Compartmentalization of Polymorphonuclear Neutrophil Delivery to Multiple Sites of Infection: The Second Front Hypothesis

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May 2003

A thesis submitted to
The Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for
the degree of *Master of Science in Experimental Surgery*

Your file Votre référence
ISBN: 0-612-88217-9
Our file Notre référence
ISBN: 0-612-88217-9

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ABSTRACT

Intra-abdominal infections are a common entity faced by general surgeons, which, when complicated by pneumonia lead to increased mortality. The susceptibility to a second infection is correlated with impaired polymorphonuclear neutrophil (PMN) migration to the remote site, perhaps due to inadequate PMN number. We tested the *second front hypothesis* that the elevated mortality arising from a second infection is due to insufficient circulating PMN, the majority being sequestered at the first site of infection. Further, augmentation of systemic PMN number by granulocyte colony stimulating factor (G-CSF) will decrease mortality.

We established a murine model for peritonitis and pneumonia. PMN counts in the blood, lung tissue, peritoneal cavity and bronchoalveolar system were measured. The results indicated significant PMN recruitment to the primary site of infection, whether lung or abdomen. Almost no PMN were found in the lung if it was the second site of infection. This was consistent with the second front hypothesis. Animals were then treated with G-CSF versus placebo and observed for mortality. Thermistor recorded temperature was used to predict death. The results were equivocal. The mortality of the infected groups was lower than predicted and therefore, although no significant difference was detected, our sample size was not powered to detect such a small magnitude of difference.

Overall, PMN recruitment to a second site of infection was shown to be diminished and may have contributed to elevated mortality. G-CSF mortality studies could be repeated with more severe infections using the thermistor model established herein.

RESUME

Les infections intra abdominales sont rencontrées fréquemment par l es chirurgiens. Ces infections, lorsque compliquées par une pneumonie, augmentent le taux de mortalité. La susceptibilité à développer un deuxième foyer d'infection est associée à la faible migration des neutrophiles polymorphonucléaires (NPM) au second site d'infection,dû possiblement au nombre inadéquat de ces derniers. Nous avons testé *l'hypothèse de second front* où le taux de mortalité élevé lié au second site d'infection est associé au nombre insuffisant de NPM circulants, ceux-ci étant retenus au foyer d'infection primaire. De plus, l'augmentation systémique de NPM par le facteur de stimulation des colonies granulocytaires (FSC-G) diminuerait le taux de mortalité.

Nous avons établi un *modèle murine* pour les péritonites et les pneumonies. Le décompte de NPM est mesuré dans le sang, les tissus pulmonaires, la cavité péritonéale et au niveau bronchoalvéolaire. Les résultats indiquent une mobilisation significative des NPM au site d'infection primaire, soit les poumons ou la cavité abdominale. Pratiquement aucun NPM ont été retrouvé au niveau du poumon lorsque celui-ci est le site d'infection secondaire. Ces résultats sont soutenus par *l'hypothèse de second front.* Par la suite, des animaux ont été traité, soit, par le FSC-G ou un placebo, puis observé afin d'établir le taux de mortalité. Un thermistor d'enregistrement de la température a été utilisé afin de prédire la mortalité. Les résultats sont équivoques. Le taux de mortalité du groupe infecté est inférieur au taux prédit, donc, malgré qu'aucune différence significative n'a été observé, la taille de notre échantillon n'a pu détecter une différence d'une si faible amplitude.

En général, il est démontré que le recrutement de NPM au site d'infection secondaire est diminué et que cela peut contribuer à l'augmentation du taux de mortalité. Des études de mortalité associées au FSC-G peuvent être répétées avec des infections plus sévères en utilisant le modèle du thermistor.

ACKNOWLEDGEMENT

First, I must acknowledge my debt to my supervisor, Dr. N.V. Christou for allowing me the privilege of working in his lab. Dr. R. Michel, the pathology lab and especially Wayne, the pathology technologist, were invaluable in obtaining the histological specimens of the lungs.

Second, I am grateful to my e-mail colleagues, Dr. C. Doerschuk and Dr. T. Takano for their aid in the details of the experiment, especially in regards to the particularly difficult issue of the myeloperoxidase stain.

Much gratitude is owed to Dan Swartz for his original work on this topic and Betty Giannias for her help in regards to technical knowledge and moral support. Of course the tireless dedication of our secretary, Ms. Bouldadakis cannot be overlooked, thanks Mary.

Un gros merci à Marie Devlin pour la belle traduction de la résumé.

Finally, special thanks to my father for his love and support during my research and throughout my career. My sincerest appreciation goes to those without whose help I could not have finished this manuscript. Thank you.

INTRODUCTION

More than fifty years after the introduction of antibiotics, infections continue to plague medicine in its care of patients. Ever advancing progress in the care of the critically ill in the fields of anesthesia, hemodynamic support and organ replacement, translates into increasing survival of patients suffering previously fatal conditions. These physiologically compromised individuals are susceptible new targets for infection. The solution initially appeared to lie in antibiotics. However, since the introduction of antibiotics there has come the evolution of resistant species of bacteria. The appearance of methicillin resistant *Staphylococcus aureus* and vancomycin resistant *Enterococcus* is an unresolved and timely issue in medicine today. Infections continue to be associated with fatal consequences. Therefore, despite the promise of new, more potent, broad spectrum antibiotics, this panacea is clearly not the predicted "magic bullet" and alone cannot overcome the infectious threat.

The basic tenet of microbiology is that there are three components determining the severity of infection: the bacteria, the inoculum and the host. The antibacterial aspect has been discussed above. There is, as yet, no procedure for detecting and so, no method of reducing the inoculum. That leaves simply the host.

The discovery of cytokines and their role in the host defense system has raised interest in combating infection by manipulating the host immune response. This excitement, however, has been tempered by the realization that as our factual knowledge of the cellular and molecular basis of the inflammatory response broadens our appreciation of its intricacies and paradoxes also expands.[1] The realization that our understanding of the process is imperfect fuels continuing research into the process whilst

acknowledging our trepidation in manipulating this incompletely delineated inflammatory response.[2]

Inflammation is, by definition, the body's response to injury or infection. The predominant cell early in this process is the polymorphonuclear neutrophil (PMN). However, cells crucial in controlling and eliminating a bacterial pathogen, cells such as the PMN, have also been implicated in systemic host tissue destruction deleterious to the organism. This paradox in the nature of the inflammatory response has been compared to a double edged sword.[3] The conditions which determine a beneficial outcome instead of a detrimental outcome remain to be elucidated.

Role of PMN

Response to bacterial invasion of the human organism is primarily mediated through PMN. This mechanism has been studied extensively and is summarized briefly here.

PMN are recruited to the site of inflammation in a five step sequence of events:

(1) rolling along the endothelium

(2) adhesion to endothelial cells

(3) transmigration through the endothelial layer

(4) migration through extracellular matrix

(5) retention at the site of inflammation.[4]

Rolling of the PMN along the endothelium is mediated by the loose binding of L-selectin, a carbohydrate moiety, on the PMN surface to an as-yet uncharacterized ligand

on the endothelial cell.[5, 6] This is followed by the high avidity binding of β-integrins, such as CD11/18, to the endothelial intracellular adhesion molecule (ICAM) receptors.[7]

Transmigration of the PMN through the endothelial layer may also be dependent on PECAM-1, but this process is not currently clearly delineated.[8] The migration of PMN through the extracellular matrix and its retention at the site of inflammation appears to be mediated by "primitive" chemokines, such as C5a, and the C-X-C chemokine family, including interleukin-8 (IL-8) and neutrophil attracting peptide-2 (NAP-2).[9, 10] The chemokine gradient determines the trajectory of the PMN, and the relatively higher concentration at the target site ensures a continuous PMN presence for the duration of the insult.

Once at the site of inflammation, the PMN contributes to the host defense system in several ways: phagocytosis, oxidative bacteriolysis and non-oxidative bacteriolysis.[4, 11, 12]

Sepsis, SIRS and MODS

While the inflammatory response is an important component of the host defense system against bacterial invasion, it can have deleterious consequences as implied above. A dysregulated hypermetabolic response to infection, trauma, acute organ injury or necrosis, can result in a spectrum of disorders including systemic inflammatory response syndrome (SIRS), sepsis, adult respiratory distress syndrome (ARDS) and multiple organ dysfunction syndrome (MODS). Previously defined as separate entities, these are now understood to represent a continuum of disease.

SIRS is defined as the presence of two or more of the following four diagnostic criteria:[13]

(1) fever or hypothermia defined as temperature greater than 38°C or less than 36°C

(2) leukocytosis or leukopenia defined as white blood cell count greater than 12,000 cells/mm^3 or less than 4,000 cells/mm^3 or >10% immature forms

(3) tachycardia defined as pulse greater than 90 beats per minute

(4) tachypnea defined as greater than 20 breaths per minute or arterial blood gas carbon dioxide tension less than 32mm Hg

SIRS is a systemic host response to sterile and/or infectious insults. When SIRS occurs in the presence of a documented infection, it is termed sepsis.[14]

Although the diagnostic value of the current definition of SIRS is debatable,[15] it epitomizes the concept that the severity of the inflammatory response and not the source of sepsis determines the outcome.[16] It can, in fact, take place in the absence of a detectable infection.

MODS represents the extreme of this pathological spectrum. MODS is associated with an ICU incidence rate of 15%[17] and mortality rate of 20-50%.[13] Originally described by Baue,[18] and elaborated by Eiseman[19] and Fry,[20] there has been much confusion in the literature concerning MODS because of the different definitions applied. The definition is the onset of remote organ dysfunction (i.e. an organ system not involved in the original inflammatory condition) in the critically ill patient as a result of a major inflammatory response.[13] An example would be ARDS. This potentially fatal complication frequently seen in ICU patients has plagued intensive care physicians for over thirty years. It is now recognized as the pulmonary aspect of MODS[21] and is often

triggered by a respiratory infection.[22] The exact etiology and process is not completely understood and there is currently no effective treatment for the condition. Much work is still being done in this field.

Since PMN are a major component of the host inflammatory response, there are several studies which attempt to delineate the role of PMN function, or rather dysfunction in MODS.[23] This topic will be discussed in more detail below.

Clinical Scenario

In the practice of general surgery, a subject of particular concern is intra-abdominal infections. Current treatment, consisting of source control by surgery or percutaneous drainage and antibiotics, is associated with a mortality rate of approximately 20%.[24-27] Prognostic factors such as severity of infection, pre-morbid condition, and other organ dysfunction have been positively correlated with an increase in morbidity and mortality. One particularly poor prognostic factor was the introduction of a second infection following the initial intra-abdominal infection. A significantly poorer outcome, independent of other factors, has been established in intra-abdominal infection (IAI) complicated by pneumonia.[24] In this study, pneumonia complicated IAI in 18% of cases resulting in a mortality rate of 53%. In contrast, recurrent IAI was found in 14% and was associated with a mortality rate of 17%, not significantly different from the 20% mortality of IAI alone. This higher mortality rate arising from remote organ injury but not recurrent IAI was supported by other papers.[26] Richardson published a retrospective study which demonstrated pneumonia in 29% of cases of IAI incurring a mortality rate of

66%. This was significantly greater than the mortality rate of 19% observed for IAI alone.[25]

Secondary or opportunistic infections in severely injured immune compromised patients have long been postulated to be a result of inefficient PMN bactericidal activity.[28, 29] Critically ill anergic patients, in experiments performed by our lab, had decreased PMN exudation in contrast to non-infected patients.[30] Compared to healthy controls, ICU patients have demonstrated a 72% inhibition in PMN delivery to skin windows: a technique used to measure PMN exudative capacity.[31] An ICU acquired infection rate of 45% with its attendant increased risk of mortality was observed by Vincent.[32]

Studies performed in this laboratory using a murine model of inflammation to parallel the clinical scenario illuminated the possible mechanism involved. Eighty mice were divided into four study groups: control, sham laparotomy, cecal manipulation and cecal ligation and puncture. Polyvinyl sponges, placed in the peritoneal cavity and dorsal subcutaneous tissue, were examined after 24 hours for PMN content. The increase in PMN exudation to the peritoneal cavity was positively correlated to the level of surgical intervention and offset by a decrease in PMN exudation to the dorsal subcutaneous tissue. This decrease in PMN exudation to the dorsal sponges was negatively correlated to the level of surgical intervention. The inference was that the intraabdominal insult attracted PMN to this primary site of inflammation, leaving relatively sparse numbers of PMN to respond to a second remote site of intervention.[33]

Why is the respiratory tract particularly vulnerable?

Both pathogen and host factors are implicated. Animal studies revealed decreased clearance of bacteria from the respiratory tract in the presence of intraperitoneal sepsis.[34, 35] In rats, endotoxemia was associated with defective recruitment of PMN to the lungs,[36] despite normal chemotactic gradients in the bronchoalveolar lavage fluid and serum of these animals. It was not just the initial inoculum size, but also the species of bacteria which was important.[37] *Staphlococcus aureus* appeared to be cleared predominantly by macrophages, whereas clearance of the gram negative rods, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* was dependent on PMN.[4, 37-39] Further evidence supporting the importance of PMN in gram negative infection was provided by Belaaouaj.[40] Mice which are genetically deficient in neutrophil elastase, a serine proteinase found in PMN granules, had decreased bacterial clearance and increased mortality when challenged with gram negative bacteria in the face of normal PMN migration. Bactericidal activity *in vitro* was decreased in elastase deficient PMN as indicated by the higher number of viable intracellular bacteria. This may explain why gram negative bacteria were the most common causes of pneumonia in the face of IAI in human studies.[25]

Given that gram negative infections of the respiratory tract appear to be dependent on PMN levels does not, however, imply a positive linear cause and effect relationship between the two. An increase in PMN function will not necessarily result in a lower rate or severity of infection. In a Jekyll and Hyde twist, PMN also appear to have the capability to destroy normal tissue. In the study cited above, mice deficient in neutrophil elastase were shown to have higher mortality with gram negative infection than normal

mice. Paradoxically, mortality was *decreased* when the mice were challenged with gram positive bacteria. The hypothesis was that the increased mortality in neutrophil elastase positive (i.e. control) mice was due to excessive proteinase action on host tissue.[40] Anderson *et al*[41] proposed a central role of PMN in MODS. Studies have demonstrated a link between PMN and ARDS, a component of MODS.[26, 42-46] Theories regarding the pathology of this syndrome have quoted the dysregulated activation of PMN releasing cytolytic substances into normal tissue, such as the endothelium.[21] The destruction of the endothelium resulted in edema and organ dysfunction, such as ARDS.

