Effects of pharmacological inactivation of adenosine 2A receptors in a murine model of polymicrobial sepsis

Helbig, Brian John

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Boston University
EFFECTS OF PHARMACOLOGICAL INACTIVATION OF ADENOSINE 2A RECEPTORS IN A MURINE MODEL OF POLYMICROBIAL SEPSIS

by

BRIAN J. HELBIG

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DEDICATION

For John and Shari
I would like to thank everyone that assisted me for the tremendous help and support that everyone provided me. A very special thank you to Agnes Bergerat for her guidance and tremendous amount of assistance. Another special thank you to Max Vaickus, Terry Hseih, and Bethany Lussier for their continued help and moments of entertainment throughout this past year. One last thank you to Weston Christensen whose assistance has gone unrecognized in the past.
EFFECTS OF PHARMACOLOGICAL INACTIVATION OF ADENOSINE 2A RECEPTORS IN A MURINE MODEL OF POLYMICROBIAL SEPSIS

BRIAN J. HELBIG

ABSTRACT

Sepsis is a worldwide health problem with an enormous economic burden and devastatingly high mortality rate. The pathophysiology and immune dysfunctions that occur during sepsis remain largely unknown, severely limiting current treatment options for sepsis. Both the innate and adaptive parts of the immune system are known to be involved in the dysfunctions that occur during sepsis. Over the last few years adenosine has been recognized as an endogenous mediator that alters both innate and adaptive immune responses. Adenosine receptors are largely expressed on many different immune cells and may serve to limit excess collateral damage in the setting of inflammation. In this study, the pharmacological effects of an A$_{2A}$ receptor antagonist on septic mice were examined using the CLP model of sepsis that results in a polymicrobial infection. Pharmacological inactivation of the A$_{2A}$ receptor significantly increased mortality in septic mice predicted to live in comparison to those given only vehicle. Treatment with the A$_{2A}$ receptor antagonist also increased expression of CD40, part of a pathway well known for its roles
in inflammation. Our data also showed increased monocyte MHCII expression after treatment with an adenosine antagonist. Our data support the role that A$_{2a}$ receptors are involved in the immune response to sepsis, and that these receptors may serve to damage excess collateral damage ensuing from the host immune response, and that additional studies on adenosine and its related purine nucleosides would be of use for better understanding of the immune dysfunctions that occur during sepsis and other diseases.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>A$_{2A}$R</td>
<td>Adenosine 2A Receptor</td>
</tr>
<tr>
<td>A$_{2B}$R</td>
<td>Adenosine 2B Receptor</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate</td>
</tr>
<tr>
<td>CARS</td>
<td>Compensatory Anti-inflammatory Response Syndrome</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>CLP</td>
<td>Cecal ligation and puncture</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T-lymphocyte Associated Antigen</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic Cell</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward Scatter</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes Simplex Virus</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>MDSC</td>
<td>Myeloid-Derived Suppressor Cell</td>
</tr>
<tr>
<td>MFI</td>
<td>Median Fluorescence Intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage Inflammatory Protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer Cell</td>
</tr>
<tr>
<td>P-die</td>
<td>Predicted to Die</td>
</tr>
<tr>
<td>P-live</td>
<td>Predicted to Live</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed Death 1</td>
</tr>
<tr>
<td>PDL1</td>
<td>Programmed Death Ligand 1</td>
</tr>
<tr>
<td>SIRS</td>
<td>Systemic Inflammatory Response Syndrome</td>
</tr>
<tr>
<td>SSC</td>
<td>Side Scatter</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-Like Receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>$T_{\text{Reg}}$</td>
<td>Regulatory CD4+ T Cell</td>
</tr>
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INTRODUCTION

Sepsis Epidemiology

Sepsis is an increasing global health problem. It is the number one cause of deaths in intensive care units and claims an estimated 250,000 deaths in the United States each year (Vincent, 2013). Sepsis is also a huge economic burden for the world, costing an estimated 24 billion dollars each year, an increase of nearly 60% from 2003 to 2007 (Lagu, 2012).

