mice (close to baseline- naïve) and was dramatically reduced in the control infected mice (3.62 vs 1.32; $p <0.05$). The total number of NK cells was reduced two-fold in control infected mice compared to the LPS treated and naïve mice, correlating with the decreased frequency of the cells. Pretreatment restored the splenic NK population and decrease their activation in LPS treated septic mice. Elevated NK cell activity during pneumococcal infection amplifies systemic inflammation and results in poor outcomes.

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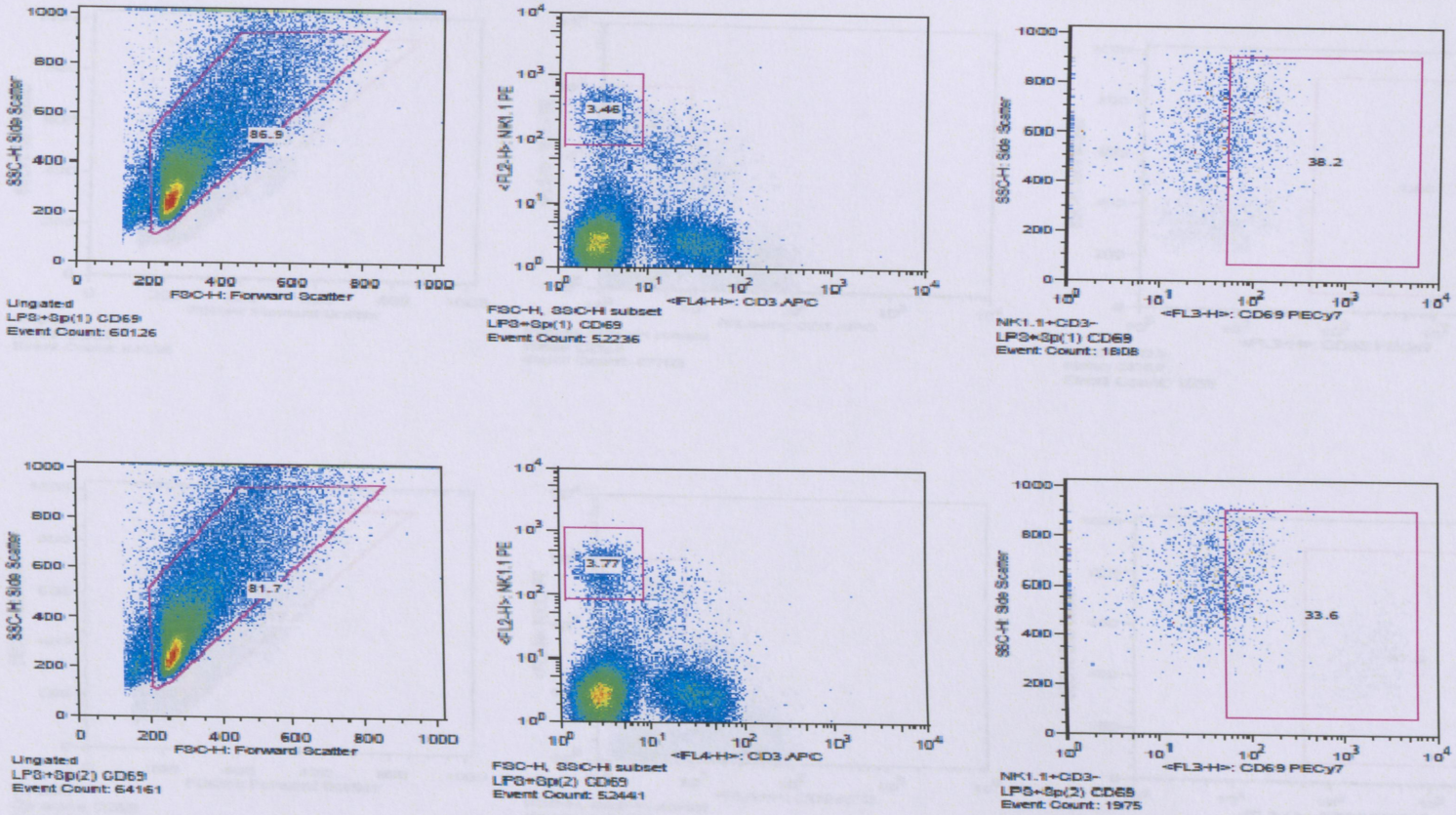


Figure 2.16a. The activation status of specific lymphocyte populations from splenocytes isolated 24 hours after bacterial challenge from naïve mice, infected experimental control mice, and LPS treated mice. Splenocytes were analysed for upregulation of CD69 and NK1.1 expression as an indication of cellular activation.

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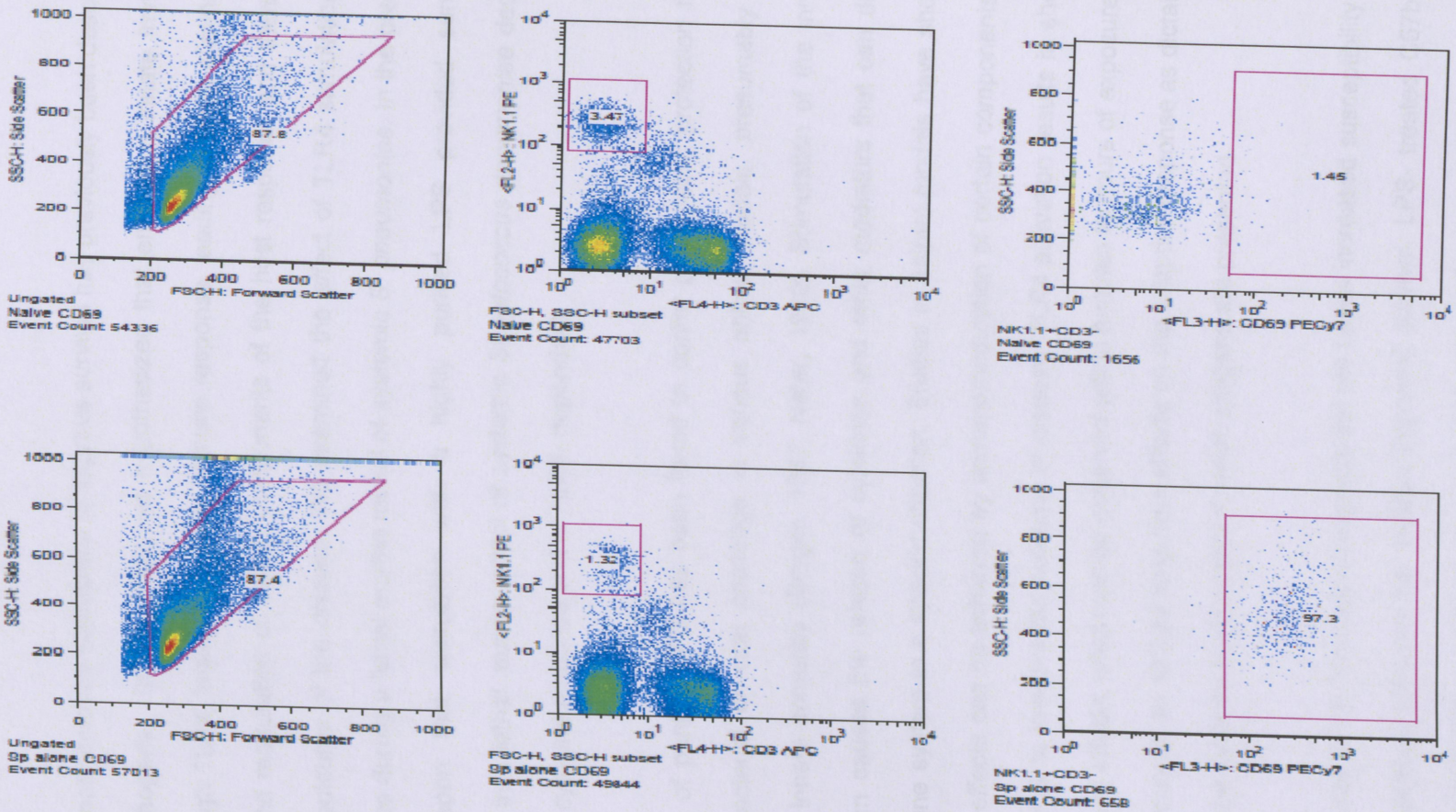


Figure 2.16b. The activation status of specific lymphocyte populations from splenocytes isolated 24 hours after bacterial challenge from naïve mice, infected experimental control mice, and LPS treated mice. Splenocytes were analysed for upregulation of CD69 and NK1.1 expression as an indication of cellular activation.

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2.4. DISCUSION AND CONCLUSIONS

The use of innate immune stimulation to improve survival has previously been described for other pathogens. In this study, we hypothesized that enhancing innate immune system, through TLR4, initiate a stronger immune response, leading to faster elimination of bacteria and modulation of certain components of the host response and therefore improve the outcome of the disease. We examined the effect of TLR4 stimulation on survival in mice during a lethal murine model of systemic *S. pneumoniae*. In the present study, we report that stimulation with a highly purified LPS provided survival enhancement in a lethal murine model of systemic *Streptococcus pneumoniae* despite attenuation of cytokine response and is TLR4- dependent.

Administration of purified LPS has been found to confer prophylactic protection from subsequent bacterial or viral challenge in various animal models, presumably via stimulation of innate immunity (Berger, 1967; Neter, 1969). Stimulation of the innate immune system causes the release of cytokines and other mediators that can drive adaptive immune system in a specific direction. Studies in animal models have shown that beneficial effects can be achieved by immunomodulation of certain components of the host response to improve the outcome of disease. TLR4 activation results in short-term antimicrobial effects, which can be observed within minutes to hours of exposure to microbial components, as well as long-term effects on the adaptive response as dictated by the tenor of the cytokine environment in which antigens are presented.

Lipopolysaccharide (LPS) hyporesponsiveness did not cause increased susceptibility of mice to pneumococcal infection but instead improved survival. LPS- treated C57bl/6 mice survived significantly compared to the control untreated group (90% versus 0%)

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and Balb/c treated groups (50% vs. 0%). The difference in the survival in different mouse strains might be due to differences in their TLR expression, reactivity to LPS, and their production of proinflammatory mediators essential to contain infection. It is now widely believed that the outcome of infection with a pathogen in mice is largely determined by whether it elicits a Th1 or Th2 response (von Stebut and Udey, 2004). In C57BL/6 mouse strains, a Th1 response prevails and enables the mice to recover from infection. In contrast, BALB/c mice mount a Th2 response, which is ineffective because only low levels of IFN-γ are produced. As a result, BALB/c mice die of infection.

In the present study, we indicate that a highly purified LPS induced tolerance in a lethal murine model of systemic *Streptococcus pneumoniae* is TLR4- dependent since tolerance could not be induced in C3H/HeJ mice. Survival in LPS-treated C3H/HeN was improved compared to no survival (100 % mortality) observed in stimulated TLR4-defective C3H/HeJ mice. C3H/HeJ LPS-treated and control mice also showed higher bacterial counts during the course of infection and there was no statistical significant difference between the control untreated and LPS treated groups but LPS pretreatment significantly reduced bacterial load by at least two-fold logs in LPS- pretreated C3HeN mice compared to the control untreated group. LPS-mediated induction of resistance to pneumococcal infection was shown to be dependent on the presence of a functional TLR4, since LPS elicited strong protection and attenuation of cytokine levels, glucose, ALT and AST in wild type C3H/HeN mice compared to control mice but not in the TLR4 mutant C3H/HeJ mice. *S. pneumoniae* induced morbidity and mortality occurs at a higher rate in C3HeJ LPS-treated mice than wild-type mice, presumably because they are unable to mount innate immune responses early enough to control the infectious burden in the period prior to the development of adaptive responses. This is because they were hyporesponsive to LPS-treatment. Similar results have been obtained when

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C3HeJ mice were evaluated in models of infection with *Escherechia coli* (Hagberg et al., 1984), *Neisseria menininginitis* (Woods et al., 1988), and *Fransiscella tularensis* (Kawai et al., 1999)

A comparison of the time course of bacterial proliferation in C57BL/6 LPS treated and untreated control mice indicated that bacteremia in LPS–treated mice were reduced significantly as early as 6 hours after infection. Since this was associated with a decrease in the bacterial load in the blood and lungs, high survival in tolerant mice was a result of an improvement in the ability of the immune system to deal with high numbers of bacteria, and reduction/elimination of bacteria due to enhanced host defense against infection. The enhanced clearance of bacteria from blood and lungs of LPS-treated mice in this study might be due to a more efficient phagocytic activity of the RES as also shown by Lehner *et al.* (2001) in their intravenous inoculation of LPS in Salmonella infection experiments. They showed enhanced phagocytosis of bacteria by Kupffer cells of LPS-tolerant animals.

We also found that the route of administration of LPS played an important role since intravenous LPS-tolerant mice showed a higher survival rate and they were much active and showed no clinical symptoms compared to control groups even though there was no statistical significant difference in terms of survival between the groups. These results suggest the importance of the route of LPS administration in this model and to induce tolerance and improve survival, LPS should be administered intravenously. The degree of enhanced resistance induced by LPS pretreatment is dependent on the route of inoculation and LPS administration. We observed greater efficacy in protection when LPS and bacterial challenge were injected by the same route in mice than employing

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heterologous inoculation routes (mice showed clinical symptoms even though there was no significant difference in the survivors in both treated groups).

It was observed that tolerance induction was dose/time dependent because it could be achieved only after at least 2 injections of 10ug at 12 hours interval and not less than that (i.e. at least 24 hours before bacterial challenge (results not shown)). We also investigated how long does tolerance last by challenging LPS-treated groups with 10^7 *S. pneumoniae* 24 hours after the last dose of LPS and improved survival benefits were achieved and this demonstrate that protection may last for at least 1 or 2 days before coming into contact with the pathogen.

To investigate some of the mechanisms of protection and also the importance of immune system in tolerance induction, CBA/CaHN-Btk^{xid}/J (B-cell deficient (*xid*) mice with normal T cell function), RAG-1 Knockout (deficient in both B and T cells) and A/J (C5-defecient strains) mice were challenged with *S. pneumoniae* after pretreatment with LPS. CBA/N and A/J treated mice also benefitted from LPS tolerance induction. These results indicated that cells of the immune system such as B cells and complement system (C-5 component) are not involved in increased resistance of LPS treated mice against pneumococcal infection. Our results are in agreement with the study conducted by Lehner et al. (2001) showing that administration of Cobra venom factor, depleting complement component C3, did not ablate survival benefits in *Salmonella typhymurium* infected LPS–tolerant mice. These results indicate that improved bacterial load in tolerant mice in our model is dependent on complement mediated uptake of bacteria by phagocytes in the course of infection not destruction of pathogens due to the formation of membrane attack complex because A/J are deficient in the C5 component. Several studies demonstrated that enhanced resistance after LPS injection was associated with

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increased bacterial activity of serum towards certain Gram-negative bacteria. In this study, we provided evidence against a role of complement system and antibodies in LPS- induced increased resistance by demonstrating LPS-induced resistance to pneumococcal infection in complement C5-deficient A/J and B cell deficient CBA/CaHN-Btk^{xid}/J strains. Greisman and Harnick (1975) indicated that endotoxin tolerance can be separated into two stages. The first stage is an antibody-independent phase which occurs hours following a LPS insult and may last for several days, is considered to be of great importance in septic patients who are unable to mount a further response against virulent and persistant pathogens. The second stage is antibody –dependent, and can appear weeks after initial LPS administration. Like many adaptive antibody-mediated immune qualities, it is restricted to the specific LPS administered in the initial setting.

We also compared the course of pneumococcal infection in LPS treated and control Rag1knockout mice (C57BL/ 6 background which have no B and T cells as a consequence of deletion of Rag1 (recombination- activating gene 1)), with infection in normal C57bl/6 animals after intravenous *S. pneumoniae* infection. LPS pretreatment failed to significantly provide survival benefits to the RAG-1 Mice. Rag-1 knockout mice were profoundly deficient in their ability to control infection with *S. pneumoniae*. There was no statistical significant difference between the LPS treated and control mice. These results indicate that immunity mediated by T and B cells is required for tolerance to be achieved and for LPS pretreatment to offer survival enhancement after pneumococcal infection. In the absence of specific, T- or B-cell- mediated immunity, chemotactic bacterial and host factors attract phagocytes that are unable to kill the organisms effectively. The inability of PMN leucocytes to kill *B. mellitensis* has been documented (Young et al., 1985; Izadjoo et al., 2000). Moreover, IFN-gamma and other cytokines

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provided by NK cells in the absence of a contribution from specifically sensitized T cells may not be sufficient to activate macrophages for effective killing of *S. pneumoniae*

Since proinflammatory cytokines were shown to be essential for activation of host defenses against *S. pneumoniae*, we were interested in whether stimulating innate immune response before bacterial challenge would affect susceptibility of mice to pneumococcal infection. We compared serum levels of cytokines following lethal challenge with *S. pneumoniae* in LPS-treated and untreated groups. Before bacterial challenge, high levels of TNF-a, IL-6, IL-10, IL-1a, Rantes, IL-12 p70, G-CSF, KC, and MIP-1a were detected in plasma of LPS-treated uninfected mice compared to naïve uninfected control mice. This may have contributed to the survival of LPS-treated mice over untreated mice following a lethal bacterial challenge, as certain amounts of endogenous TNF-a and other cytokines are almost certainly required for effective eradication of bacterial infection (Echtenacher et al., 1990; Nakane et al., 1995), and blocking cytokines accelerates death of mice challenged with bacteria. These results suggested that a stronger inflammatory response in the early hours of infection can be beneficial in controlling bacterial infection. The pivotal role of an intact cytokine response for host defense against bacterial infections has been shown and successful immune defense against infectious diseases requires a vigorous inflammatory response. TNF-α is capable of recruiting inflammatory cells to the site of infection both directly and via upregulation molecules and also stimulate the release of chemokines that are directly chemotactic for inflammatory cells (Echtenacher et al., 1990). Macrophage inflammatory protein 1 (MIP-1α) and MIP-2 are two chemokines known to be important in bacterial pneumonia (Greenberger et al., 1996). Following recruitment of phagocytic cells, TNF can also promote antimicrobial activity by activating the respiratory burst (Dusi et al., 1996) and by activating the capacity to degranulate (Klebanoff et al., 1986). These

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effects of TNF have been shown to be required for effective in vivo host defense against a range of microorganisms, including *Klebsiella pneumoniae* and *Pneumocystis carinii* (Chen et al., 1992; Laichalk et al., 1996).

Over-activation of the immune system in the course of systemic infection creates a life-threatening situation characterized by pathophysiological alteration of the microcirculation that finally can lead to multiorgan failure and death. Inhibition of inflammatory processes after a primary proinflammatory response may represent a mechanism of the host's immune system to limit autodestruction caused by sustained systemic inflammation. LPS tolerance is assosciated with suppression of several cytokines, attenuation of leukocyte infiltration and a consequently a reduction of organ damage. To combat inflammatory diseases successfully, regulation of proinflammatory mediator production would be a critical process. Endotoxin-tolerance is known to protect prophylactically against mortality and morbidity in endotoxic shock, LPS- and TNF-a mediated liver damage and various models of fulminant infection with high numbers of bacteria. This protection is associated with an attenuated inflammatory response to LPS attributable to a down-regulation of macrophage responsiveness (Mengozzi and Ghezzi, 1993; Mathison et al., 1990; Kaufmann et al., 2000).

Most studies focused on the production of TNF-a, which is almost completely downregulated during LPS tolerance, but other cytokines are also affected by endotoxin pretreatment. Our tolerant induction regimen not only blunted the release of TNF-a, but also inhibited or reduced the production of other cytokines such as IFN-g, and IL-6 in response to subsequent intravenous challenge with *S. pneumoniae*. Cytokine production was strongly attenuated in LPS- treated mice in response to *S. pneumoniae* challenge. The level of TNF-a, IFN-g, IL-12 (p40/70), IL-6, IL-1a/b, IL-10, Eotaxin, MCP-1, and

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MIP-1a/b were attenuated in C57BL/6 and C3H/HeN LPS-treated mice 12 /24 hours after infection compared to untreated group. The level of MCP-1, IL-5, IL-6 and KC were elevated at 6h time point but attenuated at 12h and 24h after infection in LPS- treated group compared to control untreated groups. IL-13, IL-17 and G-CSF were elevated 6 hours after infection but remained high 12h and 24h after infection compared to the control group. In this model, cytokine release was not abrogated completely after infection with *S. pneumoniae* suggesting that other immune stimuli, using different TLR signaling pathways, are also transmitted during infection. Pretreatment with LPS substantially downregulated release of all proinflammatory cytokines tested in this study. Attenuation of TNF-a release in this study was in agreement with the finding by Cavaillon et al. (2000) who reported suppression of TNF-alpha release in response to Zymosan, Staphylococci and streptococci after exposure of human monocytes to LPS *in vitro* (Cavaillon et al.,(2000) .

Suppression of excessive proinflammatory mediator production is a characteristic of tolerance, an adaptive innate immune response to LPS and has been linked to alterations in signal transduction pathways (Peck et al., 2004; Durando et al.; 1998). LPS tolerance is an adaptive host response and may represent a protective mechanism in response to bacterial infection. Although LPS tolerance suppresses TNF-α, IL-1β, and IL-6 production, anti-inflammatory cytokines such as IL-10 are not affected (Schade et al., 1999). Therefore, LPS tolerance appears to occur as a consequence of alterations in LPS signal transduction pathways rather than a general cellular dysfunction. Tolerance has been reported to affect various postreceptor signaling proteins. Although, down-regulation of TLR4 has been reported, over-expression of TLR4 does not block tolerance (Nomura et al., 2000).

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In this study, LPS pretreatment failed to protect IL-10 KO mice from lethal pneumococcal challenge and these mice were very sensitive to minimum dose of 10ug Ultrapure LPS used to protect normal mice from lethal bacterial challenge. The results of the study showed that the survival rate upon pneumococcal challenge was 75% in wild type C57BL/6 LPS pretreated mice as compared with 0 % in the IL-10 KO pretreated and untreated mice ($p<0.05$). These results suggest that the host's endogenous IL-10 level is a central regulator of the response to LPS or suppresses sensitization and determines the amount of ultrapure LPS tolerated without death. The high mortality rate observed in IL-10 KO mice is attributed to uncontrolled elevated or heightened production of inflammatory cytokines. The ability of IL-10 to suppress directly or indirectly proinflammatory mediators appears critical in determining the host's susceptibility to LPS and pathogenic reactions.

A common and serious consequence of overwhelming bacterial infection is generalized organ failure, such as liver, and spleen due to septic shock. To determine the effect of pretreatment with low dose of LPS on blood chemistry and determine if excessive liver injury may have contributed to increased mortality, release of the hepatocyte-associated enzymes AST and ALT into the peripheral blood were examined. In this study, LPS treatment before bacterial challenge prevented the increase or reduced the level of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in LPS treated mice compared to an elevated level of these enzymes in the control mice. The increase in the levels of AST and ALT in the control mice reflects impairment in the liver. When the liver is impaired, the liver cells release the enzymes into the blood and raise the enzyme levels. This is in line with the work of Nyblom et al., (2006), Maduka et al., (2009) and Adam et al., (2001) in which the levels of liver enzymes were increased in cirrhosis, and HIV patients, respectively. The elevation of the serum enzymes

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indigenous to the liver is an indication of hepatic dysfunction. The study also showed decreased serum glucose in the control mice compared to the normal range in the LPS treated group and this could be as a result of interference in the generation of glucose by the liver caused by pneumococcal infection. Pretreatment with low dose of LPS prevents experimental liver failure induced by *S. pneumoniae* infection. The inflammatory immune response is of crucial importance for the early containment of infection but at the same time has the potential to result in immunopathology (Pasare and Medzhitov, 2003).

Elevated NK cell activity during pneumococcal infection amplifies systemic inflammation and results in poor outcomes. NK cells are a unique T-cell subset capable of producing high levels of IL-4 and interferon gamma following activation (Godfrey et al., 2000; Gumperz and Brenner, 2000). Moreover, they have been shown to regulate other lymphocytes during the course of the immune response (Carnaud et al., 1999; Trobonjaca et al., 2001). To determine the activation status of specific lymphocyte populations, splenocytes were isolated 24 hours after bacterial challenge from naïve, infected control, and LPS treated mice and analysed for upregulation of CD69 expression as an indication of cellular activation. Flow cytometric analysis of spleen lymphocyte subsets from LPS treated and control mice substantiated the excessive activation status of these cells in pneumococcal infected mice 24 hours after bacterial challenge. A marked upregulation of CD69 expression was observed on the potential inflammatory cytokine producing lymphocyte, NK cells. Splenic NK cells were reduced in frequency and total numbers in control infected mice compared to LPS treated and naïve mice.

The total number of NK cells was reduced two- fold in control infected mice compared to the LPS treated and naïve mice, correlating with the decreased frequency of the cells.

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Combined, these data indicate dysregulated, excessive activation of the majority of cell populations involved in inflammation within the spleen. Pretreatment restored the splenic NK population and decrease their activation in LPS treated septic mice. We are currently unsure as to the mechanism responsible for the reduction of NK T cell numbers in control mice following infection but one possibility of the disappearance or of low number of NK T cells maybe the increased trafficking of NK T cells out of the spleen following infection, for the increased plasma–associated IFN-gamma levels. Previous studies by Kadioglu et al. (2000) have shown that CD3+ pulmonary lymphocytes undergo relocation to infected bronchioles in their animal pneumococcal infection.

In conclusion, this study provides evidence that induction of profound LPS tolerance, despite reducing cytokine production, improves host defense against infection with virulent strain of *S. pneumoniae.* The protective effects of LPS tolerance induction were also reported by Lehner et al. (2001) using Gram negative bacteria (*Salmonella typhimurium*) and Gram-positive bacteria (*Staphylococcus aureus, Listeria monocytogenes*). 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. These suggest that interventions designed to improve innate immunity to infection could have therapeutic benefits. 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.

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2.5. REFERENCES

Adam, S., Alaquatrah, A.A., and Alhag., E.A. 2001. Effects of various levels of dietary articial leaves on rats. *Lab animals* 34(93): 307-312.

Akira, S. 2003. Mammalian Toll-like receptors. *Curr Opin Immunol* 15:5-11.

Alexopoulou, L., Holt, A. C., Medzhitov, R., Flavell, R. A. 2001. Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3. *Nature* 413:732-738

Ayabe, T., D. P. Satchell, C. L. Wilson, W. C. Parks, M. E. Selsted, and A. J. Ouellette. 2000. Secretion of microbicidal alpha-defensins by intestinal Paneth cells in response to bacteria. *Nat Immunol* 1:113-118.

Berger, F. M. 1967. The effect of endotoxin on resistance to infection and disease. *Adv Pharmacol* 5:19-46.

Beutler, B., Milsark, I.W. and Cerami, A.C. 1985. Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. *Science 229:869-871.*

Branger, J., Knapp, S., Weijer, S., Leemans, J. C., Pater, J. M., Speelman, P., Florquin, S. & van der Poll, T. 2004. Role of Toll-like receptor 4 in Gram-positive and Gram-negative pneumonia in mice. *Infect Immun* 72: 788–794.

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Creating Future Leaders

Carnaud, C., Lee, D., Donnars, O., Park, S.H., Beavis, A., Koezuka, Y., and Bendelac, A. 1999. Cross-talk between cells of the innate immune system: NKT cells rapidly activate NK cells. *J Immunol* 163:4647-4650.

Chen, W., E. A. Havell, and A. G. Harmsen. 1992. Importance of endogenous tumor necrosis factor alpha and gamma interferon in host resistance against *Pneumocystis carinii* infection. *Infect Immun* 60:1279-1284.

Diebold, S. S., Kaisho, T., Hemmi, H., Akira, S., Reis e Sousa, C. 2004. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* 303:1529-1531

Durando, M., Meier, K.E., Cook, J.A. 1998. Endotoxin activation of mitogen-activated protein kinase in THP-1 cells; diminished activation following endotoxin desensitization. *J Leukocyte Biol* 64:259-264.

Durando, M., Meier, K.E. and Cook, J.A. 1998. Endotoxin activation of mitogen-activated protein kinase in THP-1 cells; diminished activation following endotoxin desensitization. *J Leukoc Biol* 64: 259–264.

Dusi, S., V. D. Bianca, M. Donini, K. A. Nadalini, and F. Rossi. 1996. Mechanisms of stimulation of the respiratory burst by TNF in nonadherent neutrophils: its independance of lipidic transmembrane signalling and dependance on protein tyrosine phosphorylation and cytoskeleton. *J Immunol* 157:4615-4623.

Echchannaoui, H., Frei, K., Schnell, C., Leib, S.L., Zimmerlli, W., Landmann, R. 2002. Toll-like receptor 2-deficient mice are highly susceptible to *Streptococcus pneumoniae*

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meningitis because of reduced bacterial clearing and enhanced inflammation. J Infect Dis 186: 798–806

Echtenacher, B., Falk, W., Mannel, D.N., Krammer, P.H. 1990. Requirement of endogenous tumor necrosis factor/cachectin for recovery from experimental peritonitis. *J Immunol 145:3762.*

Galanos, C., and Freudenberg, M.A. 1993. Mechanisms of endotoxin shock and endotoxin hypersensitivity. *Immunobiol 187:346-356.*

Godfery, D.I., Hammond, K.J., Poulton, L.D., Smyth, M.J., and Baxter,A.G. 2000. NKT cells: facts, function, and fallacies. *Immunol Today* 21:573-583.

Greenberger, M. J., R. M. Strieter, S. L. Kunkel, J. M. Danforth, L. L. Laichalk, D. C. McGillicuddy, and T. J. Standiford. 1996. Neutralisation of macrophage inflammatory protein-2 attenuates neutrophil recruitment and bacterial clearance in murine *Klebsiella* pneumonia. *J Infect Dis* 173:159-165.

Greisman, S.E., Young, E.J., Woodward, W.E. 1966. Mechanisms of endotoxin tolerance. IV. Specificity of the pyrogenic refractory state during continuous intravenous infusions of endotoxin. *J Exp Med 124:983.*

Gumperz, J.E., and Brenner, M.B. 2001. CD1-specific T cells in microbial immunity. *Curr Opin Immunol* 13: 471-478.

Hagberg, L., R. Hull, S. Hull, J. R. McGhee, S. M. Michalek, and C. Svanborg Eden. 1984. Difference in susceptibility to gram-negative urinary tract infection between C3H/HeJ and C3H/HeN mice. *Infect Immun* 46:839-844.

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Creating Future Leaders

Hayashi, F., Smith, K. D., Ozinsky, A., Hawn, T. R., Yi, E. C., Goodlett, D. R., Eng, J. K., Akira, S., Underhill, D. M., Aderem, A. 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* 410:1099-1103

Heil, F., Hemmi, H., Hochrein, H., Ampenberger, F., Kirschning, C., Akira, S., Lipford, G., Wagner, H., Bauer, S. 2004. Species-specific recognition of single-stranded RNA via Toll-like receptor 7 and 8. *Science.* 303:1526-1529

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., Akira, S. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature* 408:740-745

Hirschfeld, M., Kirschning, C.J., Schwandner, R., Wesche, H., Weis, J.H., Wooten, R. M., Weis, J.J. 1999. Cutting edge: inflammatory signaling by *Borrelia burgdorferi* lipoproteins is mediated by Toll-like receptor 2. *J Immunol* 163:2382-2386

Hoebe, K., Du, X., Georgel, P., Janssen, E., Tabeta, K., Kim, S. O., Goode, J., Lin, P., Mann, N., Mudd, S., Crozat, K., Sovath, S., Han, J., and Beutler, B. 2003. Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. *Nature* 424:743-748.