To further complicate the issue, not only was IAI reported to be a prime cause of MODS,[19, 20, 47] but the lung was also a major site for the sequestration of non-circulating neutrophils.[48] Therefore, given the controversy concerning the role of PMN in MODS, there has been an understandable reluctance in augmenting PMN function in this setting in the hopes of reducing the secondary infection rate, for fear of augmenting the ARDS rate and the mortality rate. In other words, fear of unleashing Mr. Hyde instead of employing Dr. Jekyll.

What therapies are available to augment PMN function and what are the data regarding its impact on infection rates and MODS?

Granulocyte-colony stimulating factor (G-CSF)

G-CSF is an 18.8 kDa glycoprotein[49] constitutively expressed by endothelial cells, fibroblasts and monocytes.[50] Acute elevation in G-CSF, dependent on the degree of injury[51] was stimulated by endotoxin, acute infections or trauma, [50, 52, 53] with gram-

negative infections associated with higher levels of G-CSF.[54] Originally described by Metcalfe in 1966[55], this CSF selectively enhanced the growth of neutrophilic colonies and has since been shown to promote growth and maturation of myeloid cells.[56] It is integral in nearly all aspects of PMN development.

Baseline levels of G-CSF were important in achieving maintenance levels of PMN.[57] G-CSF deficiency from genetic manipulation[56] or injection of antibody to G-CSF[58] resulted in neutropenia and an increased susceptibility to infections which could be reversed with exogenous G-CSF administration.

G-CSF stimulated proliferation and maturation of PMN by expanding the myeloid compartment in the marrow, halving the transit time to blood from six days to three days, and increasing the percent of immature PMN in circulating blood, effecting a left shift.[54, 59-61] The lifespan of the PMN was increased by reducing PMN apoptosis.[50, 54]

The functional activity of the mature PMN was stimulated by altering shape, cell surface receptors and phagocytosis.[50] Chemotaxis was enhanced at low doses but attenuated at large doses.[54] CD11b and CD66b were upregulated *in vivo*[62] and restoration of depressed CD64 levels in PMN from septic patients occurred when incubated with G-CSF.[63] Phagocytosis was enhanced in normal PMN and corrected in defective PMN from HIV-1 infected patients[54, 64] perhaps via upregulation of FcγRIII.[62]

G-CSF primed adherent PMN for respiratory burst and enhanced nonadherent PMN respiratory burst response to other stimuli. Again this effect has been observed in

normal as well as impaired PMN.[54, 65-67] Mobilization of secretory vesicles and specific granules due to G-CSF has been observed *in vivo*.[62]

Other effects of G-CSF include potentiating inflammation by increased production of superoxides, PGE, arachidonic acid, and TNF, as well as increased complement and antibody mediated cytotoxicity.[50]

Paradoxically, G-CSF in human volunteers has been shown to attenuate the pro-inflammatory cytokine response.[68, 69]

Granulocyte/macrophage-colony stimulating factor (GM-CSF)

GM-CSF produced by fibroblasts, endothelial cells and T lymphocytes can stimulate proliferation of myeloid cells, eosinophils, megakaryocytes and even multi-potential cells at high concentrations.[50] But, though GM-CSF has been shown to prime PMN for oxidative burst at lower concentrations than that required for G-CSF[70], no improvement in PMN chemotaxis was seen in neutropenics given GM-CSF[71] and evidence in their utility against sepsis is lacking.[51, 58, 67, 72, 73]

Filgastrim™

Exogenous administration of human recombinant G-CSF (hrG-CSF or Filgastrim) induced a transient neutropenia followed by a dose dependent neutrophilia, which returned to baseline within seven days.[51, 59] Daily injections caused an elevation of the baseline PMN count which plateau-ed after four days, unaffected by age.[56, 74] This

plateau was marked by spiking PMN counts after dosing.[54, 74] Serum G-CSF levels and absolute neutrophil counts appeared to be independent of dose.[75]

Currently Filgastrim, administered intravenously or subcutaneously at a dose of 5μg/kg/d, is used for prophylaxis and treatment of febrile neutropenia secondary to myelosuppressive chemotherapy regimens in high risk patients irrespective of age.[76] Its use is also advocated in reducing neutropenia, antibiotic use, transfusions and hospital stay in bone marrow transplant patients[77] in addition to enhancing the mobilization of peripheral blood progenitor cells for harvest with no adverse effect. Though the guidelines were drawn for the adult population, neonatal and pediatric PMN seem to respond to G-CSF in the same manner as adult PMN.[76, 78, 79]

Evidence supported the use of G-CSF in decreasing infection rates and mortality in HIV-positive neutropenics[80] and in correcting the neutropenia and decreasing infection rates in congenital agranulocytosis and cyclic neutropenia.[71, 81, 82]

Controversy exists in using G-CSF in acute myeloid leukemia, however evidence suggested the drug could prime the cells for chemotherapy without progression of the cancer.[83]

Short term toxicity of G-CSF at doses <10ug/kg/d was rare and usually resolved within days of discontinuing the medication. The main complaint was of bone pain.[59, 60, 74] The drug is contraindicated in patients receiving concomitant chemotherapy and radiotherapy.[76]

Animal Studies of G-CSF in Infection

G-CSF increased the numbers of peripheral PMN and PMN recovered in peritoneal lavage fluid after burns[84] or abdominal sepsis.[85, 86] This was likely related to the increased pool of circulating PMN as G-CSF did not act as a chemotactic agent in the peritoneum.[87] No difference was observed if the drug was given pre- or post-sepsis. This neutrophilia translated into increased bacterial clearance and decreased mortality rates from peritonitis in animals given G-CSF either prior to [58, 88-90] or after the induction of sepsis.[49, 91, 92]

The addition of G-CSF to an antibiotic regime in the treatment of infections may[88, 93] or may not[86, 90] improve mortality over antibiotics alone. Prophylactic dosing in immune compromised ethanol treated rats exposed to *K. pneumoniae* showed increased PMN in lungs and circulation, increased bacterial clearance and increased survival.[94] In contrast to this, Held showed pretreatment with G-CSF in mice subjected to intratracheal instillation of *K. pneumoniae* resulted in decreased killing of the bacteria by PMN and abscess formation in the murine lung.[95] Cirrhotic rats did not improve with G-CSF when exposed to *Streptococcus pneumonia*, however interestingly control animals in this study given G-CSF after pneumonia achieved a significant improvement in mortality.[96] More recently, rabbits infected with gram negative pneumonia responded to G-CSF with a significant neutrophilia but only a trend towards improved survival.[97]

Human Studies of G-CSF in Infection

Controversy exists over whether G-CSF decreases the rate of nosocomial infections in human subjects. A randomized double masked placebo controlled study of preterm infants by Miura showed elevated serum G-CSF levels and PMN associated with a relative risk of nosocomial infections of 0.19[98]. Mortality was unchanged in this study. In adults, an open label nonrandomized study of esophagectomy patients showed decreased nosocomial infection rates as compared to historical controls.[99] In contrast, a randomized double blind placebo controlled multicentre trial considering efficacy of G-CSF in liver transplant patients demonstrated no difference in infection rates except for significantly more nosocomial pneumonias in the group receiving G-CSF.[100]

Studies of non-neutropenic intensive care patients also gave conflicting results in terms of G-CSF decreasing infectious complications.[101, 102] In neither study was ARDS provoked by G-CSF.

A German group investigated the effect of low dose G-CSF in intubated SIRS and septic patients.[101] There was no placebo group in this study. They found increased leukocyte counts, especially in the SIRS group. Although the blood serum levels of G-CSF varied widely, there was a significant decrease in serum levels of the inflammatory cytokines IL-6 and IL-8 levels in both groups, with interestingly, an increase in IL-6 levels after termination of G-CSF in septic patients who later died. All septic patients had some evidence of lung injury which was not worsened by G-CSF treatment. None of the SIRS patients went on to develop sepsis or ARDS.

The NICU study was a randomized controlled double blind study on the use of high doses G-CSF in intubated CNS injured patients. G-CSF was found to increase

leukocyte counts and decrease the rate of bacteremia, but this did not translate into a decrease in the rate of nosocomial infections, length of stay or mortality.

A small retrospective study of G-CSF use in patients with leukopenia secondary to gastrointestinal perforation revealed a decrease in organ dysfunction rate as defined by Deitch[21] and a cause specific improvement in survival rate.[103]

G-CSF and ARDS

If ARDS is secondary to PMN mediated damage, augmenting the numbers of peripheral PMN and hence the marginated pool of pulmonary PMN by G-CSF[87, 104] will be expected to precipitate ARDS. The additional priming and activating action of G-CSF on these PMN will be predicted to be catastrophic.

There is evidence to suggest that G-CSF administration worsens lung function. In rats pretreated for two days with up to 50μg/kg/d of G-CSF, King *et al*[105] demonstrated neutrophilia, increased PMN sequestration and increased lung edema and permeability *ex vivo*. This often quoted study has three major flaws which diminish its applicability to human studies: (1) the dose of G-CSF was significantly higher than the 5μg/kg/d used in humans undergoing chemotherapy[76] (2) the initial lung injury was chemically induced and (3) examination for lung injury was via a perfusion circuit *ex vivo*. Mercer-Jones demonstrated an increased pool of marginated PMN in the liver and lungs after CD18 block, but failed to provide evidence of damage in these organs.[46] Mercer-Jones' article referred to the work of Thomas[106] in which antibody to CD18 was correlated with improved survival and no lung edema in septic rabbits. Freeman *et al* described reduced survival and worsened lung injury in rats exposed to moderate doses of intrabronchial

Escherichia coli and given G-CSF, but paradoxically improved outcomes with small or large doses of *E. coli*.[107] In a follow up study, a canine model of *E. coli* pneumonia pretreated with G-CSF revealed improved bacterial clearance with no effect, detrimental or otherwise, on pulmonary function.[108]

Significant evidence against G-CSF mediated lung damage is accumulating. Lung injury secondary to endotoxin infusion[109-112] or peritonitis[49, 91, 92] has not been worsened by G-CSF administration. In rats with sepsis from subcutaneous *E. coli*, G-CSF increased lung PMN but no injury, as measured by increased glucose uptake, was demonstrated.[113]

Human studies with G-CSF in infected patients showed no evidence of lung damage or increased incidence of ARDS with treatment.[75, 114, 115] The Phase I safety trial of G-CSF given in varying doses for non-neutropenic community acquired pneumonia revealed no decline in lung function or increased incidence of ARDS in these patients.[75] The follow up prospective randomized double blind multicentre placebo controlled study showed no benefit or harm with the use of G-CSF in non-neutropenic community acquired pneumonia. It should be noted that the patients were not severely ill, as indicated by a mortality of 6%. G-CSF appeared to accelerate the resolution of pulmonary infiltrates, especially in the multilobar pneumonia subset. ARDS was significantly less frequently seen with G-CSF compared to placebo.[115] In one prospective randomized study of low dose G-CSF in SIRS and septic patients, the APACHE II score decreased due to an improvement in the respiratory parameters. In addition, none of the SIRS patients went on to develop MODS, although this study was

limited by the small sample size of the group.[101] One case report told of resolution of ARDS in a leukopenic leukemia patient treated by G-CSF.[116]

Finally, G-CSF was linked with no major side effects in volunteer subjects, in particular no respiratory symptoms were noted.[59, 60, 62, 74] Pajkrt even described blocked passage of radiolabeled granulocytes to the lung in volunteers pretreated with G-CSF.[117] The 0.02% reported incidence of ARDS in G-CSF treated patients occurred in those with other risk factors for ARDS (from Amgen files).[75, 105]

Augmentation of the host immune defense system with G-CSF is currently indicated as prophylaxis in non-infected immunocompromised patients, however, due to concerns of activated PMN mediated host tissue damage in the infected patient, there has been reluctance in employing this therapy in this critically ill segment of the population.

Hypothesis

Animals will respond appropriately with an exudation of PMN to the primary site of infection, be it abdomen or lung. The addition of a second site of infection will render the relatively neutropenic host unable to mount an appropriate response to the insult. This second front of infection will translate into increased mortality, as animals succumb to the second infection.

The use of therapeutic doses of G-CSF during this relatively neutropenic state will curtail the mortality from a second bacterial assault by increasing overall number of neutrophils. Presumably, an overall increase in absolute neutrophil numbers will translate into an increased number of neutrophils available to the second site of infection. If the increased mortality is due to a paucity of neutrophils at the second site to combat the infection as hypothesized, mortality should decrease.

Alternatively, if the concern regarding PMN-associated ARDS is justified, an increase in mortality will result from the administration of G-CSF. This is because the absolute increase in neutrophil numbers with G-CSF translates into an increased share of these neutrophils to the second site of infection, as above. If the diversion of neutrophils from the second site is a protective physiologic mechanism and not a defect as hypothesized above, increasing the number of destructive PMN both systemically and to the second site of infection will increase mortality. This "second hit" of dysregulated function from artificially primed neutrophils in infected animals will result in ARDS/MODS and mortality.

This study will attempt to discover whether neutrophils are protective during a second infection (the second front hypothesis) or whether neutrophils contribute to the increased mortality observed after a second infection (the second hit hypothesis).

Background experiments

The murine model used for this experiment was tested in a series of background experiments performed by Dr. J. Pires. The thermistor data were collected by both Dr. Pires and I, and is interpreted here for the first time.