Defining sepsis is a difficult task because of its varied etiologies, presentation, stages, and outcomes. Historically, sepsis was defined as a systemic response to infection (Bone, 1989). To further clarify the terms associated with sepsis, an international sepsis conference met in 1991, where it was proposed that the system inflammatory response syndrome (SIRS) would be defined when two or more of the following criteria seen in table 1 were met.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Temperature</td>
<td>&gt;38°C (100.4°F) OR &lt;36°C (96.8°F)</td>
</tr>
<tr>
<td>Heart Rate</td>
<td>&gt;90 beats per minute</td>
</tr>
<tr>
<td>Tachypnea</td>
<td>Respiratory rate &gt;20 breaths per minute OR a (P_{aCO_2}&lt;32\text{ mm Hg})</td>
</tr>
<tr>
<td>Abnormal WBC Count</td>
<td>&gt;12,000 cells/(\mu\text{L}) OR &lt;4,000 cells/(\mu\text{L}), OR &gt;10% band cells</td>
</tr>
</tbody>
</table>

Table 1: Systemic Inflammatory Response Syndrome Criteria (adapted by Bone, 1992). Two or more of these criteria are required for diagnosis of SIRS.
When SIRS results from infection it is termed sepsis. Severe sepsis is defined as sepsis associated with organ dysfunction, hypoperfusion, or hypotension. Septic shock is defined as severe sepsis with hypotension that persists despite adequate fluid resuscitation (Bone, 1992).

The mortality of sepsis ranges from approximately 20% in sepsis to over 60% seen in septic shock (Brun-Buisson, 2003). Despite improvements in healthcare, the mortality in sepsis remains staggeringly high and the incidence is actually increasing over time. Many factors may contribute to the increasing incidence, such as a longer life expectancy as well as the emergence of treatment-resistant infectious agents. The six most common infection sites that result in sepsis are pneumonia, blood-stream infections (including infective endocarditis), intravascular catheter-related sepsis, intra-abdominal infections, urosepsis, and surgical wound infections (Calandra, 2005).

Pathophysiology

Based on the early initial clinical presentation of sepsis (fever, shock, and organ dysfunction), it was initially speculated that sepsis was the result of a massive inflammatory reaction from the host’s innate immune system in response to an infectious agent. This lead to intense research on anti-inflammatory treatments for sepsis aimed at controlling inflammation and dampening the so-called cytokine storm (Hotchkiss,
2003) (Freeman, 2000). Results from animal studies showed promising results, but in human phase III clinical trials only some cases improved, while many were unaffected or actually suffered increased mortality. This finding that only certain subsets of patients improved from anti-inflammatory treatments while others were harmed indicated that sepsis was a more complicated syndrome than initially thought and not simply due to an exaggerated inflammatory immune response (Fisher, 1996) (Remick, 1992) (Zeni, 1997).

**Immunosuppression**

Improved treatment strategies have resulted in most patients surviving the initial phase of sepsis. It has been found that these septic survivor patients later show signs of significant immunosuppression (Hotchkiss, 2003). Significant lymphocyte apoptosis occurs during sepsis that leaves the host vulnerable to new infections and unable to clear the initial septic insult. Septic patients experience loss of delayed hypersensitivity, increased risk for opportunistic infections, reactivation of latent viruses such as cytomegalovirus and HSV, decreased production of inflammatory mediators, and an inability to clear infection (Limaye, 2008). A post-mortem study by Torgerson found that approximately 80% of septic patients had uncleared septic foci at the time of death (Torgerson, 2009). In another study, the ability of
monocytes to secrete TNFα and IL-6 when exposed to endotoxin was severely reduced in septic patients (Heagy, 2000). These studies indicate that sepsis causes enduring immune dysfunctions that can also affect the adaptive immune system, in addition to the innate immune system, and cause significant immunosuppression. The idea of a Compensatory Anti-inflammatory Response Syndrome (CARS) was introduced to describe the host’s immune changes that attempt to limit inflammation during pathological conditions and restore homeostasis (Bone, 1996). The compensatory anti-inflammatory response syndrome involves cellular changes including an increase in lymphocyte apoptosis, decreased expression of human leukocyte antigen (HLA) receptors on antigen-presenting cells, decreased responsiveness of monocytes when stimulated, and increased production of anti-inflammatory cytokines such as IL-10 (Volk, 1996). CARS serves to limit damage caused by excessive inflammation and represents an inherent negative feedback mechanism for the immune system to control excessive inflammation.

More recent studies have shown that early sepsis is characterized by both a rapid release of pro-inflammatory and anti-inflammatory cytokines, and that this balance is important for the host response and survival (Munford, 2001). It is now commonly accepted that sepsis involves aspects of both excessive inflammation and immunosuppression from the onset. Many of the issues in treating septic patients is the
difficulty in properly characterizing the immune status of the patients, leaving physicians uncertain of whether to try to suppress or enhance the patient’s immune system. Better stratification of patients’ immune status is critical for selecting proper treatments at the correct time.

Costimulatory and Coinhibitory Receptors

T cell activation classically requires two activation signals. The primary signal resulting from the binding between the APC MHC molecule and the TCR. A secondary signal is usually required to result in T cell activation and proliferation, and often is provided by CD28 on T cells interacting with either CD80 or CD86.