Iwasaki, A., Medzhitov, R. 2004. Toll-like receptor control of the adaptive immune responses *Nat Immunol* 5:987-995

Izadjoo, M.J., Polotsky, Y., Mense, M.G., Bhattacharjee, A.K., Paranavitana, C.M., Hardfield, T.L., and Hoover, D.L. 2000. Impaired control of Brucella melitensis infection in Rag1–deficient mice. *Infect. Immun.* 68 (9):5314-5320

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Creating Future Leaders

Kadioglu, A., Gingles, N.A., Grattan, K., Kerr, A., Mitchell, T.J., Andrew, P.W. 2000. Host cellular immune response to pneumococcal infection in mice. *Infect Immun* 68: 492-501.

Kaufmann, A., Gemsa, D., Sprenger, H. 2000. Differential desensitization of lipopolysaccharide-inducible chemokine gene expression in human monocytes and macrophages. *Eur J Immunol 30:1562.*

Kawai, T., O. Adachi, T. Ogawa, K. Takeda, and S. Akira. 1999. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* 11:115-122.

Khan, A.Q., Shen, Y., Wu, Z., Wynn, T.A., Snapper, C.M. 2002. Endogenous pro and anti-inflammatory cytokines differentially regulate an *in vivo* humoral response to *Streptococcus pneumoniae. Infect Immun* 70: 749–761.

Klebanoff, S. J., M. A. Vadas, J. M. Harlan, L. H. Sparks, J. R. Gamble, J. M. Agosti, and A. M. Waltersdorph. 1986. Stimulation of neutrophils by tumour necrosis factor. *J Immunol* 136:4220-4225.

Laichalk, L. L., S. L. Kunkel, R. M. Strieter, J. M. Danforth, M. B. Bailie, and T. J. Standiford. 1996. Tumor necrosis factor mediates lung antibacterial host defense in murine *Klebsiella* pneumonia. *Infect Immun* 64:5211-5218.

Lehner, M.D., Morath, S., Michelsen, K.S., Schumann, R.R., Hartung, T. 2001. Induction of cross-tolerance by lipopolysaccharide and highy purified lipoteichoic acid via different Toll-like receptors independent of pancrine mediators. *J Immunol 166:5161-5167.*

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Creating Future Leaders

Li, L., Cousart, S., Hu, J., McCall, C.E. 2000. Characterization of interleukin-1 receptor-associated kinase in normal and endotoxin-tolerant cells. *J Biol Chem* 275:23340-23345.

Macela, A., Stulik, J., Hernychova, L., Kroca, M., Krocova, Z., and Kovarova, H. 1996. The immune response against Francisella tularensis live vaccine strain in Lps(n) and Lps(d) mice. *FEMS Immunol Med Microbiol* 13:235-238.

Maduka, C.L., Neboh, E.E., Ikekpeazu, E.J., Ureme, S.O., Umeh, C., and Ejezie, E. (2009). Assesment of liver enzymes in asymptomatic HIV seropositive patients. *Res J Bio Sci* 4(3): 360-362.

Malley, R., P. Henneke, S. C. Morse, M. J. Cieslewicz, M. Lipsitch, C. M. Thompson, E. Kurt-Jones, J. C. Paton, M. R. Wessels, and D. T. Golenbock. 2003. Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. *Proc. Natl. Acad. Sci. U. S. A.* 100:1966–1971.

Manthey, C. L., P. Y. Perera, B. E. Henricson, T. A. Hamilton, N. Qureshi, S. N. Vogel. 1994. Endotoxin-induced early gene expression in C3H/HeJ (Lps^d) macrophages. *J Immunol 153:2653.*

Manthey, C. L., S. N. Vogel. 1994. Elimination of trace endotoxin protein from rough chemotype LPS. *J Endotoxin Res 1:84.*

Mastroeni, P., S. Clare, S. Khan, J. A. Harrison, C. E. Hormaeche, H. Okamura, M. Kurimoto, and G.Dougan. 1999. Interleukin 18 contributes to host resistance and gamma interferon production in mice infected with virulent *Salmonella typhimurium. Infect Immun 67:478-483.*

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Creating Future Leaders

Mathison, J. C., G. D. Virca, E. Wolfson, P. S. Tobias, K. Glaser, R. J. Ulevitch. 1990. Adaptation to bacterial lipopolysaccharide controls lipopolysaccharide-induced tumor necrosis factor production in rabbit macrophages. *J Clin Invest 85:1108.*

Medvedev, A., Henneke, P., Schromm, A., Lien, E., Ingalls, R., Fenton, M., Golenbock, D., Vogel, S. 2001. Induction of tolerance to lipopolysaccharide and mycobacterial components in Chinese hamster ovary/CD14 cells is not affected by over expression of Toll-like receptors 2 or 4. *J Immunol* 167:2257-2267

Medzhitov, R., Janeway, C. Jr. 2000. Innate immunity. *N Engl J Med.* 343:338-344.

Mengozzi, M., Ghezzi, P. 1993. Cytokine down-regulation in endotoxin tolerance. *Eur. Cytokine Network 4:89.*

Mitchell, L., S. H. Smith, J. S. Braun, K. H. Herzog, J. R. Weber, and E. I. Tuomanen. 1997. Molecular analysis of virulence factors of *Streptococcus pneumoniae*. *J. Appl. Bacteriol.* 26:625–715.

Mogensen, T. H., Paludan, S. R. 2005. Reading the viral signature by Toll-like receptors and other pattern recognition receptors. *J Mol Med* 83:180-192

Nakane, A., M. Okamoto, M. Asano, M. Kohanawa, T. Minagawa. 1995. Endogenous γ-interferon, tumor necrosis factor, and interleukin-6 in *Staphylococcus aureus* infection in mice. *Infect Immun 63:1165.*

Nakano, M., K. Onozuka, H. Yamasu, W. F. Zhong, and Y. Nakano. 1992. Protective effects of cytokines in murine Salmonellosis. In *Microbial Infections*. H. Friedman, ed. Plenum Press, New York, p. 89-95.

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Creating Future Leaders

Neter, E. 1969. Endotoxins and the immune response. *Curr Top Microbiol Immunol* 47:82-124.

Nomura, F., Akashi, S., Sakao, Y., Sato, S., Kawai, T., Matsumoto, M., Nakanishi, K., Kimoto, M., Miyake, K., Takeda, K., Akira, S. 2000. Endotoxin tolerance in mouse peritoneal macrophages correlates with down-regulation of surface toll-like receptor 4 expression. *J Immunol* 164:3476-3479

Nyblom, H., Bjornsson, E., Simren, M., Aldenborg, F., Almer OS., and Olsson R. 2006. The AST/ALT ratio as an indicator of cirrhosis in patients with PBC. *Liver Int* 26 (7): 840-845.

Pasare, C., Medzhitov, R. 2003. Toll-like receptors: balancing host resistance with immune tolerance *Curr Opin Immunol* 15:677-682

Peck, O.M., Tempel, G.E., Halushka, P.V., Teti, G. and Cook, J.A. 2004. *Staphylococcus aureus* and lipopolysaccharide induce homologous tolerance but heterologous priming; role of interferon-gamma. *Shock* 21 (3):254–260.

Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., Beutler, B. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 282:2085-2088

Restrepo, A.V., Salazar, B.E., Agudelo, M., Rodriguez,C.A., Zuluaga, A.F., and Vesga, O. 2005. Optimization of culture conditions to obtain maximal growth of penicillin-resistant *Streptococcus pneumoniae. BMC Microbiol* 5: 34.

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Schade, F.U., Flash, R., Flohe, S., Majetschak, M., Kreuzfelder, E., Dominguez-Fernandez, E., Borgermann, J., Reuter, M., and Obertacke, U. 1999. in *Endotoxin in Health and Disease* (Brade, H. , Opal, S. M. , Vogel, S. N. , and Morrison, D. C., eds) , pp. 50-64, Marcel Dekker, Inc., New York

Toshchakov, V., B. W. Jones, P. Y. Perera, K. Thomas, M. J. Cody, S. Zhang, B. R. Williams, J. Major, T. A. Hamilton, M. J. Fenton, and S. N. Vogel. 2002. TLR4, but not TLR2, mediates IFN-beta-induced STAT1alpha/beta-dependent gene expression in macrophages. *Nat Immunol* 3:392-398

Trobonjaca, Z., Leithauser, F., Moller, P., Schirmbeck, R., and Reimann, J. 2001. Activating immuninity in the liver: Liver dendritic cells (but not hepatocytes) are potent activators of IFN-gamma release by liver NKT cells. *J Immunol* 167: 1413-1422.

von Stebut E, and Udey MC. 2004. Requirements for Th1-dependent immunity against infection with *Leishmania major. Microbes Infect 6:* 1102–1109.

Woods, J. P., J. A. Frelinger, G. Warrack, and J. G. Cannon. 1988. Mouse genetic locus Lps influences susceptibility to *Neisseria meningitidis* infection. *Infect Immun* 56:1950-1955

World Health Organizations. 1997. Gobal programmes for vaccines and immunizations (vaccine research and development). Report of the technical review group meeting, July 1997-June 1998, p26-30, WHO,Geneva, Switzerland

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Creating Future Leaders

Yoshimura, A., Lien, E., Ingalls, R.R., Tuomanen, E., Dzianski, R., Golenbock, D. (1999). Recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. *J Immunol.* 163: 1–5.

Young, E.J., Borchert, M., Kretzer, F.L., Musher, D.M. 1985. Phagocytosis and killing of Brucella by human polymorphonuclear leucocyets. *J Infect Dis* 151:682-690.

Zeigler-Heitbrock HW, Wedel A, Schraut W, Strobel M, Wndelgrass P, Sternsdorf T, Bauerle PA, Haas JG, Riethmuller G. 1994. Tolerance to lipoplysaccharide involves mobilization of nuclear factor kappa B with predominance of p50 homodimer. *J Biol Chem* 269:17001-17004.

Zeisberger, E., and Roth, J. 1998. Tolerance to Pyrogens. *Ann. N. Y. Acad. Sci.* 856: 116-131.

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CHAPTER THREE

Highly purified Lipopolysaccharide (LPS) as an adjunctive therapy provides survival enhancement in a lethal murine model of systemic *Streptococcus pneumoniae* sepsis.

Abstract

Background: Mortality from pneumococcal infections remains high, despite the development of potent antibiotics. Highly purified LPS has shown to induce protective innate immune responses when administered before bacterial challenge. The effect of reLPS as adjunctive therapy in a lethal murine model of systemic *Streptococcus pneumoniae* was investigated.

Methods: C57BL/6 and Balb/c mice were inoculated intravenously with a lethal dose of 10^7 cfu of *S. pneumoniae* serotype 2. Control groups for all strains received PBS. One group of infected mice received 25 mg/kg Ceftriaxone at 6 hours post infection. Another group received 10ug of reLPS (i.v) at 3 hours post infection and additionally every 12 hours for four more doses. Another group received a combination of 25 mg/kg Ceftriaxone (i.p) and 10ug of reLPS (i.v) at 6 hours post infection and LPS was then administered twice a day. Survival was monitored for seven days. The animals underwent cardiac puncture and ventricular blood was aspirated for bacteremia and cytokine analysis at 6h and 12h just prior sacrifice.

Results: 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) (p=0.0001) and 0% of vehicle controls (n=10) (p=0001). Treatment with a non-lethal dose of LPS only beginning at 3 hours after infection reduced mortality by 50% in

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C57BL/6 mice (n=7) and no survival benefits (100 % mortality) (p=0.03) 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=001 and 12h (0.5 ± 1.0 vs 4.9 ±1.6 log10 CFU/ml, p=0.001) after infection, compared with animals treated with ceftriaxone alone. Treatment with the combination of ceftriaxone and LPS significantly decreased 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 ($p<0.05$).

Conclusion: 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. It is noted that reLPS alone was also protective in this model. These results show the potential for innate immune stimulation as adjunctive therapy in the treatment of pneumococcal infections and these results warrant further studies.

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3.1. INTRODUCTION

S. pneumoniae is an important human pathogen and causes major complication of pneumococcal infection in poatients with underlying diseases. Despite significant advances in antibiotic therapy and critical care in the past few decades, mortality and morbidity due to pneumococcal infection and sepsis are still devastating (Nau and Eiffert, 2002).

The treatment of pneumococcal infections such as meningitis, pneumonia, and bacteremia, with beta-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 (Orman and English, 2000). Inflammatory processes are crucial for defense against pathogens but inappropriate and/or prolonged inflammation contributes to the pathogenesis of major diseases (Tarkowski and Wagner, 1998). Antibiotics can kill bacteria, but most do not strongly modulate the inflammatory response. Therefore, the addition of host response modulators to antibiotics might be beneficial for the treatment of severe pneumococcal pneumonia (Levy et al., 2001).

The innate immune system is important for the elimination of bacteria and one of the first steps in activating host defense mechanisms is recognition of pathogens by phagocytic cells. Phagocytes recognize highly conserved motifs (pathogen-associated molecular patterns [PAMPs]) shared by large groups of microorganisms, leading to intracellular signaling and ultimately resulting in the production of cytokines and chemokines and activation of the adaptive immune system (Takeda et al., 2003). One of the best-known PAMPs is endotoxin (lipopolysaccharide [LPS]), which is part of the outer membrane of

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gram-negative bacteria and responsible for activating innate host defense mechanisms in Gram negative infections (Wright et al., 1990). Gram-positive bacteria do not contain LPS in their cell walls but express other PAMPs such as lipoteichoic acid (LTA), peptidoglycan (PGN), and lipoproteins. Recognition of and responses to PAMPs are controlled by several pattern recognition receptors (PRRs). Toll-like receptors emerged as the key regulators of innate immune responses to infection in mammals (Takeda et al., 2003) and TLR4 has been shown to mediate LPS responsiveness, implying that TL4 is the PRR for LPS (Shimazu et al., 1999).

Stimulation of the innate immune system causes the release of cytokines and other mediators that can drive adaptive immune system in a specific direction. The use of innate immune stimulation to improve survival has previously been described (Kaufmaunn et al., 2000). Studies in animal models have shown that beneficial effects can be achieved by immunomodulation of certain components of the host response to improve the outcome of disease. In our previous chapter (Chapter 2), we induced LPS tolerance in mice and studied the response of the mice to a subsequent challenge with the live *Streptococcus pneumoniae*. It was observed that endotoxin pretreatment improved bacterial clearance and decreased mortality in mice challenged with *Streptococcus pneumoniae*. In the current study, it was hypothesized that enhancing the innate immune response, through TLR4, would initiate a stronger immune response, leading to a faster elimination of bacteria and modulation of certain components of the host response after antibiotic treatment and therefore improve the outcome of the disease.

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3.2. The main objective of the study

The main objective of the study was to determine the effect of enhancing the innate immune response, through TLR4 after antibiotic treatment on the progression of pneumococcal infection in a lethal murine model of systemic *Streptococcus pneumoniae* sepsis.

3.2.1. The specific objectives of the study were:

3.2.1.1. To examine the effects of LPS treatment as an adjunctive therapy to antibiotics on the survival of mice after pneumococcal challenge

3.2.1.2. To determine the mechanism of protection or effect of LPS treatment as an adjunctive therapy to antibiotic ceftriaxone on bacterial load and cytokine production after pneumococcal challenge

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3.2 MATERIALS AND METHODS

3.2.1 Experimental animals

Female C57bl/6 (approximately 20g: Jackson laboratories, Bar Harbor, ME) were housed at 22-25 ^{0}C with a 12h light/dark cycle, fed standard laboratory food and water *ad libitum*, and were kept under specific pathogen-free conditions. The protocol was approved by the Animal Care and Use Committee of the University of Virginia, USA.

3.2.2 Experimental inoculums

Before each experiment, several colonies of *S. pneumoniae* were inoculated into Brain-heart infusion broth, then subcultured on to agar plates and incubated for 24 hours at 37^{0}C in 5% CO_2. Five to ten colonies of an overnight culture were then inoculated into 10 ml of Brain-heart infusion broth, serially diluted and then incubated for 4-6 hours at 37^{0}C and then subcultured on agar plates for 18 h at 37^{0}C in 5% CO_2 to obtain a final concentration of 9 log10 cfu/ml, as determined by optical density reading (Vitek).

3.2.3 Survival studies

Female C57bl/6 mice were inoculated intravenously with 1x10^7 CFU of *S. pneumoniae* and treated with 10ug of purified LPS, or PBS for controls, at t=3h, 6hrs and then every 24 hours spanning 48 hours. A dose of ceftriaxone (25 ug/kg) at t=6hrs was given to a subset of treated and control mice to create a total of four groups (bacterial infection +PBS; bacterial infection + 10ug LPS given 3hrs post infection; bacterial infection + 10ug LPS at 3hrs+ Ceftriaxone at 6hrs post infection; bacterial infection + ceftriaxone at 6hrs post infection).

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3.2.4 Time course studies

Test (Ceftriozone alone treated mice and LPS plus Ceftriaxone treated mice) mice were sequentially sacrificed at 6h and 12h after bacterial challenge. The mice were anesthetised with ketamine and xylazine and killed by cervical dislocation. The animals underwent cardiac puncture and ventricular blood was aspirated for bacteremia and cytokine analysis just prior sacrifice.

3.2.5 Quantification of bacterial load from blood and lungs

Serial ten-fold dilutions were performed to determine bacterial counts at each time point. The detection limit with this method was 10^2 cfu/ml, and a value of 0 (log 10 =1) was assigned to the first and subsequent sterile cultures. Two hundred microliters of each dilution was aseptically plated and cultured on Tryptose blood agar plates at 37^0C overnight. The number of bacterial colonies was counted and quantitative cultures expressed as CFU per milliliter of blood.

3.2.6 Cytokine quantification

Blood samples from untreated, ceftriaxone treated, and Ceftriaxone plus LPS-treated mice were collected 6h after bacterial infection. Serum was separated from blood by centrifugation and stored at -70^0C until cytokine assays were performed. Serum cytokines were determined by using protein-based multiplex immunoassay system (Bio-Rad Laboratories, Hercules, CA). The system has capacity to quantify 23 cytokines, namely, IL-1 alpha and Beta, TNF-alpha, IFN-gamma, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12 p40 and p70, IL-13, IL-17, KC, MIP-1 alpha, MIP-1 beta, MCP-1, G- CSF, RANTES.

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3.2.7 Statistical Analysis

Statistical comparisons of cytokines levels were determined by a 2 tailed Student's T-test (Microsoft Excel software, Microsoft corporation,Redmond, WA). Survival curves were generated and compared with a log-rank test (GraphPad PRISM software, San Diego, CA). Data was displayed as means+/-SEM unless otherwise stated. Differences were considered significant when $p<0.05$.

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3.3. RESULTS

3.3.1 LPS treatment as an adjunctive therapy improves survival in systemic pneumococcal model

The effect of LPS treatment post infection to increase survival in mice challenged intravenously with live $1x10^7$ cfu *S. pneumoniae* was examined. Subsets of mice were treated with LPS alone 3h post infection, ceftriaxone alone or combination of ceftriaxone and LPS. The 5 days survival rate of mice challenged with *S. pneumoniae* without antibiotic treatment was 0% and LPS treatment post infection beginning 3h after bacterial challenge had 40% (n=7) survival benefits when administered without ceftriaxone. When highly purified LPS was administered as from 6 hours after bacterial challenge, there was no survival benefits compared with the control group (0% survival; p=0.0720). But when administered in combination with ceftriaxone the survival of mice increased significantly compared to ceftriaxone alone treated mice. The survival of mice treated with a combination of ceftriaxone and LPS was 80% (n=20), compared with 0 % of mice that received the vehicle control (n=10) and 40% (n=20) of mice that received cefriaxone alone (p=0.0001 v/s control and p=0.0001 v/s ceftriaxone). Treatment with LPS plus ceftriaxone protected mice better than treatment with ceftriaxone alone (p<0.0001) (Fig. 3.1 and 3.2).

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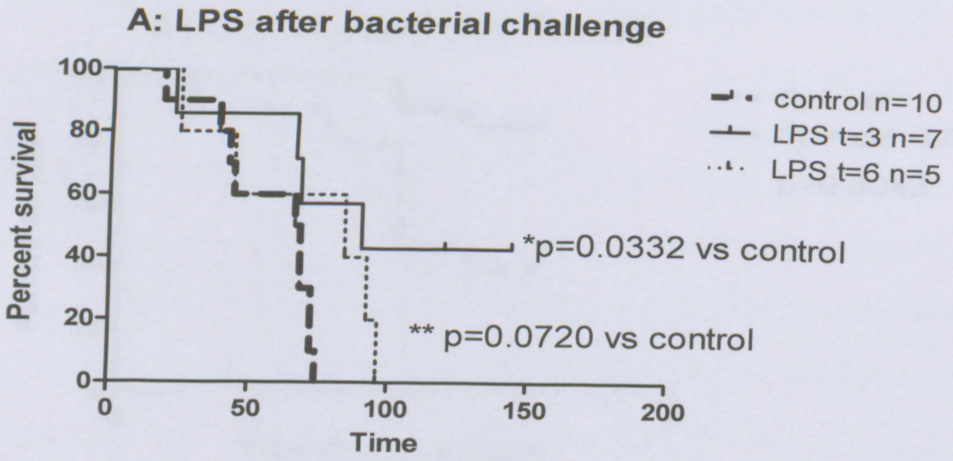


Figure 3.1. Survival curves for C57BL/6 mice treated with LPS 3h and 6h after 1 x 10^7 CFU bacterial challenge. (A) Mice were treated with LPS 3h and/or 6h and then 12 hours after bacterial challenge for 2 days. Treatment of mice with LPS starting at 3h after bacterial challenge showed significant survival benefits (42% (n=7) vs 0% (n=10)) with mean survival time of 90h compared with 67h in the control group (p=0.0332). No survival benefits were observed when treatment was started 6h after bacterial challenge (n=5). Mean survival time was 80h compared to mean survival time of 67h of control mice (p=0.0720).

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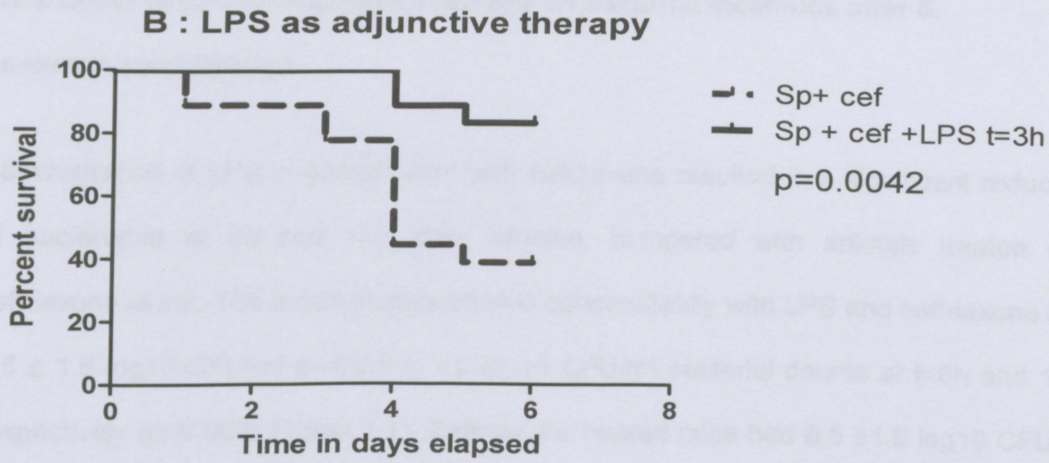


Figure 3.2 Survival curves for C57BL/6 mice treated with LPS and ceftriaxone after 1 x 10^7 CFU bacterial challenge. Survival of mice treated with the combination of LPS and ceftriaxone was improved significantly (80%; n=20) and mean survival time of 144h compared with 40% (n=20) survival and mean survival time of 72h of mice that received ceftriaxone alone (p=0.0042).

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3.3.2 Effect of LPS as adjunctive therapy on bacterial clearance after S. pneumoniae challenge.

Administration of LPS in combination with ceftriaxone resulted in a significant reduction of bacteremia at 6h and 12h after infecton, compared with animals treated with ceftriaxone alone. The blood of mice treated concomitantly with LPS and ceftriaxone had 2.5 ± 1.6 log10 CFU/ml and 0.5 ± 1.0 log10 CFU/ml bacterial counts at t=6h and 12h, respectively (p=0.001) (Table 3.1). Ceftriaxone treated mice had 6.6 ±1.6 log10 CFU/ml and 4.9 ±1.6 log10 CFU/ml bacterial counts at 6h and 12h, respectively. Mice treated concomitantly with LPS and ceftriaxone had 4 log10 fewer bacteria at t=6h and 12h, respectively, than those of mice treated with ceftriaxone alone. The difference between ceftriaxone plus LPS and ceftriaxone-treated groups reached statistical significance ($p<0.05$) at t=7 and 12h after bacterial challenge. These data indicate that treatment with LPS alone enhance bacterial clearance by the immune system and also promote the containment or clearance of bacteria when administered in combination with Ceftriaxone.

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Table 3.1: Bacteremia in mice 6h and 12h after bacterial challenge

	Bacterial counts log CFU/ml mean ± STDEV	
	6 hours	12 hours
Sp alone	7.9 ± 1.89	9.1 ± 1.1
Sp + LPS	6.8 ± 1.3	7.9 ± 1.3
Sp + cef	6.6 ± 1.5	4.9 ± 1.6
Sp + cef+ LPS	2.5 ± 1.6	0.5 ± 1.0

P value =0.001, Sp+cef+LPS vs Sp+ cef group at 6h and 12h after bacterial challenge

Key notes

Sp = *Streptococcus pneumoniae*

Cef = ceftriaxone

LPS = Lipopolysaccharide

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3.3.3 Effect of LPS as adjunctive therapy on circulating cytokine concentrations after S. pneumoniae challenge

Treatment of pneumococcal infections with the beta-lactam antibiotic ceftriaxone leads to a rapid increase in tumor necrosis factor alpha (TNF-a), and interleukin -1 beta (IL-1 beta), and other proinflammatory and anti-inflammatory cytokines/chemokines (Echtenacher et al., 1990). To understand the underlying protective effect of LPS treatment, the impact of LPS on the cytokine profile of mice infected with *S. pneumoniae* with or without LPS, and treated with ceftriaxone was examined. In control and ceftriaxone treated mice, all cytokines levels were greatest at 6h and 12h after infection. All these cytokines were reduced significantly in LPS plus ceftriazone treated mice compared to the infected untreated mice and ceftriaxone alone treated mice. Mice treated with the combination of ceftriaxone and LPS showed a significant decrease in the level of TNF-alpha, IFN-gamma, IL-12p70, MIP-1 alpha, IL-1 beta, and RANTES 12h after bacterial challenge compared to those treated with ceftriaxone alone ($p<0.05$; Table 3.2). However, Th2 cytokines (IL-2, IL-3, IL4, and KC) were elevated 12h after bacterial challenge. There was no statistical significant difference observed in levels of IL-1a, IL-5, IL-6, IL-10, 1L-12p40, IL-17, G-CSF, GM-CSF between the LPS treated and ceftriaxone alone treated groups ($p>0.05$). The results show that LPS treatment as an adjunctive therapy reduced Th1 cytokine levels and elevated Th2 cytokine levels.

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Table 3.2. Cytokine levels in control, ceftriaxone alone and LPS plus ceftriaxone treated mice at 12 hours after bacterial challenge.

cytokines	Sp + Cef	Sp + cef + LPS	P value
* TNF-α	9303 ± 2484	660 ± 190.5	p<0.05
IL-1α	1586 ± 339	1196 ± 176	p>0.05
*IL-1β	1450 ± 1074	436 ± 67.2	p<0.05
**IL-2	132 ± 59.9	398 ± 87	p<0.05
**IL-3	172 ± 19.9	355 ± 126.9	p<0.05
**IL-4	95 ± 43.2	281 ± 32.9	p<0.05
IL-5	2251 ± 141.8	1536 ± 665	p>0.05
IL-6	13978 ± 829	17245 ± 1546	p>0.05
IL-10	1330 ± 225.1	1074 ± 170	p>0.05
IL-12p40	12042 ± 5242	4571 ± 4343.5	p>0.05
*IL-12p70	6395 ± 3185	1219 ± 1083	p<0.05
IL-17	809 ± 390	760 ± 193	p>0.05
G-CSF	11514 ± 1145	13234 ± 1461	p>0.05
GM-CSF	281 ± 224	601 ± 185	p>0.05
*IFN-γ	12930 ± 6886	2347 ± 1200	p<0.05
**KC	19595 ± 429	26129 ± 832	p<0.05
*MIP-α	9200 ± 1411	1819 ± 243	p<0.05
*RANTES	23364 ± 4056	1792 ± 604	p<0.05

*indicates cytokines which were reduced significantly in LPS plus ceftriaxone group compared to the ceftriaxone-alone treated group, p<0.05

**indicates cytokines which were elevated significantly in LPS plus ceftriaxone group compared with ceftriaxone- alone treated group, p<0.05

Key notes

Sp = *Streptococcus pneumoniae* Cef = ceftriaxone LPS = Lipopolysaccharide

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3. 4. DISCUSION AND CONCLUSION

Modern therapeutic approaches in infectious diseases have focused on modulation of the host response (Roilides and Pizzo, 1992). Of the many adjuvant immunomodulatory strategies proposed for pneumococcal infection (bacterial meningitis), only the use of dexamethasone has gained access to clinical practice (Odio et al., 1991). In our previous chapter (Chapter 2), it was demonstrated that innate stimulation is a promising approach toward reducing early mortality in pneumococcal infections. TLR4 has been implicated as playing an essential role in host defense by virtue of its capacity to signal LPS-induced inflammatory response (Chow et al., 1999). The current study showed that stimulation of TLR4 by low dose of highly purified LPS as an adjunctive therapy to the antibiotic Ceftriaxone achieved a survival benefit in the reduction of mortality in severe *S. pneumoniae* infections compared with control infected and ceftriaxone treated mice but did not improve significantly (40%) survival rate when administered 3h post infection without antibiotic. Bacterial load and cytokine levels were reduced significantly in LPS plus ceftriaxone treated group compared with Ceftriaxone alone treated mice.

It was observed that the beneficial effect of purified LPS as adjunctive therapy in a murine model of pneumococcal infection is greatly affected by the timing and severity of infection or inflammation. In the presence of a severe infection produced by high inoculums, treatment with LPS initiated 6h after bacterial challenge did not improve survival in severe *S. pneumoniae* infections. It was noted that administration of LPS 3h post infection was protective in this model. Our results were in agreement with the study conducted by Deallaire et al. (2001) which showed that when LPS was injected into infected mice to determine whether a stronger early inflammatory response would improve survival rates in their pneumococcal pneumonia model, LPS improved survival

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to 100%. The current results showed less survival benefits maybe because of high dose of infection (1x 10^7 CFU) used and also the route of bacterial challenge employed in our systemic model. In this study, mice were challenged intravenously compared to intranasal challenge used in the other study. The increased survival observed when proinflammatory compound, LPS, was injected concomitance with the inoculum suggests that a stronger inflammatory response in the early hours of infection can be beneficial. Treatment with LPS initiated 6h after bacterial challenge did not improve survival in severe *S. pneumoniae* infections. Our results were in contrast with the study conducted by Rayhane et al. (2000) where they investigated whether delayed LPS injection could be beneficial in protecting mice against fungal infection. Their results indicated that a protective effect was induced when a larger amount of LPS (25 ug) was injected 1 day after a fungal challenge. No protective effect was seen when the injection was performed 3 days later, and the injection even proved to be lethal when performed 8 days after infection, reminiscent of the increased toxicity of LPS when delivered with other microbial agents (Nansen et al., 1997) or even with TNF (Rothstein and Schreiber, 1988). The beneficial effect of LPS was associated with delivery either before or soon after the inoculation, further supporting the concept of priming. Administration of LPS 6h after bacterial challenge proved to be lethal.