The animal model used needed to reflect the clinical scenario. Mortality with a second source of infection should be higher than that with a single source for the selected model. One hundred and fifty mice were divided into different intervention groups and observed for mortality. Half this number underwent cecal ligation and puncture (CLP), the other half did not. Each half was then divided into groups having intratracheal (IT) injections of normal saline or *Klebsiella pneumoniae* at doses of $10^{1.5}$, 10^{2} or 10^{3} organisms per animal. The animals were monitored for a week to observe mortality. Post-mortem examination was performed to rule out bowel obstruction as a cause of death.

In addition, as per a requirement by the Animal Ethics Committee, the temperature of the animals was monitored at regular intervals using subcutaneous temperature probes (thermistor). Results were analyzed to determine if temperature could be used as a reliable predictor of mortality.

Dr. Pires' data is summarized in the following figures and established the validity of the murine model of CLP with and without IT *Klebsiella* for the experiments in this thesis (see Table 1 and Figures 1 and 2). Pearson chi square tests were used to determine the statistical significance of the observed differences.

The results showed increased mortality with peritonitis versus without (see Figure 1). There was no difference in mortality between control animals and those undergoing a sham operation (i.e. simple laparotomy). Mortality rates were seen to increase with

increasing doses of IT *Klebsiella*. This effect was even more pronounced in the presence of peritonitis (see Figure 2).

Mortality Rates for Various CLP and/or IT Techniques

CLP	Klebsiella dose	Mort. (%)	# of Animals that Died	Mean survival time	Median survival time	SD for survival time
CLP (-)	0	0%	0/16	152.00	152.00	0.00
CLP (-)	$10^{1.5}$	26%	6/23	134.43	152.00	27.82
CLP (-)	10^{2}	65%	24/37	95.13	80.00	45.11
CLP (-)	10^{3}	89%	16/18	72	64.00	30.22
CLP (+)	0	27%	4/15	124.27	152.00	40.67
CLP (+)	$10^{1.5}$	91%	21/23	78.83	72.00	27.96
CLP (+)	10^{2}	91%	31/34	68.47	60.00	26.07
CLP (+)	10^{3}	100%	16/16	47.73	44.00	14.08

Table1:

This table demonstrates the mortality rate and ratio as well as survival time (in hours) for animals that had undergone either cecal ligation and puncture (CLP) and/or intratracheal instillation of *Klebsiella*. There were three doses of *Klebsiella* used for this experiment. CLP(-) and a *Klebsiella* dose of zero indicate a sham operation. *(Courtesy of Dr. J. Pires)*

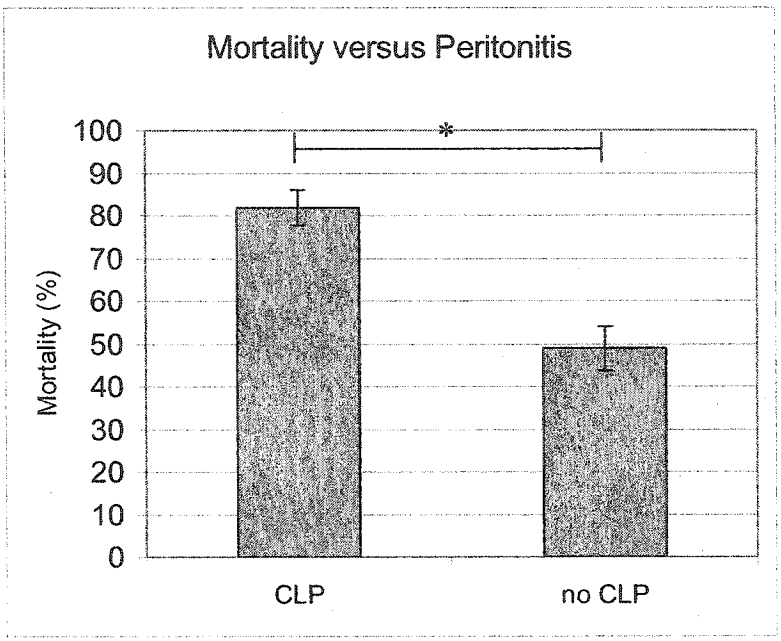

Figure 1:

Mortality rates in the presence or absence of CLP. The presence of CLP is associated with increased mortality. Error bars show standard error of the mean (SEM).
* $p = 0.000$, $n = 94$ (control) $n = 88$ (CLP)
(Courtesy of Dr. J. Pires)

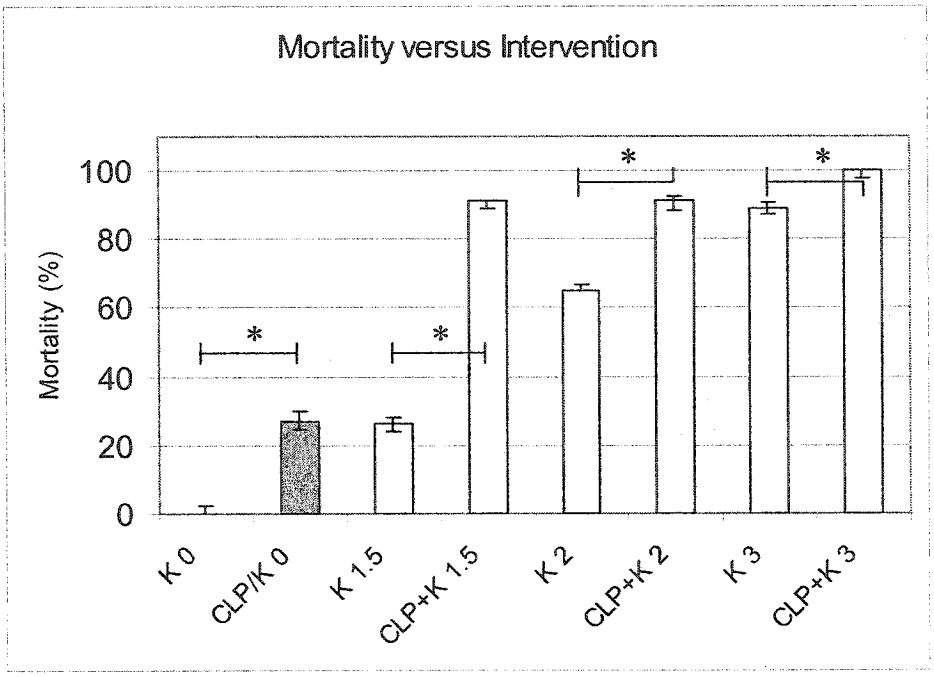

Figure 2:

Mortality rate of control versus peritonitis in the presence of varying doses of *Klebsiella pneumoniae*. The dose of *Klebsiella* (K) is indicated by the exponential value, 0 is control, 1.5 equals $10^{1.5}$, 2 equals 10^2 and 3 equals 10^3. Increasing doses of *Klebsiella* are associated with increasing rates of mortality. Peritonitis (CLP) increases the mortality for each dose of *Klebsiella*. Error bars show standard deviation. * $p < 0.050$
$n = 31$ (0 group) $n = 46$ (1.5 group) $n = 71$ (2 group) $n = 34$ (3 group)
(Courtesy of Dr. J. Pires)

The results of the thermistor data were analyzed using the receiver operating characteristics curve (see Figure 3). The temperature closest to the left upper hand corner was considered the "best fit" balancing specificity and sensitivity for this test. In this case the best fit was a temperature of 34°C.

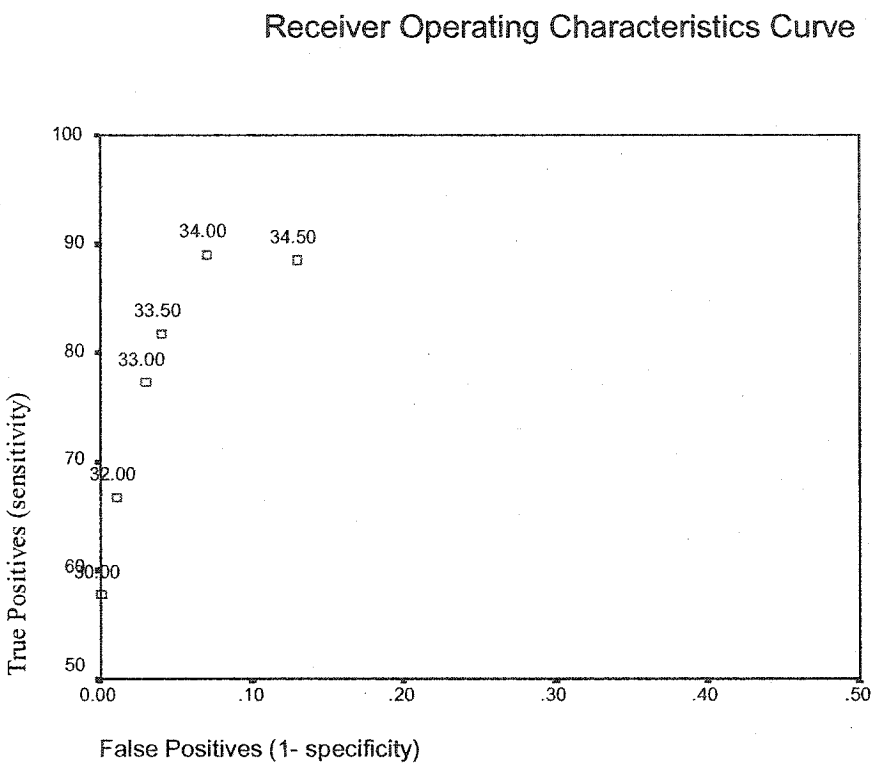

Figure 3:

The receiver operator curve illustrates the sensitivity and specificity for the different temperatures. The X axis represents 1-specificity or the false positive fraction (FPF). The Y axis equals the sensitivity or the true positive fraction (TPF). Above, a temperature of 34°C is the optimal point.

Study Design

There are three components to this study:

1. Neutrophil distribution will be examined in the selected animal model to establish the compartmentalization of neutrophils in the presence of one or two sites of infection. Groups of ten mice will be subjected to CLP or *K. pneumoniae* (at a dose of 10^4 organisms per animal) or both. One group of animals will have bronchoalveolar lavage (BAL) performed. Other animals will have their lungs inflated with 3% gluteraldehyde/formaldehyde in preparation for slide fixation. Another group will have direct peritoneal lavage (DPL) performed and the lungs will be removed, weighed and processed to determine myeloperoxidase (MPO) content as a reflection of neutrophil content.
2. To corroborate the effect of *K. pneumoniae,* groups of ten mice will be given intratracheal injections of lipopolysaccharide (LPS) at a dose of 50μg with and without CLP. The groups will then be subjected to the same post-mortem analyses detailed above. This component will serve to show that the *K. pneumoniae* results are due to inflammation and not merely due to bacteremia. LPS is a component of the cell wall in gram-negative bacteria responsible for endotoxic shock.[118] LPS causes an ICAM-1 mediated neutrophil influx, alveolar macrophage induction of TNF-α, IL-1 and CINC. As well, permeability of lung to albumin was demonstrated.[119]
3. The effect of post-peritonitis administration of hrG-CSF on animal survival in the presence or absence of pneumonia will be evaluated. One hundred and forty mice will be divided into three groups: control, CLP and CLP plus *K. pneumoniae* at a

dose of 10^2 organisms per animal. These groups will be treated with a daily subcutaneous dose of either placebo or hrG-CSF at a dose of 50μg/kg. The animals will be monitored for a week to observe mortality. Subcutaneous temperature probes will be inserted pre-operatively and the animals monitored at regular intervals and sacrificed when temperature drops below the selected threshold.

An increased survival with hrG-CSF would support the second front hypothesis. A decreased survival with hrG-CSF would validate the second hit hypothesis.

MATERIALS AND METHODS

Unless otherwise indicated, all chemicals used in this experiment came from Fisher Chemical, Montreal, Quebec, Canada.

Mice

Specific pathogen-free white CD-1 male mice weighing 30-35g were used in all experiments (Charles River Laboratories). The animals were acclimatized in our facilities a minimum of 3 and a maximum of 7 days before use. They were housed 3-5 animals per sterilized cage in a controlled 12 hour light/dark cycle with an ambient temperature of 22-24°C. Standard Purina laboratory rat chow and water were available *ad libitum*. After intervention, animals were kept in a quarantine room under the same conditions. Unless otherwise indicated, intervention groups consisted of ten mice each.

Anesthesia for the experiments consisted of chamber induction of pure isoflurane (Abbott Laboratories, Montreal, QC), followed by nose-cone ventilation with 2-4% isoflurane titrated to optimal sedation.

Animals were sacrificed via cervical dislocation, and blood samples drawn into heparinized syringes from the inferior vena cava (IVC) or by cardiac puncture, if BAL was not to be performed. The animals were killed 24 hours after the first operation, be it CLP or intratracheal instillation of bacteria.

Cecal Ligation and Puncture

Cecal ligation and puncture was performed under a modified technique of Chaudry[120] which has been used by other investigators.[26, 46, 49, 121] Under anesthesia, the abdomen was prepped with proviodine and a midline incision was made in

the skin. Scissors were used to cut the peritoneum ~ 1cm in the avascular linea alba exposing the peritoneal cavity. The cecum was brought through the wound from its typical location in the left upper quadrant. Feces was milked into the cecum, which was tied at the base with a 2-0 silk tie (Davis & Geck), taking care not to obstruct the bowel. A 22 gauge needle was used to pierce the cecum once or twice depending upon the protocol. For the long term mortality studies two punctures of a 22 gauge needle separated by 1cm were performed in order to effect a sublethal dose of peritonitis.[121] Feces were extruded via the puncture site to ensure patency, and the cecum was carefully returned into the abdominal cavity, taking care not to spill more stool. The incision was closed in one layer with 2-0 silk suture.