Costimulatory and inhibitory receptors play a key role in the activation and suppression of the adaptive immune system. These receptors are cell-surface proteins that are involved in the interaction between APCs and T cells. They are expressed on APCs, which regulate adaptive immunity by regulating immune cell activation and inhibition, and therefore play a significant role in infections and the inflammatory response seen in sepsis.

The most well-known costimulatory members are CD80 (B7-1) and CD86 (B7-2), part of the B7 family. CD86 is constitutively expressed at low levels and its expression is rapidly increased after APC activation, whereas CD80 is induced hours or days after APC activation. CD80 and
CD86 interact with both the stimulatory CD28 and inhibitory CTLA-4 receptors on T cells. CD28 signaling on T cells promotes T cell activation and survival. A study that used an agonistic CD28 monoclonal antibody on humans resulted in overwhelming inflammation and even death and showed many characteristics similar to those seen in sepsis (Suntharalingam, 2006). In contrast to CD28, CTLA-4 is an inhibitory receptor that is upregulated on T cells following activation and serves to limit the immune response. CTLA-4 expression is increased on T lymphocytes in patients with sepsis and is correlated with the downregulation of CD86 on monocytes (Roger, 2009). Whereas CD28 is constitutively expressed on naïve T cells, CTLA-4 is induced only after T cell activation, serving to limit the adaptive immune response by competing with the lower-affinity CD28 on APCs, as well as exerting a direct inhibitory impact on cells to inhibit T cell activation. (Alegre, 2001). CTLA-4 is also strongly expressed on immunosuppressive T Regulatory (T_{Reg}) cells, where it enhances their activity and proliferation (Che, 2007).

**CD40 and CD40L**

CD40 and CD40L(CD154) are a costimulatory pair that are part of the TNF superfamily and serve as a regulator of lymphocyte function, especially CD4 T cell activation (Grewal, 1996). CD40 is constitutively
expressed on APCs and B cells. Its ligand, CD40L (CD154) is predominantly expressed on activated T cells, macrophages, and platelets (Henn, 1998) (van Kooten, 2000). Classical activation of the CD40/CD40L pathway results in the development of APC function and increased expression of costimulatory molecules that serve to activate T cells during an immune response. The pathway also activates the NF-κB transcription factor, which is critical for its roles in inflammation. Activation of the CD40/CD40L pathway also results in production of IL-6, an important inflammatory cytokine and its level is strongly correlated with disease severity in sepsis (Clark, 1990) (Remick, 2005).

**PD-1/PDL1**

PD-1 is a more recently discovered inhibitory receptor of the B7 family that is found primarily on activated CD4 and CD8 T cells, but also B cells and monocytes. The PD-1 receptor has 2 known ligands: PD-L1 (B7-H1) and PDL2 (B7-DC). PD-L1 is currently thought to be the more important of the two ligands and is expressed on both hematopoietic and non-hematopoietic cells. PD-L1 expression has been characterized on dendritic cells (DCs), macrophages, monocytes, B cells, endothelial cells, and other cell types (Keir, 2008). Lymphocytes and monocytes are thought to be primarily involved in the PD-1/PDL1 pathway especially during infection (Sharpe, 2007). When the PD-1 receptor is activated by
its ligand it exerts strong inhibitory effects on the immune response and is involved in regulating cytokine production, T cell activation, tolerance, and apoptosis. There is currently intense interest in the PDL1-PD1 pathway as it has been shown to play roles in sepsis, cancer, HIV, and other diseases. Recent studies have indicated that blocking of the PDL1-PD1 pathway improves survival in sepsis by increasing monocyte responsiveness and decreasing lymphocyte apoptosis (Zhang, 2010). PD-1 is also highly expressed on the immunosuppressive T Regulatory cells, which play a major role in inducing CD8+ T cell anergy (Csoka, 2007).

In the past several years, the role of neutrophils as solely innate immune cells has been called into question. Many studies have presented significant data suggesting the involvement of neutrophils in regulating the adaptive immune system (Ostanin, 2012) (Culshaw, 2008). Specifically, recent studies have provided support that neutrophils can express PDL1 and even alter the adaptive immune response by interacting with T cells (Huang, 2014). A recent 2014 study indicated that neutrophil PDL1 expression was induced by IFN-α, HIV-1 virions, and multiple TLR ligands including LPS (Bowers, 2014).

**Adenosine**

Adenosine was first postulated to act as an extracellular signaling molecule in 1929 when Szent-Györgyi and Drury injected heart muscle
extracts into animals and noted a decreased heart rate in those subjects (Drury & Szent-Györgyi, 1929). The molecule responsible for decreased heart rate was identified as adenosine, and adenosine has been used therapeutically since the 1980s to decrease heart rate in humans (diMarco, 1985).