To obtain more insight into the effect of LPS treatment on host defense, bacterial loads in the blood of Ceftriaxone and LPS plus ceftriaxone treated mice were compared. After infection with 1X10^7 cfu of *S. pneumoniae,* bacteria were counted at 7h and 12h. LPS plus Ceftriaxone reduced bacterial load by 4 logs and 3 logs in blood, respectively. The difference between ceftriaxone plus LPS and ceftriaxone-treated groups reached statistical significance ($p<0.05$) at both time points. These data indicate that treatment

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with LPS enhance bacterial clearance by the immune system and also promote the containment or clearance of bacteria when administered in combination with ceftriaxone. Events that take place in the first hours are crucial to determine whether the infection would be controlled and cleared or, on the contrary, would progress to eventually end up in an accurate fatal infection. This observation suggests that the rapid bacterial growth outpaced the development of the inflammatory response. The use of LPS, confirmed that it is possible to increase the early inflammatory response and that this increase has a beneficial effect on survival through increased neutrophil recruitment at the site of infection (Alonso et al., 1995). A poor host response with low neutrophil recruitment in the early hours after bacterial challenge is detrimental for the outcome of pneumococcal infection. The failure of the host's immune system to control the growth of bacteria despite a strong neutrophil recruitment stresses the fact that the pneumococci must be eliminated before their growth reaches trigger points from which even a powerful inflammatory response will not be able to contain the infection (Alonso et al., 1995). To make matters worse, this overwhelming inflammation at the late stage of infection could contribute to tissue injury, shock, and death (Bergeron et al., 1998). Treatment with Ceftriaxone 6h after bacterial challenge greatly reduced bacterial load but did not improve survival. However, treatment with LPS plus Ceftriaxone showed a statistical significant improved survival rate compared with ceftriaxone alone treated and control infected mice. The fact that LPS improved survival, compared with that in ceftriaxone treated infected mice not exposed to LPS, suggest that a stronger inflammatory process is beneficial in this model.

Local production of cytokines and chemokines plays a role in the protective immune response to pneumococcal infection (Strieter et al., 2002). Therefore we determined the effect of LPS treatment as adjunctive therapy on cytokine concentration compared with

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the ceftriaxone alone treated group. Cytokine levels measured in blood 12h after bacterial challenge were reduced significantly in LPS plus Ceftriaxone treated group compared with high levels of cytokine levels in ceftriaxone treated mice. Our results show that *S. pneumoniae* induce severe inflammation, as evidenced by elevated proinflammatory cytokines. The addition of LPS to antibiotic down-regulated levels of inflammatory cytokines and by reducing inflammation, contributes to a better outcome in this model.

Our previous studies showed that pretreatment with LPS may be of some advantage in preventing the development of an infection. The results of this study showed that delayed treatment with low dose of LPS does not provide survival benefit but use of LPS as an adjunctive therapy provided survival enhancement in a lethal murine model of systemic *Streptococcus pneumoniae*. TLR4 agonists may have a unique therapeutic role in the modulation of innate immune responses when administered with antibiotic ceftriaxone. We believe that our study is the first to demonstrate the successful use of LPS as adjunctive therapy to antibiotics against severe pneumococcal infection that generates overwhelming inflammation. In our study, cefriaxone in association with LPS (initiated 3h after infection), reduced both the magnitude and duration of inflammation and significantly improved survival.

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3. 5. REFERENCES

Alonso De Velasco, E., Verheul, A.F., Verhoef, J., Snippe, H. 1995. Streptococcus pneumoniae virulence factors, pathogenesis, and vaccines. *Microbiol Rev* 59:591-603.

Bergeron, Y., Ouellet, N., Deslauriers, A.M., Simard, M., Olivier, M., Bergeron, M. 1998. Cytokine kinetics and other host factors in response to pneumococcal pulmonary infection in mice. *Infect Immun* 66:912-22

Chow, J.C., Young, D.W., Golenbock, D.T., Christ, W.J., and Gusovsky, F. 1999. Toll-like receptor -4 mediates lipopolysaccharide-induced signal transduction. *J Biol Chem* 274: 10689-10692.

Kaufmann, A., D. Gemsa, H. Sprenger. 2000. Differential desensitization of lipopolysaccharide-inducible chemokine gene expression in human monocytes and macrophages. *Eur J Immunol. 30:1562.*

Levy, B.D., Clish, C.B., Schmidt, B., Gronert, K., and Serhan, C.N. 2001. Lipid mediator class switching during acute inflammation: signals in resolution. *Nat Immunol* 2:612-619.

Nansen, A., Pravsgaard Christensen, J., Marker, O., and Randrup Thomsen, A. 1997. Sensitization to lipopolysaccharide in mice with asymptomatic viral infection: role of T-cell-dependent production of interferon gamma. *J Infect Dis* 176:151-157.

Nau, R., Eiffert, H. 2002. Modulation of release of proinflammatory bacterial compounds by antibacterials: potential impact on course of inflammation and outcome in sepsis and meningitis. *Clin. Microbiol. Rev* 15:95-110.

Odio, C.M., Faingezicht, I., Paris, M. 1991. The beneficial effects of early dexamethasone administration in infants and children with bacterial meningitis. *N Engl J Med* 324:1525-31

Orman, K.L., English, B.K. 2000. Effects of antibiotic class on the macrophage inflammatory response to *Streptococcus pneumoniae*. *J Infect Dis* 182:1561-5.

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Creating Future Leaders

Rayhane, N., Fitting, C., Lortholary, O., Dromer, F., and Cavaillon, J. 2000. Administration of Endotoxin associated with Lipopolysaccharide tolerance protects mice against fungal infection. *Infect Immunol* 3748-3753.

Roilides, E., and Pizzo, P.A. 1992. Modulation of host defense by cytokines: evolving adjuncts in prevention and treatment of serious infections in immunocompromised hosts. *Clin Infect Dis* 15:508-524

Rothstein, J.L., and Schreiber, H. 1988. Synergy between tumor necrosis factor and bacterial products causes hemorrhagic necrosis and lethal shock in normal mice. *Proc. Natl. Acad. Sci.* USA 85:607-611

Schuchat, A., Robinson, K., Wenger, J.D., et al. 1997. Bacterial meningitis in the United States in 1995. *N Engl J Med.* 337:970-976.

Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K., and Kimoto, M. 1999. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J Exp Med* 189:1777-1782.

Strieter, R.M., Belperio, J.A., and Keane, M.P. 2002. Cytokines in innate host defense in the lung. *J Clin Invest* 109:699-705.

Takeda, K., Kaisho, T., and Akira, S. 2003. Toll-like receptors. *Annu Rev Immunol* 21:335-376.

Tarkowski, A., Wagner, H. 1998. Arthritis and sepsis caused by Staphylococcus aureus: can the tissue injury be reduced by modulating the host's immune system? *Mol Med Today* 4:15-18.

Wright, S.D., Ramos, R.A., Tobias, P.S., Ulevitch, R.J., and Mathison, J.C. 1990. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* 249:1431-1433.

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CHAPTER FOUR

Adenosine A_{2A} receptor agonist as an adjunctive therapy improves survivalin a lethal murine model of systemic *Streptococcus pneumoniae*

Abstract

Background: *S. pneumoniae* is currently the leading cause of invasive bacterial disease in children and elderly worldwide. 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} 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*.

Methods: 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. A dose of ceftriaxone (25 ug/kg) at t=6hrs was given to a subset of treated and control mice to create a total of six groups (bacterial infection plus PBS [n=30]; bacterial infection + ATL313 alone [n=15]; bacterial infection+ATL313+ Ceftriaxone [n=26]; bacterial infection+ceftriaxone [n=31]). 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 coinjected intraperitoneally with ATL313 and ZM243185. Survival was monitored for 7 days. Blood samples were

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collected at 7h and 12h after bacterial challenge for bacteriological and cytokine measurements.

Results: ATL 313 (2.5- 25ug/kg), doses beginning 1, 6 and 24h after bacterial challenge 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 of mice compared to ceftriaxone alone treated mice. The survival of mice treated with the combination of ceftriaxone and ATL313 was 89% (n=26) compared to 0 % survival of mice that received the vehicle (n=30) and 23% (n=31) of mice that received cefriaxone alone (p=0.0001). 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 (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).

Conclusion: 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. More investigations are warranted to further assess the therapeutic benefit of A_{2A} receptor agonists as an adjunctive agent to antibiotics in the treatment of pneumococcal infection and also further investigate the mechanism of protection involved in the ATL 313 protection.

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4.1 INTRODUCTION

Despite significant advances in antibiotic therapy and critical care in the past few decades, the mortality rate of pneumococcal infection is substantial, ranging between 10% and 30%. Patients may die despite eradication of bacteria from lungs by antibiotic therapy. This is due in part to the ability of pneumococcal cell walls to generate exaggerated inflammation and tissue damage (Cundell et al., 1995). Poor outcomes from severe pneumococcal pneumonia occur partly because of overwhelming inflammation elicited by bacterial components. 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 (Orman and English, 2000; Nau et al., 2002).

Inflammatory processes are crucial for defense against pathogens but inappropriate and/or prolonged inflammation contributes to the pathogenesis of major diseases (Tarkowski and Wagner, 1998). The demonstration that the extracellular adenosine 2A receptor ($A_{2A}R$) participates in an endogenous immunosuppressive loop that serves to down-regulate inflammation in vivo by a nonredundant mechanisms (Ohta and Sitkovsky, 2001) highlighted the interest in naturally occurring purines in the regulations of inflammation and tissue protection.

The purine nucleoside adenosine is released into the extracellular space by a wide variety of stimuli and is one of the best characterized endogenous immunomodulator molecules. Adenosine binds to four different types of G-protein –coupled cell surface molecules, termed the A_1, A_{2A}, A_{2B}, and A_3 adenosine receptors (Linden, 1994; Ongini

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and Fredholm, 1996). Ligation of these receptors located on immune cells has important effects on a variety of immune cell functions including antigen presentation (Xaus et al., 1999), phagocytosis (Salmon et al., 1993), negative selection or target cell killing (Resta et al., 1997). An important feature of the regulation of immune processes by adenosine is that adenosine alters immune cell production of soluble mediators, such as cytokines (Ongini and Fredholm, 1996) and free radicals. Such an interference with cell-to-cell communication within the immune system can modify the outcome of the immune response. The A_{2A} adenosine receptors are present on inflammatory cells including neutrophils , mast cells, monocytes, macrophages, eosinophils, platelets, and T-cells , and are involved in antiinflammatory activities. Activation of A_{2A} AR results in an increase in cyclic AMP concentration in inflammatory cells (Sullivan et al., 2001).

The nucleoside adenosine is regarded as a key regulatory molecule that serves to balance cellular oxygen supply and demand during metabolic stress. Adenosine is released by cells into the extracellular space during hypoxia, exercise and ischemia (Mabley et al., 2003). In addition, neutrophils release nuclear DNA as a means to trap pathogens during acute inflammation, providing a rich source for extracellular nucleotides and nucleosides (Brinkmann et al., 2004).

Adenosine possesses anti-inflammatory properties and that adenosine analogs can protect mice from a variety of inflammatory diseases such as septic shock (Parmely et al., 1993; Hasko et al., 1998) and rheumatoid arthritis (Szabo et al., 1998) by inhibiting the release of free radicals and proinflammatory cytokines from different immune cells. The selective $A_{2A}R$ agonist CGS21680 was shown to protect liver from ischemia/reperfusion injury in rats and from ConA-induced tissue damage in mice (Ohta and Sitkovsky, 2001). Other used anti-inflammatory drugs, such as methotrexate and

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sulfasalazine, also to exert their effects by inducing the release of endogenous adenosine, which then signals through adenosine receptor (Cronstein et al., 1993; Cronstein et al., 1995; Gadangi et al., 1996; Marabito et al., 1998). Therefore, the triggering of a natural anti-inflammatory pathway by ligands of adenosine receptors represents an attractive immunomodulation strategy for the treatment of inflammatory diseases. The accumulation of extracellular adenosine in inflamed and damaged tissues (Winn et al., 1981; van Belle et al., 1987) and the immunosuppresive properties of c-AMP-elevated adenosine receptors indicate that signaling by A_{2A} adenosine receptor on immune cells is a possible natural mechanism of inhibition of inflammation. Extracellular adenosine and adenosine A_{2A} receptors act as both antiinflammatory signals and sensors of excessive inflammatory tissue damage (Linden, 1994).

4.2. The main objective of the study

The aim of the study was to determine whether A_{2A} receptor agonist, ATL313 as an adjunctive therapy, can increase the survival of mice exposed to live *Streptococcus pneumoniae* in a sepsis model.

4.2.1 The specific objectives of the study were:

4.2.1.1 We examined the effect of A_{2A} AR agonist ATL313 on survival in mice challenged with *S. pneumoniae*.

4.2.1.2 To test whether the effect of ATL313 was through functional A_{2A} adenosine receptors, by using C57BL/6 A_{2A}-receptor-deficient mice were used and co-injecting groups of C57BL/6 mice intraperitoneally with ATL313 and ZM243185 (A_{2A} antagonist).

4.2.1.3 To determine if hematopoietic cells are important in the protective effect of A_{2A}AR agonists ATL313 using Chimeric C57BL/6 mice with A_{2A} KO bone marrow transfer

4.2.1.4. To determine the effect of ATL 313 on bacterial clearance and circulating cytokine concentrations after *S. pneumoniae* challenge

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4.3 MATERIALS AND METHODS

4.3.1 Mice

Female C57bl/6 and C57BL/6 A_{2A}-receptor-deficient mice (approximately 20g; Jackson laboratories, Bar Harbor, ME) were housed at 68-72^{0}F with a 12h light/dark cycle, fed standard laboratory food and water *ad libitum*, and were kept under specific pathogen-free conditions. The protocol was approved by the Animal Care and Use Committee of the University of Virginia, USA.

4.3.2 Creating Chimeras: C57BL/6 mice with A_{2A} KO bone marrow transfer

Another strategy to identify the importance and whether A_{2A} receptors expressed on inflammatory cells and/or endothelial cells is the cellular target(s) of A_{2A}AR agonists in protection from death following bacterial challenge in the murine pneumococcal sepsis model is through the use of chimeric animals whereby A_{2A} KO bone marrow is transplanted into WT C57Bl/6 mice. The Chimeras were created as described by Linden et al., 1998). Briefly, the marrow from tibia and femur of A_{2A} KO donor mice were harvested to obtain a suspension of approximately 50 million nucleated bone marrow cells. Recipient mice were irradiated twice at 4-h intervals with 600 rad. immediately following the second irradiation period, 3 million bone marrow cells from A_{2A} Knock-out mice were injected intravenously into wild type C57Bl/6. Chimeras were confirmed using CD45.1/45.2 markers.

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4.3.3 Reagents and drugs

The $A_{2A}AR$ agonist, ATL313, (4-{3-(6-amino-9-(5-cycloproprylcarbamoyl-3,4-dihydroxytetrahydrofuran-2-yl)-9H-purin-2-yl)prop-2-ynyl}piperidine-1-carboxylic acid methyl ester) was supplied by Adenosine Therapeutics,LLC (Charlottesville,VA) and Adenosine 2a receptor antagonist ZM 241385 were purchased from Adenosine Therapeutics,LLC (Charlottesville,VA). Ketamine and xylazine were purchased from Vedco, Inc., (St. Joseph, MO). In all cases vehicle used was phosphate buffered saline (PBS).

4.3.4 Dose effect

The appropriate dose of ATL313 for survival studies was determined by a dose response curve where increasing doses of ATL313 (0.5-25 ug/kg) were administered to mice after intravenous (IV) injection of a lethal dose of *Streptococcus pneumoniae*.

4.3.5 Survival studies

Female C57bl/6 were inoculated intravenously with 10^7 colony forming units (CFU) *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 spanning 48 hours. A dose of ceftriaxone (25 ug/kg) at t=6hrs was given to a subset of treated and control mice to create a total of six groups (bacterial infection plus PBS; bacterial infection + ATL313 alone; bacterial infection+ATL313+ Ceftriaxone; bacterial infection+ceftriaxone). To test whether the effect of ATL313 was through functional A_{2A} adenosine receptors, C57BL/6 A_{2A}-receptor-deficient mice, and Chimera were used and groups of C57BL/6 mice were coinjected intraperitoneally with ATL313 and ZM243185.

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4.3.6 Quantification of bacterial CFUs from blood.

Experimental and control animals were sequentially sacrificed at 7, 12, and 24h after bacterial challenge. The mice were anesthetized with ketamine and xylazine and killed by cervical dislocation. Blood was collected by cardiac punture for bacteremia. Serial ten-fold dilutions were performed to determine bacterial counts at each time point. One hundred microliters of blood was diluted serially in 10ml sterile physiological saline (PBS). The detection limit with this method was 10^2 cfu/ml, and a value of 0 (log 10 =1) was assigned to the first and subsequent sterile cultures. Two hundred microliters of each dilution was aseptically plated and cultured on Blood agar plates at 37^0C overnight. The number of bacterial colonies was determined and quantitative cultures expressed as CFU per milliliter of blood.

4.3.7 Cytokine quantification

Cytokine Investigations were carried out using a protein bead-based multiplex immunoassay system (Bio-Rad Laboratories, Hercules, and CA). The levels of the following cytokines were determined namely, IL-1 alpha and Beta, TNF-alpha, IFN-gamma, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12 p40 and p70, IL-13, IL-17, KC, MIP-1 alpha, MIP-1 beta, MCP-1, G- CSF, RANTES. This panel allows for the evaluation of early and late appearing as well as pro-and anti-inflammatory cytokines.

4.3.8 Statistical Analysis

Statistical comparisons of cytokines values were determined by a 2 tailed Student's t-Test (Microsoft Excel software, Microsoft corporation,Redmond, WA). Survival data were plotted and the survival curves compared with a log-rank test (GraphPad PRISM software, San Diego, CA). Data are displayed as means+/-SEM unless otherwise stated. Differences were considered significant at $p<0.05$.

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4.4. RESULTS

4.4.1 ATL 313 improves survival in systemic pneumococcal model

We examined the effect of A_{2A} AR agonist ATL313 on survival in mice challenged with *S. pneumoniae*. The 5 days survival rate of mice challenged with *S. pneumoniae* with no treatment was 0%. ATL 313 (2.5- 25ug/kg), doses beginning 1, 6 and 24h after bacterial challenge 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 administered at t=1h, 6h, 12h increased the survival of mice compared to ceftriaxone alone treated mice. The survival of mice treated with the combination of ceftriaxone and ATL313 was 89% (n=26) compared to 0 % survival of mice that received the vehicle (n=30) and 23% (n=31) of mice that received cefriaxone alone ($p=0.0001$ v/s control and $p=0.0001$ v/s ceftriaxone). Treatment with ATL plus ceftriaxone protected mice better than treatment with ceftriaxone alone ($p<0.0001$ vs ceftriazone alone) (Fig 4.1).

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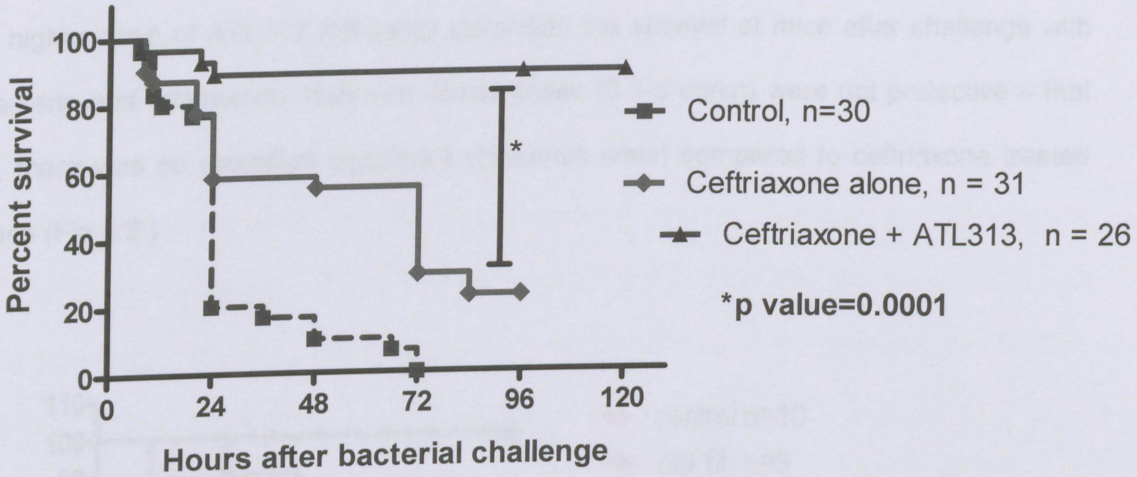


Figure 4.1. Survival curve of C57BL/6 treated with ATL 313 after bacterial challenge. Mice were inoculated with 1x 10^7 cfu *S. pneumoniae*. Mice (n=30) were treated with PBS vehicle (triangle), the antibiotic ceftriaxone (n=31; diamond), or a combination of ceftriaxone and ATL 313 (n=26; triangle). A single dose of 25ug/kg ceftriaxone was administered 6 hours after ceftriaxone. ATL 313 or vehicle were injected intraperitoneally at t=1h, 6, and 24h, 48h. Treatment with ATL 313 plus ceftriaxone protected mice better than ceftriaxone treatment alone (p=0.0001).

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A higher dose of ATL313 (25ug/kg) increased the survival of mice after challenge with bacteria and ceftriaxone. However, lower doses (0.5-5 ug/kg) were not protective – that is, there was no statistical significant difference when compared to ceftriaxone treated mice (Fig 4.2.)

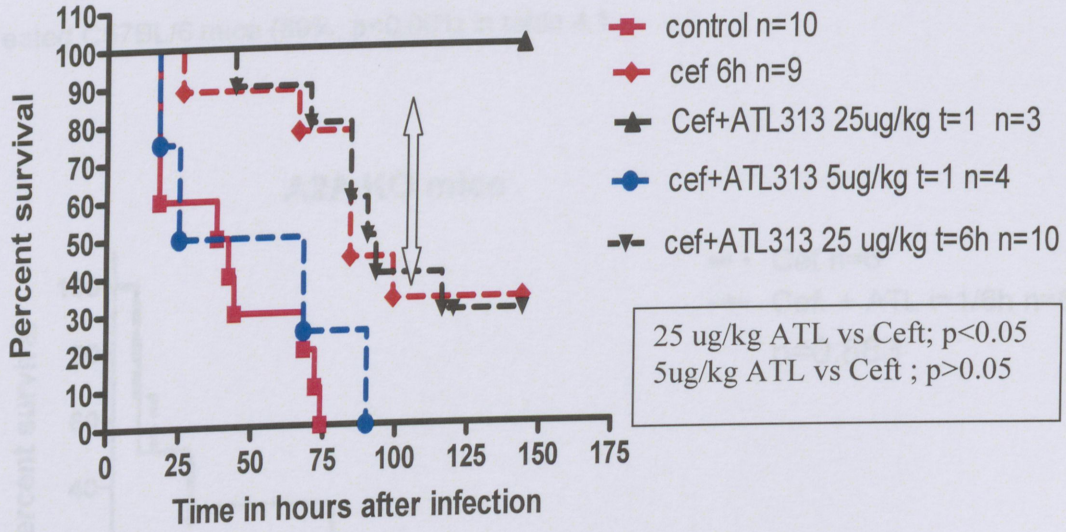


Figure 4.2. Survival curve of mice treated with ATL 313 after bacterial challenge. Mice were inoculated with 1x 10^7 cfu *S. pneumoniae*. Mice were treated with PBS vehicle (n=10, square), the antibiotic ceftriaxone (n=9; diamond), or a combination of ceftriaxone and 5ug/kg ATL 313 (n=4; circle) or a combination of ceftriaxone and 25ug/kg ATL 313 (n=10; triangle). A single dose of 25ug/kg ceftriaxone was administered 6 hours after ceftriaxone. ATL 313 or vehicle were injected intraperitoneally at t=1h, 6, and 24h, 48h. A higher dose of ATL313 (25ug/kg) increased the survival of mice after challenge with bacteria and ceftriaxone.

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To test whether the effect of ATL313 was through functional A_{2A} adenosine receptors, C57BL/6 A_{2A}-receptor-deficient mice were used and groups of C57BL/6 mice were coinjected intraperitoneally with ATL313 and ZM243185 (A_{2A} antagonist). Survival was reduced or reversed after ATL313 treatment in A_{2A} AR KO (17%; N=6/group) (Fig 4.3) and after treatment with ZM241385 (25%; n=8/group) (Fig. 4.4) compared to ATL 313 treated C57BL/6 mice (89%; p<0.001) in table 4.1

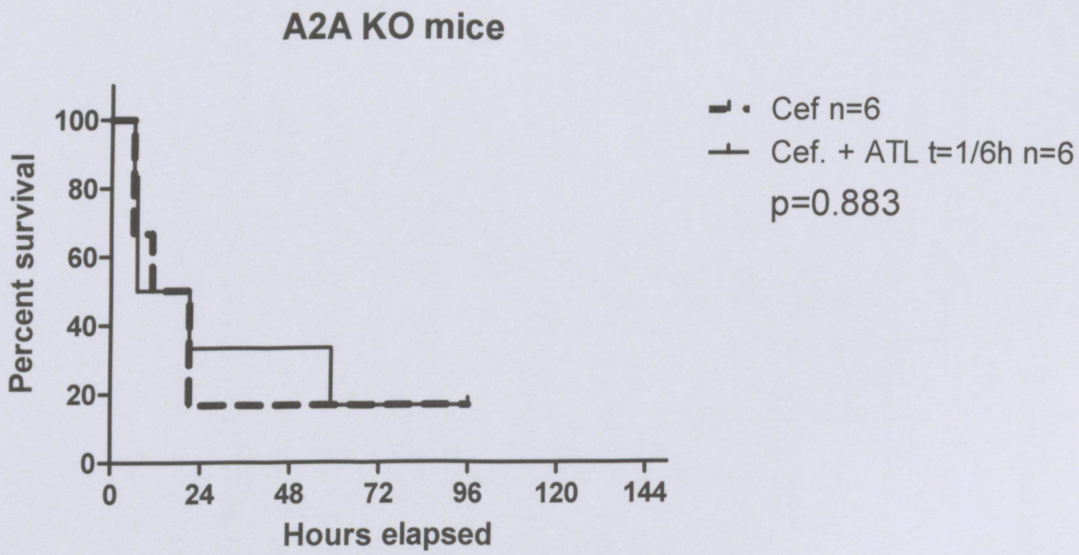


Figure 4.3. Survival curves of A2A KO mice treated with ATL313 after bacterial challenge. Mice were treated the antibiotic ceftriaxone , or a combination of ceftriaxone and ATL3131 (n=6 per group). A single dose of 25ug/kg ceftriaxone was administered 6 hours after ceftriaxone. ATL 313 or vehicle were injected intraperitoneally at t=1h, 6, and 24h, 48h. Survival was reduced after treatment ATL313 treatment in A_{2A} AR KO compared to ATL 313 treated C57BL/6 mice (p=0.883).

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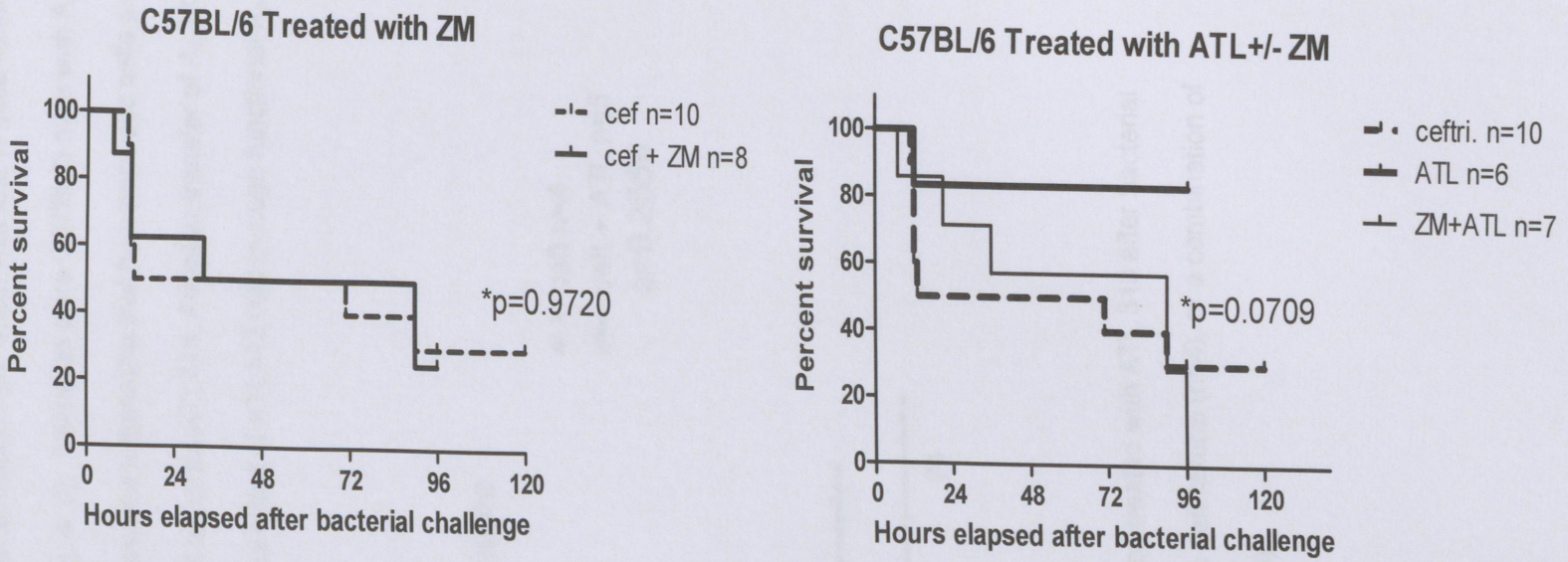


Fig. 4.4. Survival curve of mice treated with ATL 313 +/- ZM241385 after bacterial challenge. Mice were inoculated with 1x 10^7 cfu *S. pneumoniae* . **(Left)**Mice (n=10) were treated with ceftriaxone, or a combination of ceftriaxone and ZM241385 (N=8). Treatment with ZM241385 plus ceftriaxone did not protect mice from *S. pneumoniae* challenge when compared with the ceftriaxone alone treated group (p=0.9720) . **(Right)** Mice (n=10) were treated the ceftriaxone, or a combination of ceftriaxone and ATL3131, or a combination of ceftriaxone plus ATL 313 plus ZM241385 (N=7). Treatment with ATL 313 plus ceftriaxone protected mice better than ceftriaxone treatment alone (p=0.0001) and the effect of ATL313 was reversed by ZM241385 (p=0.0709).

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The ability of A_{2A} AR agonist ATL313 to protect mice from death was lost in mice lacking the A_{2A} AR (Chimera mice) (p=0.257) (Fig. 4. 5). Chimeras were C57BL/6 mice with A_{2A} KO bone marrow transfer and were not rescued suggesting that hematopoietic cells are important in the protective effect of A_{2A}AR agonists ATL313. Adoptive transfer of A_{2A}KO bone marrow into C57Bl/6 showed that the effect of ATL313 was through functional A_{2A} adenosine receptors.