Six hours later, under anesthesia, the abdomen was reopened along the same incision and the ischemic cecum distal to the tie excised. The stump and abdominal cavity was irrigated with 10cc warmed normal saline (Baxter Corp., Toronto, ON) and the abdomen was closed in two layers. Subcutaneous injection of 3cc warmed normal saline was performed before rousing from anesthesia to prevent dehydration in the post-operative period.

Previous experiments performed in this laboratory indicated no difference in mortality from sham laparotomies in the presence or absence of pneumonia. Therefore no sham laparotomy group was included in the following experiments.

Intratracheal Instillation of Bacteria

Intratracheal instillation of bacteria (IT) was undertaken according to published procedures.[94, 122] A dose of 10^4 organisms of *K. pneumoniae* (American Type Standard

Collection, MD,) was injected intratracheally[37] in short term experiments in order to assure an impressive inflammatory response[122] A dose of 10^2 was used for the longer G-CSF mortality experiments. *K. pneumoniae* was grown in tryptic soy broth (Difco, Detroit, IL) for 18h at 37°C. The concentration of bacteria per volume was quantified by a standard pour plate method on sheep's blood agar and frozen at -70°C in 0.2cc aliquots. The bacterial concentration was verified with each set of experiments, again using a standard pour plate method on sheep's blood agar. Under anesthesia, after prepping the skin with proviodine (Rougier Inc, Chambly, QC), a transverse cut was made in the skin overlying the trachea, gaining exposure via blunt dissection in the midline between the muscles. The anterior strap muscles were divided to facilitate exposure. The bacteria were injected between two tracheal rings via a 25 gauge needle mounted on an Eppendorf pipette calculated to deliver 25μL. Signs of accurate delivery were hyperventilation or expulsion of suspension via the nares.

When IT was performed in conjunction with CLP, it was performed after excision of the cecum and closure of the abdominal wall, under the same anesthetic.

Previous experiments performed in this laboratory indicated no difference in mortality, hence presumably no marked effect, from saline inoculations in the presence of peritonitis so these groups were not tested further.

Intratracheal Instillation of Lipopolysaccharide

A dose of 50μg LPS (~1.5mg/kg) per animal was chosen since this dose has been demonstrated to achieve an inflammatory reaction in murine lungs.[123-125] Intratracheal instillation of LPS follows the same steps as detailed above, except that instead of the

bacterial suspension, 25μL of sterile normal saline containing 50μg of LPS was injected instead.[119]

The injection of LPS took place 4 hours prior to sacrifice, as opposed to the 18 hour delay for *K. pneumoniae*, because of the Animal Ethics Committee. The animal laboratory technician had observed that the mice were in apparent distress. Since there was published data showing pulmonary reaction in mice 4 hours post-injection, the protocol was modified so that animals were sacrificed 4 hours after intratracheal injection of LPS.[119]

Bronchoalveolar Lavage

Bronchoalveolar lavage was performed as follows. After sacrifice, the trachea was exposed as per the method for IT and then isolated by dividing all surrounding muscles. A 22 gauge intravenous catheter, bevel cut to a length of 0.5cm was inserted intratracheally and secured with a 2-0 silk tie. Aliquots of 0.5cc of ice cold Dulbecco's phosphate buffered saline (dPBS) + 3mM EDTA (GIBCO, Grand Island, NY and Becton Dickinson, Franklin Lanes, NJ) were delivered slowly from 1cc syringes, then aspirated after massaging the chest wall for 10 seconds. This was repeated twice. The syringe was replaced twice for a total of nine washes: three syringes with three washes per syringe (Dr. C. Doerschuk personal communication). The samples were pooled and the volume recorded before centrifugation at 250g (IEC Centra-8R centrifuge) for 10 minutes.

The supernatant was subjected to the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) of the spectrophotometer (Beckman DU530 spectrophotometer, Montreal, QC) to determine protein levels.

The remaining pellet was resuspended in 100μL of PBS solution. A 50μL sample was diluted in 450 μL Turk's stain or 0.01g gentian violet and cell counts were performed using the haemocytometer. The formula used to calculate the number of PMN per milliliter of bronchoalveolar lavage fluid (# PMN /cc BAL) is as follows:

$$\#\ \text{counted} \times (2.5 \times 10^4) \times 100\mu L \div \text{BAL volume}$$

Direct Peritoneal Lavage

This was performed by a modified method described by others.[35, 84] Anesthetized animals had a nick made through the skin and linea alba large enough so as to introduce a 16 gauge angiocatheter which had been perforated distally multiple times with the needle of the angiocatheter. Irrigation of the peritoneal cavity with 5cc flush of ice cold PBS + 5mM EDTA from a 10cc syringe and followed by gentle aspiration was carried out three times per syringe for two syringes, giving a total of six flushes of the peritoneal cavity. The samples were combined and the volume recorded before analysis of protein and PMN content, as described above for BAL.

Myeloperoxidase Assay (MPO)

Tissue, in this case right mouse lung was weighed then processed for MPO using 3,3'5,5' tetramethylbenzidine as per Nathens *et al*[119] and Dr. T. Takano (personal communication). This method, attributed to Suzuki[126] and used by other laboratories[127-130] was preferable to the carcinogenic o-dianisidine method described by Bradley.[131, 132] The tissue was homogenized for 45 seconds at high speed using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) in 3cc of 10mM

potassium phosphate solution, pH = 7.4. The homogenate was centrifuged at ~12,000g at 4°C for 20 minutes (Beckman J2-21M Induction Drive Centrifuge) and the supernatant was discarded. The pellet was re-homogenized in 3cc of 50mM potassium phosphate solution + 0.5% HTAB, pH = 6.0. The samples were frozen overnight at -70°C and then thawed and re-homogenized the next morning. Sonication at 30W for 45 seconds (Fisher Sonic Dismembranator 400) then centrifugation resulted in MPO rich supernatant. The supernatant was examined for protein (Pierce BCA protein assay) and for MPO content.

MPO was measured by the spectrophotometrical change in optical density at 655nm (OD_{655}) over 45 seconds (Beckman DU 530 spectrophotometer). The reaction was effected in microcuvettes (Starstedt, Newton, NC) containing 350µL of 220mM potassium phosphate buffer with 110mM sodium chloride, 50µL of supernatant and 50µL of 16mM 3,3',5,5' tetramethylbenzidine (Sigma, St. Louis, MO) in N,N-dimethylformamide. The reaction was catalyzed by 50µL of 3.0mM hydrogen peroxide.[126, 129]

Harvesting PMN for standard curve

Mice were injected intraperitoneally with 2cc of 2% casein hammarsten (BDH Chemicals, Poole England).[127, 133] This was prepared by initially dissolving the casein in 1N sodium hydroxide and then gradually titrating to neutral pH using 1N hydrochloric acid to avoid precipitation. DPL as described above was performed 4 hours later. The number of PMN was counted by hemocytometer and calculated for the sample volume. Two to four mice were required to obtain adequate numbers of PMN. The sample was serially diluted to obtain decreasing numbers of PMN per dilution and the different

dilutions were analyzed for MPO content. Both septic and control mice were used to obtain these neutrophils. There was no demonstrable difference in MPO expression from septic versus control PMN.[134]

Several studies have demonstrated the linear relationship of MPO to PMN. Our results corroborated these findings (see Figure 4).[127, 132, 135]

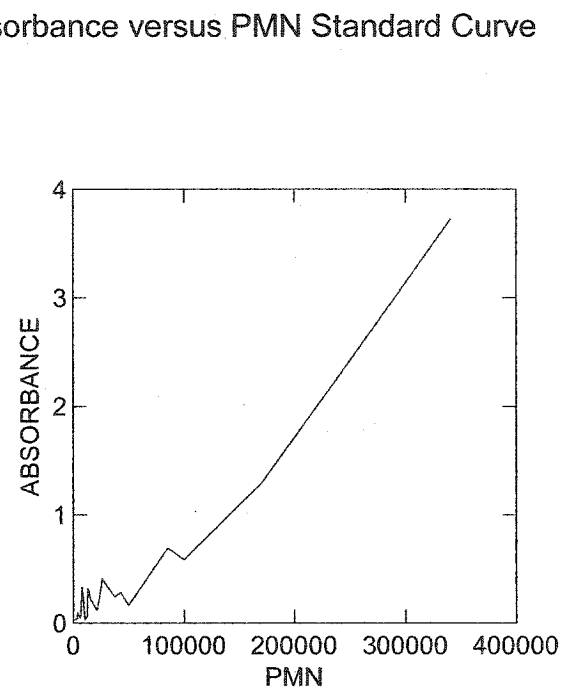

Figure 4:

Absorbance versus neutrophil count demonstrating a linear relationship. The PMN were isolated from healthy and septic mice, counted and serially diluted to give a wide range of values.

The equation obtained from this graph was:

$$\text{absorbance} = -0.0407 + 9.98 \times 10^{-6} \div \text{PMN}$$

There was significant variability seen in the absorbance at levels below 4000 PMN. This non-linear pattern may have been due to the sensitivity of the readings and probably indicated the threshold limit for this test. Analysis of the linear graphs from the three previously mentioned studies[127, 132, 135] revealed that the smallest number of neutrophils used in these studies was $3.5 \times 10e4$. This number was much higher than the 4000 PMN threshold noted above.

Given this limitation, the equation for the least square regression line was recalculated. When values of absorbance at levels below 4000 PMN were omitted in the calculations, the following equation was the result:

$$\text{absorbance} = -0.05847 + 1.01 \times 10^{-5} \div \text{PMN}$$

This second equation was used to calculate PMN from absorbance in the final analysis.

PMN counts in mouse blood

Heparinized samples of 0.5cc mouse blood drawn from the IVC or via cardiac puncture were sent to the hematology laboratory (Royal Victoria Hospital, Montreal, QC) for processing. The samples were processed in the machine and the results were reported as WBC counts $\times 10^9$ and PMN counts $\times 10^6$. PMN counts were added to lymphocyte counts as per the printout because of concerns that the differential count calculated by the machine underestimated PMN in mouse blood since it was calibrated for human and not murine blood.

PMN separation of mouse blood

After loading 1cc of mouse blood on 3cc of Ficoll Hypaque (Pharmacia Inc., Piscataway, NJ), it was placed in the centrifuge for 400g at 4°C for 25 minutes (IEC Centra-8R centrifuge). The top and middle layers were discarded. Vigorous shaking was used to break up the pellet. Red cells were lysed by 6cc sterile water for 20 seconds before adding 2cc of 3.6% hypotonic saline. This was centrifuged for 5 minutes at 400g at 4°C. The supernatant was discarded and to the remaining pellet was added 8cc of PBS without calcium or magnesium.

The resulting solution was centrifuged again for 5 minutes at 400g at 4°C. The supernatant was discarded and 100μL of media was added to the pellet. An aliquot of 50μL was diluted in 450μL of Turk's and this solution was counted by haemocytometer. The final count was calculated as per the formula for BAL to arrive at the number of PMN per mL of blood (# PMN / cc blood).

Histological analysis of lungs

After sacrifice, the trachea was isolated and the bevel cut intravenous catheter secured as per the protocol for BAL. The sternum was split to visualize the lungs. The left lung was inflated with 3% glutaraldehyde/formaldehyde solution (McGill Pathology, Royal Victoria Hospital, Montreal, QC) at 25cm of water pressure. Then the hilum of the lung was clamped to prevent deflation of the specimen. The lung was left to bathe in a 3% gluteraldehyde/formaldehyde solution for 24-48 hours before sectioning the specimen along the midsagittal plane. Epoxy fixation was performed. The slides were examined under a microscope by myself blinded to the coded investigation groups, for evidence of

pneumonia and PMN sequestration. PMN were tallied in the intravascular, interstitial and intraalveolar spaces.

Blood and lung cultures

Blood samples were drawn by cardiac puncture after proviodine preparation of the skin, into aseptic heparinized syringes. Three 50μL samples were applied to sheep's blood agar under aseptic conditions, and colony counts were made after a 24 hour incubation period at 37°C.

The right lung of the animals was harvested under the fume hood of the quarantine room after cleaning the skin with proviodine. The surgical instruments were soaked in alcohol between animals. Wet weights of the lungs were recorded and the samples were homogenized in 1cc of sterile normal saline. The homogenizer tip was soaked in alcohol between samples. Three 50μL samples were applied to sheep's blood agar under aseptic conditions, and colony counts were taken after a 24 hour incubation period at 37°C. For the above, serial dilutions were performed where required in order to obtain reasonable counts.

Neutrophil CD11b adhesion molecule expression

As per the protocol of Wang,[136] 100μL sample of PMN incubated with either LPS, TNFα or normal saline was mixed with 10μL of FITC-conjugated anti-CD11b mouse anti-rat antibody or 10μL of FITC-conjugated murine IgG2a isotypic control mAb and incubated for 15 minutes at 25°C. The results were analyzed by flow cytometry.

Activity of LPS was measured by mean channel fluorescence for neutrophil surface CD11b receptor expression and shown to be slightly less than TNFα, reading 180 versus 212, but higher than the control value of 106. The LPS in this experiment was shown to be able to prime and activate PMN.

G-CSF mortality studies

Filgastrim, a nonglycosylated form of hrG-CSF used for this study, was generously donated by Amgen, Canada. Homology between mouse and human G-CSF is 50-65%.[54] Temperature probes (IPTT 100, BioMedic Data Systems Inc. Delaware, USA) were inserted into the dorsal subcutaneous tissue during the initial anesthetic. Previous studies with implantation of the probes 12 hours before intervention had demonstrated no adverse effect or significant temperature variations due to these probes alone. The intervention groups were operated on as above, using a single puncture of the cecum and/or 10^2 organisms of *K. pneumoniae* intratracheally. Subcutaneous injections of G-CSF or vehicle (millipore filtered preparation of 100μL D5W + 2.0mg/cc bovine albumin) were administered immediately after CLP/sham operation and daily for six days in a blinded fashion. A dose of 50μg/kg was chosen as it was demonstrably effective in murine models.[85, 88, 94-96, 113] Temperature readings were performed every four hours or eight hours overnight (DAS-5007 Pocket Scanner System, BioMedic Data Systems Inc, Delaware, USA) and animals were sacrificed if observed to be agonal or if body temperature dropped below 32°C, an indication of 99% mortality. Data confirming the predictive nature of body temperature in this murine model was performed in a series of background experiments. The receiver operating characteristics curve[137-139] used to

analyze this data showed the optimal temperature to be 34°C (see Figure 3) however in order to minimize the false positive sacrifices, a temperature of 32°C was chosen. Only one animal with a temperature <32°C survived the week, giving a false positive rate of 0.01%.