More recent studies have supported the role of adenosine as a potent immune regulator. During normal conditions extracellular concentrations of adenosine are negligible, however adenosine accumulates extracellularly in response to stresses such as inflammation and hypoxia (Bodin & Burnstock, 1998). In line with this evidence, it has been shown that septic patients have significantly elevated levels of adenosine in their blood compared to controls (Kaufmann, 2007).

Adenosine binds to four different cell-surface receptors: A1, A2A, A2B, and A3. All four of these receptors exert their effects mainly through G-coupled proteins, although some G-protein-independent mechanisms have been suggested (Fredholm, 2007). The adenosine pathway has been implicated to play major roles in the immunosuppression seen in infection and in the tumor microenvironment, both of which are known to have increased adenosine accumulations. Although a detailed look at all four of these adenosine receptors is beyond the scope of this work, the reader is highly encouraged to consult two excellent reviews on purinergic signaling and receptor subtypes (Burnstock, 2013) (Bours,
The \( \text{A}_2 \)-receptors (\( \text{A}_{2\text{A}} \) and \( \text{A}_{2\text{B}} \)) are coupled to \( \text{G}_\alpha \text{S} \)-proteins and induce adenylyl cyclase and cAMP buildup inside cells (Fredholm, 2007). The high-affinity \( \text{A}_{2\text{A}} \)-receptor has received significant attention and is speculated to be the most important adenosine receptor for regulating immune responses. It is highly expressed on many different immune cells including: neutrophils, macrophages, monocytes, mast cells, T cells, MDSCs, eosinophils, basophils, NK cells, dendritic cells, and others. Studies have shown that agonists of adenosine exert a range of immunosuppressive responses on these cells mainly via the \( \text{A}_{2\text{A}} \) receptor (Blackburn, 2009). Figure 1 depicts some of the various effects that adenosine has on immune cells when it activates the \( \text{A}_{2\text{A}} \) receptor pathway.

One of the main suppressive effects of \( \text{A}_{2\text{A}} \text{R} \) activation occurs due to the increased production of IL-10, which is a key immunosuppressive cytokine. IL-10 inhibits the production of pro-inflammatory cytokines such as IL-6, IL-12, and TNF\( \alpha \). IL-10 also decreases antigen presentation by decreasing MHC-II expression, down-regulates costimulatory molecule expression, and decreases immune cell recruitment by down-regulating chemokine production (Couper, 2008). The adenosine pathway seems to exist to limit excess collateral damage in the setting of inflammation,
which indicates it is an important inborn control mechanism of the immune system.
FIGURE 1: Cellular Effects of A2AR Activation on Immune Cells
**Current Study**

Sepsis is an extremely complicated syndrome that presents many incompletely understood immune dysfunctions and is characterized by a high mortality rate. An important goal in sepsis research is to further understand the pathophysiology and the immune alterations that occur in the different stages of sepsis, and how these alterations impact survival outcome and long-term immune alterations.

Recent data have demonstrated a role for adenosine as a signaling molecule in pathological environments such as inflammation, tumor microenvironments, and in hypoxic/ischemic tissues. Accumulating evidence suggests a potent role for adenosine molecules in altering the immune response and serving as an inborn negative feedback mechanism during inflammation.

This study will use a well-regarded technique to induce a mixed polymicrobial infection that elicits immune effects similar to those seen in peritonitis. This technique, Cecal Ligation and Puncture (CLP) is considered a top-notch research technique for emulating sepsis.

There were three primary aims in undertaking this study.

#1: Does antagonism of the adenosine 2A receptor alter mortality in sepsis?
#2: Does administration of an adenosine 2A receptor antagonist alter the expression of CSMs during sepsis?

#3: Does adenosine 2A receptor antagonism alter the plasma cytokine profile of septic mice in comparison to untreated septic mice at 24 hours post CLP?

We plan to examine these specific aims through the utilization of a murine CLP model of sepsis. We will then administer an adenosine 2A receptor antagonist and examine the overall effect on mortality by following the survival of the mice and analyzing their survival by log-rank analysis. At 24 hours post-surgery we will take a small amount of peripheral blood and separate the cells from the plasma. The plasma will be used to study cytokine expression in septic mice, either treated or untreated, at 24 hours post-surgery. We will then use the cell pellet to stain for CSMs expression to be analyzed by flow cytometric analysis.
METHODS

Animals
Female 8-12 week old (~20-25g) outbred ICR mice (Harlan-Sprague Dawley, Indianapolis, IN) were used for all studies. Mice were acclimated in the animal housing room for a minimum of 24 hours before experiments in a temperature and humidity-controlled room on a 12 hour light-dark cycle. Mice were allowed access to food and water ad libitum. All experiments were approved by the Boston University Animal Care and Use Committee.