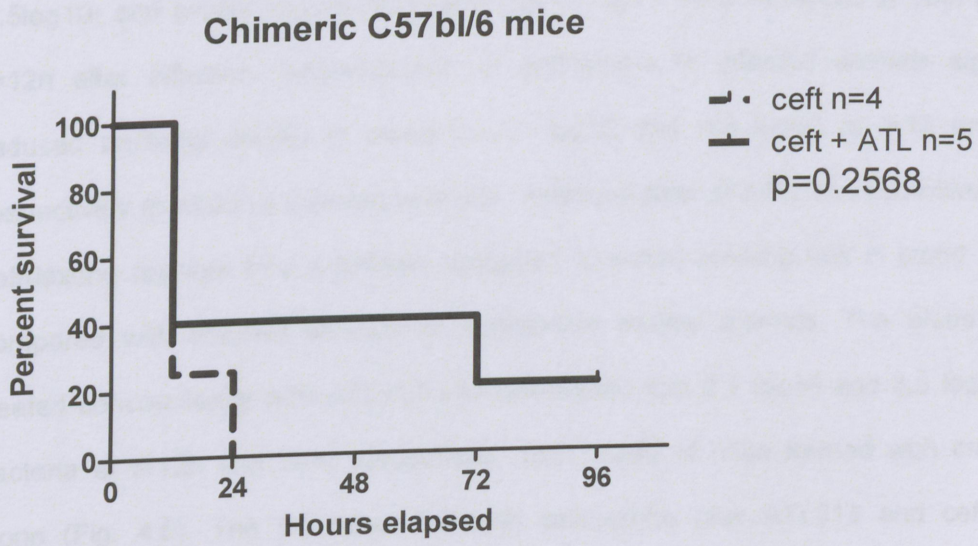


Figure 4.5. Survival curve of Chimera mice treated with ATL 313 after bacterial challenge. Mice were treated the antibiotic ceftriaxone (n=4), or a combination of ceftriaxone and ATL3131 (n=5). (p=0.256)

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4.4.2 Effect of ATL 313 on bacterial clearance after S. pneumoniae challenge.

Bacteria burden in blood from infected control animals gradually increased from 7.2 log10 to > 8 log 10 cfu until the animal died 48hrs after infection. Infected animals treated with ATL313 alone had bacterial counts similar or slightly higher than those of untreated infected animals at t=7h and t=12h and they died within 24h after infection. The colony-forming unit count in the bloodstreams of mice receiving ATL 313 alone (n=6) was 8.4 log10 at t=7h and was slightly higher than that in infected controls (n=8; 7.5log10) and similar counts of greater than 8 log10 were observed in both groups at t=12h after infection. Administration of ceftriaxone to infected animals significantly reduced bacterial counts in blood to 3.7 log10 and 4.6 log10 at t=12 and t=24h, respectively ($p<0.05$ vs infected animals). Administration of ATL313 in combination with ceftriaxone resulted in a significant reduction of colony-forming unit in blood at t=24h, compared with infected animals or ceftriaxone treated animals. The blood of mice treated concomitantly with ATL313 and ceftriaxone had 2.1 log10 and 3.3 log10 fewer bacteria at t=12h and 24h, respectively, than those of mice treated with ceftriaxone alone (Fig. 4.6). The difference between ceftriaxone plus ATL313 and ceftriaxone-treated groups reached statistical significance ($p<0.05$).

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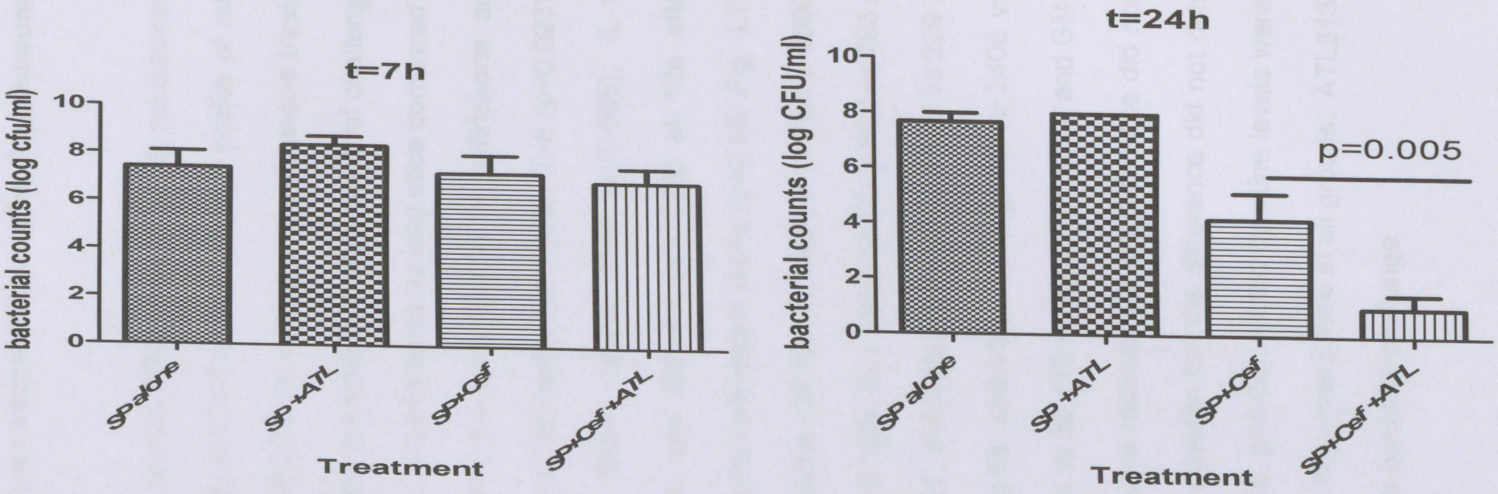


Figure 4.6. Mean blood bacterial counts after 1x 10^7 cfu *S. pneumoniae* and ATL treatment. Mice were treated with PBS vehicle, ATL 313 alone, the antibiotic ceftriaxone, or a combination of ceftriaxone and ATL 313. A single dose of 25ug/kg ceftriaxone was administered 6 hours after ceftriaxone. ATL 313 or vehicle were injected intraperitoneally at t=1h, 6, and 24h, 48h. Blood samples collected at different time points t=7h and t=24h. (**A**) The colony-forming unit count in the bloodstreams of mice receiving ATL 313 alone (n=6) at t=7h and was slightly higher than that in infected controls (n=8). There was no statistical significant difference in all the groups 7h after infection. (**B**) Administration of ceftriaxone to infected animals significantly reduced bacterial counts in blood at t=24h, ($p<0.05$ vs infected animals). Administration of ATL313 in combination with ceftriaxone resulted in a significant reduction of colony-forming unit in blood at t=24h, compared with infected animals or ceftriaxone treated animals. (n=5-9 per group, p=0.0055).

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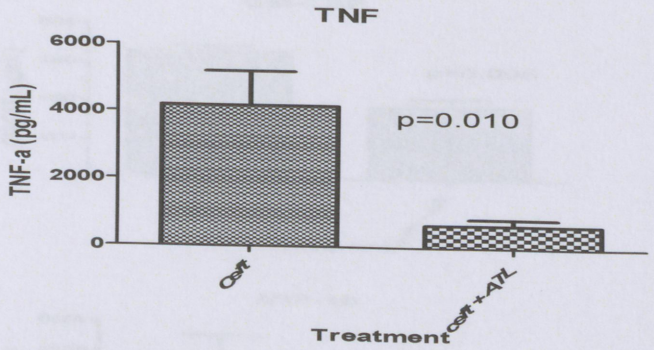
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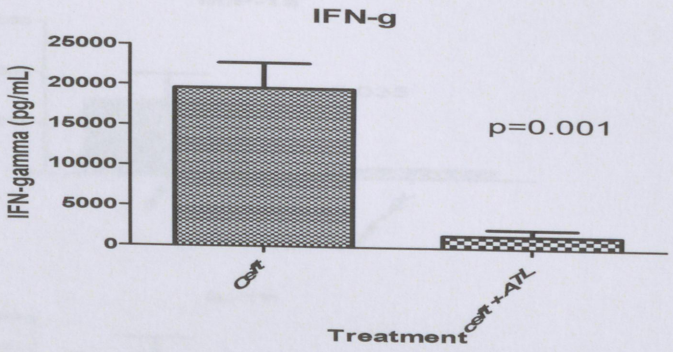
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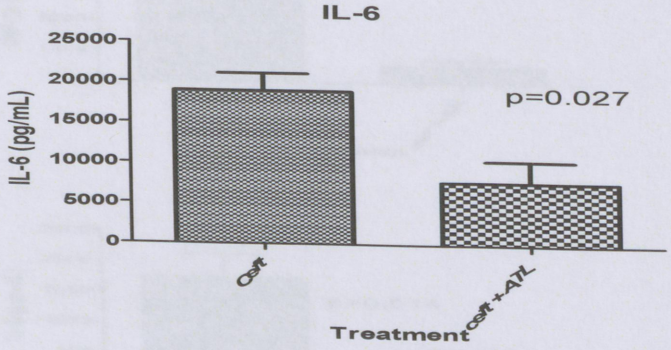
4.4.3 Effect of ATL 313 on circulating cytokine concentrations after S. pneumoniae challenge

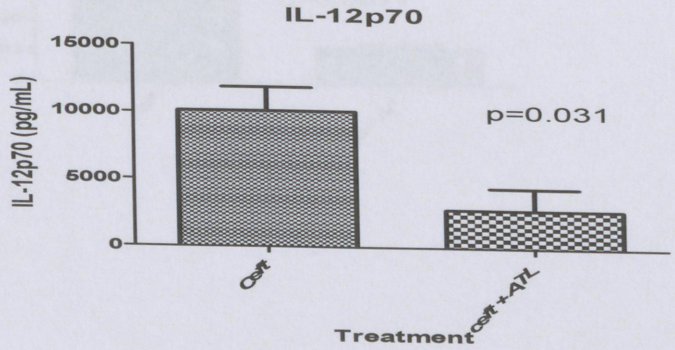
To understand the underlying protective effect of A2A AR agonist ATL313, we examined the impact of A2A adenosine receptor agonist ATL313 on the cytokine profile of mice infected with *S. pneumoniae* with or without ATL313. In control and ceftriaxone treated mice, the levels of the majority of cytokines were greatest 12h after bacterial challenge. All these cytokines were reduced significantly in ATL313 alone treated mice compared to the infected untreated mice. Mice treated with the combination of ceftriaxone and ATL313 showed a significant 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 (0.0071), IL-5 ($p=0.014$), and Eotaxin ($p=0.023$) at 12h after bacterial challenge compared to those treated with ceftriaxone alone ($p<0.05$; Fig. 4.7). However, the cytokines concentration (MCP-1 alpha (24,550 ± 1045 vs 13,200 ± 6090, $p=0.12$), KC (26,028 ± 962 vs 21,516 ± 2015, $p=0.132$), IL-1 alpha (2,171 ± 652 vs 793 ± 225.8, $p=0.073$), IL-2 (3,39 ± 36.1 vs 288 ± 61.27, $p=0.053$), IL-3 (287 ± 11.35 vs 229 ± 42.44, $p=0.309$), IL4 (333 ± 17.17 vs 186 ± 40.64, $p=0.033$), IL-17 (5,837 ± 2408 vs 1,230 ± 445.1, $p=0.077$), G-CSF (25,777 ± 1,036 vs 26,412 ± 250.2, $p=0.521$), and GM-CSF (660.6 ± 29.4 vs 378 ± 45.2, $p=0.0049$)) were reduced but the difference did not reach statistical significance. IL-13 was slightly elevated but the difference did not rich statistical significance (346.3 ± 32.8 vs 385 ± 72.8, $p=0.68$). All the cytokine levels were very low (returned to baseline) 24h after infection in survived mice in all groups. ATL313 influences circulating cytokine concentrations after bacterial challenge.

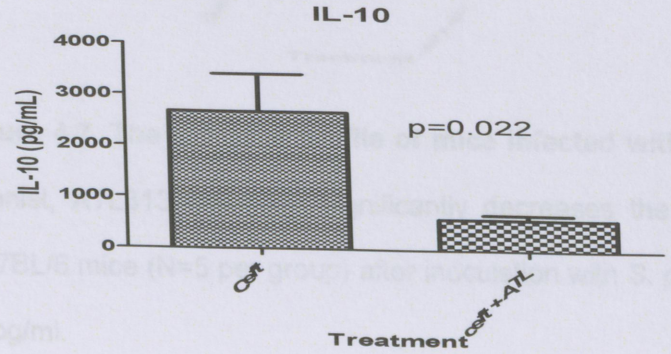
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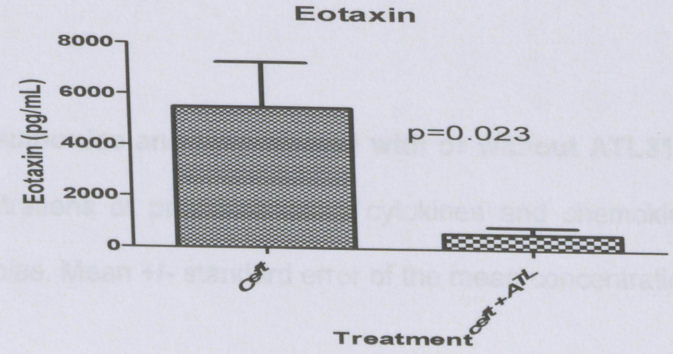












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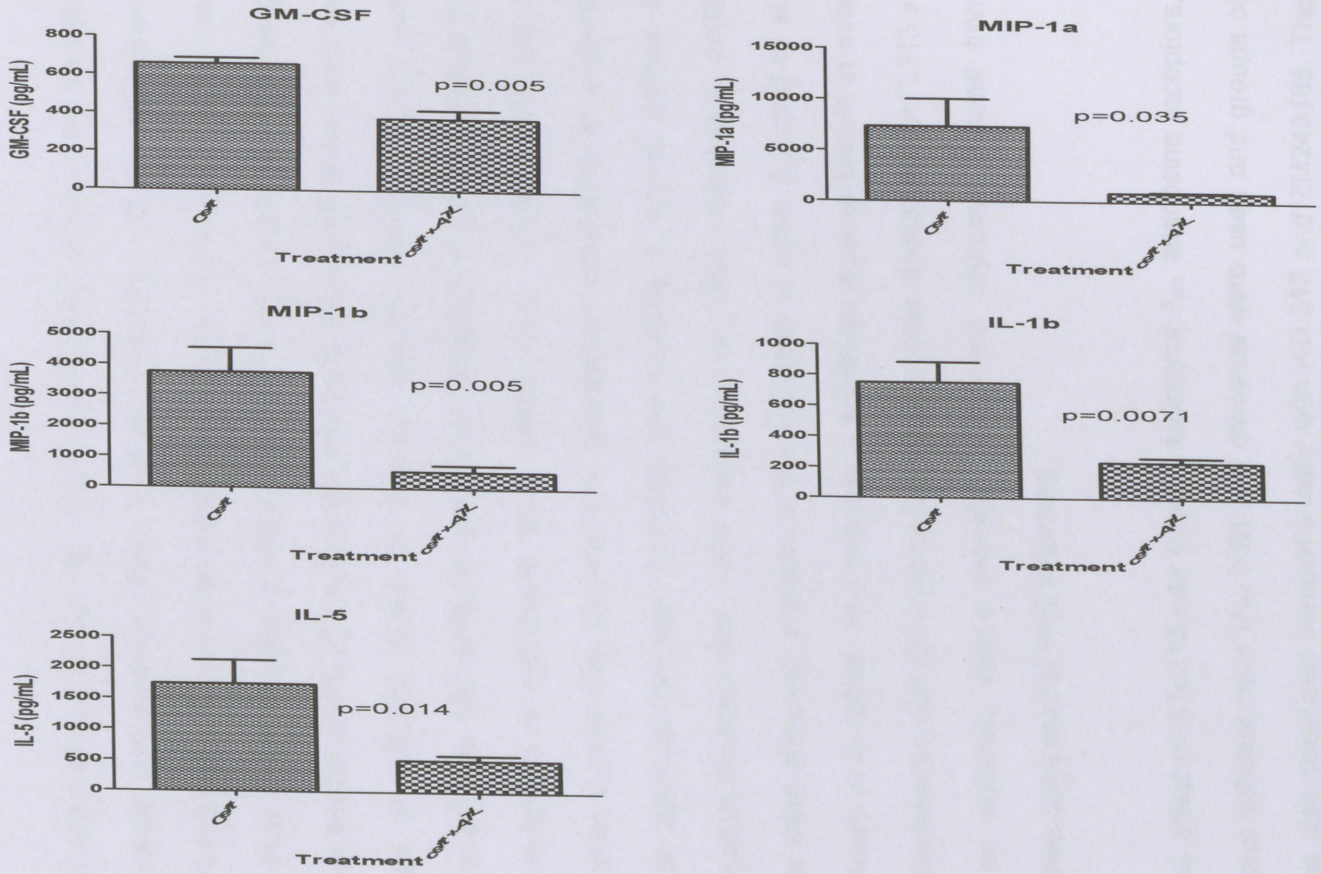


Figure 4.7. The cytokine profile of mice infected with *S. pneumoniae* and then treated with or without ATL313. The $A_{2A}AR$ agonist, ATL313 (25ug/kg) significantly decreases the concentrations of proinflammatory cytokines and chemokines in female C57BL/6 mice (N=5 per group) after inoculation with *S. pneumoniae*. Mean +/- standard error of the mean concentrations are shown in pg/ml.

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4.5. DISCUSION

We examined the effect of A_{2A} AR agonist, ATL313, to increase survival, and its effect on bacterial clearance and cytokine profile in mice challenged with *S. pneumoniae* serotype 2. We found that ATL313 as an adjunctive therapy improves survival reduces bacteremia and influences circulating cytokine concentrations in pneumococcal sepsis model. The survival benefit of ATL313 was dose- and -time-dependent as low dose and late administration of ATL313 failed to improve survival. When ATL313 was administered in combination with antibiotic ceftriaxone, 25ug/kg ATL313 increased the survival of mice compared to ceftriaxone alone treated mice. These results are in agreement with other studies that showed that endogenous adenosine or synthetic adenosine receptor agonists decrease morbidity and mortality in animal models of endotoxemia and sepsis (Parmely et al., 1993; Firestein et al., 1994; Adanin et al., 2002) and protection has been attributed primarily to the activation of either A_{2A} (Noji et al., 2002) or A_3 (5). Moore *el al* (2008) also reported a significant survival benefit in mice infected with live *Escherichia coli*, *Staphylococcus aureus* when treated with ATL313 in conjunction with an antibiotic versus antibiotic alone and improved survival from endotoxic shock when compared to PBS treatment.

To test whether the effect of ATL313 was through functional A_{2A} adenosine receptors, C57BL/6 A_{2A}-receptor-deficient mice (A_{2A} KO) and chimeras were used and groups of C57BL/6 mice were also coinjected intraperitoneally with ATL313 and ZM243185. The injection of a selective A_{2A} AR antagonist, ZM241385, did not improve survival or reduce mortality in the absence of A_{2A} AR agonist, but it greatly attenuated the protective effect of ATL313. The protective effects of ATL313 were greatly attenuated in mice that lack the A_{2A} AR gene (A_{2A} knockout mice and Chimera mice. The A_{2A} AR antagonist

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ZM241385 counteracts the effects of the agonists (CGS21680) and IB-MECA to inhibit LPS-stimulated release of TNF from human monocytes (Sullivan and Linden, 1998) and also reverses the effects of the adenosine uptake inhibitor 3-[1-(6,7-diethoxy-2morpholinoquinazolin-4-yl)piperidin-4-yl]-1,6-dimethyl-2,4(1H,3H)-quinazoline-dione hydrochloride (KF24345) in vivo (Noji et al., 2002). The results of ZM241385 and A_{2A} KO mice study implicate agonist protection via A_{2A}, and not A_3, AR-mediated mechanisms.

We also studied the effect of ATL 313 on the clearance of bacteria to correlate with the survival of ATL 313 treated mice. Our results showed that mice treated with A_{2A} AR agonist ATL313 alone had high bacteremia compared to control untreated mice 12h after infection. In this experiment, treatment with ATL313 alone did not modify bacterial clearance in blood, but it accelerated bacterial dissemination to bloodstream early after infection. This suggests that administration of ATL313 without antibiotics may be detrimental to early host defense against *S. pneumoniae*. These results are in agreement with findings by Hardant et al., indicating that at high concentrations of adenosine receptor agonist ATL313; A_{2A} AR agonists inhibit neutrophil bactericidal function (Hardart et al., 1991) and allows bacteria to survive. In contrast, administration of ATL313 with ceftriaxone facilitated bacterial clearance from blood more effectively than the use of ceftriaxone alone. We observed a decrease in the number of bacteria in blood with treatment with ceftriaxone and ATL313.

These findings are consistent with the results of a model of sepsis (*E. coli*) in mice in which they observed a decrease in the number of *E. coli* colony-forming units in blood with the treatment with A_{2A} AR agonist ATL146e and ceftriaxone (Sullivan et al. 2004) and they observed an increase in the number of neutrophils in the site of initial infection. Although A_{2A} AR agonists inhibit neutrophil recruitment into LPS-induced inflamed

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tissues (Sullivan et al., 1999), the protective effect and reduced bacterial load in the ceftriaxone plus ATL313 treated mice might result as an increase in neutrophil in the circulation or may arise from the actions of agonists to promote neutrophil chemotaxis and enhanced phagocytosis. Enhanced neutrophil migration to the initial infection has the potential to prevent the spread of infection to the blood and other organs.

A_{2A} AR agonists have effects on multiple cell types that are involved in inflammation and coagulation. On platelets, agonist binding to A_{2A} AR s inhibits aggregation (Hourani, 1996) and in Neutrophils, adenosine inhibits adhesion and oxidative activity (Fredholm et al., 1996), and on monocytes and macrophages, it decreases the release of proinflammatory cytokines (Bouma et al., 1994; Eigler et al., 1997; Sullivan et al., 1998). We have shown that ATL313 as an adjunctive therapy improves survival and also reduces bacterial load which correlated with survival of mice. To understand the underlying protective effect of A_{2A} AR agonist ATL313, we examined the impact of A_{2A} adenosine receptor agonist ATL313 on the cytokine profile of mice infected with *S. pneumoniae* and treated with ceftriaxone with or without ATL313.

Cytokines can cause inflammation via upregulation of other pro-inflammatory cytokines in the case of TNF, MIP-1a, 1L-17, modulation of T-cells by IFN-y, and activation of monocytes by MCP-1 (Sica et al., 1990; Fahey et al., 1992; Jovanovic et al., 1998; Mountford et al., 1999). Treatment of pneumococcal infections with the beta-lactam antibiotic ceftriaxone led to a rapid increase in tumor necrosis factor alpha (TNF-a), IL-6, IFN-γ and interleukin -1 beta (IL-1 beta), and other proinflammatory and anti-inflammatory cytokines/chemokines which were profoundly decreased by the co-administration of ATL313. This is likely due to the release of bacterial components that activated toll-like receptor pathway which results in NFkB and release of more

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proinflammatory cytokines. A_{2A} AR agonist activates A_{2A} AR on immune cells (Neutrophil, NK cells, etc.) and inhibits production of proinflammatory cytokines by increasing production of cyclic AMP. By decreasing proinflammatory cytokine such as TNF-α expression, decrease recruitment of neutrophil, NK cells, macrophages which produces deleterious reactive oxygen species and therefore inflammatory damage to host tissues and organs.

These data are in accord with the results of studies in which adenosine agonist was shown to suppress or down regulate the expression of proinflammatory cytokine with a concurrent survival benefit in sepsis/ endotoxin model (Moore et al., 2008, Eigler et al., 2000). Hasko et al. (2000) also showed that adenosine inhibits IL-12 and TNF-alpha production via adenosine A_{2A} receptor dependent and independent mechanisms. Eigler et al. (2000) reported that adenosine exerts potent anti-inflammatory activities through inhibition of cytokine synthesis by activated monocytes and an adenosine kinase inhibitor suppress TNF-alpha production in human mononuclear cells (Eigler et al., 1997; Eigler et al., 2000). An important feature of the regulation of immune processes by adenosine is that adenosine alters immune cell production of soluble mediators, such as cytokines (Salmon et al., 1993) and such an interference with cell-to-cell communication within the immune system can modify the outcome of the immune response. These proinflammatory mechanisms result in recruitment and activation of neutrophils, NK cells, and macrophages which produce deleterious reactive oxygen species and lysosomal enzymes (Wolpe et al., 1988; Huffnagle et al., 1995; Fossiez et al., 1998; Wang et al., 1999). A_{2A} AR ATL313 agonist down-regulate the expression of cytokines with a concurrent survival benefits. These effects may be due to a direct effect of decreased activation of cytokine-producing cells i.e. activation status of PMNs and Natural killer (NK) cells.

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A_{2A} Adenosine Receptor (AR) agonists interrupt the pro-inflammatory responses of immune cells and protect against morbidity and mortality associated with sepsis. There is a deleterious alteration in the regulatory balance of NK cell production of pro-(e.g. IFN-, TNF-, perforin) vs. anti-inflammatory (IL-10) mediators during the course of the sepsis which leads to increased NK cell activation, cytotoxicity, and ultimately, host mortality. Although, IFN-, IL-12 and IL-10 are important mediators of NK cell function in general, it is not known how the influence of each cytokine impacts NK cell function during sepsis. The relative concentration of each cytokine in the serum changes depending on the stage of sepsis with TNF- and IFN- noted early on and IL-10 predominant in the latter stages of sepsis. This variability in cytokine concentration during the course of sepsis is likely to have an impact on NK function.

Our results show that A_{2A}AR agonists decrease circulating proinflammatory cytokines, (e.g. TNF-α, IFN-γ, etc.) and anti-inflammatory (IL-10, IL-2, IL-5) cytokine after sepsis induction in mice that go on to survive the septic challenge. A_{2A}AR agonists may tilt the NK cell's balance towards increased anti-inflammatory cytokine release during the early predominantly proinflammatory phase of sepsis. This early inflammatory effect would then dampen the positive feedback related to IL-12 signaling by dendritic cells and subsequent NK cell activation and detrimental IFN- release. This intervention would then prevent the late counterproductive release of IL-10 which is associated with poor outcomes. We propose or speculate that A_{2A}AR agonists curtail the initial proinflammatory response of NK cells during sepsis thus preventing a transition to the late immunosuppressive stage of sepsis.

We believe that our study is the first to demonstrate the successful use of A_{2A} receptor agonist ATL313 against severe systemic pneumococcal infection. In our study, ATL313

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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. We show that the protective mechanism(s) of adenosine and A_{2A} AR agonists in this model arise from A_{2A} AR activation and may be particularly effective because they produce anti-inflammatory actions that reduce proinflammatory cytokines and promote bacterial clearance.

The model described here indicates that the protective effects are pronounced when ATL313 is given early after bacterial infection and therefore may be more relevant to treatment of early infection. Thus, the results of these preliminary animal studies and other studies point to the promising use of A_{2A} receptor agonists as therapeutic agents to treat infection-associated inflammation. We speculate that ATL 313 improved survival of mice by interrupting with the pro-inflammatory response of immune cells and also decreasing NK cell activation during pneumococcal sepsis. It is unlikely that the survival and decrease in cytokines completely explains the protective efficacy of A_{2A} AR agonists in experimental murine pneumococcal sepsis. It will be important to delineate which cells are the effectors of this anti-inflammatory effect of A_{2A} AR agonist ATL313. More investigations are warranted to further assess the therapeutic benefit of A_{2A} receptor agonists as an adjunctive agent to antibiotics in the treatment of bacterial infections and also further investigate the mechanism of protection involved in the ATL 313 protection.

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4.6 REFERENCES

Adanin, S., Yalovetskiy, I.V., Nardulli, B.A., Sam, A.D II, Jonjev, Z.S., Law, W.R. 2002. Inhibiting adenosine deaminase modulates the systemic inflammatory response syndrome in endotoxemia and sepsis. *Am J Physiol Regul Integr Comp Physiol* 282:R1324–32

Bouma, M.G, Stad, R.K., Van den Wildenberg, F.A.J.M., Buurman, W.A. 1994. Differential regulatory effects of adenosine on cytokine release by activated human monocytes. *J Immunol* 153:4159–68.

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann,Y., Weiss, D.S., Weinrauch, Y., Zychlinsky, A. 2004. Neutrophil extracellular traps to kill bacteria. *Science* 303:1532-1535.

Cronstein, B. 1995. The antirheumatic agents sulphasalazine and methotrexate share an anti-inflammatory mechanism. *Br J Rheumatol* 34:30-32.

Cronstein, B., Naime, D., and Ostad, E. 1993. The antiinflammatory mechanism of methotrexate: increased adenosine release at inflamed sites diminishes leukocyte accumulation in an in vivo model of inflamation. *J Clin Invest* 92: 2675-2682.

Cundell D., Masure H.R., Tuomanen E.I. 1995. The molecular basis of pneumococcal infection: a hypothesis. *Clin Infect Dis 21 (suppl 3)*: S204-11.

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Creating Future Leaders

Eigler, A., Greten, T.F., Sinha, B., Haslberger, C., Sullivan, G.W., Endres, S. 1997. Endogenous adenosine curtails lipopolysaccharide-stimulated tumour necrosis factor synthesis. *Scand J Immunol* 45:132–9.

Eigler, A., Matschke, V., Hartmann, G., Erhardt, S., Boyle, D., Firestein, G.S., and Endres S. 2000. Suppression of TNF-alpha production in human mononuclear cells by an adenosine kinase inhibitor. *J Leuckoc Biol* 68:97-103.

Fahey, T.J. 3rd, Tracey, K.J., Tekamp-Olson, P., Cousens, L.S., Jones, W.G., Shires, G.T., Cerami, A., Sherry, B. 1992. Macrophage inflammatory protein 1 modulates macrophage function. *J Immunol* 148: 2764-9.

Firestein, G.S., Boyle, D., Bullough, D.A. 1994. Protective effect of an adenosine kinase inhibitor in septic shock. *J Immunol* 152:5853–9.

Fossiez F, Banchereau J, Murray R, van Kooten C, Garrone P, Lebecque S. 1998. Interleukin-17. *Int Rev Immunol* 16:541-51.

Fredholm, B.B., Zhang, Y., van der Ploeg, I. 1996. Adenosine A2A receptors mediate the inhibitory effect of adenosine on formyl-Met-Leu-Phe–stimulated respiratory burst in neutrophil leucocytes. *Naunyn Schmiedebergs Arch Pharmacol* 354:262–7.

Gadangi, P., Longaker, M., Naime, D. 1996. The antiinflammatory mechanism of sulfasalazine is related to adenosine at inflamed sites. *J Immunol* 5: 1937-1941.

© University of Venda

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Creating Future Leaders

Hardart, G.E., Sullivan, G.W., Carper, H.T., Mandell, G.L. 1991. Adenosine and 2-phenylaminoadenosine (CV-1808) inhibit human neutrophil bactericidal function. *Infect Immun* 59:885–9.

Hasko, G., Kuhel., D.G., Chen, J., Schwarzchild, M.A., Deitch, E.A., Mabley, J.G., Marton, A., and Szabo, C. 2000. Adenosine inhibits IL-12 and TNF-alpha production via adenosine A2A receptor-dependent and independent mechanisms. *FASEB J* 14:2065-2074.

Hasko, G., Nemeth, Z.H., Vizi, E.S., Salzman, A.L., and Szabo, C. 1998. An agonist of adenosine A3 receptors decreases interleukin-12 and interferon gamma production and prevents lethality in endotoxemic mice. *Eur. J. Pharmacol.* 358:261-268.

Hourani, S.M. 1996. Purinoceptors and platelet aggregation. *J Auton Pharmacol* 16:349–52.

Huffnagle GB, Strieter RM, Standiford TJ, McDonald RA, Burdick MD, Kunkel SL, Toews GB. 1995. The role of monocyte chemotactic protein- 1 (MCP-1) in the recruitment of monocytes and CD4+ T cells during a pulmonary *Cryptococcus neoformans* infection. *J Immunol*, 155:4790-7.