Statistics

Data was analyzed using two group *t*-test with Bonferroni correction. The exception was the analysis of mortality results from the G-CSF experiments using Pearson chi square test. Results were considered statistically significant if $p \leq 0.05$. Analysis of the slope for the standard curves of MPO per PMN was calculated by linear regression. The optimal temperature threshold for estimating mortality was plotted on a receiver operating characteristics curve using Systat 8.0 for Windows software (SPSS, Chicago, IL). All data was analyzed using Systat 8.0 for Windows software. Preparation of the figures and tables was from Microsoft ® Excel 2000.

RESULTS

Distribution of PMN

Serum blood count

The second front theory hypothesizes that the first site of infection sequesters PMN, rendering the host neutropenic for the second infection. Serum blood counts were performed to test for neutropenia (see Figure 5).

The serum PMN levels for control and saline IT, both non infected groups, were compared. No significant difference was seen between these two groups (p-value = 0.436). The slightly higher mean number of PMN in serum seen with the saline group may be secondary to demargination from the stress of surgery.

Serum PMN of the control group was then compared to the septic groups. With a single site of infection present (i.e. CLP, IT, LPS) serum PMN levels were significantly less than control (p-value = 0.019, 0.015 and 0.019 respectively). Serum PMN counts between the groups with a single site of infection were not significantly different from each other.

Serum PMN levels of animals with two sites of infection (CLP+IT and CLP+LPS) were significantly lower than control as well (p-value = 0.001 and 0.000 respectively). Again there was no difference between the two infected groups.

Comparison of serum PMN between those animals with only intratracheal insult versus those animals with peritonitis preceding the intratracheal insult showed diminished levels of circulating PMN in those with antecedent peritonitis. Animals with only IT had significantly higher serum PMN levels than CLP+IT, similarly animals with LPS had significantly higher serum PMN levels than CLP+LPS (p-values = 0.029 and 0.012 respectively).

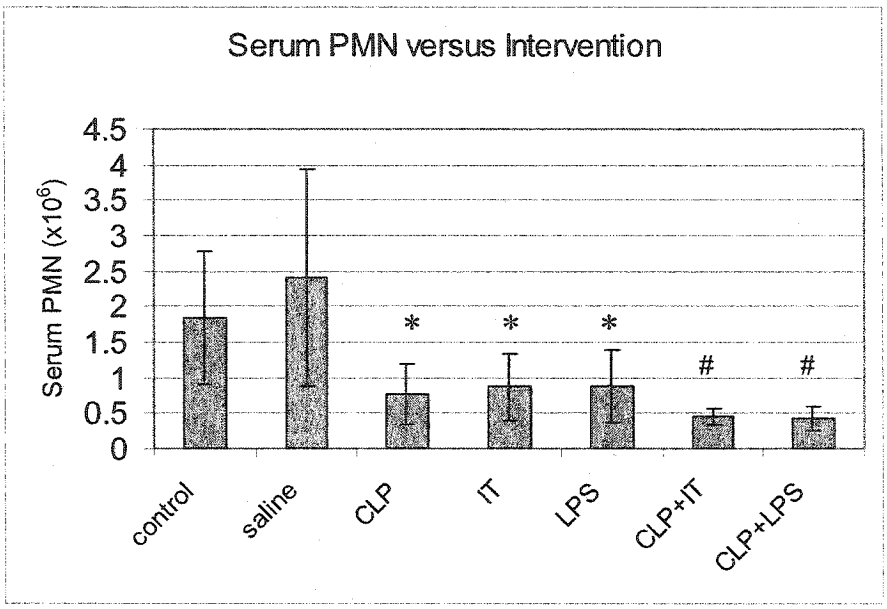

Figure 5:

Neutrophil (PMN) content in arterial blood in intervention groups. The single intervention groups do not differ from each other and are lower than control or saline. The double intervention groups do not differ from each other and are lower than IT or LPS respectively. Values are mean ± SD. * $p < 0.05$ compared to control, # $p < 0.05$ compared to IT or LPS
CLP = cecal ligation and puncture, IT = intratracheal pneumonia, LPS = intratracheal lipopolysaccharide, saline = intratracheal normal saline. n = 7-10 per group

DPL data

The hypothesis postulates the observed drop in circulating PMN to be a result of sequestration. The DPL fluid was analyzed as the first site of infection, the peritoneum, to determine if PMN sequestration in the peritoneal cavity had occurred. Elevated levels of PMN in the DPL fluid of animals experiencing peritonitis are expected. Control animals and those with only intratracheal intervention would not be expected to have significant numbers of PMN in the peritoneal cavity.

DPL fluid demonstrated markedly elevated counts of PMN in the lavage of those animals subjected to peritonitis (see Figure 6). The numbers of PMN in the DPL fluid of

control animals was significantly less than in the CLP, CLP+IT and CLP+LPS groups (p-value = 0.000). To determine if this was due to a simple increase in the cellularity of DPL fluid, the ratio of PMN to other cells was calculated. The same three groups had a ratio greater than two, compared to the others which had ratios less than one, indicating a minority of PMN. Further, there is no statistically significant difference between the infected groups in the number of other cells present in the DPL fluid (data not shown).

Interestingly, while the saline and IT group had PMN present in the lavage in numbers no different from control (p-value = 0.318 and 0.505 respectively), the LPS group had significantly more PMN than the control, saline and IT groups but significantly less than the CLP, CLP+IT and CLP+LPS groups (p-value = 0.000).

Analysis of the three peritonitis groups showed significantly more PMN in the CLP+LPS group than the CLP+IT group (p-value = 0.002). The CLP group was not different from either.

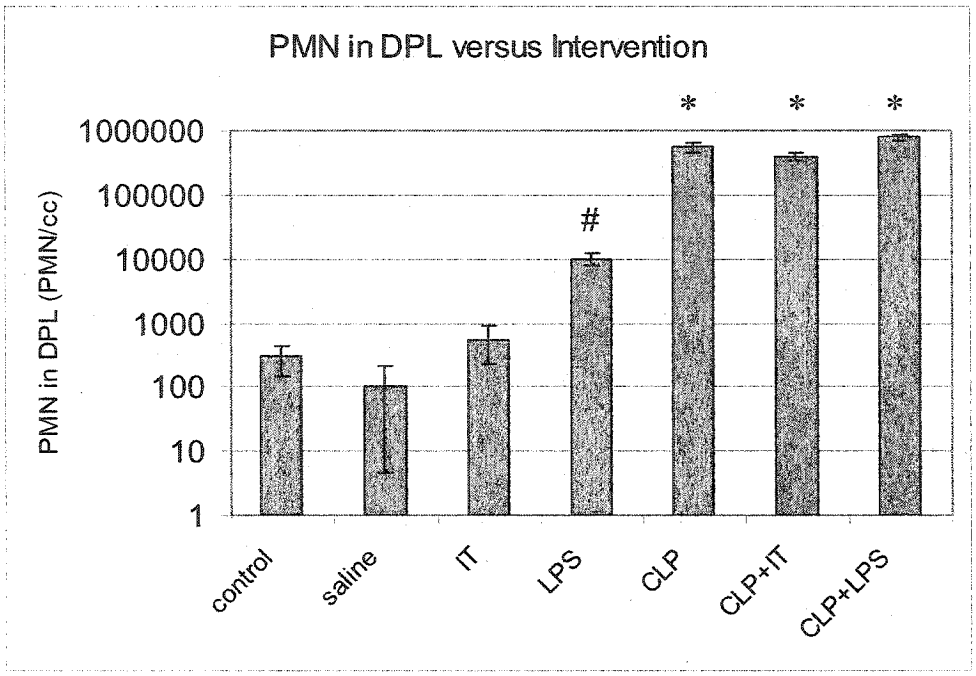

Figure 6:

Logarithmic graph of neutrophils (PMN) per cc of peritoneal lavage fluid in intervention groups. All CLP groups had significantly increased PMN compared to non-CLP groups Values are mean ± SEM. * $p < 0.05$ compared to control, saline, IT, LPS, # $p < 0.05$ compared to all other groups.
CLP = cecal ligation and puncture, IT = intratracheal pneumonia, LPS = intratracheal lipopolysaccharide, saline = intratracheal normal saline. $n = 12$ per group

Information regarding volume returns for DPL was collected to see if PMN sequestration was associated with increased fluid sequestration. Indeed, the peritonitis groups had significantly higher volume return of DPL fluid than control, saline or IT (see Figure 8). There was no difference among the peritonitis groups. Also showing a high volume of return was the LPS group. The volume was equivalent to the CLP+LPS group.

Protein levels of this DPL fluid were collected and analyzed to determine whether the increased volume was secondary to increased permeability. Again the peritonitis groups had significantly more protein than the control, saline or IT groups. The protein

level of the LPS group this time however was similar to control. The DPL volume and protein data are shown together for easy comparison (see Figure 7).

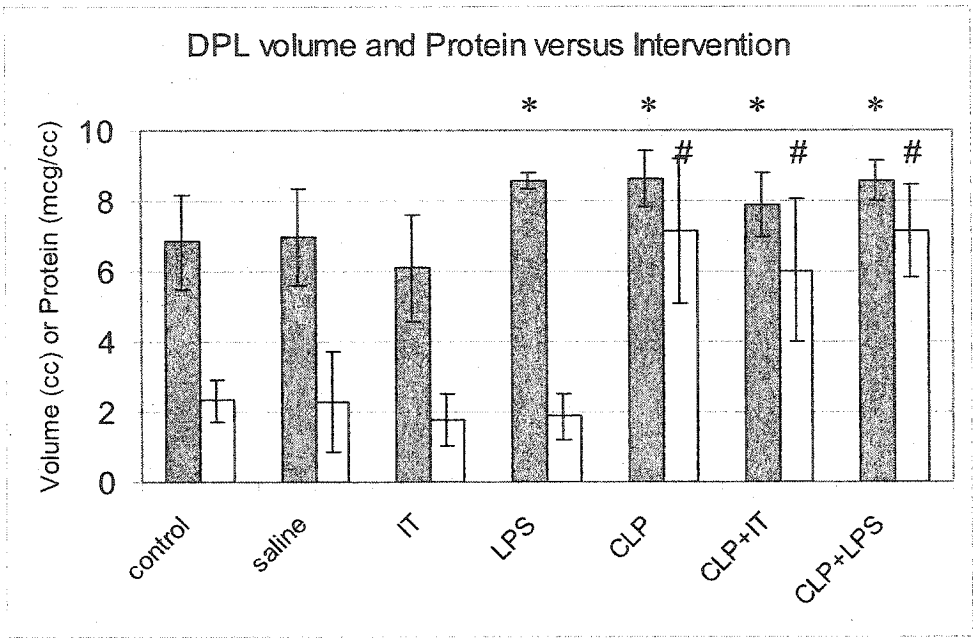

Figure 7:

DPL volume (solid column) and protein concentration (striped column) in intervention groups. All CLP groups and LPS had more DPL volume than control, saline or IT. Only CLP groups had more protein. Values are mean ± SD. * $p < 0.05$ compared to control, saline, IT, # $p < 0.05$ compared to control, saline, IT and LPS.
CLP = cecal ligation and puncture, IT = intratracheal pneumonia, LPS = intratracheal lipopolysaccharide, saline = intratracheal normal saline. $n = 12 - 14$ per group

BAL data

The hypothesis further predicts that as the second site of infection, the lung would have fewer PMN than if it were the only site of infection. The number of PMN in the lungs was measured in two ways: BAL and MPO. The BAL results measured the number of intraalveolar PMN. MPO measured PMN residing in the entire lung, i.e. the sum of the intraalveolar, interstitial and intravascular compartments.

BAL PMN counts were collected for the different intervention groups (see Figure 8). The hypothesis suggests that IT or LPS alone would result in high numbers of BAL PMN because it is the primary site of infection. The CLP, control and saline groups would have comparatively low baseline numbers of BAL PMN. The double intervention group would be expected to have diminished levels of PMN compared to its counterpart, in other words, CLP+IT would be less than IT and CLP+LPS would be less than LPS.

PMN counts were found to be markedly elevated in all groups versus control (p-value < 0.015) except for control versus CLP+IT (p-value $= 0.190$). The CLP and saline groups were equally elevated (p-value $= 0.458$).

The IT group was significantly elevated as expected compared to all groups (p-value $= 0.000$). As predicted, the CLP+IT group had significantly diminished numbers of PMN compared to the IT group (p-value $= 0.000$).

The LPS group however did not have PMN counts similar to IT as was expected. Instead the PMN count was similar to saline, CLP, and CLP+IT (p-value > 0.486) and significantly less than IT (p-value $= 0.000$). However, CLP+LPS did show a trend toward fewer PMN compared to LPS alone, although this difference was not statistically significant (p-value $= 0.069$).

When compared as the ratio of PMN to other cells, all groups had a ratio less than one, except the IT group whose ratio approached five. There was no difference in the number of other cells for the different groups (data not shown).