Sepsis Model
The Cecal Ligation & Puncture (CLP) model as originally developed by Wichtereman and Chaudry with slight modifications was utilized to simulate a septic state resulting from intra-abdominal peritonitis (Wichterman & Chaudry, 1980). Mice were briefly anesthesized with 4% isoflurane and a midline abdominal incision of ~3cm was created. The cecum was exposed and ligated distal to the ileocecal valve with a 4-0 USP braided silk suture. The cecum was then punctured twice longitudinally with a 16-gauge needle, and the cecum was gently squeezed to extrude a small amount of fecal content to ensure wound patency. The cecum was returned to the abdomen, the abdominal wall
closed with sutures, and the skin closed with wound glue. This CLP method resulted in ~50% mortality in the first 5 days after surgery, representing an acute model of sepsis. Pain management consisted of Buprenorphine (.05 mg/kg; sc) every 12 hours for the first 2 days post surgery. Broad-spectrum antibiotics were administered every 12 hours for 5 days (Imipenem 25 mg/kg; sc) beginning 1.5 hours after CLP to emulate the standard treatment for human sepsis.

For A2A receptor antagonist studies, mice were administered SCH-58261 (10 mg/kg, dissolved in DMSO) at 6-hours post CLP by s.c. injection once daily for 2 days, and control mice were given vehicle (DMSO + saline).

**Telemetry Live/Die Stratification**

Prior work done by our lab has indicated that mice that have undergone CLP can be stratified into those predicted to live (P-live) and those predicted to die (P-die) at 6 hours post-CLP based on heart rate. Mice with a heart rate of >700 b.p.m. were predicted to survive and those with a heart rate of <550 b.p.m. are predicted to die. Mice with heart rates between 550 and 700 were not stratifiable, and thus excluded from analyses. Mice that were treated with the A2A receptor antagonist were treated at this time, after the stratification into P-live and P-die groups,
and then treated one additional time 24 hours after this initial treatment.

**Blood Sampling**

Sampling occurred at 24 hours post-CLP surgery via facial vein puncture. 50µL blood was aspirated via a pipette with a pre-rinsed tip to prevent clotting. The blood was diluted in a 150µL PBS solution (1X PBS, 3.38mM EDTA, and 2% FCS) and centrifuged at 300g at 4°C, and the plasma was collected and stored at -80°C until further analysis. The cells were then resuspended and the red blood cells were lysed, centrifuged, the supernatant was aspirated off, and the remaining cell pellet was collected for flow cytometric analysis.

**Flow Cytometry**

The following antibodies were used for flow cytometry

- The lymphocyte staining antibodies consisted of: FITC-conjugated anti-CD3, PerCP-conjugated anti-CD4, APC-H7-conjugated anti-CD8, BUV395-conjugated anti-CD25, BV737-conjugated anti-CD69, BV421-conjugated anti-CD28, PE-conjugated anti-PD1, PE-Cy7-conjugated anti-
CD40L, and APC-conjugated anti-CTLA-4. Compensation beads were also utilized. All antibodies were purchased from BD Biosciences (San Diego, CA). Flow cytometry was performed on a BD LSRII flow cytometer (BD Biosciences) and the data was analyzed with FlowJo software (Tree Star).

**Cytokine Analysis**

IL-6, MIP-2, and IL-12 were assayed from commercially available enzyme-linked immunosorbent assay (ELISA) kits (R & D Systems, Minneapolis, Minn.) All samples were run in duplicate.

**Statistics**

Statistics were performed using Prism 6 (GraphPad, San Diego, CA). For comparison between two groups, an unpaired *t* test for normally distributed data or the Mann-Whitney *U* test for data that did not follow a normal distribution was used. For group analyses one-way ANOVA was used for normally distributed data and the Kruskal-Wallis test was used for groups that did not display a normal distribution. Values presented are expressed as means ± SEM. Survival was analyzed by Kaplan-Meier analysis.
RESULTS

A2AR antagonism increases mortality in an acute model of sepsis

Mice predicted to live that were untreated had an overall survival rate of ~85% at the end of 14 days. In contrast, mice that were originally predicted to survive and subsequently treated with the A$_2$A$_R$-antagonist had a survival rate of ~23% at the end of 14 days. This difference was significant (p=.0003). Mice that were predicted to die in both the untreated group and the group treated with the A$_2$A$_R$-antagonist had a 0% survival rate.