Jovanovic DV, Di Battista JA, Martel-Pelletier J, Jolicoeur FC, He Y, Zhang M, Mineau F, Pelletier JP. 1998. IL-17 stimulates the production and expression of proinflammatory cytokines, IL-beta and TNF-alpha, by human macrophages. *J Immunol* 160:3513-21.

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Creating Future Leaders

Linden, J. 1991. Structure and function of A1 adenosine receptors. *FASEB* 5:2668-2676.

Linden, J. 1994. Cloned adenosine A3 receptors: pharmacological properties, species differences and receptor functions. *Trends Pharmacol* Sci 15:298-306.

Mabley, J., Soriano, F., Pacher, P., Hasko, G., Marton, A., Wallace, R., Salzman, A., Szabo, C. 2003. The adenosine A3 receptor agonist, N6-(3-iodobenzyl)-adenosine-5'-N-methyluronamide, is protective in two murine models of colitis. *J Immunol* 154:4793-802.

Marabito, L., Montesinos, M.C., Schreibman, D.M. 1998. Methotrexate and sulfasalazine promote adenosine release by a mechanism that requires ecto-5'-nucleotidase-mediated conversion of adenine nucleotides. J Clin Invest 2: 295-300.

Moore, C.C., Martin, E.N., Lee, G.H., Obrig, T., Linden, J., and Scheld, W.M. 2008. An A_{2A} adenosine receptor agonist, ATL313, reduces inflammation and improves survival in murine sepsis models. *BMC Infec Dis* 8:141.

Mountford AP, Coulson PS, Cheever AW, Sher A, Wilson RA, Wynn TA. 1999. Interleukin-12 can directly induce T-helper 1 responses in interferon-gamma (IFN-gamma) receptor-deficient mice, but requires IFN-gamma signalling to downregulate Thelper 2 responses. *Immunology* 97:588-94.

Nau, R., Eiffert, H. 2002. Modulation of release of proinflammatory bacterial compounds by antibacterials: potential impact on course of inflammation and outcome in sepsis and meningitis. *Clin Microbiol Rev* 15:95-110.

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Creating Future Leaders

Noji, T., Takayama, M., Mizutani, M. 2002. KF24345, an adenosine uptake inhibitor, suppresses lipopolysaccharide-induced tumor necrosis factor–a production and leukopenia via endogenous adenosine in mice. *J Pharmacol Exp Ther* 300:200–5.

Ohta, A., Sitkovsky, M. 2001. Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. *Nature* 414:916-920.

Ongini, E., and Fredholm, B.B. 1996. Pharmacology of adenosine A2A receptors. *Trends Pharmacol Sci.* 17:364-372.

Orman, K.L., and English, B.K. 2000. Effects of antibiotic class on the macrophage inflammatory response to *Streptococcus pneumoniae*. *J Infect Dis* 182:1561-5.

Parmely, M.J., Zhou W-W, Edwards C.K., Borcherding, D.R., Silverstein R, Morrison, D.C. 1993. Adenosine and a related carbocyclic nucleoside analogue selectively inhibit tumor nrcrosis factor-alpha production and protect mice against endotoxin challenge. *J Immunol* 151:389-396.

Parmely, M.J., Zhou W-W, Edwards, C.K III, Borcherding, D.R., Silverstein, R., Morrison D.C. 1993. Adenosine and a related carbocyclic nucleoside analogue selectively inhibit tumor necrosis factor–a production and protect mice against endotoxin challenge. *J Immunol* 151:389–96.

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Resta, R., Hooker, S.W., Laurent, A.B., Jamshedur Rahman, S.M., Franklin, M., Knudsen, T.B., Nadon, N.L., and Thompson, L.F. 1997. Insights into thymic purine metabolism and adenosine deaminase deficiency revealed by transgenic mice overexpressing ecto-5'-nucleotidase (CD73). *J Clin Invest* 99: 676-683.

Salmon, J.E., Brogle, N., Brownlie, C., Edberg, J.C., Kimberly, R.P., Chen, B.N., and Erlanger, B.F. (1993). Human mononuclear phagocytes express adenosine A1 receptors. *J Immunol* 151: 2775-2785.

Sica A, Wang JM, Colotta F, Dejana E, Mantovani A, Oppenheim JJ, Larsen CG, Zachariae CO, Matsushima K. 1990. Monocyte chemotactic and activating factor gene expression induced in endothelial cells by IL-1 and tumor necrosis factor. *J Immunol* 144:3034-8.

Sullivan, G.W., Fang, G., Linden, J., Scheld, W.M. 2004. A2A Adenosine receptor activation improves survival in mouse of endotoxemia and sepsis. *J Infect Dis*189: 1897-1904.

Sullivan, G.W., Linden, J. 1998. The role of A_{2A} adenosine receptors in inflammation. *Drug Dev Res* 45:103–12.

Sullivan, G.W., Linden, J., Buster, B.L., Scheld, W.M. 1999. Neutrophil A_{2A} adenosine receptor inhibits inflammation in a rat model of meningitis: synergy with the type IV phosphodiesterase inhibitor, rolipram. *J Infect Dis* 180:1550–60.

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Creating Future Leaders

Sullivan, G.W., Linden, J., Hewlett, E.L, Carper, H.T., Hylton, J.B., Mandell, G.L. 1990. Adenosine and related compounds counteract tumor necrosis factor–a inhibition of neutrophil migration: implication of a novel cyclic AMP independent action on the cell surface. *J Immunol* 145:1537–44.

Sullivan, G.W., Rieger, J.M., Scheld, W.M., Macdonald, T.L., Linden, J. 2001. Cyclic AMP-dependent inhibition of human neutrophil oxidative activity by substituted 2-proprynylcyclohexyl adenosine A_{2A} receptor agonists. *Br J Pharmacol* 132: 1017-1026.

Szabo, C., Scott, G.S., Virag, L., et al. 1998. Suppresion of macrophage inflammatory protein (MIP) 1-alpha production and collagen-induced arthritis by adenosine receptor agonists. Br J Pharmacol 125: 379-387.

Tarkowski, A., Wagner, H. 1998. Arthritis and sepsis caused by Staphylococcus aureus: can the tissue injury be reduced by modulating the host's immune system? *Mol Med Today* 4:15-18.

Van Belle, H., Goossens, F. and Wynants, J. 1987 Formation and release of purine catabolites during hypoperfusion, anoxia, and ischemia. Am. J. Pathol. 252: H886-H893.

Van de Beek, D., de Gans, J., McIntyre, P., Prasad, K. 2003. Corticosteroids in acute bacterial meningitis. In the Cochrane Library, Issue 3.

Wang, J., Wakeham, J., Harkness, R., Xing, Z. 1999. Macrophages are a significant source of type 1 cytokines during mycobacterial infection. *J Clin Invest* 103:1023-9.

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Winn, H.R., Rubio, R and Berne, R.M. 1981. Brain adenosine concentration during hypoxia in rat. AM. *J. Physiol.* 241: H235-H242

Wolpe SD, Davatelis G, Sherry B, Beutler B, Hesse DG, Nguyen HT, Moldawer LL, Nathan CF, Lowry SF, Cerami A. 1988. Macrophages secrete a novel heparin-binding protein with inflammatory and neutrophil chemokinetic properties. *J Exp Med* 167:570-81.

Xaus, J., Mirabet, M., Lloberas, J., Soler, C., Lluis, C., Franco, R., and Celada, A. 1999. IFN-gamma upregulates the A2B adenosine receptor expression in macrophages: a mechanism of macrophage deactivation. *J Immunol* 162: 3607-3614.

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CHAPTER FIVE

Adenosine A_{2A} receptor activation reduces pneumococcal inflammation by inhibiting NK cell activation in a murine pneumococcal sepsis model

Abstract

Background: 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 also 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 (Heremans et al., 1990). 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. In this study we sought to better characterize the effects of A_{2A} R activation on the NK cell activity in pneumococcal sepsis model.

Materials and methods: Mice were inoculated intravenously (i.v) with a lethal dose (10^7 cfu) of *S. pneumoniae* serotype 2. Mice were separated into groups (n= 4-5 per group) receiving 1) PBS, 2) ceftriaxone alone, and 3) a combination of ceftriaxone and 25ug/kg ATL313 given 1hour, 6 hours thereafter for 2 days after infection. Ceftriaxone was administered as a single dose of 25mg/kg intraperitoneally (i.p) 6 hours after infection. To assess the effects of depleting NK or NKT cells or blocking their activation in pneumococcal infection, 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.

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Survival was monitored for seven days. Spleens were harvested and processed to assess NK cell activation (expression of markers CD69, NK1.1, perforin and intracellular interferon gamma) using flow cytometry.

Results: The mean percentage of splenic NK cells assessed by percentage of CD3-NK1.1+ was reduced in the experimental control mice when compared with mean percentage of untreated mice (0.967 ± 0.1250 vs 3.142 ± 0.3171; p=0.0096) and ATL 313 treatment restored but not statistically significantly, the splenic NK population (2.000 ± 0.522 vs 0.967 ± 0.1250; p=0.226). Treatment with the combination of ATL and ceftriaxone (n=4) also showed a higher 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%)($p<0.05$), reduced the level of intracellular and plasma IFN-γ (0.55% compared to 12.1%) ($p<0.05$). Blockade of NK cell activation with the treatment with a combination of PK136 and antibiotics resulted in 80% survival compared with 40% of mice that received ceftriaxone alone ($p<0.05$) in pneumococcal animal sepsis model.

Conclusion: A_{2A} AR agonist (ATL313) decrease NK cell activation, reduce production of proinflammatory cytokine IFN- γ and release of perforin and improve survival in a murine pneumococcal sepsis model. NK cells contribute to the lethality of a murine model of *Streptococcus pneumoniae* infection. A further study to improve understanding on the negative impact of ATL313 on NK cell function during sepsis is needed.

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5.1. INTRODUCTION

The fatality rate associated with *Streptococcus pneumoniae* pneumonia still approximates 20-30% despite the use of potent antibiotics (Pallares et al., 1995). Death can occur days after initiation of antibiotic therapy, when tissues are sterile and pneumonia is clearing. There is growing evidence that aspects of the immune response greatly contribute to the high mortality rate. Immunosuppressed patients die as a consequence of poor host response while immunocompetent hosts face may overwhelm inflammatory reactions that contribute to tissue injury, shock, and death (Johnston, 1991). The majority of patients recover uneventfully; however, despite modern antimicrobial chemotherapy, the fatality rate of severe infection remains high. The morbidity and mortality are related to sepsis, septic complications and septic shock (Georges et al., 1999).

Sepsis syndrome, or sepsis, is an adverse systemic response to infection that includes fever, rapid heartbeat and respiration, low blood pressure and organ dysfunction associated with compromised circulation (Annane et al., 2005). Sepsis is also called Systemic inflammatory response syndrome (SIRS). Sepsis is a severe illness caused by overwhelming infection of the bloodstream by toxin-producing bacteria. The bacterial infection can originate anywhere in the body. In hospital patients, bacterial infection may originate from intravenous lines, surgical wounds, surgical drains, and sites of skin ulcers or bedsores. Sepsis can also be triggered by events such as pneumonia, trauma, surgery and burns, or by conditions such as cancer or AIDS. Sepsis occurs through infection with Gram-positive bacteria and even fungi and viruses, or as a consequence of secreted toxins. However, the sepsis syndrome occurs commonly in response to lipopolysaccaharide (LPS) from Gram-negative bacteria (Annane et al., 2005).

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The annual incidence of sepsis is 750,000 cases in the United States and is increasing by 9% each year (Annane et al., 2005). Sepsis is a major burden on the US healthcare system resulting in annual costs of $16.7 billion (Angus et al., 2001). In Germany, an annual sepsis case load of 76 to 110 per 100,000 inhabitants has been estimated and held responsible for approximately 60,000 deaths per year (Engel et al., 2007). Despite these startling figures from high-income countries, the largest part of the global burden of sepsis still occurs unrecognized by the Western medical community. Given that approximately 80% of the world's population lives in low- or middle-income countries, it can be assumed that most sepsis cases occur outside the more economically developed world. While few reports on the outcome of sepsis in these countries exist, low hygienic standards, widespread malnutrition and a high incidence of bacterial, parasitic and HIV infection suggest a disproportionally high morbidity and mortality from sepsis in low- and middle-income countries (Cheng et al., 2008). Indeed, the latest global burden of disease report of the World Health Organization found that three infectious diseases (lower respiratory tract infection, diarrhoeal diseases, HIV/AIDS) range among the four most frequent causes of death in low-income countries (World Health Organization, 2004).

During recent years, sepsis care in high-income countries has substantially improved due to extensive research efforts allowing novel insights into the pathophysiology and treatment of sepsis (Martin et al., 2003). Current scientific evidence to improve the care of severe sepsis or septic shock patients is summarized in the Surviving Sepsis Campaign (SSC) guidelines (Dellinger et al., 2004, Dellinger et al., 2008) which are considered the gold standard of care in many countries.

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As sepsis escalates to severe sepsis and septic shock, defined by end organ damage and refractory hypotension, respectively, mortality can be as high as 40-70%. Despite many clinical studies of adjunctive therapies, activated protein C is the only therapeutic option available to clinicians (Regnault and Levy, 2007). Unfortunately, activated protein C is controversial due to questions of efficacy, increased risk of bleeding, and high costs that accompany its use (Regnault and Levy, 2007). The pathophysiology of sepsis is complex and poorly understood. It involves bacterial components as well as host cellular factors, including various cytokines (Annane et al., 2005). Although the immune system is essential for combating invading pathogens, there are examples of diseases caused or worsened by inappropriate activation of that system resulting in inflammation, debilitating tissue damage through cell death, and sometimes death, as in anaphylactic or septic shock.

Phagocytes mediate a protective innate host response during pneumococcal infection. Resident macrophages within the alveoli and interstitium are ideally situated to act as primary defenses against pneumococcal pneumonia (Jonsson et al., 1985). The recruitment of significant numbers of neutrophils to the site of infection within hours provides the second line of defense (Vial et al., 1984). The immune response against pneumococci is also orchestrated by cytokines and chemokines such as tumor necrosis factor (TNF-α) and interferon gamma (IFN-γ). These mediators recruit and activate inflammatory cells to the site of infection. The depletion or genetic ablation of specific cytokines (Knock-outs mice) has revealed roles for many of these mediators in pneumococcal pneumonia (van der Poll et al., 1997). Several pneumococcal components induce cytokine production in vitro or in vivo. Stimulation of macrophages in vitro with pneumococcal lipoteichoic acid releases TNFα (Keller et al., 1992), monocytes stimulated in vitro with individual pneumococcal cell wall constituents release IL-1 and

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IL-6, whilst the pneumococcal toxin, pneumolysin, induces TNFα and IL-1 (Heumann et al., 1994).

Nasopharyngeal colonization with *S. pneumoniae* is present in 25% of the population, yet only a small proportion of these develop pneumonia, of which a minority develop sepsis. The explanation for the relative rarity of pneumonia and sepsis is likely to be influenced by the host's innate immune response to *S. pneumoniae* (Brown et al., 2002). The fact that mortality rates from sepsis remain high (35-45%), highlights the need to understand the host response during Gram-positive sepsis in order to identify novel methods of control (Bone, 1992).

NK cells are part of the innate immune system and thus do not require any specific sensitization for the expression of their function (Lodoen and Lanier, 2006; Orange and Ballas, 2006). NK cells are considered an important first line of defense against microbial infections because their effector functions are not antigen specific and are activated immediately following contact with infected target cells. These effector functions of cytolysis and cytokine secretions are augmented by IL-15 and IL-12 cytokines produced by activated manocytes as part of the early response to infection (Carson et al., 1995). NK cells are lymphocytes that do not belong to the T- or B-cell lineage; they are often referred to as large granular lymphocytes, although not all NK cells are large. NK cells are developmentally related to T cells, but are clearly distinct in that they lack membrane expression of T-cell receptor/CD3 complex and do not rearrange any of the T-cell receptor genes. NK cells express in common with T cells the ζ chain, which associates with CD16 (FcγRIIIA) and an array of other NK cell surface molecules.

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NK cells are a subpopulation of bone marrow-derived large, granular lymphocytes that lack T cell- and B cell-specific subset markers (TCR-, CD4-, CD8-, CD3γδε-, and Ig-), but express some specific markers , such as NK1.1 and ASGM1 (Lanier et al., 1992). NK cells have been shown to play a critical role in innate immunity against a variety of viruses, bacteria, fungi, and parasites (Trinchieri, 1989). The protective role of NK cells has been attributed to their ability to secrete immunoregulatory cytokines, such as IFN-γ (Dunn and North, 1991), lyses host cells infected with the intracellular pathogens, and directly inhibit growth of microorganisms (Levitz and Dupont, 1993; Murphy et al., 1993). Their function is a tightly regulated process, involving activating (CD16, CD2, CD28 and NKR-P1) and inhibitory (Ly-49, KIR) membrane receptors, the latter recognizing polymorphic MHC class I molecules. NK cells are unique in their constitutive expression of a number of receptors for cytokines that are produced by activated macrophages, such as IL-12, IL-15, and IL-18 and they stimulate NK cell proliferation and activation (Tomura et al., 1998). IFN-γ is the prototypic macrophage-activating factor and is able to enhance a variety of macrophage effector functions. Thus, it appears that NK cells can be recruited by activated macrophages to secrete cytokines that are essential for macrophage function. This feedback loop has been shown to be important in the clearance of certain intracellular pathogens; however it is not known whether NK cells participate in the desregulated macrophage response to overwhelming bacterial infections, particularly those due to extracellular pathogens (Tripp et al., 1993).

NK cells have been demonstrated to be involved in the first line of defense against viruses, bacteria, parasites. Previous studies have demonstrated that depletion of NK cells using anti-asialo GM1 antiserum significantly reduces early IFN-γ production in resistant C3H/HeN mice and renders them susceptible to cutaneous L. *major* infection, suggesting that NK cells are involved in host defense against this parasite (Scharton and

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Scott, 1993). Furthermore, a study indicated that NK cells are involved in protection and healing of cutaneous Leishmaniasis in humans (Maasho et al., 1998).

The importance of NK cells in early antibacterial immunity has been demonstrated by a study showing that SCID mice that lack αβ and γδ T cells but have NK cells develop activated macrophages and partially control *Listeria monocytogenes* (Newborg and North, 1980). Later it was demonstrated that spleen cells from naïve SCID (Severe combined immunodefiency syndrome) and nude mice produced significant levels of IFN-γ following incubation with heat-killed *L. monocytogenes* (Bancroft et al., 1987). Furthermore, administration of neutralizing anti-IFN-γ mAb or NK cell depletion before infection abolished macrophages activation in SCID mice. Together, these data indicate that NK cell-derived IFN-γ is involved in vivo macrophage activation following *L. monocytogenes (*Bancroft et al., 1989*)*. Similarly, many studies have demonstared that NK cells also play a critical role in immunity against viruses such as murine CMV, Coxsackievirus B4, and infleuenza virus (Vella and Festenstein, 1992; Karupiah et al., 1992; Orange et al., 1995). This has been attributed to their cytolytic capability and ability to produce type 1 IFNs that have antiviral activity.

Lymphocytes are a major immune cell besides macrophages in the development of chronic inflammatory diseases (Jonasson et al., 1986). Therefore, a direct interaction between lymphocytes and this pathogen, if it occurs, may contribute to the pathogenesis of chronic inflammatory diseases. The activation of T lymphocytes, both in vivo and in vitro, induces expression of CD69. This molecule, which appears to be the earliest inducible cell surface glycoprotein acquired during lymphoid activation, is involved in lymphocyte proliferation and functions as a signal-transmitting receptor in lymphocytes, including natural killer (NK) cells, and platelets (Cambiaggi et al., 1992; Ziegler et al.,

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1993). NK cells express CD69 after activation by different stimuli such as IL-12, Interferon-α or anti-CD16 monoclonal antibodies (mAbs). CD69 is a disulphide –linked homodimer with two chains contitutively phosphorylated belonging to the type II intergral protein with an extracellular C- type lectin superfamily domain (Ziegler et al., 1993). In humans, the CD69 gene is located in chromosome 12 at bands p13-p12 in aregion of known as natural killer complex, associated with other C-type lectin genes that control NK-cell activity, such as NKG2, CD94 genes, suggesting that CD69 may have a particular significant in NK- cell function (Lopez-Cabrera et al., 1993; Renedo et al., 1997). Once expressed on T cells, CD69 acts as a costimulatory molecule leading to cell proliferation, secretion and/or cytotoxicity. CD69 is rapidly induced in NK cells shortly after activation (Borrego et al., 1993).

Natural killer cells are widely believed to be important for the elimination of tumor cells and virally infected cells. Upon attacking to a target cell, the NK cell exocytoses the contents of its cytoplasmic granules in a vectorial fashion into the contact-bounded extracellular space between the NK and target cell membrane (Bakker et al., 2000). The cytoplasmic granules of NK cells contain several proteins implicated in cytolysis, including various serine esterases (granzymes) and perforin.

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. Once its activating receptor is bound by a ligand, recruitment and activation of tyrosine kinases and adapter proteins and activation of gene transcription and cytoskeletal reorganization occurs. Activation can also occur through stimulation by effector cytokines such as interleukin (IL)-12 and IL-18. Lipopolysaccharide (LPS) and other pathogen –associated molecular patterns (PAMPs) have also been shown to

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directly stimulate isolated NK cells in an IL-12 and IL-10 dependent manner (Tomura et al., 1998).

NK cells have several functions, most of which can be assigned to their ability to secrete cytokines or the ability to exert cytotoxicity (Yokoyama and Kim, 2006). NK cells are a major source of interferon gamma (IFN-γ). NK cells are also able to kill certain tumor target cells directly with no need for prior sensitization and it is this ability that earned NK cells their name (Lodoen and Lanier, 2006; Orange and Ballas, 2006). NKT cells are a newly defined lymphocytes subset that shares the characteristics of NK cells and T cells. NKT cells express certain markers unique to NK cells but also express some T-cell markers, including CD3 and a restricted pattern of TCR expression. NKT cells also are a major source of cytokines, including IFN-γ, and IL-4. NKT cells, however, are quite heterogeneous. Some NKT cells subsets are thymically derived whereas others arise from an extrathymic pathway (Kronenberg, 2005).

The activity of most inflammatory cells, including but not limited to macrophages, monocytes, T lymphocytes, platelets, and polymorphonuclear leukocytes, is inhibited by the activation of the inflammatory Gs-coupled $A_{2A}R$, resulting in reduced proinflammatory cytokine production and diminished endothelial adhesion molecule expression (Cronstein, 1994). Tissue hypoxia, as occurs in sepsis, enhances breakdown of adenosine triphosphate (ATP) to adenosine monophosphate (AMP), which is then dephosphorylated by the cytosolic 5'nucleotidase to adenosine (Thiel et al., 1997). Adenosine can bind to four G protein coupled receptors, A_1, A_{2A}, A_{2B}, and A_3. The $A_{2A}AR$ is present on inflammatory cells including, neutrophils, mast cells, monocytes, macrophages, eosinophils, platelets, and T-cells, and is involved in antinflammatory activities (Burnstock et al., 1998). Activation of $A_{2A}ARs$ results in an increase in cyclic

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AMP concentration in inflammatory cells which is increased further by concurrent type IV phosphodiesterase inhibitors. A_{2A}AR agonists decrease superoxide production in neutrophils, degranulation of neutrophils, TNF- production by monocytes and macrophages, and neutrophil –endothelial cell adherence (Sullivan et al., 2001). Death occurs in mice deficient for *Adora2a*, the A_{2A}AR gene, after exposure to Concavalin A due to liver injury within 8 hours compared to complete survival in wild-type mice. Pro-inflammatory cytokines are present in higher concentration in *Adora2a-/-* mice when compared to WT mice. Similar finding are observed in experimental animals exposed to LPS (Ohta et al., 2001).

It is well established that extracellular adenosine inhibits T lymphocyte activation and effector function, including T cell adhesion to tumor cells and cytotoxic activity, by signaling primarily through A_{2A} and A3 adenosine receptors on the surface of T cells. Importantly, A_{2A} adenosine receptor-deficient mice exhibit enhanced anti-tumor immune responses by CD8+ T cells, as well as a dramatic reduction in the growth of experimental tumors in comparison to wild-type controls. A_{2A} adenosine receptor signaling has also 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 (Lappas et al., 2005).

In the absence of or inhibition of adenosine deaminase, the substrate adenosine accumulates (Hirschhorn, 1985). Since these nucleosides are growth inhibitory or cytotoxic to numerous cells, it has been assumed that the accumulation might play a role in the severe combined immunodefiency associated with adenosine deaminase deficiency (Seegmiller, 1985). Treatment of mice with the adenosine analogue produced

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marked inhibition of NK cell activity while stimulating that of antibody production (Priebe et al., 1988; Priebe et al., 1990). NK cells appear to play an important role in the immunomodulation produced by these adenosine analogues. One mechanism by which adenosine-related compounds might regulate NK cell activity involves adenosine receptors. Although evidence suggests the presence of an adenosine A_{2A} receptor on murine and human T-and B- lymphocytes, T-helper and T-cytotoxic cells, basophils, mast cells, and neutrophils, the presence of this or other adenosine receptors on NK cells has been reported (Ullberg et al., 1983). It has been reported that various chemical manipulations that mimic cyclic AMP or lead to its accumulation are inhibitory to NK cell function (Ullberg et al., 1983). Thus, adenosine receptor A_{2A} agonists (stimulatory for adenyl cyclase) would be anticipated to inhibit NK cell function whereas adenosine receptor A1 agonists (inhibitory for cyclase) might stimulate this immune response and inhibition of NK cell activity is mediated via adenosine receptors.

The functional experiments demonstrated that the adenosine receptor agonists modify NK cell activity in the manner anticipated from known effects of cyclic AMP on this function (Priebe et al., 1990). 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 (Heremans et al., 1990). 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.

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5.2. The main objective of the study

In this study we sought to better characterize the effects of A_{2A} R activation on the NK cell activity in pneumococcal sepsis model.

5.2.1 The specific objectives of the study were:

5.2.1.1 To assess the effects of A_{2A} Receptor activation on NK cell activation by their prevalence and numbers and by their surface phenotype, such as expression of CD69, NK1.1, perforin and intracellular interferon gamma.

5.2.1.2 To examine the effects of depleting NK or NKT cells or blocking their activation in pneumococcal infection to determine the importance of NK cell in pneumococcal infection

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5.3. MATERIALS AND METHODS

5.3.1 Animals

Female C57BL/6 (Jackson laboratory Bar Harbor, Maine), Jα281$^{-/-}$ (gift from Dr Masaru Taniguchi, RIKEN Research Center for Allergy and Immunology, Department of Molecular Immunology, Ciba university, Chiba, Japan) mice, 8-10 weeks of age, weighing approximately 20 grams, were kept in a sterile environment prior to start of experiments. The mice were maintained on standard lab chow and water *ad libitum* with a 12-hr light/dark cycle. The protocol used in this study was approved by the Animal Care and Use Committee at the University of Virginia.

5.3.2 Reagents and drugs

The A2AAR agonist, ATL313 was supplied by adenosine Therapeutic, LCC (Charlottesville, VA). Anti-NK1.1, PK136 was a gift from M. Brown, University of Virginia, Charlottesville, VA). Ketamine and xylazine were purchased from Vedco, Inc., (St. Joseph, MO). In all cases vehicle used was phosphate buffered saline (PBS)

5.3.3 NK1.1 cell depletion

To study the role of NK cells during sepsis, mice were injected intraperitoneally with 200 µg of anti-N.K 1.1 mAb (PK136) 48h before and immediately before sepsis induction. Successful depletion was confirmed by FACS analysis of splenocytes collected from the naïve mice at the time of infection. Anti-NK1.1 (PK136) was purified from hybridomas in the University of Virginia hybridoma core.

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5.3.4 Infection

Mice were inoculated intravenously (i.v) with a lethal dose (10^7 cfu) of *S. pneumoniae* serotype 2. Mice were separated into groups receiving ceftriaxone, a combination of ceftriaxone and 25ug/kg ATL313 given 1hour after infection, and PBS injected into the control mice. Ceftriaxone was administered as a single dose of 25mg/kg intraperitoneally (i.p) 6 hours after infection. ATL313 was given every 6hours thereafter for 2 days. Animals were anesthetized and then killed by cervical dislocation at various time points (6 hours and 12 hours after bacterial challenge) and spleens were removed or harvested.

5.3.5 Flow cytometry of cell surface T cell markers: NK cell activation

Experimental and control animals were sacrificed 24 hours after bacterial challenge. The mice were anesthetized with ketamine and xylazine and killed by cervical dislocation. Spleens were harvested from untreated and experimental treated mice, passed through a 40-mm nylon cell strainer (BD Biosciences) and collected in phosphate-buffered saline. Red blood cells were lysed. Cells were washed and resuspended at $5x10^6$ cells/ml in PBS supplemented with 5% FBS and 0.1 sodium cyanide (NaN_3). 0.1-ml aliquots were placed on ice and labeled for 30 min in the dark with anti-mouse CD3, anti-mouse NK1.1, anti-CD69-FITC, anti-perforin, anti-IFN-γ, anti-mouse DX5 (eBioscience). Control samples were labeled with isotype-matched control antibodies. Stained cells were washed with 1ml iced PBS and resuspended in PBS containing 1% paraformaldehyde. The fluorescence intensity was measured with a dual laser benchtop flow cytometer (FACSCalibur; Becton Dickinson) with a minimum of 10, 000 events

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being collected. An excitation wavelength of 488nm and an emission wavelength of 530 nm were used for FITC-stained cells; an excitation wavelength of 488nm and an emission wavelength of 585 nm were used for PE-stained cells; an excitation wavelength of 635 nm and an emission wavelength of 661 nm were used for APCs; and an excitation wavelength of 488 nm and an emission wavelength of 670 nm were used for PE-Cys5.5-stained cells. Analyses were performed with FlowJo software (Tree Star, Inc).

5.3.6 Detection of intracellular IFN-γ

The analysis for IFN-γ production by NK cells was done by intracellular cytokine staining as per the method described previously (Joyee et al., 2007). Intracellular IFN-γ was detected in spleen NK cells by FACS analysis using FIX & PERM cell permeabilization reagents (Caltag Laboratories) according to the manufacturer's protocol.

5.3.7 Detection of Serum interferon gamma (IFN-γ) concentration

Blood samples from untreated, infected control, Ceftriaxone treated, Ceftriaxone plus ATL treated C57BL/6 mice were collected at different time points after bacterial infection. Individual sera were separated from blood by centrifugation and stored at -70^{0}C until cytokine assays were performed. Serum interferon gamma (IFN-γ) cytokine level was determined by using protein-based multiplex immunoassay system (Bio-Rad Laboratories, Hercules, CA).

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5.3.8 Statistical analysis

Survival data were plotted and the survival curves compared with a log-rank test (GraphPad PISM software, San Diego, CA). Prism software (GraphPad) was also used for all statistical analyses. Unpaired t test or one-way analysis of variance (ANOVA) with post-hoc Dunnett's multiple comparisons was used to compare experimental groups with controls.

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5.4 RESULTS

5.4.1 NK cells become activated in vivo by bacterial challenge

We evaluated cellular inflammation by quantifying the total population of spelnic natural killer cells and measuring their activation. Activation of NK cells was assessed by their prevalence and numbers and by their surface phenotype, such as expression of CD69, an early activation marker, CD3-NK1.1^{+}, perforin and intracellular interferon gamma. Analysis was performed 12 hours after bacterial challenge.