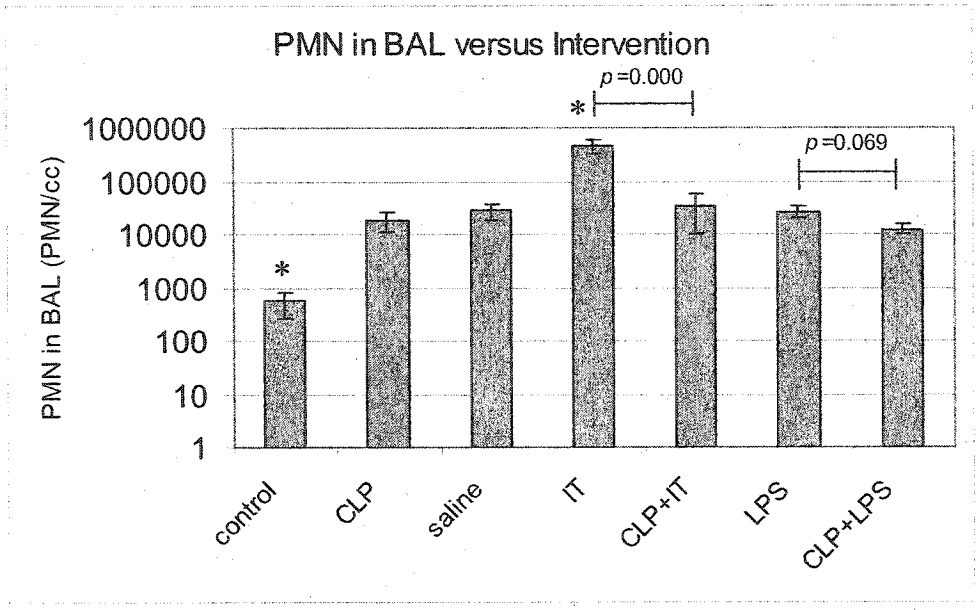

Figure 8:

Logarithmic graph of neutrophil (PMN) in bronchoalveolar lavage fluid in the intervention groups. IT had more PMN than all groups and control had less than all groups. Values are mean ± SEM. * $p < 0.05$ compared to all groups except control versus CLP+IT.
CLP = cecal ligation and puncture, IT = intratracheal pneumonia, LPS = intratracheal lipopolysaccharide, saline = intratracheal normal saline. $n = 10$ per group

BAL volume was collected and analyzed for protein content as done for DPL (see Figure 9). This was done to determine whether the influx of PMN was associated with increased fluid from increased permeability of the endothelium.

Results from BAL revealed volume returns that were consistent for all groups except LPS. This group had significantly less volume compared to control, saline, IT and CLP+IT groups (p-value < 0.047). The IT group itself had statistically greater volume only when compared to LPS (p-value < 0.010).

Results from the protein content of the BAL fluid did not mirror the results from BAL volume. Protein in the IT group was significantly higher than all groups (p-value <

0.024) except CLP+LPS. CLP+LPS group however, did not differ from the other groups (p-value > 0.076). The results of BAL volume and protein concentration are graphed together. The results did not support increased permeability of the lung endothelium in any of these animals. The increased protein level of the IT group could reflect the presence of acute phase reactants or chemotactic factors in the lung. This raises an interesting point which is expanded upon in **Future Projects**.

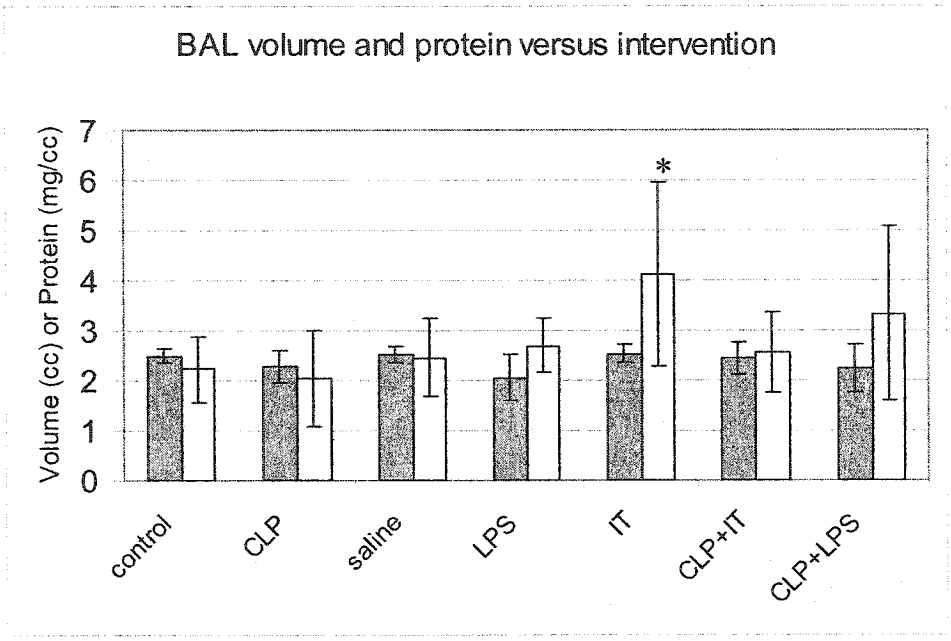

Figure 9:

Volume (solid column) and protein concentration (striped column) of intervention groups. LPS volumes were less than all groups except CLP and CLP+LPS (p-value < 0.047). Protein level in the IT group was higher than all groups except CLP+LPS. Values are mean ± SD. * $p < 0.05$ IT compared to all groups
CLP = cecal ligation and puncture, IT = intratracheal pneumonia, LPS = intratracheal lipopolysaccharide, saline = intratracheal normal saline. $n = 10$ per group

Lung tissue neutrophil content

Gross examination of the lungs revealed no visible difference between the right and the left lung, septic or control groups. Right lung wet weights were consistent between all groups (see Figure 10).

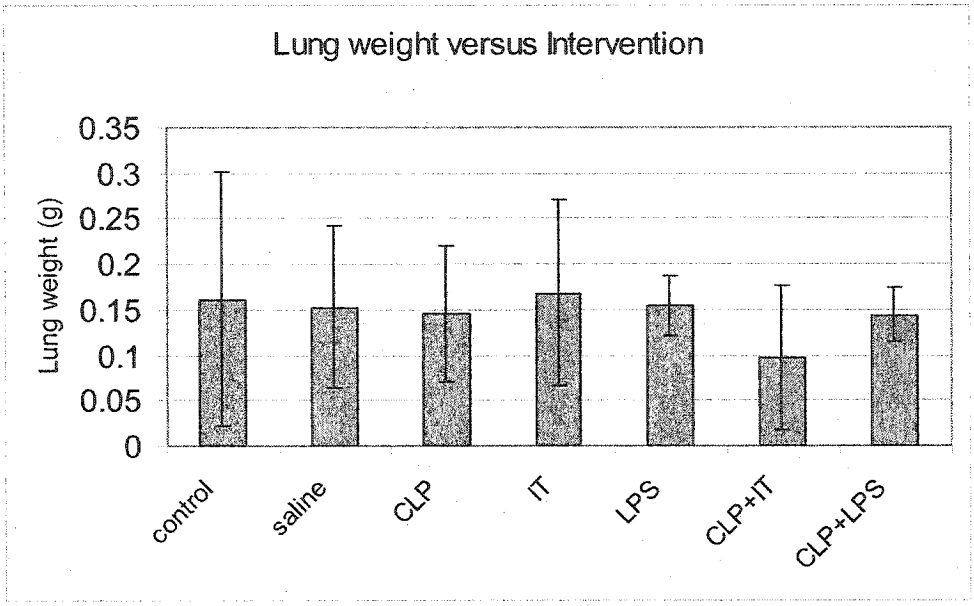

Figure 10:

Wet lung weight for the intervention groups. There was no significant difference between groups ($p > 0.106$). Values are mean ± SD. CLP = cecal ligation and puncture, IT = intratracheal pneumonia, LPS = intratracheal lipopolysaccharide, saline = intratracheal normal saline. $n = 10$ per group

Myeloperoxidase data was collected from processing the entire right mouse lung to calculate PMN sequestration in the lung. This count would include not only alveolar PMN, measured above in the BAL fluid, but also intravascular and interstitial PMN. One may therefore expect the MPO results to mirror those of the BAL PMN since BAL PMN is a component of MPO. However this would only be true if PMN distribution in the lung

remained the same. Any discrepancy between MPO and BAL results would imply a difference in the amount of interstitial or intravascular PMN of the lungs.

Absorbance data reflecting the amount of myeloperoxidase enzyme in the lung was converted to numbers of PMN using the equation obtained from the least squares linear regression of the standard curve derived in the **Materials and Methods** section. Figure 11 illustrates the numbers of calculated PMN versus intervention groups. CLP+LPS and IT groups were significantly higher than CLP, CLP+IT, saline and control groups. The LPS group was not significantly different from either group. These results are compromised by the fact that they are not standardized.

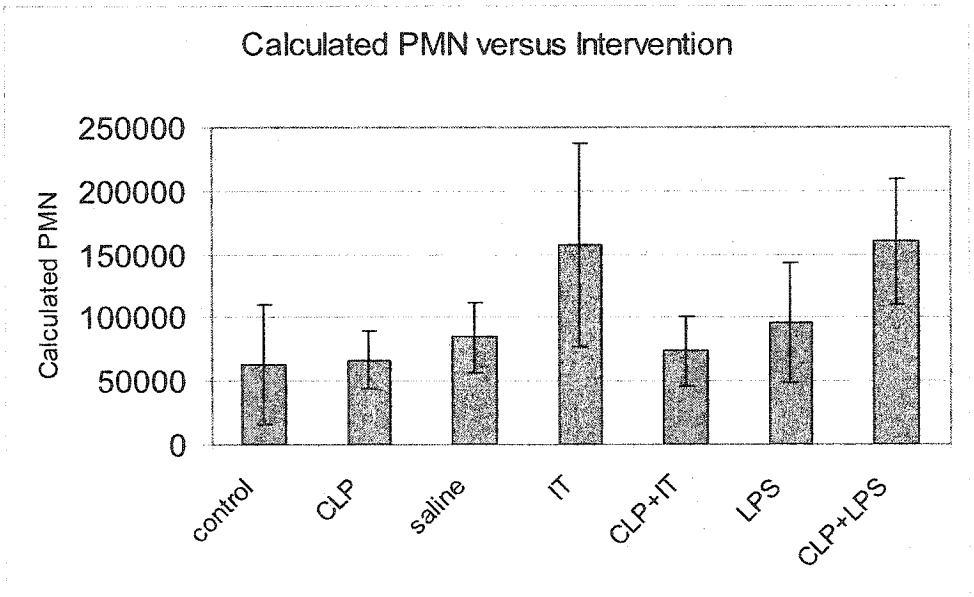

Figure 11:

Calculated number of PMN from absorbance OD_{655} levels in the intervention groups. PMN was calculated from the least squares linear regression equation obtained using the standard curve. CLP+LPS and IT groups show significantly more PMN compared to all groups except LPS. Values are mean ± SD. CLP = cecal ligation and puncture, IT = intratracheal pneumonia, LPS = intratracheal lipopolysaccharide, saline = intratracheal normal saline. n = 12 per group

Despite the insignificant difference between groups for the wet lung weights, these results in Figure 11 cannot be taken at face value and therefore further calculations were performed.

In order to standardize the absorbance OD_{655} levels for each animal, the absorbance OD_{655} level was divided by the protein content for each sample to give a result of absorbance per gram of protein defined as MPO. This was consistent with results published by other laboratories.[119] Total lung protein levels were not different between the groups (data not shown) and were significantly higher (g/μL) than protein levels found in DPL (μg/cc) or BAL (mg/cc). Although there were no differences in protein levels between the groups, the calculated MPO units for the different intervention group demonstrated significant differences between the IT group and control, CLP, CLP + IT and LPS (see Figure 12).

Control and CLP were similar to each other (p-value = 0.311). Of the intratracheal intervention groups, IT was elevated compared to saline (p-value = 0.065) and both were elevated compared to control (p-value <0.018). The MPO value for the LPS group was less than IT (p-value = 0.017) somewhere between control and saline (p-value = 0.106 and 0.906 respectively)

The level of MPO measured in the lungs when CLP was performed prior to the induction of IT pneumonia dropped significantly. The MPO found in the CLP+IT group was less than that found in the IT group alone (p-value = 0.007). In fact it was no different than saline or control (p-value = 0.154 and 0.199 respectively). Conversely MPO level in the CLP+LPS group was higher than the LPS group although this was not statistically significant (p-value = 0.322).

When the two double intervention groups were compared CLP+LPS had relatively more MPO than CLP+IT (p-value 0.081).

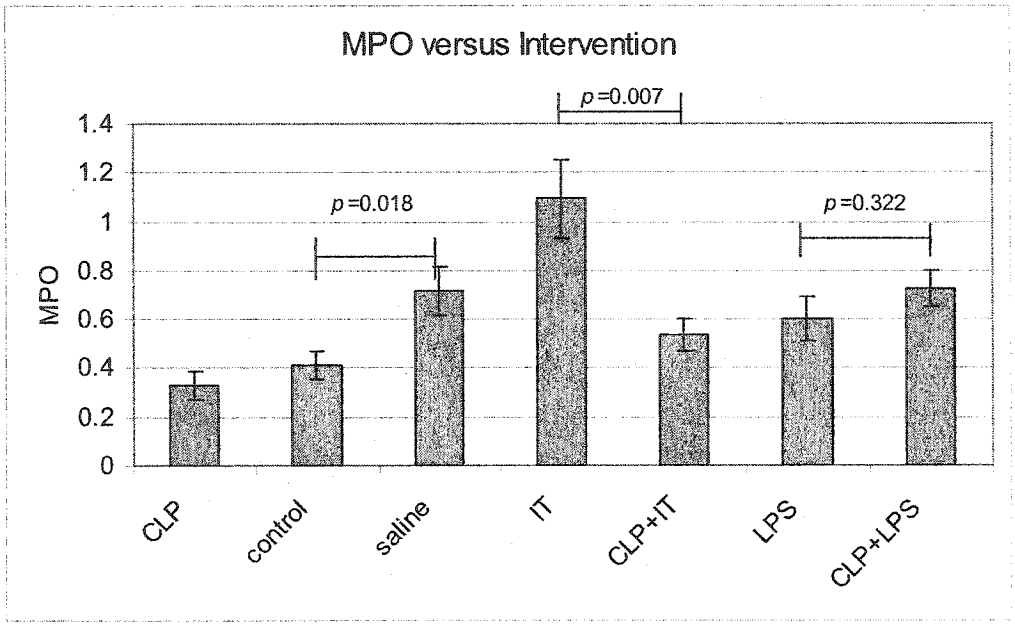

Figure 12:

MPO units compared to intervention. MPO was calculated as absorbance OD_{655} divided by protein content. Control and CLP were similar. The IT and saline groups had significantly greater MPO units than control. LPS did not. The MPO of CLP+IT was less than IT but CLP+LPS appeared greater than LPS. Values are mean ± SEM. CLP = cecal ligation and puncture, IT = intratracheal pneumonia, LPS = intratracheal lipopolysaccharide, saline = intratracheal normal saline. n = 12 per group

Lung Histology

Microscopic examination of the lungs was undertaken in order to further analyze the results of the BAL and MPO data. These data suggested increased numbers of PMN in the intra-alveolar space as well as the whole lung compared to control. Histologic study would help to determine whether the PMN were in the intra-alveolar, intravascular or interstitial space and if so in what proportion.