FIGURE 2: Survival Curve P-live mice treated with the A$_2$A$_R$ antagonist experienced increased mortality in comparison to P-live mice treated solely with vehicle (p=.0003). Mortality was 100% in both P-die control and P-die treated mice. Mice were monitored for survival for 14 days, and no additional deaths occurred after this period when survival was monitored for 30 days.
PDL1 expression is increased on neutrophils in P-die mice

Neutrophils from P-die mice showed higher expression of PDL1 than P-live mice after CLP. This difference was statistically significant (p=.0034) (Figure 3). Flow cytometry data indicated that there were two distinct neutrophil populations, one subset expressing high amounts of PDL1 and the other subset with low PDL1 expression (FIGURE 4). This subset of PDL1-high expressing neutrophils was not seen in control healthy mice as can be seen in FIGURE 5.

FIGURE 3 Neutrophil PDL1 Expression PDL1 expression was significantly increased (p=.0034) on mice that were predicted to die after CLP when compared to P-live mice.
FIGURE 4 Neutrophil Flow PDL1 Expression on P-die Mice

P-die mice showed two subsets of neutrophils that expressed different levels of PDL1. Neutrophils were gated by Ly6G, a rather specific neutrophil marker.

FIGURE 5 Neutrophil Flow PDL1 Expression of Healthy mice

Healthy mice did not show a PDL1-high subset like the septic mice.
**A₂₅R antagonism increases CD86 expression on inflammatory monocytes**

Treatment with the A₂₅R antagonist increased the expression of CD86 on monocytes. P-live mice treated mice showed a CD86 expression of 1407±59 compared to P-live control mice 1124±16, and this difference was statistically significant \( p < .0001 \). **Figure 6**

P-die mice treated with SCH-58261 also showed an increase in CD86 expression (1540±99) compared to P-die control mice (1213±52), and this difference was also statistically significant (\( p = .0425 \)). However, there was no statistically significant difference in CD86 expression between the P-live control mice and P-die control mice.

**FIGURE 6 CD86 Expression on Inflammatory Monocytes**

Treatment with the A₂₅R-antagonist significantly increased CD86 expression in both treatment groups, however P-live control and P-die control mice showed no significant difference in CD86 expression.
**A$_{2A}$R antagonism increases MHCII expression on peripheral blood monocytes**

Mice that were predicted to live and treated with the A$_{2A}$R antagonist showed a significant (p=.0025) elevation in MHCII expression on peripheral blood monocytes (174.5±15) in comparison to control mice predicted to live (125.51±3). P-die A$_{2A}$R antagonist treated mice also showed increased expression of MHCII compared to the P-die control mice, but this difference was not statistically significant.

**FIGURE 7 MHCII Expression on Monocytes** P-live mice treated with the A$_{2A}$R-antagonist showed significantly increased MHCII expression in comparison to P-live control mice (p=.0025).
P-die mice show elevated PDL1 expression on peripheral blood monocytes

PDL1 expression was increased on the inflammatory monocyte subset of P-die mice (3453±1384) in comparison to P-live control mice (359.5±174) and this difference was significant (p=.0081). Figure 8

Treatment with SCH-58261 did not appear to affect levels of PDL1 18 hours after treatment.

**FIGURE 8 PDL1 Expression on Inflammatory Monocytes** PDL1 expression was significantly greater on P-die mice than P-live mice (p=.0081), however treatment with SCH-58261 did not appear to affect PDL1 expression in peripheral blood monocytes.
**A2A R antagonism increases CD40 expression on monocytes**

P-live control mice showed decreased CD40 expression (393.5±48.55) in comparison to P-die control mice (1039±269.9), and this difference was statistically significant (p=.0042) **Figure 9**

CD40 expression was also increased in P-live mice treated with SCH-58261 and this result was significant in P-live mice (p=.0063). P-live control mice had a level of CD40 expression of 393.5±48.55, whereas P-live mice treated with the A2A R antagonist exhibited an increased CD40 expression of 1226±337.9.

**FIGURE 9 CD40 Expression on Monocytes** CD40 expression on P-live control mice was lower than P-die control mice. Treatment with SCH-58261 increased CD40 expression in both groups, however it was only significant in the P-live group (p=.0063).
**A2AR antagonism increases cell activation marker CD69 in CD4 T cells**

P-live mice treated with A2AR antagonist showed increased expression of the cell proliferation marker CD69 (93.63±14.99) on CD4 T cells in comparison to P-live control mice (56.06±4.557), and this difference was significant (p=.0077). **Figure 10**

**FIGURE 10 CD69 Expression on CD4 T Cells** P-live mice treated with the A2A-antagonist showed increased expression of CD69 on CD4 T lymphocytes in comparison to P-live control mice, and this increase was statistically significant (p=.0077).
**A2AR antagonism increases cell activation marker CD69 in CD8 T cells**

Mice treated with A2AR antagonist showed increased expression of the cell proliferation marker CD69 on CD8 T cells in comparison to control mice, but this difference was not statistically significant. **Figure 11**

**FIGURE 11 CD69 Expression on CD8 T Cells** While mice treated with the A2A-antagonist showed increased expression of CD69 on CD8 T lymphocytes, the increase was not significant (p >.05)
**A2AR antagonism increases IL-12 in plasma**

IL-12 levels were increased in both P-live treated and P-die treated mice, with respect to their controls. For the P-die mice this difference was statistically significant (p=.0397). **Figure 12**

Nonparametric ANOVA analysis revealed that the median between each group varied significantly (p<.0120).