A decrease in NK cell populations assessed by CD3- NK1.1^{+} cells was observed in the spleens of mice infected with *S. pneumoniae* bacteria and treated with ceftriaxone compared to the NK cell numbers in the spleens of mice treated with ceftriaxone plus ATL313 (Fig.5. 1). The mean percentage of splenic NK cells assessed by percentage of CD3-NK1.1^{+} was reduced in the experimental control mice (n=4) when compared with mean percentage of untreated mice (n=5) (0.967 ± 0.1250 vs 3.142 ± 0.3171; p=0.0096). ATL 313 treatment restored the splenic NK population and showed a higher percentage of splenic NK cells compared to the reduced percentage of NK cells in the experimental control mice even though the difference observed did not reach statistical significance (2.000 ± 0.522 vs 0.967 ± 0.1250; p=0.226). Mice challenged with *S. pneumoniae* and then treated with antibiotics (n=4) showed low level of NK cell population (1.648 ± 0.566) and treatment with the combination of ATL and ceftriaxone (n=4) showed a higher level of NK cell populations (2.063 ± 0.277) compared to ceftriaxone alone treated group (p=0.535; ATL vs ceftriaxone alone treated group). Bacterial challenge and ceftriaxone treatment resulted in a substantial loss of NK cells (sustained downregulation of NK1.1) and ATL313 restored the splenic NK population and also decreased their activation in septic mice receiving ceftriaxone.

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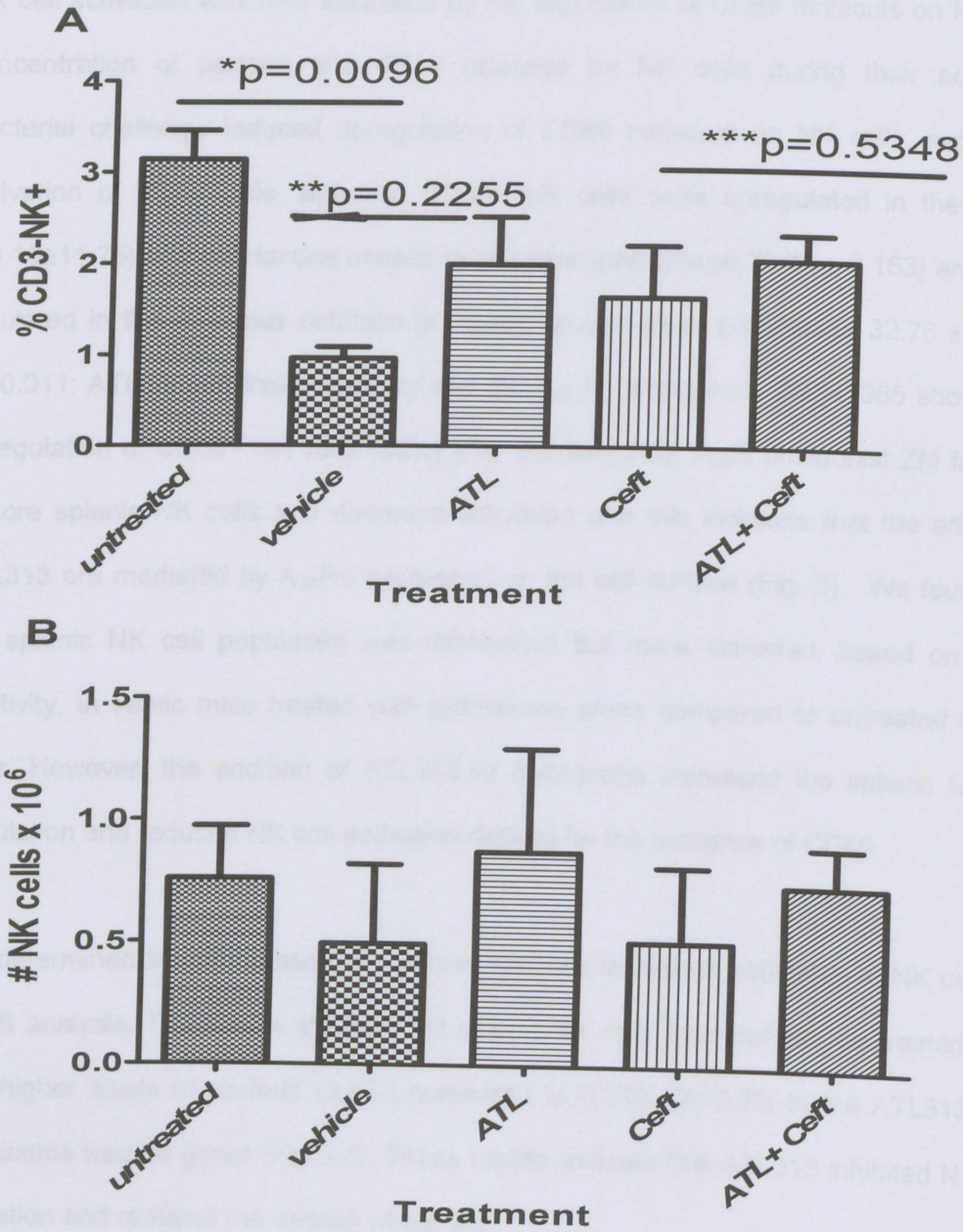


Figure 5.1: Mean percentage of splenic CD3-NK+ NK (A) and (B) absolute number of NK cells after experimental pneumococcal sepsis. Mice were challenged with *S. pneumoniae* and then treated with ceftriaxone and ATL313 (n=4-5 per group). * indicates untreated vs Vehicle, ** indicates vehicle vs ATL treated group; *** indicates ceft vs ATL313 treated group.

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NK cell activation was also assessed by the expression of CD69 molecule on NK cells, concentration of perforin and IFN-γ released by NK cells during their activation. Bacterial challenge induced upregulation of CD69 molecule on NK cells, suggesting activation of these cells. Spleenic CD69+ NK cells were upregulated in the control (48.15±11.75) and ceftriaxone treated mice (mean percentage 70.03 ± 8.163) and down regulated in the ATL plus ceftriaxone treated group (mean percentage 32.78 ± 6.327) ($p=0.011$; ATL vs ceft treated group) and the A_{2A} R antagonist ZM241385 showed an upregulation of CD69+ NK cells (80%) (Fig. 5.2 and 5.3). A_{2A}R antagonist ZM failed to restore splenic NK cells and decrease activation and this indicates that the effects of ATL313 are mediated by A_{2A}Rs expressed on the cell surface (Fig. 3). We found that the splenic NK cell population was diminished but more activated, based on CD69 positivity, in septic mice treated with ceftriaxone alone compared to untreated control mice. However, the addition of ATL313 to ceftriaxone increased the splenic NK cell population and reduced NK cell activation defined by the presence of CD69.

We determined the percentage of perforin released to assess activation of NK cells by FACS analysis. The results showed that splenic NK cells from ceftriaxone treated mice had higher levels of perforin (3,4%) compared to 0.21% ($p<0.05$) in the ATL313 plus ceftriaxone treated group (Fig 5.4). These results indicate that ATL313 inhibited NK cell activation and reduced the release of perforin.

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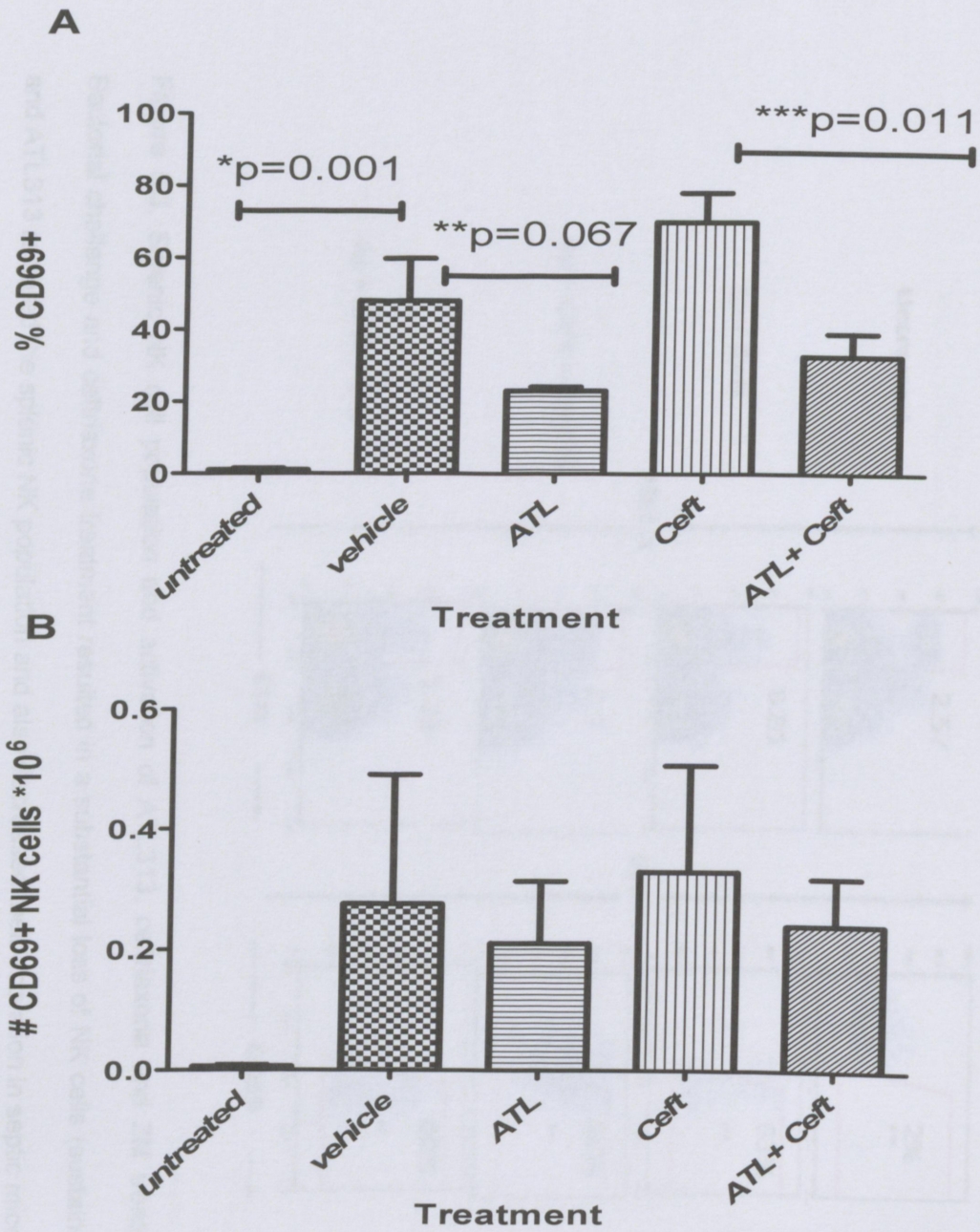


Figure 5.2: Mean percentage (A) and absolute number (B) of splenic CD69+ NK cells after experimental sepsis. Mice were challenged with *S. pneumoniae* and then treated with ceftriaxone and ATL313. * indicates untreated vs Vehicle, ** indicates vehicle vs ATL treated group; *** indicates ceft vs ATL313 treated group.

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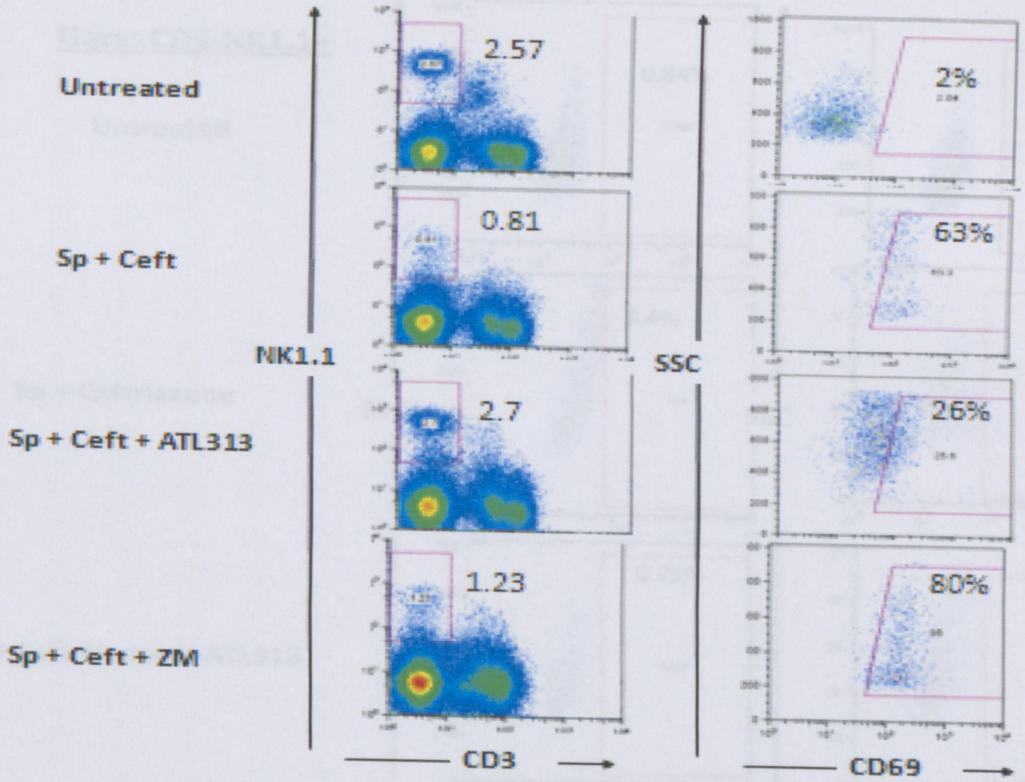


Figure 5.3: Splenic NK cell population and activation of ATL313, ceftriaxone and ZM treated after bacterial challenge. Bacterial challenge and ceftriaxone treatment resulted in a substantial loss of NK cells (sustained downregulation of NK1.1) and ATL313 restored the splenic NK population and also decreased their activation in septic mice receiving ceftriaxone.

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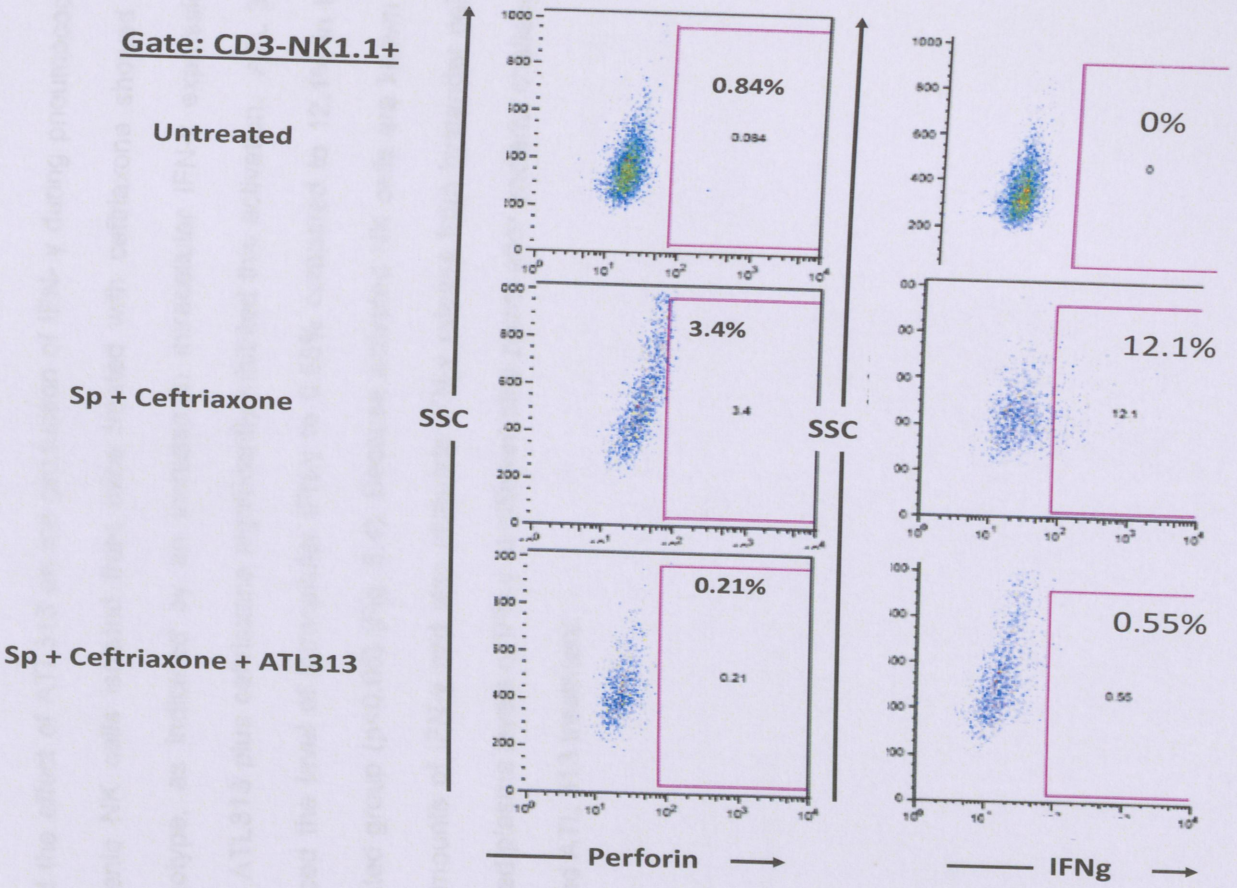


Figure 5.4: Percentage of concentration of perforin and intracellular IFN-γ from spleen cells isolated from mice treated with ATL3131 and ceftriaxone after experimental sepsis. Intracellular IFN-γ and perforin were detected in spleen NK cells by FACS analysis.

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5.4.2 A2AR agonist ATL313 treatment inhibits interferon gamma production and is dependent on NK cell activation

We determined the effect of ATL313 on the production of IFN- γ during pneumococcal infection. Splenic NK cells isolated from mice treated with ceftriaxone showed an activated phenotype, as indicated by an increase in intracellular IFN-γ expression. Treatment with ATL313 plus ceftriaxone significantly inhibited this activation. ATL 313 treatment reduced the level of intracellular IFN-γ to 0.55% compared to 12.1% in the ceftriaxone treated group ($p<0.05$) (Fig. 5.4). Because activated NK cells are known to release large amounts of IFN-γ and also stimulate IFN-γ release from bystander cells, we also examined plasma levels of IFN-γ at different time points after bacterial challenge and antibiotic and ATL 313 treatment.

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5.4.3 Blockade of NK cell activation improves or enhanced survival benefits

To test the hypothesis that NK cell activity contributes to pneumococcal infection, we examined the effects of depleting NK cells or blocking their activation. Treatment of C57BL/6 mice with anti-NK1.1 (PK136) 2 days before bacterial challenge substantially depletes NK cells and NKT cells (both express NK1.1 surface marker) in the spleen and liver as assessed by FACS analysis (data not shown). The splenic NK cell activation was determined using DX5 marker and the results showed that PK136 depleted or decreased NK cell activation by almost 70 % compared with the PBS treated mice (0.72 vs 3.94; $p<0.005$) (Fig. 5.5A) during pneumococcal sepsis. This depletion or NK cell inactivation provided survival benefit and results in 25% survival and also delayed death by 24 hours in the PK136 treated infected group compared with PBS treated infected group which died within 24 hours. Treatment with ceftriaxone resulted in 80% survival in PK136 plus ceftriaxone treated group compared with 40% of mice that received ceftriaxone alone ($p<0.05$) (Fig. 5.5B).

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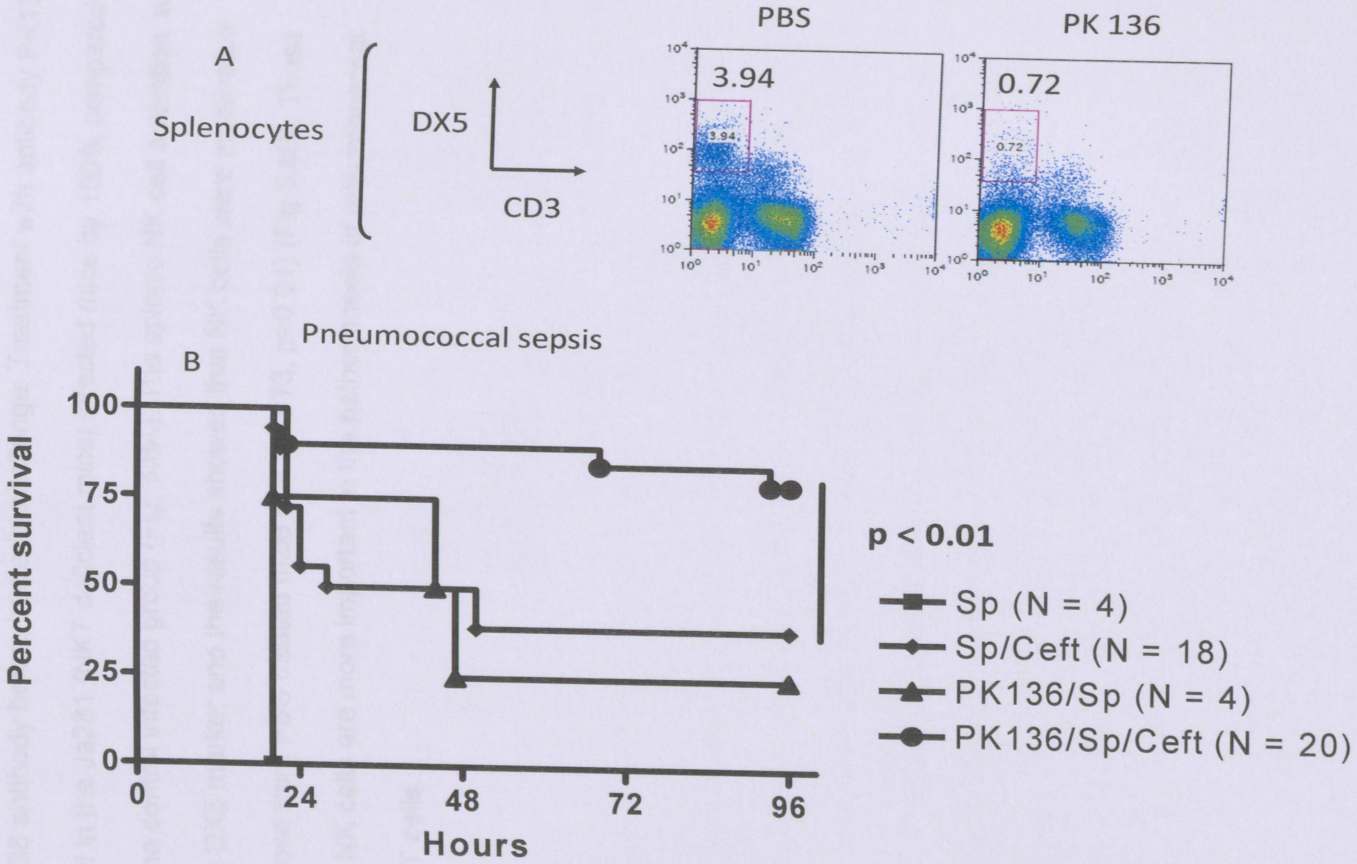


Figure 5.5: Mean percentage (A) of splenic NK activation assessed by DX5 marker after experimental sepsis by FACS analysis. (B) Survival curves of C57Bl/6 mice after bacterial challenge. Mice were injected with anti-NK1.1 (PK136) antibody 2 days before bacterial challenge. Mice were challenged with *S. pneumoniae* and then treated with ceftriaxone. (P<0.01; ceft vs PK136/ceft treated group.)

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To determine which cell type (whether NK or NKT cells) are important in the pathogenesis of pneumococcal infection, Ja281 mice (NKT cell deficient mice) were treated with PK136 antibody before bacterial challenge. Treatment with antibody PK136 improved survival in the Ja281 (NKT deficient mice) infected mice by 100% compared to 45% survival in the control infected group (Fig. 5.6A). The splenic NK cell activation was determined using DX5 marker and the results showed that NK cells were inactivated compared with those from PBS treated mice (1.4 vs 3.73, p=0.01) (Fig.5.6B). These results show that NK cells are more important in the pathogenesis of pneumococcal infection than NKT cells.

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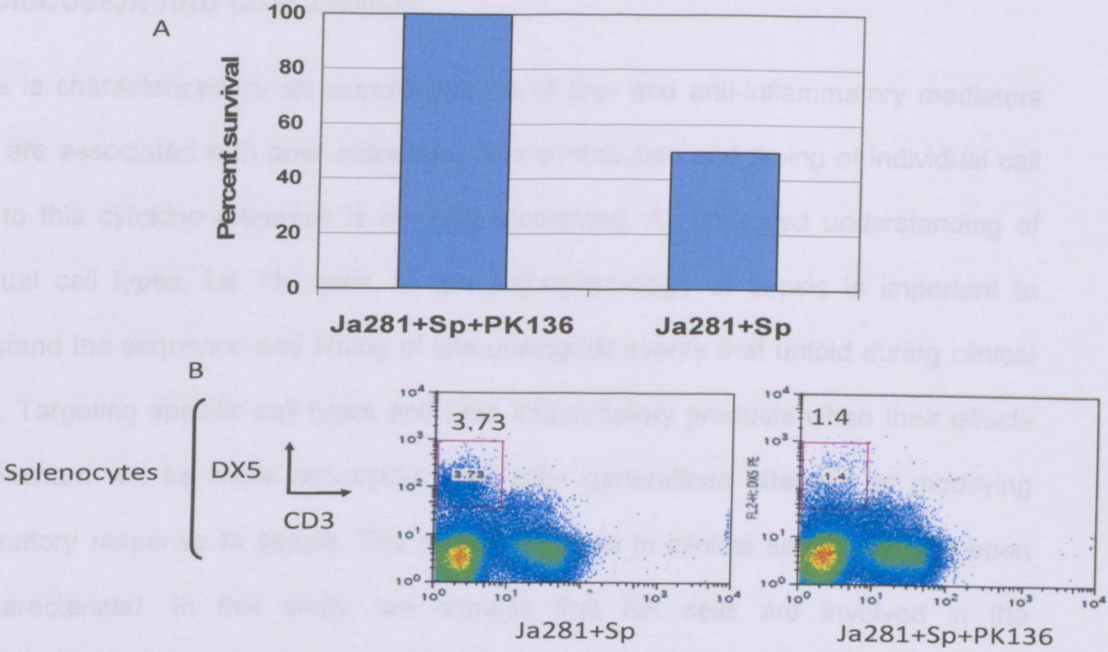


Figure 5.6: (**A**) Survival curves and splenic NK cell activation of Ja281 mice after bacterial challenge. (**B**) Mean percentage of splenic NK activation assessed by DX5 marker after experimental sepsis by FACS analysis. Mice were injected with anti-NK1.1 (PK136) antibody 2 days before bacterial challenge. Mice were challenged with *S. pneumoniae* and then treated with ceftriaxone. ($P<0.01$; ceft vs PK136/ceft treated group. N=5 per group)

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5. 5. DISCUSION AND CONCLUSION

Sepsis is characterized by an overabundance of pro- and anti-inflammatory mediators which are associated with poor outcomes. The contribution and timing of individual cell types to this cytokine response is not fully elucidated. An improved understanding of individual cell types, i.e. NK cells, to the pathophysiology of sepsis is important to understand the sequence and timing of immunological events that unfold during clinical sepsis. Targeting specific cell types and their inflammatory products when their effects are dominant will be more successful than prior generalized attempts at modifying inflammatory response to sepsis. The role of NK cells in clinical sepsis has not been well-characterized. In this study, we showed that NK cells are involved in the pathogenesis of pneumococcal sepsis model and adenosine A_{2A} AR activation reduced inflammation by inhibiting NK cell activation. Our data shows that depletion of NK cells improves survival from experimental sepsis and that an A_{2A}AR agonist (ATL313) maintains the splenic NK cell population, decrease NK cell activation, and improves survival in a murine pneumococcal sepsis model.

Decrease in NK cell populations were observed in the spleens of mice infected with *S. pneumoniae* bacteria and treated with ceftriaxone compared to the NK cell numbers in the spleens of mice treated with ceftriaxone plus ATL313. Bacterial challenge and ceftriaxone treatment resulted in a substantial loss of NK cells and ATL313 restored the splenic NK population and also decreased their activation in septic mice receiving ceftriaxone. Because there was no evidence of NK cell death during sepsis, our data suggest that there is active trafficking out of the spleen during sepsis. However, the extent of NK cell trafficking during sepsis is not known. We speculate that NK cells traffic

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to sites of organ damage such as the liver and their local lymph nodes (e.g. mesenteric lymph nodes). It is also important to note that activated NK cells will exhibit increased adherence to vascular endothelium (Allavena et al., 1991). This could be an important destination for NK cells under septic conditions. We expect that there will be increased circulating NK cells in proportion to increasing severity of sepsis. But, given the negative impact of NK cells during murine sepsis it is important to know not only how the NK cells are causing inflammation and damage but also where this effect of NK cells occurs. For example, are NK cells required at the site of end-organ damage or does circulation and release of effector mediators such as perforin and cytokines suffice?

A decrease in NK cell numbers in sepsis could indicate that this cellular population has been activated in the course of bacterial infection. Previous studies have shown that treatment of mice with the potent NK cell agonist results in the rapid activation and proliferation of these cells, followed by homeostatic contraction of the NK cell population and acquisition of an anergic phenotype. NK cell activation can have a number of detrimental effects in mice, including liver damage and exacerbated inflammatory response (Parekh et al., 2006).

Previous studies showed that treatment with agonists of $A_{2A}R$ or depletion of CD4+ lymphocytes effectively reduces inflammatory processes (Day et al., 2006), and the amount of tissue damage that occurs during reperfusion (Savransky et al., 2006). Of the total tissue necrosis that occurs in models of heart, kidney, skin, liver Ischemia reperfusion injury (IRI), 30-75% of the tissue injury occurs during reperfusion and can be prevented by treatment with $A_{2A}R$ agonists (Linden, 2005).

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Prior studies have shown that NK or NKT cells can be activated in response to various infectious agents, either through direct recognition of microbial glycolipid antigen or indirectly through cytokines secreted by DCs in conjuction with endogenous antigens expressed by activated DCs (Lin et al., 2005). Activation of NK cells was assessed by their prevalence in the spleen (NK1.1, which becomes down- regulated on activated NK cells and remains expressed at low levels on NK cells rendered anergic), and by their surface phenotype, such as expression of CD69 (an early activation marker), concentration of perforin and intracellular interferon gamma. In this study we showed that the activation of NK cells after pneumococcal bacterial challenge plays a central role in initiating the inflammatory cascade responsible for pneumococcal infection. Induced upregulation of CD69 by spleen NK cells was observed in ceftriaxone treated mice but less evident in the ATL313 plus ceftriaxone treated group. We determined the level of perforin released to indicate activation of NK cells. The results of this study showed that ATL313 inhibited NK cell activation and reduced the level or the release of perforin. Upon attaching to a target cell, the NK cell exocytoses the contents of its cytoplasmic granules in a vectorial fashion into the contact-bounded extracellular space between the NK and target cell membrane (Bakker et al., 2000). The cytoplasmic granules of NK cells contain several proteins implicated in cytolysis, including various serine esterases (granzymes) and perforin which are released during NK cell activation.