Examination of the slides under light microscopy failed to detect any significant number of PMN. Dr. Michel studied a random sample of slides and failed to detect any evidence of pneumonia on IT or CLP+IT slides. Discussions with Dr. Doerschuk, a pathologist published in this technique, failed to reveal any discrepancy between my technique and hers. There were no intraalveolar PMN observed in any of the slides and the interstitial PMN counts were negligible in all groups (data not shown).

Blood Culture

In an attempt to validate the presence of pneumonia in the animals, blood and lung cultures were taken as described under **Material and Methods**. Unfortunately, neither blood cultures nor lung cultures could be attained aseptically. CLP groups in both sets were contaminated by *Klebsiella*, the same strain as that used in the experiments. The control group was not affected but this group was harvested at a separate time from the intervention groups.

For the blood collection this contamination may have occurred from the fur. The instrument used to perform cervical dislocation on the mice was not cleaned between animals and perhaps the single wash with proviodine solution prior to cardiac puncture was not adequate to sterilize the puncture site. More appropriate shave and skin preparation was not feasible due to the quick coagulation time after cervical dislocation was performed resulting in little to no blood return upon cardiac puncture.

Contamination of the lung tissue was less likely to arise from the fur since there was adequate time to perform three washes with proviodine. Possible sources of the contamination were the instruments: the scissors, forceps or likely the homogenizer. These instruments were dipped in alcohol solution between animals and the homogenizer

was run for 30 seconds in alcohol solution between specimens. The homogenizer however has many interstices at its tip which was impossible to sterilize completely without dismantling the machine. For MPO, a simple rinse was all that was described in the protocol and the results seemed to support this method as adequate, but perhaps for lung cultures a longer rinse for sterilization is required.

G-CSF mortality study

Of 140 mice for the G-CSF study, 2 died under anesthesia, 3 were excluded because of mesenteric bleeding (2 from the placebo CLP+IT group, 1 from the G-CSF CLP group) and 1 was excluded because of cecal rupture from the G-CSF CLP group. This leaves a total of 134 animals for analysis. The mortality is illustrated in Figure 14. There were no deaths in either control group. There were no abscesses found in any of the dead animals. Bowel obstruction was found in 3 animals.

The results of Pearson chi square analysis for survival versus intervention and/or G-CSF follow. There was a significant relation (p-value = 0.008) noted for survival versus intervention. Individual analysis of survival between the intervention groups revealed that this difference was between control and CLP+IT. There was no significant difference in mortality between control and CLP or between CLP and CLP+IT.

Survival versus G-CSF for all intervention groups was not significant when analyzed individually (see Figure 13). Analysis of survival versus G-CSF within the CLP and within the CLP+IT groups individually failed to uncover any statistically significant difference (p-value = 0.328 and 0.089 respectively). The risk ratio for survival based on G-CSF or placebo was not significantly different between these groups, with the 95% confidence interval straddling one.

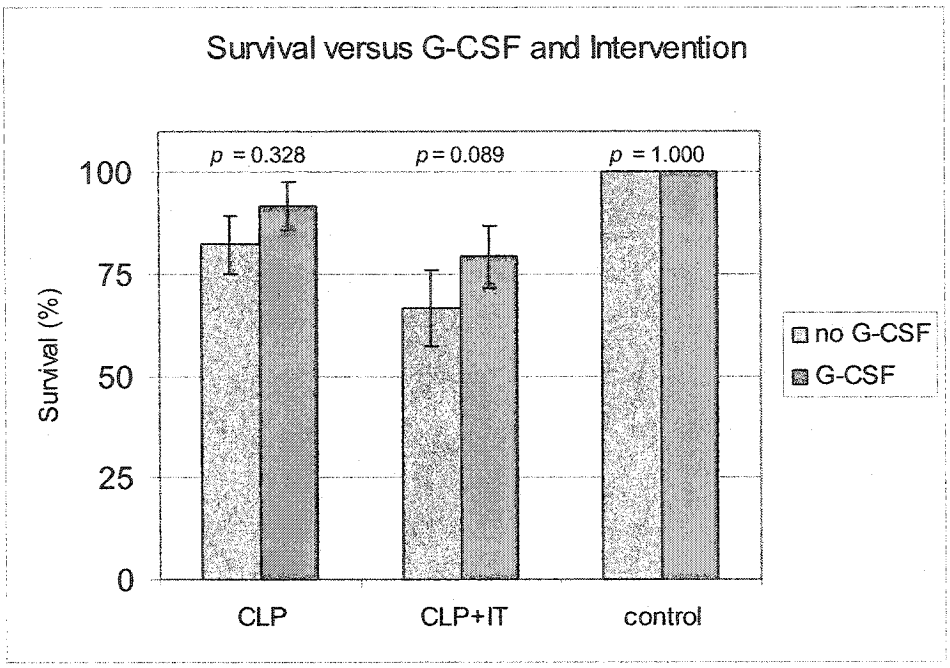

Figure 13:

This graph illustrates the difference in survival rates within the different intervention groups: CLP, CLP+IT and control for placebo (solid column) versus hrG-CSF (striped column). Values are mean ± SD. Despite the visual disparity, there was no statistical difference upon analysis. $n = 45$ (CLP) $n = 48$ (CLP+IT) $n = 26$ (control)

DISCUSSION

Studies have shown nosocomial infections to be associated with PMN dysfunction.[28, 140] This study was designed to test the theory that the increased mortality observed after a second infection was due to insufficient PMN recruitment to this second site. Furthermore, if the higher mortality observed with a second site of infection was due to insufficient PMN numbers, would administration of hrG-CSF after the initial insult but prior to the second infection attenuate this mortality rate by augmenting systemic PMN levels?

Data from the *Klebsiella pneumoniae* experiments demonstrated patterns of PMN sequestration in this animal model to validate this hypothesis. Data from the LPS experiments did not do so and possible explanations are discussed further below.

Klebsiella pneumonia

The hypothesis was supported by the results of the *Klebsiella* subgroup of experiments. Significant neutropenia was achieved versus control and saline in both CLP and IT groups. A further decrease was seen when CLP and IT was combined. The neutropenia observed in the infected groups was consistent with expectations of sepsis.[13, 113, 141] The twice infected groups had even less circulating PMN than the singly infected group as predicted. Similar findings were recently published.[142] This decline in PMN could be due to decreased production, increased destruction, and/or sequestration.

The results further show that neutropenia was associated with significant sequestration of neutrophils in the affected compartment. For CLP and CLP+IT this compartment was the peritoneum. For IT and CLP+IT this compartment was the lung. In

the CLP+IT group, the initial insult was CLP and the distribution of sequestered neutrophils reflected this. Neutrophil levels in the DPL of CLP and CLP+IT mice were not significantly different whereas neutrophil levels in the BAL of CLP+IT mice were significantly lower than that seen for IT alone. It is interesting to note that all intervention groups including CLP and saline had significantly more BAL PMN than control animals. This may be secondary to redistribution of PMN in the lung and will be discussed in more detail alongside the MPO findings.

MPO readings reflected the sequestration of PMN in the lung whereas BAL reflected PMN numbers in only one compartment of the lung: the intraalveolar space. Combining these data we can draw some inferences about the distribution of PMN in the lung.

The MPO level in control was not expected to be zero but was performed to establish a baseline level for normal animals. This baseline value reflected the MPO from the PMN normally found in the lung. The lung is a known reservoir for PMN.[104, 143] The baseline value may also reflect trace amounts of MPO from macrophages and erythrocytes.[132, 144, 145]

The IT group had elevated numbers of PMN in the lung as reflected by increased absorbance OD_{655} compared to the CLP group which was on par with control. This rise signified an increased influx of PMN to the lung as the primary site of infection. The previously mentioned presence of MPO in macrophages and erythrocytes is unlikely an important source of the increased MPO. No increased numbers of macrophages or erythrocytes were noted in the analysis of BAL and these cells have not been shown to be a significant source of MPO.[132, 144, 145]

The elevated BAL PMN seen in the CLP group versus control group was not seen in the MPO results. The MPO levels of CLP and control were not different. If total PMN in the lung as reflected by MPO was no different between CLP and control groups, this implied that the elevation of BAL PMN of the CLP group was the result of intraalveolar redistribution of PMN residing within the lung. Hence, there were presumably less interstitial and intravascular PMN in the CLP group. Several researchers have found that animals with a non-pulmonary source of sepsis have increased numbers of PMN in the lungs,[46, 109, 110] however this was not an universal finding. [113] In a pig model three hours after endotoxemia, BAL PMN was significantly elevated compared to placebo although MPO was not.[110]

In contrast, the elevated BAL PMN seen in the saline group was associated with an elevated MPO reading compared to control. This increased PMN sequestration in the lung of saline injected compared to control animals may have been due to trauma from the intratracheal fluid. The results for the saline group were less than that for the IT group, so the integrity of the experiment was not jeopardized.

Support for the hypothesis came from the CLP+IT data. As foreseen, the readings for CLP+IT animals were significantly less than IT for both the BAL and MPO experiments. This meant that animals exposed to peritonitis prior to intratracheal *Klebsiella* not only had less PMN in the intraalveolar space but less PMN in the lung compared to animals with *Klebsiella* alone. In fact, the PMN sequestration in the lung seen with CLP+IT was not just less than IT, but appeared to be less than that of lungs exposed to saline alone. This was a key finding. Although saline and CLP+IT were statistically equivalent in terms of BAL PMN and MPO, when compared to control

values, saline animals showed statistically more BAL and MPO. The CLP+IT animals did not. These results demonstrated a state in which an infectious source (IT) that in a normal animal caused a significant migration of PMN to the lungs, drew not simply an attenuated reaction (compared to IT) but an ablated reaction (compared to saline) from the target organ in the presence of an underlying remote infection (CLP).

Serum PMN in CLP+IT was significantly lower than serum PMN in both saline and control, presumably secondary to the observed sequestration of PMN in the peritoneal compartment. The diminished sequestration of PMN to the lung in CLP+IT animals, as reflected by the MPO data, could therefore be due to inadequate numbers of available PMN.

This is the crux of the hypothesis: sequestration of PMN to a second site of infection is diminished due to significant sequestration of PMN at the first site reducing the number of available PMN.

Whether this defect is deleterious to the host, allowing pneumonia to overcome the animal (the second front hypothesis) or whether this is a protective mechanism minimizing exposure of the lung to activated destructive neutrophils (the second hit hypothesis) was the question to be answered by the G-CSF experiment. By reversing the observed neutropenia, would mortality be reduced (the second front hypothesis) or increased (the second hit hypothesis)?

Interpretation of the BAL and MPO results would have been helped with lung histology. Unfortunately, the slides had virtually no PMN in the intraalveolar space and few in the intravascular space for all groups. This could be due to a sampling error, since only the left lung and not both lungs were fixed,[95] or perhaps a technical error, although

the protocol was followed as described. It is curious to see in another study using similar sized mice that histologic sections of the liver were performed to correlate neutrophil sequestration with liver MPO but that histologic sections of the lung were not performed. The explanation given was that: "Lung histologic studies were not performed, since the MPO assay is a well accepted method for quantitating lung neutrophil sequestration." [46]

LPS findings

Although, LPS did induce systemic neutropenia in mice which was more severe in the CLP+LPS mice, the pattern of PMN sequestration deviated from that shown for *Klebsiella* animals.

Analysis of DPL revealed significant PMN in the CLP+LPS mice as predicted, in fact, significantly more than CLP+IT. However there was also a lesser but significant increase seen in the LPS mice. This was associated with increased volume of DPL but not increased protein levels in both groups. Only CLP+LPS had both increased protein, implying that the volume increase for the LPS group was not due to increased permeability. It was not likely due to increased experience with the lavage process either since the LPS group also had the least volume of BAL fluid. Was this due to an infiltrative process? Why were there PMN present in the DPL of LPS animals? This difference in activity between LPS and *Klebsiella* is investigated further below.

Analysis of BAL PMN in LPS and CLP+LPS showed it to be no different from saline. While LPS was greater than CLP+LPS in numbers that bordered on significance (p-value = 0.069) this relationship was not supported by the MPO data. Results of MPO

showed LPS and CLP+LPS to be no different from saline. In fact, MPO in CLP+LPS appeared to be slightly greater than LPS.

Another interesting comparison was CLP+IT to CLP+LPS. The neutropenia observed in these two groups were equivalent, but CLP+LPS showed significantly more PMN in DPL and slightly more MPO in the lungs (p-value = 0.081) compared to CLP+IT. This means a greater overall number of PMN was observed for CLP+LPS whether secondary to increased production, decreased destruction, or recruitment from another compartment is unknown.