**FIGURE 12 Plasma IL-12 Concentration at 24 Hours** P-die treated mice showed increased IL-12 concentrations in peripheral blood at 24 hours post-CLP, and this elevation was significant (p=.0397).
**P-die mice show elevated pro-inflammatory IL-6 in plasma**

Control mice that were initially predicted to die showed significantly increased IL-6 levels in comparison to the control mice that were predicted to live. P-live vehicle treated mice had a plasma IL-6 concentration of 1060±483.3, whereas P-die control mice IL-6 levels were elevated to 45725±15848. The difference in means was statistically significant between these two groups (p=.0149). **Figure 13**

The A$_{2A}$ receptor antagonist did not appear to significantly affect plasma IL-6 levels at 24 hours (18 hours after treatment). There were no statistically significant differences between the P-live treated and P-live control mice, nor between the P-die treated and P-die control mice.

![Plasma IL-6](image)

**FIGURE 13 Plasma IL-6 Concentration at 24 Hours** P-die mice had higher concentrations of IL-6 in peripheral blood at 24 hours Post-CLP.
**P-die mice show elevated MIP-2 in plasma at 24 hours after CLP**

Control mice that were predicted to die had a concentration of 110800±32901, whereas mice that were predicted to live had significantly less MIP-2 with a mean 3340±1413 in their plasma at 24 hours after CLP. This difference in MIP-2 concentrations was statistically significant (p=.0115). **Figure 14**

The A$_{2A}$ receptor antagonist did not appear to significantly affect plasma MIP-2 levels at 24 hours (18 hours after treatment). There were no statistically significant differences between the P-live treated nor P-die treated mice with their respective controls.

**FIGURE 14 Plasma MIP-2 Concentration at 24 Hours** P-die mice showed increased MIP-2 in peripheral blood at 24 hours post-CLP with regard to P-live mice.
A$_{2A}$R antagonism increased mortality in septic mice that were initially predicted to survive at 6 hours. This is in contrast to findings from another study in which inactivation of the A$_{2A}$ receptor increased survival in septic mice (Nemeth, 2006). However, our findings are in agreement with a study by Sullivan in which A$_{2A}$ receptor activation improved survival in a mouse model of endotoxemia (Sullivan, 2004). These differences may result from differing degrees of severity in the CLP model used. Additionally, our model utilized antibiotics to emulate the standard treatment for human sepsis, but could result in a rapid drug-induced killing of vast quantities of bacteria, which could cause a rapid exaggerated immune response. In this situation, A$_{2A}$R activation may prove beneficial by controlling the exaggerated response brought on by the drug-induced bacterial killing. Normally A$_{2A}$R activation results in significant immunosuppressive effects, and it seem like that by blocking the adenosine receptor there was significantly increased inflammation. This increased inflammation would be harmful during an acute hyper-inflammatory stage seen with our CLP model.

Our data show that MHCII expression was increased on mice treated with the A$_{2A}$R antagonist. This increased MHCII expression suggests improved antigen presentation. It is well known that decreased
antigen presentation is commonly seen during the immunosuppressive aspects of sepsis. Antagonism of adenosine receptors could be used therapeutically to enhance antigen presentation in order to enhance immune functions during an immunosuppressed state.

The role of the PDL1/PD1 pathway is incompletely understood at this time. Upregulation of PD-1 and PD-L1 on T cells, B cells and monocytes during sepsis has been regularly reported and blocking of this pathway has appeared to be beneficial in reducing lymphocyte apoptosis and T cell anergy commonly seen in sepsis (Zhang 2010).

A study showed that during sepsis CD40 expression is increased on peripheral blood monocytes and that the expression was positively correlated with survival (Sugimoto, 2003). However, another group reported decreased mortality in CD40−/− mice compared to WT mice after CLP (Gold et al, 2003). Based on our results that P-live control mice actually had lower expression of CD40 in comparison to P-die control mice, it seems plausible that in CD40 could exacerbate inflammation and increase mortality in an acute model of sepsis that is characterized by an hyper-inflammatory immune response. Additionally, because the A2A antagonist significantly increased CD40 expression in our P-live mice, it seems likely that this increase also resulted in exacerbated inflammation and the subsequent increased mortality in the P-live treated mice.