In order to determine whether NK cells were responsible for the elevated cytokine levels in mice and thus may play a detrimental role in the pathology of pneumococcal infection, the level of IFN-γ were determined. Splenic NK cells isolated from mice treated with ceftriaxone showed an activated phenotype, as indicated by an increase in intracellular IFN-γ expression. Treatment with ATL313 significantly inhibited this activation and ATL 313 treatment reduced the level of intracellular IFN-γ. Because activated NK cells are

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known to release large amounts of IFN-γ and also stimulate IFN-γ release from bystander cells, we also examined plasma levels of IFN-γ at different time points after bacterial challenge and antibiotic and ATL 313 treatment. These findings indicate that NK cells are activated rapidly after bacterial challenge and initiation of antibiotic treatment release large amounts of IFN-γ as a result of NK cell activation, and this is inhibited by $A_{2A}R$ activation by $A_{2A}R$ agonist ATL313. These findings demonstrate that the production of IFN-γ by NK cells in response to activation is inhibited by the activation of the $A_{2A}R$ and this activity is dependent on functional cell surface expression of the $A_{2A}R$. IFN- has been described as an important mediator of sepsis pathophysiology. Early studies showed a survival benefit and decreased hepatotoxicity in mice deficient of IFN- receptors challenged with LPS (Heremans et al., 1990). NK cells are known to produce IFN- when activated by IL-12 and intracellular IFN- is increased in NK cells after CLP (Heremans et al., 1994).

NK cells can exert an influence on macrophages during the course of certain intracellular infections, and we were interested to determine whether they played a role in the inflammatory response associated with Gram positive bacterial infection *(Streptococcus pneumoniae*). To test the hypothesis that NK cell activity contributes to pneumococcal infection, we examined the effects of depleting the cells or block their activation. It was observed that depletion of NK cells before the administration of a lethal dose of *Streptococcus pneumoniae* resulted in markedly improved survival. The results of this study were in agreement with the study conducted by Heremans et al. (1994). They have demonstrated that depletion of NK cells before the induction of the generalized Schwartzman reaction leads to a 70% reduction in mortality and significantly lower levels of IFN-γ and TNF-α after the systemic injection of LPS. Depletion of NK cells also improved survival after cecal-ligation and puncture (CLP) suggesting their importance in

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sepsis pathophysiology (Sherwood et al., 2004). Most of these CLP studies also documented a decrease in circulating proinflammatory cytokines and improvement in tissue histology scores after NK cell depletion. Similar results occurred in a combined experimental trauma/sepsis model. Although NK cells are increasingly recognized as important in the control of bacterial infections, they have also recently been implicated in the pathophysiology of sepsis. Depletion of NK cells from mice conferred protection after intraperitoneal (i.p) challenge with *Escherichia coli* (Badgwell et al., 2002). This decrease in inflammation after NK cell depletion was accompanied by improvement in survival, reductions in serum cytokines, less severe tissue damage, and decreased activation of STAT1 and NFκB. These results suggest that NK cells can promote the septic process in vivo, possibly via interactions with the macrophage compartment (Badgwell et al., 2002).

The C-type lectin receptor, NK1.1, is expressed on NK cells and NKT cells, and both can be depleted competently by anti-NK1.1 antibodies as assessed by FACS analysis of splenocytes and liver leukocytes. The protective effect of anti-NK1.1 antibodies PK136, therefore, indicates that NK cells or NKT cells are involved in the inflammatory cascade for pneumococcal infection.

Ja281 mice were treated with PK136 before bacterial challenge to determine whether NK or NK T cells or both are important in the pathogenesis of pneumococcal sepsis. The results with the Ja281 mice indicate that NK not NK T cells are important or involved in the pathology of pneumococcal infection. PK136 antibody depleted NK cells and provided increased survival benefit in pneumococcal infection as achieved in the C57bl/6 mice.

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The results of the study showed that the administration of anti-NK 1.1 PK136 to deplete NK cells provided similar survival benefits as does ATL313 treatment. Co-treatment with antibody plus ATL 313 afforded no additional protection beyond that achieved by NK.1.1 cell depletion (anti-NK1.1 PK136) or activation of A_{2A} receptor (ATL313) alone (data not shown). Treatment with ATL313 inhibits NK cell activation. These results show that depletion by administration of PK136 antibody before bacterial challenge or activation of $A_{2A}R$ cells provides survival benefits and NK cells are involved in the pathogenesis of pneumococcal infection.

The results of the study implicate NK cells as predominant mediators of inflammation that are 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 cytokines levels more substantially by targeting an upstream event in the cascade (NK cell activation). The activity of ATL313 to potertly inhibit the production of IFN-γ and to dramatically protect mice indicates that $A_{2A}R$-selective agonists may be a useful tool in the treatment of sepsis. It will be interesting to see if activation of $A_{2A}R$ proves to be a clinically viable treatment for sepsis.

Overall it appears that the increased inflammatory response to pneumococcal infection is due to elevated NK cell activity and the production of proinflammatory cytokines by these cells. Stimulation of these cells, at least in part, by pneumococci challenge induces an overwhelming inflammatory response that promotes bacteraemia and poor outcome. Given the importance of NK cells in the pathophysiology of sepsis, as evidenced by our preliminary data which details improved survival from sepsis after NK cell depletion, it is

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important to understand how $A_{2A}AR$ agonists impact NK cell function. It is also important to understand whether the decreased activation of NK cells by $A_{2A}AR$ agonists is directly linked to improved survival and decreased organ failure. We determined the impact of adenosine $A_{2A}AR$ on NK cell function during pneumococcal sepsis. Our data show that $A_{2A}AR$ agonists improve survival and decrease NK cell activation. However, whether the decrease in NK cell activation is directly related to improved survival or outcomes from experimental sepsis is not clear. It is possible that NK cells do not cause damage to the host directly through cytotoxicity but indirectly through permissive growth of bacteria, for example. Because NK cells are known to be prodigious producers of IL-10 it is possible that NK cells actively contribute to the immunoparalysis of late sepsis which increase susceptibility to ongoing, nosocomial, and reactivated infections.

In conclusion, we have demonstrated that A_{2A} AR agonist (ATL313) decrease NK cell activation, reduce production of proinflammatory cytokine IFN- γ and improve survival in a murine pneumococcal sepsis model. NK cells contribute to the lethality of a murine model of *Streptococcus pneumoniae* infection and depletion of NK cells before the administration of live bacteria led to improved survival from experimental sepsis. Reduced inflammation (reduced early proinflammatory cytokines) and NK cell inactivation correlated with the increase in the survival in this model. These results suggest that analysis of NK cell activation during the septic process may yield insights into the interactions that occur between these 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 in the improved outcomes from experimental sepsis. We need to study the impact of $A_{2A}AR$

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agonist on NK cell trafficking during sepsis. To study why, how and where 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 allow us to determine 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.

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5.6. REFERENCES

Allavena, P., Paganin, C., Martin-Padura, I., Peri, G., Gaboli, M., Dejana, E. 1991. Molecules and structures involved in the adhesion of natural killer cells to vascular endothelium. *J Exp Med* 173: 439-48.

Angus D.C, Linde-Zwirble W.T, Lidicker J., Clermont G., Carcillo J., Pinsky M.R. 2001. Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. *Crit Care Med* 29:1303-1310.

Annane D., Bellissant E., Cavaillon J.M. 2005. Septic shock. *Lancet* 365:63-78.

Badgwell, B., Parihar, R., Magro, C., Dierksheide, J., Russo, T., Carson, W.E. 2002. Natural killer cells contribute to the lethality of a murine model of Escherichia coli infection. *Surgery* 132 (2):205-12

Bakker, A.B.H., Wu, J., Phillips, J.H., and L.L. Lanier. 2000. NK Cell activation: Distinct stimulatory pathways counterbalancing inhibitory signals. *Human Immunol* 61:18-27

Bancroft, G.J., Schreiber, R.D., Bosma, G.C., Bosma, M.J., and Unanue, E.R. 1987. A T-cell independent mechanism of macrophage activation by interferon gamma. *J Immunol* 139:104

Bancroft, G.J., Sheehan, K.C.F., Schreiber, R.D., and Unanue, E.R.1989. Tumornecrosis factor is involved in the T cell independent pathway of macrophage activation in SCID mice. *J Immunol* 143:127

Bone, R.C. 1991. Pathophysiology of sepsis. *Ann Intern Med* 115:457-469

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Creating Future Leaders

Bone, R.C., Balk, R.A., Cerra, F.B., Dellinger, R.P., Fein, A.M., Knaus, W.A., Schein, R.M., Sibbald, W.J. 1992. Definations for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. *Chest* 101:1644-1655

Borrego, F., Pena, J., Solana, R. 1993. Regulation of CD69 expression on human natural killer cells: differential involvement of protein kinase C and protein tyrosine kinases. *Eur J Immunol* 23: 1039

Brown, J.S., Hussell, T., Gilliland, S.M., Holden, D.W., Paton, J.C., Ehrenstein, M.R., Walport, M.J., Batto, M. 2002. The classical pathway is the dominant complement pathway required for the innate immunity to *Streptococcus pneumoniae* infection in mice. *PNAS* 99(26): 16969-16974.

Burnstock, G., Liang, B.T., Linden, J. 1998. Cardiovascular Biology of purines. 1st edition. London:Kluwer Academic Publishers.

Cambiaggi C, Scupoli MT, Cestari T. 1992. "Constitutive expression of CD69 in interspecies T-cell hybrids and locus assignment to human chromosome 12.". *Immunogenetics* 36 (2): 117–20.

Carson, W.E., Yu, H., Dierksheide, J., Pfeffer, K., Bouchard, P., Clark, R. 1999. A fatal cytokine-induced systemic inflammatory response reveals a critical role for NK cells. *J Immunol* 162:4943-51

Cheng A.C., West T.E., Limmathurotsakul D., Peacock S.J. 2008. Strategies to reduce mortality from bacterial sepsis in adults in developing countries. *PLOS Medicine* 5:1173-1179.

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Creating Future Leaders

Dellinger PR, Carlet JM, Masur H, Gerlach H, Calandra T, Cohen J, Gea-Banacloche J, Keh D, Marshall JC, Parker MM, Ramsay G, Zimmerman JL, Vincent JL, Levy MM. 2004. Suriving Sepsis Campaign guidelines for management of severe sepsis and septic shock. *Crit Care Med* 32:858-873.

Dellinger, R.P., Levy, M.M., Carlet, J.M., Bion, J., Parker, M.M., Jaeschke, R., Reinhart, K., Angus, D.C., Brun-Buisson, C., Beale, R., Calandra, T., Dhainaut, J.F., Gerlach, H., Harvey, M., Marini, J.J., Marshall, J., Ranieri, M., Ramsay, G., Sevransky, J., Thompson, B.T., Townsend, S, Vender, J.S., Zimmerman, J.L., Vincent, J.L. 2008. Surviving Sepsis Campaign: International guidelines for management of severe sepsis and septic shock. *Crit Care Med*, 36:296-327.

Dunn, P.L., and North, R.J. 1991. Early IFN-γ production by natural killer cells is important in defense against muine Listeriosis. *Infect Immun* 59:1892

Engel, C., Brunkhorst, F.M., Bone, H.G., Brunkhorst, R., Gerlach, H., Grond, S., Gruendling, M., Huhle, G., Jaschinski, U., John, S., Mayer, K., Oppert, M., Olthoff, D., Quintel, M., Ragaller, M., Rossaint, R., Stuber, F., Weiler, N., Welte, T., Bogatsch, H., Hartog, C., Loeffler, M., Reinhart, K. 2007. Epidemiology of sepsis in Germany: results from a national prospective multicenter study. *Intensive Care Med* 33:606-618.

Georges, H., Leroy, O., Vandenbussche, C., Guery, B., Alfandari, S., Tronchon, L., Beaucaire, G. 1999. Epidemiological features and prognosis of severe community-acquired pneumococcal pneumonia. *Intensive Care Med* 25:198–206.

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Creating Future Leaders

Heremans, H., Dillen, C., van Damme, J., Billiau, A. 1994. Essential role for natural killer cells in lipopolysaccharide-induced Shwartzman-like shock reactions in mice. *Eur J Immunol* 24:1155-60.

Heremans, H., van Damme, J., Dillen, C., Dijkmans, R., Billiau, A. 1990. Interferon gamma, a mediator of lethal lipopolysaccharide-induced Shwartzman-like shock reactions in mice. *J Exp Med* 171:1853-69.

Heumann, D., Barras, C., Sverine, A., Glauser, M.P., Tomasz, A. 1994. Gram positive cell walls stimulate synthesis of tumor necrosis factor alpha and interleukin-6 by human monocytes. Infect *Immun* 62:2715-2721.

Hirschhorn, R. 1985. Complete and partial adenosine deaminase deficiency. Relationship of the immune function to metabolite concentrations, enzyme activity, and effects of therapy. *Ann NY Acad Sci* 451: 20-25.

Houldsworth, S., Andrew, P.W., Mitchell, T.J. 1994. Pneumolysin stimulates production of tumor necrosis factor alpha and interleukin-1 beta by human mononuclear phagocytes. *Infect Immun* 62:1501-1503.

Johnston, R.B.J. 1991. Pathogenesis of pneumococcal pneumonia. *Rev Infect Dis* 13: S509-S517.

Jonasson, L., J. Holm, O. Skalli, G. Bondjers and G.K. Hansson. 1986. Regional accumulations of T cells, macrophages, and smooth muscle cells in the human atherosclerotic plaque. *Arteriosclerosis* 6: 131–138.

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Creating Future Leaders

Jonsson, S., Musher, D.M., Chapman, A., Goree, A., Lawrence, E.C. 1985. Phagocytosis and killing of common bacterial pathogens of the lung by human alveolar macrophages. *J. Infect Dis* 152:4-13.

Karupiah, G., Ramsay, A.J., Ramshaw, I.A., and Bladen, R.V. 1992. Recombinant vaccine vector induced protection of athymic, nude mice from influenza A virus infection: analysis of protective mechanisms. *Scand J Immunol* 36:99.

Keller, R., Fischer, W., Keist, R., Bassett, S. 1992. Macrophage response to bacteria: induction of marked secretory and cellular activities by lipoteichoic acids. *Infect Immun* 60:3664-3672.

Lanier, I., Spits, H., and Phillips, J.H.1992. The developmental relationship between NK cells and T cells. *Immunol Today* 13:392

Levitz, S.M., and Dupont, M.P. 1993. Phenotypic and functional characterization of human lymphocytes activated by interleukin-2 to directly inhibit growth of Cryptococcus neoformans in vitro. *J Clin Invest* 91:1490

Lopez-Cabrera, M., Santis, A.G., Fernandez-Ruiz, E. 1993. Molecular cloning, expression, and chromosomal localization of the human earliest lymphocyte activation antigen AIM/CD69, a new member of the C-type animal lectin superfamily of signal-transmitting receptors. *J.Exp Med* 178: 537

Maasho, K., Sanchez, F., Schurr, E., Hailu, A., and Akuffo. 1998. Indications of the protective role natural killer cells in human cutaneous leishmaniasis in an area of endemicity. *Infect Immun* 66:2698

© University of Venda

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Creating Future Leaders

Marrie TJ. 2000. Community-acquired pneumonia in the elderly. *Clin Infect Dis* 4:1066–1078.

Martin GS, Mannino DM, Eaton S, Moss M. 2003. The epidemiology of sepsis in the United States from 1979 through 2000. *N Engl J Med* 348:1546-1554.

Menges, T., Engel, J., Welters, I., Wagner, R.M., Little, S., Ruwoldt, R. 1999. Changes in blood lymphocyte populations after multiple trauma: association with posttraumatic complications. *Crit Care Med* 27:733-40

Murphy, J.W., Hidore, M.R., and Wong, S.C., 1993. Direct interactions of human lymphocytes with the yeast-like organism, *Cryptococcus neoformans*. *J Clin Invest* 91:1553

Newborg, M.S., and North R.J. 1980. On the mechanism of T cell independent anti-Listeria resistance in nude mice. *J Immunol* 124:571

Ohta, A., Sitkovsky, M. 2001. Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. *Nature* 414:916-920.

Orange, J.S.,Wang, B., Terhorst, C., and Biron, C.A. 1995. Requirement for natural killer cell-produced interferon gamma in defense against murine cytomegalovirus infection and enhancement of this defense by interleukin 12 administration. *J Exp Med* 182:1045

Pallares, R., Linares, J., Vadillo, M., Cabellos, C., Manresa, F., Viladrich, P.F.,Martin, R., and F. Gudiol. 1995. Resistance to penicillin and cephalosporin and mortality from severe pneumococcal pneumoniae in Barcelona, Spain. *N Engl J Med* 333:474-486

© University of Venda

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Creating Future Leaders

Priebe, T., Kandil, O., Nakic, M., Pan, B.F., and Nelson, J.A. 1988. Selective modulation of antibody response and natural cell activity by purine nucleoside analogues. *Cancer Res* 48:4799-4803.

Priebe, T., Platsoucas, C.D., and Nelson, J.A. 1990. Adenosine receptors and modulation of Natural killer cell activity by purine nucleosides. *Cancer Res* 50:4328-4331.

Regnault, V., and Levy, B. 2007. Recombinant activated protein C in Sepsis: endothelium protection or endothelium therapy. *Crit care* 11:103

Renedo, M., Arce, I., Rodriguez, A. 1997. The human natural killer gene complex is located on chromosome 12p12-p13. *Immunogenetics* 46:307

Scharton, T.M., and Scott, P. 1993. Natural killer cells are a source of interferon gamma that drives differentiation of CD4+ T cell subsets and induces early resistance to Leishmania major in mice. *J Exp Med* 178: 567

Seegmiller, J.E. 1985. Overview of possible relation of defects in purine metabolism to immune deficiency. *Ann NY Acad Sci* 451: 9-19.

Sherwood, E.R., Enoh, V.T., Murphey, E.D., and Lin, C.Y. 2004. Mice depleted of CD8+ T and Natural killer cells are resistant to injury caused by cecal ligation and puncture. *Laboratory investigation* 84:1655-1665

© University of Venda

University of Venda
Creating Future Leaders

Sullivan, G.W., Rieger, J.M., Scheld, W.M., Macdonald, T.L., Linden, J. 2001. Cyclic AMP-dependent inhibition of human neutrophil oxidative activity by substituted 2-propynylcyclohexyl adenosine A_{2A} receptor agonists. *Br J Pharmacol* 132:1017-26.

Sullivan,G.W., I.J. Sarembock and J. Linden. 2000. The role of inflammation in vascular diseases. *J Leukoc Biol* 67: 591–602.

Thiel, M., Caldwell, C.C., Sitkovsky, M.V. 2003. The critical role of adenosine A2Areceptors in downregulation of inflammation and immunity in the pathogenesis of infectious diseases. Microbes Infect 5: 515-526.

Tomura, M., Zhou, X.Y., Maruo, S., Ahn, H.J., Hamaoka, T., Okamura, H., Nakanishi, K., Tanimoto, T., Kurimoto, M., Fujiwara, H. 1998. A critical role for IL-18 in the proliferation and activation of NK1.1+CD3- cells. *J Immunol* 160:4738-4746.

Trinchieri, G.1989. Biology of natural killer cells. *Adv Immunol* 47:187

Tripp, C.S., Wolf, S.F., Unanue, E.R. 1993. Interleukin 12 and tumor necrosis factor alpha are costimulators of interferon gamma production by natural killer cells in severe combined immunodefiency mice with Listeriosis, and interleukin 10 is a physiologic antagonist. *Proc Natl Acad Sci* USA 90:3725-9.

Ullberg, M., Jondal, M., Lanefelt, F., and Fredholm, B.B. 1983. Inhibition of human NK cell cytotoxicity by induction of cyclic AMP depends on impaired target cell recognition. *Scand J Immunol* 17:365-373.

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Creating Future Leaders

Van der Poll, T., Keogh, C.V., Buurman, W.A., Lowry, S.F. 1997. Passive immunization aginst tumor necrosis factor-α impairs host defense during pneumococcal pneumonia in mice. *Am J Respir Crit Care Med* 155:603-608.

Vella, C., and Festein, H. 1992. Cocksakievirus B4 infection of the mouse pancreas: the role of natural killer cells in control of virus replication and resistance to infection. *J Gen Virol* 73:1379.

Vial, W.C., Toews, G.B., Pierce, A.K. 1984. Early pulmonary granulocyte recruitment in response to streptococcus pneumoniae. *Am Rev Respir Dis* 129:87-91.

World Health Organization. 2004. Global Burden of Disease Report - Update

Yoshimoto, T.A., Bendelac, A., Watson, C., Hu, L.J., Paul, W.E. 1995. Role of NK1.1^{+} T cells in a Th2 response and immunoglobulin E production. *Science* 270:1845

Ziegler, S.F., Ramsdell, F., Hjerrid, K.A. 1993. Molecular characterization of the early activation antigen CD69: a type II membrane glycoprotein related to a family of natural killer cell activation antigens. *Eur J Immunol* 23:1643

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CHAPTER SIX

Etanercept as Adjunctive Therapy Provides Survival Enhancement in a clinically relevant Murine Model of lethal Systemic *Streptococcus pneumoniae*

Abstract

Background: Growing antibiotic resistance and paradoxical enhancement of lethality as a result of the release of proinflammatory cell components prompted our study of Etanercept, a tumor necrosis factor alpha (TNF-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.

Methods: Five (5) groups of Balb/c mice were inoculated intravenously with a lethal dose (10^7 cfu) of *S. pneumoniae* serotype 2. (1) Sham (n=20) received PBS. (2) One group (n=20) received 25 mg/kg Ceftriaxone alone 5 hours after challenge. (3) One group (n=5) received 100ug of Etanercept (i.v) alone at the time of challenge. (4) One group (n=6) received a combination of Ceftriaxone (i.p) t=5h and 100ug of Etanercept (i.v) at the time of challenge. (5) One group (n=16) received a combination of Ceftriaxone (i.p) t=5h and 100ug of Etanercept (i.v) at 4 hours after challenge. Survival was monitored for several days. Serum samples from group 2 and 3 mice at 24 hours after challenge were analyzed for inflammatory cytokines with bead-based multi-analyte flow cytometry.

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Results: Sham and Etanercept alone treated mice died within 48 hours. 75% of ceftriaxone alone treated mice died within 72 hours and 50% of ceftriaxone plus early (t=0) Etanercept mice died within 24 hours. 70 % of mice treated with a combination of ceftriaxone and late (t=4h) Etanercept survived. 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 –related or treated mice compared with ceftriaxone treated mice ($p<0.05$).

Conclusion: The use of Etanercept 4 hours after *S. pneumoniae* challenge followed by ceftriaxone provided a survival benefit in our model. 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 a survival benefit. The reduction in inflammatory cytokines correlates positively with survival. Timely neutralization of TNF-alpha deserves close scrutiny as a survival aid in systemic *S. pneumoniae* infection.

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6.1 INTRODUCTION

The fatality rate associated with *Streptococcus pneumoniae* pneumonia still approximates more than 23%, despite the use of potent antibiotics and aggressive intensive care support (Pallares et al., 1995). Therapeutic challenges include the development of more effective vaccines that protects against colonization by pneumococci, the development of antibiotics that bypass the widespread emergence of multiresistant strains, the strengthening of the immune response in immunosuppressed patients, and the control of overwhelming inflammatory reactions that are associated with tissue injury, shock, and death in immunocompetent hosts. In fact, there is growing evidence that aspects of the immune response greatly contribute to the high mortality rate associated with this threatening infection (Bergeron et al., 1998).

During infection, inflammation is essential for host defense, but it can injure tissues and organ function. Pneumococcal infection is characterized by an intense inflammatory response that is coordinated mainly by cytokines. Many different cytokine responses are generated during bacterial sepsis, either as the direct result of activation of macrophages, neutrophils, and endothelial cells or by other inflammatory mediators. Some cytokines, such as TNF-alpha, IL-1, IL- 6, IL-8, and interferon (IFN) gamma, are proinflammatory; others, such as IL-4, IL-10, 1L-13 and transforming growth factor (TGF) beta, have counter-regulatory activities.

Tumor necrosis factor alpha (TNF-alpha) is a 17-kDa cytokine that has been shown to mediate many of the detrimental pathophysiologic events that occur in bacterial sepsis and sepsis syndrome but however, mounting evidence suggests that TNF is a critical component of effective antibacterial host defense (Gosselin et al., 1995). It is also a

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potent activator of both PMN and macrophages, leading to enhancement of protease release, stimulation of the respiratory burst, and induction of leukocyte and vascular adhesion molecule expression, which are essential for transmission of these cells into the sites of infection (Gosselin et al., 1995).

Tumor necrosis factor (TNF) plays an important role in innate immunity and is a pluripotent proinflammatory cytokine that exerts powerful effects on the immune system, including the release of other proinflammatory cytokines, activation of neutrophils, and the induction of adhesion molecules – which lead to a rapid attraction of inflammatory cells to the inflammatory site (van der Poll and Lowry, 1995). TNF is capable of recruiting inflammatory cells to the site of infection both directly and via upregulation of adhesion molecules. It is also capable of stimulating the release of chemokines, cytokines that are directly chemotactic for inflammatory cells and can also promote antimicrobial activity by activation respiratory burst and by activating the capacity to granulate. These effects of TNF have been shown to be required for effective in vivo host defense against a range of microorganisms, including *Pneumocystis carinni* (Chen et al., 1992).

TNF has been implicated as a central mediator of the host response to bacterial infection, in which it may play a dual role. Systemic and excessive release of TNF into the circulation is harmful to the host, as documented by numerous laboratory studies in which anti-TNF strategies prevented death during otherwise rapidly fatal sepsis (Tracey et al., 1987; Hinshaw et al., 1990). However local production of TNF, at the site of an infection, is important for adequate antibacterial defense. This importance has been demonstrated, in particular, in murine models of pneumonia, in which treatment with anti-TNF impaired host defense against various respiratory pathogens, including

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Streptococcus pneumoniae (Brieland et al., 1995; Laickalk et al., 1996; van der Poll et al., 1997; Rijneveld et al., 2001). The cytokine tumor necrosis factor alpha functions as an endogenous alarm signal that coordinates gene expression and cellular activity, driving inflammatory responses to infection, injury, or irradiation.

The treatment of pneumococcal infections-such as meningitis, pneumonia, and bacteremia- with beta-lactam antibiotics can result in the paradoxical enhancement of inflammation as a result of the release of proinflammatory cell-wall products, such as lipoteichoic acid (LTA) and peptidoglycan (PGN) (Orman and English, 2000; Nau and Eiffert, 2002). Ceftriaxone is a widely used antibiotic against pulmonary infection as its broad spectrum of activity and prolonged elimination half-life in plasma make it a drug of choice for once daily administration. Antibiotics can kill bacteria, but most do not strongly modulate the inflammatory response. Therefore, the addition of host response modulators to antibiotics might be beneficial for the treatment of severe pneumococcal pneumonia. In light of its presumed detrimental role in the pathogenesis of overwhelming sepsis, anti-TNF has been evaluated in a fairly large number of controlled clinical trials of patients with sepsis. Although individual trials did not reveal a significant benefit for patients treated with anti-TNF, pooled data from trials that evaluated monoclonal antibodies directed against TNF demonstrated a significant reduction in mortality (Bernard et al., 1997; Marshall, 2000).

There is growing evidence that aspects of the immune response greatly contribute to high mortality rate: while immunosuppresed patients die as a consequence of poor host response, immunocompetent host face overwhelming inflammatory reactions that contribute to tissue injury, shock and death (Tuomanen et al., 1995)

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Side effects with anti-TNF antibodies include transient respiratory tract infections, formation of human anti-chimeric antibodies with associated acute and delayed hypersensitivity infusion reaction (Hanauer et al., 1999), formation of autoantibodies with rarer instances of drug- induced lupus, and a few cases of lymphoma (Weinblatt et al., 1999). The immunogenicity of these treatments with anti-TNF antibodies in many cases partial or complete loss of efficacy has stimulated the search for alternative TNF blockers. One attractive molecule is the dimeric fusion protein etanercept, with which extensive clinical experience has been collected in the treatment of rheumatoid arthritis, juvenile rheumatoid arthritis, psoriatic arthritis (lovell et al., 2000).

Etanercept (Enbrel, Immunex, Seattle, WA), an injectable tumor necrosis factor (TNF) receptor fusion protein that binds and inactivate human TNF, is used in active rheumatoid arthritis. Etanercept consists of the extracellular ligand-binding portion of the human p75 TNF receptor linked to the Fc portion of the human IgG1. Because it does not contain nonhuman material, it is theoretically less immunogenic. It is a genetically engineered TNF alpha receptor fusion protein, which is able to bind TNF alpha and reduce its bioavailability. Etanercept was first approved for the treatment of moderate to severe rheumatoid arthritis (RA) in November 1998 and is now used for juvenile RA. TNF is expressed in increased amounts in the airspace of humans with bacterial pneumonia and in the lungs of mice challenged with bacterial pathogens (Gosselin et al., 1995). Recent studies suggest that neutralization of TNF in vivo can impair neutrophil influx and lung bacterial clearance in response to aerosolized or intratracheally administred *P. aeruginosa* (Gosselin et al., 1995). To our knowledge, investigations examining the effect that treatment with anti-TNF, as an adjunctive therapy, has during either clinical or experimental pneumonia have not been performed.

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6.2 The main objective of the study

In this study, we sought to determine the effect that etanercept (tumor necrosis factor (TNF) receptor fusion protein) therapeutically administered together with antibiotic ceftriazone has in mice with ongoing systemic pneumococcal infection. We tested the hypothesis that etarnecept could be a promising adjunctive agent to antibiotic ceftriaxone for the treatment of severe pneumococcal pneumonia.

6.2.1 The specific objectives of the study were:

6.2.1.1 The effect of Etanercept as an adjunctive therapy to ceftriaxone on the survival of mice after *S. pneumoniae* challenge

6.2.1.2 To determine bacterial load in blood 24hrs after bacterial challenge in order to evaluate antibacterial host defense after the different treatment strategies

6.2.1.3 To measure concentration of inflammatory cytokines in serum samples from Ceftriazone treated group and Ceftriazone plus Etanercept treated group to obtain insight into the effect of anti-TNF Etanercept and/or Ceftriazone on the inflammatory response

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6.2 MATERIALS AND METHODS

6.2.1 Mice

Eight to ten week- old BALB/c and C57BL/6 mice weighing (18-20g) obtained from Jackson Laboratory (USA) were used in all experiments. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Virginia, Charlottesville, USA. Mice had free access to mouse chow and water throughout the experiment and were exposed to alternate standardized light and dark periods every day.

6.2.2 Experimental inoculums

The bacteria culture was prepared according to the procedure described by Restrepo et al., 2005. Briefly before each experiment, several colonies of *S. pneumoniae* were inoculated into 10ml Brain Heart infusion broth (serial 10- fold dilutions prepared) and the tubes were incubated for 16 hours at 37 ^{0}C in 5% CO2. After incubation, 1ml of overnight culture, from the most diluted tube with growth, were inoculated into 9ml of Brain heart infusion broth and then incubated for 4 hours at 37 ^{0}c in 5 % CO_2, in order to obtain a final concentration of 10 ^{9} CFU/ml. This concentration was first determined by optical density measurement (standardized by hourly determination of Optical density and viable CFU/ml count), with reference to a standard curve, and confirmed bacterial concentration by using successive dilution cultures to ensure final concentration of inoculums.

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6.2.3 Infection and treatment

S. pneumoniae serotype 2 obtained from American Type Culture Collection (ATTC 6302, Mannasas, Virginia) was grown for 4-6h at 37 ^{0}C (as describe above), to a midlogarithmic phase, by use of Brain Heart infusion broth (Remel). Mice were inoculated intravenously with 50ul of bacterial suspension (approximatetly 5x 10^{7} cfu). Five groups of BALB/c and C57Bl/6 mice were inoculated with a lethal dose of *S. pneumoniae* serotype 2. Mice received a single intravenous injection (total volume 100ul) of one of the following treatments: sterile saline (100ul) (group 1); or sterile saline with 100ug Etanercept (Sigma) (group2); or 25mg/kg ceftriaxone (CEF, Roche) in 50 ul saline intraperitoneally (group 3); or combination of CEF (25mg/kg) in 50ul saline and with 100 ug Etanercept (100ul) (group 4, 5). Briefly, Control mice received sterile saline ; group 2 received 100ug of Etanercept intravenously at the time of bacterial challenge; group 3 received 25 mg/kg of cefriazone alone 5 hours after bacterial challenge; group 4 received a combination of 100ug of Etanercept (i.v) at the time of challenge and 25mg/kg Ceftriaxone (i.p) 5 hours after bacterial challenge; group 5 received a combination of 100ug of Etanercept (i.v) 4 hours after challenge and 25mg/kg ceftriaxone 5 hours after bacterial challenge. The timing of ceftriaxone administration was based on preliminary studies (titration not showed here) in which we aimed to established an antibiotic –treatment schedule that would rescue approximately 20-30% of the mice to allow the evaluation of effects of immunomodulatory treatments in the context of antibiotic treatment i.e. effect of Etanercept as an adjunctive therapy in the treatment of systemic pneumococcal infection. We chose to administer Etanercept at the time of infection and close to antibiotic treatment to assess the effect that Etarnecept has in a therapeutic clinical setting. Survival was monitored for 6 days.