As mentioned above, BAL volume results for LPS were diminished. Perhaps the increased DPL fluid found in the LPS group resulted in a relative hypovolemia, but this was only observed in the LPS group. The other groups with large DPL volume did not have significantly diminished volume of BAL. Protein was not elevated in the LPS group, only in the IT group. Therefore the volume difference was not due to permeability.

The discrepancy of the LPS data from the *Klebsiella* data could be due to either the modification of protocol required by the severity of the LPS induced inflammation or a true difference between LPS and *Klebsiella* in this model.

The methodology used for this set of experiments was modified so that LPS mice were sacrificed after four hours instead of the twenty four in the IT group. The CLP+LPS group had intratracheal injections of LPS twenty hours after CLP (instead of six) or four hours (instead of eighteen) prior to sacrifice. This modification was made at the request of the Animal Care Committee who felt that clinically these animals were critically ill and suffering and requested their sacrifice within a shorter time frame. The dose of LPS given to these mice was calculated from the mg/kg dose used in rats according to a

previously published article.[125] In that protocol, animals were sacrificed four hours after injection. A recent study showed that differences in time interval of interventions such as intratracheal LPS produced significant changes in cytokines and PMN counts in BAL fluid.[142] The change in timing of the interventions could have resulted in some of the observed differences.

For example, the lack of PMN found in the BAL fluid of the LPS group, contradicts previous findings in rat models[36, 125] but may be explained by Harmsen.[124] That study found that aerosol exposure of mice to LPS resulted in significant PMN in bronchoalveolar lavage fluid six hours post-exposure. Although PMN were noted in the lavage fluid at three hours post-exposure, it was significantly less than that at six hours. Our results from BAL fluid were obtained four hours after LPS compared to eighteen after IT.

The departure in protocol may also explain PMN migration in the CLP+LPS group. Instead of inciting a second inflammatory response in the wake of an acute peritonitis, the twenty hour delay may have allowed the animal time to recover and redistribute or produce PMN. This could explain the increased number of PMN in the DPL of the CLP+LPS animals compared to the CLP+IT animals. The delayed onset of LPS in the lung would allow the PMN to concentrate on the unique infectious site in the peritoneum. The twenty hour delay would allow time to make available more neutrophils, so that the LPS exposure, when it occurred, resulted in aggregation of more PMN to the lung than seen in CLP+IT. This delay could also explain the inverse correlation of BAL PMN and MPO seen in CLP+IT versus CLP+LPS. CLP+LPS had relatively more MPO but less BAL PMN than CLP+IT. Because of the shorter time to

sacrifice, PMN in the CLP+LPS group may not have had time to migrate to the BAL compartment, instead remaining in the interstitium.

There may also be a true difference between LPS and *Klebsiella*. LPS is the endotoxin resulting from degradation of bacteria such as Klebsiella. It reproduces the cellular and hemodynamic effect found in septic shock[118] and has been used in murine models.[123-125] However, in a recently published article, rats receiving intratracheal LPS (200μg/kg) alone or six hours after CLP, showed no significant difference in the number of BAL PMN when sacrificed twenty four hours later.[142] The *Klebsiella* results in this thesis followed a similar protocol and found a significant difference in these two groups. This difference between LPS and *Klebsiella* may indicate a true difference between these two stimuli and may be the answer to why there were PMN found in the peritoneum of LPS animals. LPS introduced into the peritoneal cavity has been used as a model of murine peritonitis.[34, 146] Did intratracheal LPS migrate to the peritoneal cavity causing PMN migration? Or was this a systemic reaction to LPS? Hirano noted an unexpected increase in LPS inducible PMN in peritoneal lavage after intratracheal LPS.[123] This augmenting effect of LPS on PMN migration to the peritoneal cavity could explain the slightly greater number of PMN in CLP+LPS compared to CLP+IT in the DPL fluid. It may also relate to the increased volume seen in the DPL of LPS animals.

G-CSF mortality study

This experiment confirmed neither the *second front* nor the *second hit* hypothesis. There are a couple of explanations for this.

The model was developed to test the effect of a secondary site of infection on mortality. Both procedures individually are acceptable replications of clinically occurring peritonitis or pneumonia. During the period that these experiments were conducted, no published study had combined the two methods. Previous unpublished studies had, however lead us to expect mortality in the untreated groups to range from 27% for CLP to 91% for CLP and IT. The placebo animals did not follow this trend.

The mortality for untreated groups was 17.9% for CLP and 33.3% for CLP+IT in this study. This resulted in two problems.

Clinically, although the mortality of control animals was statistically significantly different from the CLP + IT group, there was no significant difference between the CLP and the CLP + IT group, or even for control and CLP. Failure to live up to the expected increase in mortality, questions the propriety of the model in testing the *second front* or the *second hit* hypothesis since the implied second site did not affect mortality as is found clinically.

Statistically, the low mortality rate in the CLP and CLP+IT group may account for the lack of effect with subsequent administration of G-CSF. Even when mortality was analyzed within the septic groups to determine the effect of G-CSF intervention on mortality in CLP mice alone or CLP+IT mice alone, there was no significant difference detected. This experiment was designed for higher expected mortality rates and therefore was not powered to detect small changes. The sample size may simply not have been large enough to detect a change that small. Most studies compared G-CSF administration in models where the untreated group's mortality rate approached 100%. Ours fell far short. The fact that our model did not reflect clinical rates of mortality due to peritonitis

makes it impossible to say whether a statistically insignificant change in this model is clinically significant.

One may be tempted to say that since G-CSF did not statistically *increase* mortality we can refute the *second hit* hypothesis. However without more data, such as demonstration of increased activated neutrophils in the lungs of G-CSF treated animals compared to control animals, we cannot say with any certainty that activated neutrophils do not damage the lungs. All we can conclude is that a dose of 50 μg/kg of G-CSF in a mouse model of peritonitis and pneumonia did not affect the observed mortality compared to placebo treated animals. This same dose of G-CSF was adequate in previous studies to cause significant neutrophilia in mice.[86, 95] This contradicts Held's finding that pretreatment with G-CSF in a murine *K. pneumoniae* model resulted in increased mortality.[95] His experimental model was a simple pneumonia and therefore his mice were presumably non-neutropenic compared to our model.

The background experiments which had demonstrated 91% mortality for the CLP+IT group, found the mean time to death to be 60 hours. This short time to death could be explained by the onset of bacterial pneumonia, although no histology was performed upon these animals. Longer time to death could imply survival past the bacterial inoculation, instead succumbing to dehydration or malnutrition due to ileus or intraabdominal abscess.

No intraabdominal abscesses were found in any of the autopsies, but 3 bowel obstructions were discovered. These may have been the cause of death. Treatment with G-CSF did not give a statistically significant difference in lifespan within the individual

septic groups. The observed decrease in lifespan was due to the effect of CLP+IT rather than the absence of G-CSF.

FUTURE PROJECTS

A repeat of the LPS model using a lesser, but still effective, dose of LPS can be done following the protocol of the bacterial studies regarding the timing of the interventions. The results obtained would determine if our interpretation of the LPS data is correct.

The observed redistribution of PMN in this study is presumably due to sequestration however decreased production or increased destruction could contribute. Analysis of circulating PMN for immature band forms and analysis of bone marrow could be performed to assess production. Destruction of PMN could be assessed by measuring apoptosis of the sequestered PMN in the DPL and BAL.

Exciting new information has come from this laboratory regarding the migration of PMN along a C5a gradient. It would be interesting to analyze blood and BAL fluid to determine the C5a chemotactic gradient of the different intervention groups. Analysis of PMN from DPL, blood and BAL for activity, L-selectin, TNF-α, IL-1 and IL-10 levels could be measured at the same time.

The suggestion of decreased mortality in the G-CSF treated mice from this experiment, although insignificant, could be probed further. Since G-CSF requires time to reach therapeutic levels and our model allows only six hours between interventions, leukocyte transfusion given at the time of IT could be performed to determine whether this reverses the neutropenia and diminished MPO to the lungs of twice infected animals.

If PMN transfusion reverses neutropenia but not MPO, then investigations regarding PMN characteristics as well as chemotactic gradients are indicated (see above).

If PMN transfusion results in increases in BAL PMN and MPO then the G-CSF studies could be repeated. The mortality studies would have to be modified so as to increase the mortality in the infected groups in order to detect a difference. A subgroup of animals receiving G-CSF could be analyzed as per the methods in this experiment to compare to the PMN transfusion results. Terminal animals, as per the thermistor data, could be sacrificed and compared to surviving animals as regards PMN distribution.

CONTRIBUTION TO KNOWLEDGE

The temperature probe model presented in this thesis has never before been published. Temperature as a predictor of mortality is a much more reliable and reproducible measure than the previously used clinical signs of deterioration. Temperature probes will likely become mandatory in future murine projects requiring mortality data to acquire the approval of the Animal Ethics Committee.

The use of the double pronged septic focus in mice is also a new model. A recent paper combined these two procedures in a rat model but they did not excise the cecum.[142] It is a durable, easy to learn model which can be modified to different degrees of severity of infection. Certainly much more information can be obtained from this model, the limitation currently being the small size of the animal and therefore small tissue and blood samples available.

Although ultimately left with the lack of a clear cut answer to the proposed hypothesis, I am encouraged by the words of scientist Dr. Richard P. Feynman. As the watchdog concerning the integrity of Science and a role model for those who pursue it, his remarks to the Caltech class of 1974 remain *à propos* today.

"...If you've made up your mind to test a theory, or you want to explain some idea, you should always decide to publish it whichever way it comes out. If we only publish results of a certain kind, we can make the argument look good. We must publish *both* kinds of results..."[147]

APPENDIX

McGill University
Animal Use Protocol

P.I.		
FACC		
RGO		
REM		
INVEST		
PROTO		

FOR OFFICE USE ONLY

Project #	3832
Investigator #	UVH-014
Approval End Date	Aug 31, 1999
Facility Committee	UVH

☐ New Application

☒ Renewal of Project # 3832

1. Investigator Information

Principal Investigator: Nicolas V. Christou, MD, PhD

Department: Surgical Research Laboratories, Dept. of Surgery

Address:

E-mail:

Animal Use: Research ☒ Teaching ☐ Specify Course number:

Project Title: Mechanisms of decreased neutrophil exudation and microbicidal activity in sepsis mice

2. Funding Source

External ☒ Internal ☐

Source(s): MRC

Peer Reviewed source: Yes ☒ No ☐ *If no, see instructions - section 2

Awarded ☒ Pending ☐

Funding Period: From: April 1998 To: April 1999

Proposed Start Date of Research: (Day/Month/Year) April 1, 1998

Expected Date of Completion: (Day/Month/Year) April 1, 1999

3. Emergency: Person(s) designated to handle emergencies (2 emergency telephone numbers must be indicated)

Name: Dr. Nicolas V. Christou Phone #: Work: Alternative #:

Name: Dr. Felicia Huang Phone #: Work: Alternative #:

Certification:

The information in this application is exact and complete. I agree to follow the policies and procedures set forth by the Facility Animal Care Committees and McGill University, as well as those described in the "Guide to the Care and Use of Experimental Animals" prepared by the Canadian Council on Animal Care. I shall request the Animal Care Committee's approval prior to any deviations from the procedures described within.

Principal Investigator/Course Director		Date	APRIL 24/98

Approval:

Chairperson, Facility Animal Care Committee		Date	June 16 1998
University Animal Care Officer		Date	June 29/98
Approved period for animal use	Beginning 01-09-98	Ending	31-08-99

☐ NOTE REVIEWER'S MODIFICATION(S) ON PAGE 2

Revised 02/97

JUN 18 1998

4. Research Personnel and Qualifications (See Instructions - Section 4)

Give the names of all individuals who will work with animals in this study (including the Principal Investigator) and their employment classification (investigator, technician/research assistant, under graduate or graduate student, fellow or student supervisor). The Principal Investigator certifies that all personnel listed in this section will be provided with the specific training and/or experience which qualifies them to perform the procedures described in the protocol. Each person listed in this section must initial to indicate s/he have read this Application.

NAME	CLASSIFICATION	INITIAL
Nicolas V. Christou, MD PhD	Principal Investigator	
Daniel E. Swartz, MD	Graduate Student/ Surgery Resident	
Andrew J.E. Seely, MD	Graduate Student/ Surgery Resident	
Felicia Huang, MD	Graduate Student/ Surgery Resident	
Lorrenzo Ferri, MD	Graduate Student/ Surgery Resident	
Betty Giannias	Research Assistant	PG

5. Reviewer's Modification(s): The following modification(s) have been made to this Application during the review process. Please make these changes to your original documentation. You must comply with the recommended changes as a condition of approval for this Application.

6. Summary (See Instructions - Section 6)

BACKGROUND INFORMATION: (include here the potential benefit to human or animal health or to the advancement of scientific knowledge) - LAY TERMINOLOGY

> Neutrophils are the first line of defense against bacterial invasion. Patients with life-threatening infections have a 70-90% reduction of neutrophils to inflammatory sites. One possible explanation is the "second front" hypothesis, which states that in the presence of a major intraabdominal infection (the "first front") a "second front" such as the lung remains defenseless! We have support for this hypothesis with our previous work in mice (Protocol #3324 since these experiments cannot be done in humans). The scenario of intraabdominal infections followed a week later by pneumonia that kills the patient is not infrequent. We will try to determine if this is due to inadequate PMN migration or cytotoxicity of PMN that do migrate.

GLOBAL AIM OF THE STUDY: (include here the principal direction of the study) - LAY TERMINOLOGY

> We wish to follow up on the above concept, by using an animal model of abdominal infection followed by pneumonia, in order to simulate the common clinical scenario of an ICU patient recovering from an abdominal infection who develops a secondary infection. If we confirm that animals with intraabdominal infection are more susceptible to pneumonia because of insufficient neutrophils delivered to the lungs we wish to test the hypothesis that increasing neutrophil delivery by administration of G-CSF will reduce the mortality, which follows pneumonia in the lung.

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