These finding suggest that an adenosine receptor antagonist might be
better suited for application during a more chronic model of sepsis that is characterized by significant immunosuppression.

Upregulation of CD25 on treated P-live mice as expected. This is in agreement with other studies that indicated a decrease in CD25 expression when mice were treated with an adenosine agonist (Sevigny, 2007). However, an increase in CD25 could represent an increase in \( T_{\text{Regs}} \) that are involved in immune suppression. Future studies that can stain for \( T_{\text{Regs}} \) in flow cytometry via the intracellular FOXP3 protein or another T regulatory cell-specific marker will be required to examine this possibility.

The increased expression of the cell activation marker CD69 support our hypothesis that by antagonizing the A\(_{2A}\) receptor more cells become activated and proliferate. This finding is compatible with many studies that show that A\(_{2A}\)R agonists increase lymphocyte apoptosis and anergy. Treatment with A\(_{2A}\)R antagonists should be more carefully examine as a therapeutic option for increasing lymphocyte activity and decreasing apoptosis to combat immune suppression.

MIP-2 levels were significantly elevated in mice that were predicted to die in comparison to mice predicted to live. This is in agreement with a previous study by Ebong in which MIP-2 levels were also elevated in mice that subsequently died of sepsis (Ebong, 1999). Treatment with SCH-58261 did not appear to impact MIP-2 levels in the plasma 18 hours
after its administration. This could be due to timing of plasma sampling or SCH-58261 dosing effects, and future experiments will be needed to clarify these results.

Adenosine plays a significant role in the immune response and offers a unique therapeutic strategy that should be researched more in the future. It will be important in any future application of adenosine agonists and antagonists to consider the timing and phase of sepsis. During an intense hyper-inflammatory condition the use of an adenosine antagonist might exacerbate inflammation and organ damage, whereas the use of an antagonist would be better suited for a patient that exhibits immunosuppression. Patients with sepsis display marked heterogeneity, and better stratification is critical to the application of any future sepsis treatment. Future studies of the A2AR-antagonist that utilize a more chronic model of sepsis that results in longer survival time and significant immunosuppression will be interesting to examine. Additionally, it will be worthwhile to examine the effects of adenosine receptor antagonism at a local level, by studying cells directly from the peritoneum. It has become very clear that adenosine receptors are critically involved in regulating the immune response and that treatments focused on modulating adenosine pathways may provide remarkable results for the treatment of immune dysfunctions seen in sepsis and other diseases.
REFERENCES


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VITA

Brian J. Helbig

(636) 222-2766  Birth Year 1990  130 W. Newton St.
bhelbig@bu.edu  Boston, MA
02118

EDUCATION:

TULANE UNIVERSITY  New Orleans, LA  May 2012
Bachelor of Science in Cell & Molecular Biology
Minor: Chemistry

BOSTON UNIVERSITY  Boston, MA  May 2016
Masters of Science in Medical Sciences

HONORS & AWARDS:

Founder’s Scholarship
Dean’s List

RELEVANT COURSEWORK

Cell & Molecular Biology  Neuroscience  Organic Chemistry
Immunology  Disease Neurobiology  Biochemistry
Cancer Biology  Psychopharmacology  Heterocyclic Synthesis
Anatomy & Physiology  Behavioral Endocrinology

LABORATORY SKILLS

Spectroscopy: NMR, UV/VIS, IR, ESI-MS
Aseptic Technique
Chromatography: TLC, column, GC
Mammalian Cell Culture
SDS-PAGE  ELISA

EXPERIENCE:

Research Lab
Tulane Biology Department  New Orleans, LA  2012
• Successfully maintained a thorough scientific laboratory notebook.
• Maintained and manipulated mammalian cell cultures.
• Applied principles of pharmacological manipulation and various assay designs to in vitro systems.

Research Lab
Tulane Neuroscience Department  New Orleans, LA  2012
• Examined the endocrine effects of gonadal hormones on
male and female Long-Evans rats.
• Successfully and ethically performed gonadectomies on the rats, while maintaining a sterile perimeter.
• Demonstrated ethical responsibility in scientific issues and use of animals in research.

**Researcher**
Tulane Chemistry Department  New Orleans, LA  2012
• Synthesized and purified organic molecules utilizing various cross-coupling reactions.
• Elucidated structures via NMR, IR, UV, GC-MS, ESI-MS.
• Collaborated with Ph.D. candidates in their research projects.

**OUTSIDE ACTIVITIES:**
MALEcenter Health Center Volunteer  2015
Tutor for Boys & Girls Club  2015