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6.2.4 Enumeration of bacteria from blood

Blood samples were harvested from Sham mice (group 1), ceftriaxone –treated mice (group 3), and group 5 mice that received a combination of ceftriaxone and etanercept 4 hours after bacterial challenge. Blood samples were harvested by cardiac puncture from mice anaesthetized with Isoflurane anaesthesia 24 hours after bacterial challenge. Serial 10 –fold dilutions in sterile PBS were made from blood and then 200ul volumes of dilutions were plated onto Sheep blood agar plates. Inoculated plates were incubated at 37 ^{0}C overnight. After incubation, colonies were counted to determine Colony forming units per ml (CFU/ml).

6.2.5 Determination of serum cytokine

Blood samples were harvested from Sham mice (group 1), ceftriaxone –treated mice (group 3), and group 5 mice that received a combination of ceftriaxone and etanercept 4 hours after bacterial challenge. Blood samples were collected by cardiac puncture from mice euthanized with Isoflourine 24 hours after bacterial challenge and analyzed for inflammatory cytokines with bead-based multi-analyte flow cytometry. Blood samples were spun at 10 000rpm for 10 minutes and serum samples were collected and frozen at -70 ^{0}C until cytokine analysis. Mouse inflammatory cytokines (TNF-a, IL-1 alpha, IL-1 beta, IL-2, IL-4, IL-5, IL-6, IL-10, 1L-12 (p40 and p70), IL-13, IL-17, granulocyte macrophage colony stimulating factor (GM-CSF), Interferon gamma, etc) in blood or serum were simultaneously measured using a protein bead-based multi-analyte flow cytometer (Multiplex immunoassay system Bio-Rad laboratories, Hercules, and CA). Briefly, antibodies that are highly specific for individual cytokine are immobilized on beads with different bead color code and added to samples. Bead bound cytokines are detected using a mixture of biotinylated cytokine specific monoclonal antibodies and

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Streptavidin-PE. Fluorescence signals from the bead-coding dyes and the reporter molecules attached to detection reagents are recorded and digitally processed to translate the signals into quantitative data for each reaction.

6.2.6 Data and statistical analysis.

Statistical comparisons of cytokine values were determined by a 2 tailed Student's t-Test (Microsoft Excel software, Microsoft Corporation, Redmond, WA). Survival data were plotted and the survival curves compared with a log-rank test (GraphPad PRISM software, San Diago, CA). Data are displayed as means+/- SEM unless otherwise stated. Differences were considered significant at $p<0.05$.

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6.3 RESULTS

6.3.1 Survival rate studies

All mice that did not receive Ceftriaxone died soon after *S. pneumoniae* bacterial challenge, irrespective of Etanercept treatment (Figure 6.1). All Sham mice (n=20) died within 3 days and Etanercept alone treated group (n=5) mice all died within 24 hours. Treatment with ceftriaxone resulted in 80% (n= 20) of the mice died within 72hrs, whereas treatment with combination of Ceftriaxone and Etanercept increased survival i.e 50% of mice that received Etanercept at the time of challenge and ceftriazone 5hrs after challenge (group 4, n=6) and of interest, 80% of mice (group 5 n= 16) that received combination of Etanercept at t=4hrs and Cefttriaxone at t=5hrs after bacterial challenge survived bacterial challenge ($P<0.05$, vs treatment with Cef alone), which is a significantly better protection than treatment with ceftriaxone alone. Mice surviving for 6 days after bacterial challenge were assumed to be permanent survivors in this study because there were no late deaths and mice were very active and healthy.

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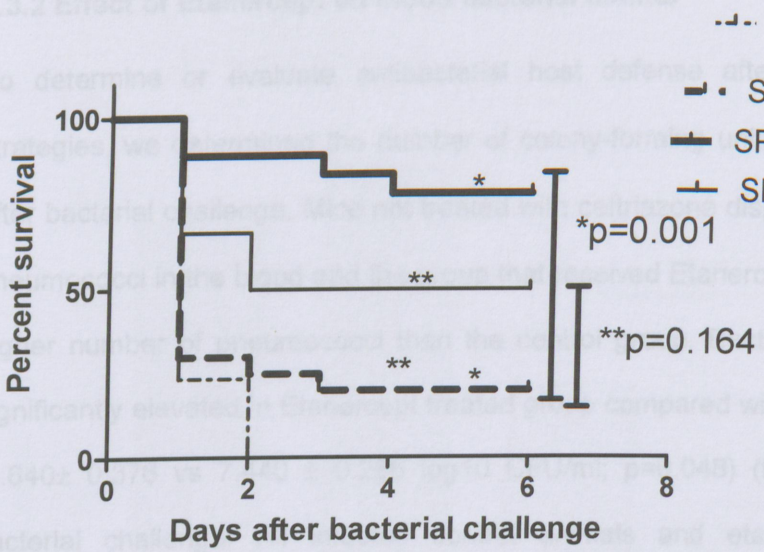


Figure 6.1. Survival curves for C57bl/6 mice treated with Etanercept with/out ceftriaxone after 10^7 cfu acterial challenge. Treatment with ceftriaxone resulted in 80% (n= 20) mortality 72hrs, whereas treatment with combination of Ceftriaxone and Etanercept increased survival i.e 50% of mice that received Etanercept at the time of challenge and ceftriazone 5hrs after challenge (group 4, n=6) and of interest, 80% of mice (group 5 n= 16) that received combination of Etanercept at t=4hrs and Cefttriaxone at t=5hrs after bacterial challenge survived bacterial challenge (P<0.05, vs treatment with Cef alone

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6.3.2 Effect of Etanercept on blood bacterial counts

To determine or evaluate antibacterial host defense after the different treatment strategies, we determined the number of colony-forming units (CFU/ml) in blood 24hrs after bacterial challenge. Mice not treated with ceftriazone displayed a higher number of pneumococci in the blood and the group that received Etanercept alone had significantly higher number of pneumococci than the control group. Bacterial counts in blood was significantly elevated in Etanercept treated group compared with untreated control group (8.640± 0.376 vs 7.440 ± 0.286 log10 CFU/ml; p=0.048) (figure 6.2) at 24hrs after bacterial challenge. All infected control animals and etanercept treated animals developed septicemia within 24hrs after bacterial challenge (>5log10 CFU in the blood streams of both groups). As expected, administration of ceftriaxone to infected animals significantly reduced bacterial counts in blood to 4.2 log 10 CFU /ml($p<0.05$ vs. infected animals). Remarkably, mice treated with combination of Etanercept and Ceftriaxone had 3 log10 fewer *S. pneumoniae* organisms than those of mice treated with ceftriazone alone (1.34 ± 0.491 vs. 4.22±0.961 log10 CFU/ml, p=0.016) (Fig 2). The differences between ceftriaxone plus etenercept and ceftriaxone –treated groups reached statistical significance ($p<0.05$) 24 hours after bacterial challenge.

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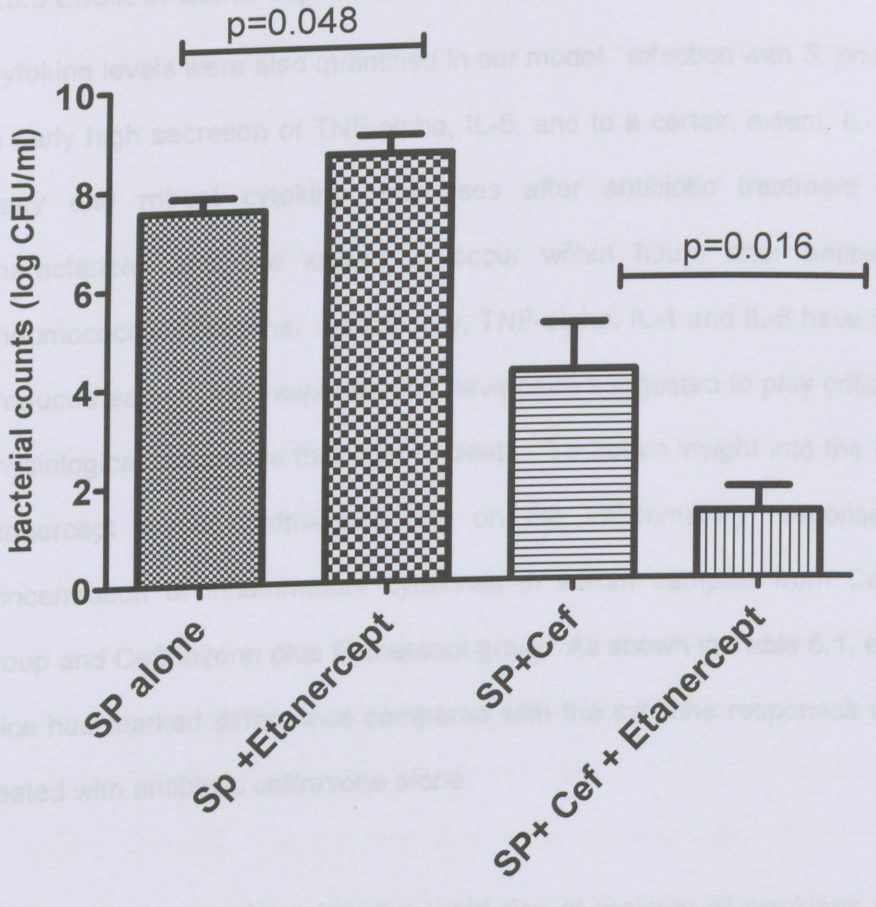


Fig 6.2. Bacteremia in mice treated with Etanercept with/out ceftriaxone 24 hours after 10^7 cfu bacterial challenge. Mice treated with combination of Etanercept and Ceftriaxone had 3 log10 fewer *S. pneumoniae* organisms than those of mice treated with ceftriazone alone (1.34 ± 0.491 vs. 4.22 ± 0.961 log10 CFU/ml, p=0.016).

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6.3.3 Effect of Etanercept on Inflammatory mediators

Cytokine levels were also quantified in our model. Infection with *S. pneumoniae* resulted in early high secretion of TNF-alpha, IL-6, and to a certain extent, IL-1 alpha in serum. Early and robust cytokine responses after antibiotic treatment have been well characterized and are known to occur within hours after antibiotic treatment in pneumococcal infections. Specifically, TNF-alpha, IL-1 and IL-6 have been shown to be produced early in this response and have been suggested to play critical roles in driving physiological responses that lead to death. To obtain insight into the effect of anti-TNF Etanercept and/or Ceftriazone has on the inflammatory response, we measured concentration of inflammatory cytokines in serum samples from Ceftriazone treated group and Ceftriazone plus Etanercept group. As shown in Table 6.1, etanercept treated mice had marked differences compared with the cytokine responses of mice that were treated with antibiotic ceftraxone alone.

Ceftriaxone treatment produced a rapid rise of majority of cytokines at 24 hours after treatment and was significantly higher than that obtained in untreated controls (TNF 5,802 vs. 1289; IFN gamma 2,641.5 vs. 995; IL-3 1,498 VS. 331.5; IL-17 6,074.5 vs. 2,179.5, GM-CSF 1,703 vs. 547; MIP-1 beta 2,381 vs. 598; RANTES 3,791.5 vs. 1,003) ($p<0.05$). IL-6 (4,957 vs. 2,806.5), IL-10 (983 vs.512), G-CSF (26,189 vs. 23,981.5), KC (22,311 vs. 5,832), MCP-1 (8,687.5 vs. 791) levels were significantly higher in the control untreated group and were significantly reduced in ceftriaxone alone. Simultaneous administration of anti-TNF Etanercept with ceftriaxone at 5 hours after bacterial challenge resulted in a significant reduction in blood TNF compared with values in the ceftriaxone-treated group. These data suggest that initiation of ceftriaxone

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therapy in experimental pneumococcal infection exacerbates inflammatory response, which can be modulated by concurrent anti-TNF Eternercept administration.

Etanercept consistently and significantly reduced levels of the majority of inflammatory cytokine. The respective values 24 hours after challenge for treatment with ceftriazone plus etanercept compared with treatment with ceftriaxone alone were TNF-alpha (787.5 vs. 5,801), IL-6 (255.5 vs. 2,806.5), IFN gamma (504.5 vs. 2,641.5), IL-12 (750 vs. 3,182), IL-3 (220 vs. 1,498), IL-17 (1,066 vs. 6,074.5), G-CSF (12,985.5 vs. 23,981.5), GM-CSF (284.5 vs. 1,703), KC (1,210 vs. 5,832), MIP-1 beta (218.5 vs. 2,381), RANTES (551 vs. 3,791.5). Cytokine measurements performed at 48 h showed that levels had returned close to baseline undetectable values (Data not shown). Inflammatory cytokine serum levels were lower in the etanercept (higher survival) group than either the sham or ceftriaxone alone groups. All reported results were statistically significant ($p<0.05$). Etanercept decreased inflammatory cytokines and improved survival in a clinically relevant murine model of lethal systemic *Streptococcus pneumoniae*.

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Table 6. 1. Levels of proinflammatory cytokines in blood collected 24h after bacterial challenge in control, ceftiaxone and ceftriaxone plus etanercept treated mice.

Cytokines	Controls	Ceft	Ceft + Etan	*P values	**P values
TNF-α	1289	5801	787	<0.05	<0.05
IL-6	4957	2806	255	>0.05	<0.05
IFN-γ	995	2641	504	<0.05	<0.05
IL-12	2352	3182	720	>0.05	<0.05
IL-17	2179	6074	1066	<0.05	<0.05
GM-CSF	547	1703	284	<0.05	<0.05
MIP-1β	598	2381	218	<0.05	<0.05
RANTES	1003	3791	551	<0.05	<0.05
KC	2231	5832	1210	<0.05	<0.05
IL-3	331	1498	220	<0.05	<0.05
IL-1α	871	1063	609	>0.05	<0.05
EOTAXIN	716	1081	427	>0.05	<0.05
MIP-α	1239	1265	615	>0.05	<0.05
G-SCF	26189	23981	12985	>0.05	<0.05
MCP-1	8687	718	230	<0.05	>0.05
IL-10	983	512	195.5	>0.05	>0.05

The results are expressed as the means for 5 mice in each group. * indicates p values for Ceftriaxone vs control and ** p values for Etanercept vs Ceftriaxone treated group based on student T-test

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6.4 DISCUSION AND CONCLUSION

Despite the introduction of numerous therapeutic advances, mortality and morbidity associated with bacterial sepsis remain significant. In patients with sepsis, the success of treatment with anti-TNF is limited, in spite of an abundance of experimental data indicating that, during overwhelming sepsis in animals, elimination of endogenous TNF activity exerts strong protective effects (Hinshaw et al., 1990; van der Poll and Lowry, 1995). These studies focus on the role that TNF plays in the pathogenesis of pneumococcal infection and do not explain how treatment with anti-TNF, i.e treatment administered to animals with already -ongoing pneumococcal infection, influences the course of pneumococcal infection in the context of treatment with antibiotics.

In this study, we sought to determine the effect of Etanercept (tumor necrosis factor (TNF) receptor fusion protein), therapeutically administered together with antibiotic ceftriazone, has in mice with ongoing systemic pneumococcal infection. We tested the hypothesis that etanercept could be a promising adjunctive agent to antibiotic Ceftriazone for the treatment of severe pneumococcal pneumonia. We have shown in this study that anti- TNF etanercept administered just before treatment with ceftriaxone 4 hours after bacterial challenge increases or provides a survival benefit in our systemic pneumococcal murine model and Etanercept can be used as an adjunctive therapy in the treatment of pneumococcal infection, as reflected by a trend toward increased survival, less pneumococci recovered from blood (low bacterial count), and low serum inflammatory cytokine levels compared with mice treated with Ceftriaxone alone.

All mice that did not receive Ceftriaxone died soon after *S. pneumoniae* bacterial challenge, irrespective of Etanercept treatment (Fig. 1). All Sham mice died within 3

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days and all Etanercept alone treated mice died within 24 hours and the lower survival rate observed in Etanercept treated group was associated with insufficient bacterial clearance. The present finding that, in the absence of concurrent treatment with Ceftriaxone, administration of anti-TNF influenced bacterial growth which suggests that in these mice, antibacterial host defense was impaired. The administration of Etanercept at the time of bacterial challenge impaired local host defense and reduced survival and this finding indicates that TNF produced at the site of infection is required for an adequate antibacterial defense during pneumococcal infection. Our results were consistent with those reported using anti-TNF monoclonal antibodies during pneumococcal pneumoniae in mice (van der Poll, 1997). The previous study demonstrated that treatment with an anti- TNF antibody 2 hours before inoculation resulted in fourfold increase in *S. pneumoniae* CFU isolated from lungs. Anti-TNF treated mice died significantly earlier from pneumococcal pneumoniae than control mice. TNF receptor –deficient mice also showed high mortality and impaired bacterial clearance (Lee et al., 2003). A recent study demonstrated that lymphocyte proliferation in response to blood monocytes and alveolar macrophages infected with *Histoplsama capsulatum* was inhibited by treatment with the anti-TNF alpha antibody infliximab, whereas control antibodies had no effect. This result suggests that patients receiving anti-TNF antibodies or therapy may be at a great risk for developing disseminated histoplasmosis because of immunosuppressive effects (Wood et al., 2003). The current findings are also in line with previous studies that used anti- TNF in the model of murine pneumococcal pneumonia (van der Poll et al., 1997; Rijneveld et al., 2001), a finding indicating that animals treated with anti-TNF had increased bacterial load relative to that in control animals which determines the extent of the inflammatory response and TNF is important mainly during the early phases of the infection. This suggests that administration of etanercept without antibiotics may be detrimental to early host defense

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against pneumococcal pneumonia. These data add to our knowledge of potentially adverse effects of treatment with anti- TNF in patients with severe infection, particularly in patients with pneumococcal infection. Lee and coworker (2003) showed that TNF-alpha gene-deficient mice showed higher mortality than wild type (TNF-alpha) mice after inoculation of intranasal bacteria. An impaired bacterial clearance in TNF-alpha $^{-/-}$ mice was associated with decreased systemic concentration of chemokine macrophage inflammatory protein -2 and reduced pulmonary neutrophil recruitment.

In contrast, administration of etanercept with ceftriazone facilitated bacterial clearance from blood more effectively than use of ceftriaxone alone. Remarkably, mice treated with combination of Etanercept and Ceftriaxone had lower bacterial counts than mice treated with CEF alone (vs, $P<0.05$) (Fig 2). In recent study, treatment with the anti-inflammatory cytokine IL-10 was reported to improve the efficacy of CEF during pneumococcal pneumonia in mice (Wang et al., 2000). IL-10 reduced both the extent and the duration of inflammation, outgrowth of pneumococci, mortality, and findings suggesting that a decrease of the inflammation, by adjunctive anti-inflammatory immunotherapy, may result in a beneficial effect on outcome (Wang et al., 2000). The results of this study, in which anti-TNF Etarncept (anti-inflammatory strategy directed at the cytokine network) was combined with CEF, support these findings. Of note both IL-10 and Etanercept impaired host defense against pneumococcal infection when administered simultaneously with the infectious challenge in mice not treated with antibiotics (8, 9, and 15). As expected, treatment with CEF strongly reduced the number of pneumococci CFU recovered from the blood.

To obtain insight into the effect of anti-TNF Etanercept and/or Ceftriazone has on the inflammatory response, we measured concentration of inflammatory cytokines in serum

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samples from Ceftriazone treated group and Ceftriazone plus Etarnercept group. More substantial and persistent inhibition of proinflammatory mediators in blood was observed with the concomitant administration of cefriaxone and etanercept than with the administration of ceftriaxone alone. Treatment with ceftriaxone and etanercept induced a significant ($p<0.05$) decrease in TNF-alpha, IL-6, IFN gamma, IL-12, IL-3, IL-17, G-CSF, GM-CSF, KC, MIP-1 beta, RANTES response, and had little or no effect on IL-1 alpha and beta, MIP-1 alpha, MCP-1, Eotoxin, IL-13, IL-10, IL-9, IL-4, IL-5, IL-2 response in this model (figure), compared with treatment with ceftriaxone alone. Etanercept decreased inflammatory cytokines and improved survival in a clinically relevant murine model of lethal systemic *Streptococcus pneumoniae*. Inflammatory cytokine serum levels were lower in the etanercept (higher survival) group than either the sham or ceftriaxone alone groups. All reported results were statistically significant ($p<0.05$). Treatment with Ceftriazone was also associated with increased levels of inflammatory cytokines or enhanced inflammatory response which was reduced in Etanercept plus ceftriazone treated group and a finding that agrees with the strong anti-inflammatory effect of anti-TNF Etanercept prophylactically administered to animals with severe sepsis (Hinshaw et al., 1990). A similar phenomenon was observed in vitro when *Streptococcus pneumoniae* was treated with penicillin and numerous cell wall fragments were released (Fischer and Tomasz, 1984). Several anti-TNF strategies have been evaluated in clinical trials involving patients with severe sepsis, many of whom suffered from pneumonia. These data suggest that initiation of ceftriaxone therapy in experimental pneumococcal infection exacerbates inflammatory response, which can be modulated by concurrent anti-TNF Etanercept administration. We have demonstrated here that treatment with Ceftriaxone and anti-TNF administered 4hrs after bacterial challenge in systemic murine model of pneumococcal infection is associated with an increased

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survival benefit and the reduction in inflammatory cytokines correlates positively with survival.

The rationale for the use of adjunctive pro-inflammatory cytokines and certain antibodies for treating infectious disease is to enhance or dampen or reduce the host inflammatory response. Anti-cytokine therapy shows remarkable promise for ameliorating the clinical symptoms and perhaps halting the disease progression. Etanercept and Infliximab, both now approved for the treatment of RA, are effective antagonists of tumor necrosis factor alpha and these agents, used in combination with antibiotics, demonstrate powerful anti-inflammatory activity and may slow progression of the disease (Lovell et al., 200). Because bacterial sepsis is a dynamic and evolving condition, different strategies may be needed at different stages in the pathogenesis of sepsis and through carefully performed trials and thoughtful selection of combination therapy aimed at different points in the pathological process, it may be possible in the future to modify the course of pneumococcal infection. Administration of anti-TNF etanercept plus ceftriaxone improved survival rate of mice ($p < 0.05$ compared with therapy with ceftriaxone alone). Etanercept should be considered as an adjunctive therapy to antibiotics against severe pneumococcal infections. The future use of adjunctive immunomodulators for infectious disease requires a better understanding of microbial pathogenesis and the relative need for immune activation versus immune modulation in the context of the immune response of the affected individual. The damage response framework can be a useful tool when considering the type of intervention that might be successful, but such predictions require experimental validation to be translated to the bedside and clinic.

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

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provides a survival benefits when combined with ceftriaxone. 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. Moreover, the current wave of enthusiasm regarding the treatment of patients with anti- TNF 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.

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6.5 REFERENCES

Bergeron, Y., Oullet, N., Deslauriers, A.M., Simard, M., Olivier, M., Bergeron, M.G. 1998. Cytokine kinetics and other host factors in response to pneumococcal pulmonary infection in mice. *Infect Immun* 66:912-922.

Bernard, G.R., Wheeler, A.P., Russell, J.A. 1997. The effects of Ibuprofen on the physiology and survival of patients with sepsis. The Ibuprofen in Sepsis Study Group. *N Engl J Med* 336:912-918.

Bernstein, J.M., Campbell, G.D. 1999. Treatment of pneumonia and its implications for antimicrobial resistance-introduction. *Chest* 115:S1-S2

Brieland, J.K., Remick, D.G., Freeman, P.T, Hurley, M.C., Fantone, J.C., Engleberg, N.C. 1995. In vivo regulation of replicative Legionellapneumophilia lung infection by endogenous tumor necrosis factor alpha and nitric oxide. *Infect Immun*: 63:3253-3358.

Chen, W., Havell, E.A., and Harmsen, A.G. 1992. Importance of endogenous tumor necrosis factor alpha and gamma interferon in host resistance against Pneumocystis carinni infection. *Infect Immun* 60:1279-1284.

Fischer, H., Tomasz, A. 1984. Production and release of peptidoglycan and wall teichoic acid polymers in pneumococci treated with beta-lactam antibiotics. *J Bacteriol* 157:507-513.

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Gamble, J.R., Harlan, J.M., and Klebanoff, S.J. 1985. Stimulation of the adherence of neutrophils to umbilical vein endothelium by human recombinant tumor necrosis factor. *Proc Natl. Acad. Sci* USA 82:8667-8671.

Gosselin, D., DeSanctis, J., Boule, M., Skamene, E., Matouk, C., and Radzioch, D. 1995. Role of tumor necrosis factor alpha in innate resistance to mouse pulmonary infection with Pseudomonas aeruginosa. *Infect Immun* 63:3272-3278.

Hanauer, S.B., Rutgeerts, P.J., D'Haens, G.R. 1999. Delayed hypersensitivity to infliximab (Remacade) infusion after 2-4 year interval without treatment. *Gastroenterology* 116:G3174.

Hinshaw, L.B., Tekamp-Olson, P., Chang, A.C. 1990. Survival of primates in LD100 septic shock following therapy with antibody to Tumor necrosis factor (TNF alpha). *Circ. Shock* 30:279-292.

Laickalk, L.L., Kunkel, S.L., Strieter, R.M., Danforth, J.M., Bailie, M.B., Standiford, T.J. 1996. Tumor necrosis factor mediates lung antibacterial host defense in murine *Klebsiella pneumonia*. *Infect Immun* 64:5211-5218.

Lee, J.H., Del Sorbo, L., Khine, A.A., de Azavedo, J., Low, D.E., Bell, D., Uhling, S., Slutsky, A.S., Zhang, H. 2003. Modulation of bacterial growth by tumor necrosis factor – alpha in viro and in vivo. *Am J Respir Crit Care Med* 168:1462-1470.

Lovell, D.J., Giannini, E.H., Reiff, A. 2000. Etanercept in children with polyarticular juvenile rheumatoid arthritis. *N Engl J Med* 342:763-769

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Marshall, J.C. 2000. Clinical trials of mediator-directed therapy in sepsis: what have we learned? *Intensive care Med* 26: S75-S83.

Nau, R, Eiffert, H. 2002. Modulation of release of proinflammatory bacterial compounds by antibacterials: potential impact on course of inflammation and outcome in sepsis and meningitis. *Clin Microbiol Rev* 15:95-110.

Neiderman, M.S., Bass, J.B., Campbell, G.D. 1993. Guidelines for the initial management of adults with community –acquired pneumonia: diagnosis, assessment of severity, and initial antimicrobial therapy. *Am Rev Respir Dis* 148: 1418-26.

Orman, K.L., English, B.K. 2000. Effects of antibiotic class on the macrophage inflammatory response to Streptococcus pneumoniae. *J Infect Dis* 182:1561-1565.

Pallares, R., Linares, J., Vadillo, M., Cabellos, C., Manresa, F., Viladrich, P.F., Martin, R., Gudiol, F. 1995. Resistance to penicillin and Cephalosporin and mortality from severe pneumococcal pneumonia in Barcelona, Spain. *N. Engl J Med* 333:474-486.

Restrepo, A.V., Salazar, B.E., Agudelo, M., Rodriguez, C.A., Zuluaga, A.F., and Vesga, O. 2005. Optimization of culture conditions to obtain maximal growth of penicillin-resistant *Streptococcus pneumoniae*. *BMC Microbiol* 5:34

Rijneveld, A.W., Florquin, S., Branger, J., Speelman, P., Van Deventer, S.J., van der Poll, T. 2001. TNF-alpha compensates for the impaired host defense of IL-1 type 1 receptor-deficient mice during pneumococcal pneumonia. *J Immunol.:* 167: 5240-5246.

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Creating Future Leaders

Tracey, K.J., Fong, Y., Hesse, D.G. 1987. Anti-cachectin/TNF monoclonal antibodies prevent septic shock from during lethal bacteremia. *Nature* 330:662-664

Van der Poll, T., Keogh, C.V., Buurman, W.A., Lowry, S.F. 1997. Passive immunization against tumor necrosis factor-alpha impairs host defense during pneumococcal pneumonia in mice. *Am J Respir Crit Care Med* 155:603-608.

Van der Poll, T., Lowry, S.F. 1995. Tumor necrosis factor in sepsis: mediator of multiple organ failure or essential part of host defense? *Shock* 3:1-12.

van der Poll, T., Marchant, A., Buurman, W.A. 1995. Endogenous IL-10 protects mice from death during septic peritonitis. *J Immunol* 155:5397-5401.

Wang, E., Simard, M., Ouellet, N., Bergeron, Y., Beauchamp, D., Bergeron, M.G. 2000. Modulation of cytokines and chemokines, limited pulmonary vascular bed permeability, and prevention of septicemia and death with ceftriaxone and interleukin-10 in pneumococcal pneumonia. *J. infect Dis* 182: 1255-1259.

Weinblatt, M. E., Kremer, J.M., Bankhurst, A.D. 1999. A trial of etanercept, a recombinant tumor necrosis factor receptor:Fc fusion protein, in patients with rheumatoid arthritis receiving methotrexate. *N Engl J Med* 340:253-259.

Wood, K.L., Hage, C.A., Knox, K.S., Kleiman, M.B., Sannuti, A., Day, R.B., Wheat, L.J., Twigg, H.L III. 2003. Histoplasmosis after treatment with anti- tumor necrosis factor – alpha therapy. *Am J Respir Crit Care Med* 167:1279-1282.

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CHAPTER SEVEN

CONCLUSIONS AND RECOMMENDATIONS

In the present study, we report that

1. Stimulation with a highly purified LPS initiates a stronger immune response, leading to a faster elimination of bacteria, provided survival enhancement in a lethal murine model of systemic *Streptococcus pneumoniae* despite attenuation of cytokine response and is TLR4- dependent.
2. Treatment with LPS modulates components of the host immune response after antibiotic treatment and therefore improves the outcome of the disease.
3. ATL313 as an adjunctive therapy improves bacterial clearance and influences circulating cytokine concentrations in pneumococcal sepsis model and therefore improves survival in pneumococcal sepsis model.
4. Adenosine A_{2A} AR activation by ATL313 reduced inflammation by inhibiting NK cell activation and NK cells are involved in the pathogenesis of pneumococcal sepsis
5. Anti- TNF eternecept administered just before treatment with ceftriaxone 4 hours after bacterial challenge increases or provides survival benefit in our systemic pneumococcal murine model and may be used as an adjunctive therapy in the treatment of pneumococcal infection

These results show the potential for innate immune stimulation, adenosine A2AR agonist ATL313 and tumor necrosis factor (TNF) receptor fusion protein Etanecept, as adjunctive therapy in the treatment of pneumococcal infections.

As the treatment of infections becomes increasingly complicated by aggressive immunosuppressive regimens and the emergence of resistant bacteria strains,

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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 recommended to explore therapeutic benefits of immunomodulation or antiinflammatory agents in a lethal murine model of systemic *S. pneumoniae* infection for the treatment of pneumococcal infections.

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