2009

Histone Deacetylase Inhibitors and Innate Immunity in Septic Shock

Joseph Bertsche
Virginia Commonwealth University

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Histone Deacetylase Inhibitors and Innate Immunity in Septic Shock

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

Joseph Bertsche
B.S., Virginia Commonwealth University, Richmond, VA, 2007

Director: PAUL M. FAWCETT PH.D.
ASSISTANT PROFESSOR, DEPARTMENT OF INTERNAL MEDICINE, DIVISION OF INFECTIOUS DISEASE AND DIRECTOR OF RESEARCH RESOURCES, MASSEY CANCER CENTER AND VIRGINIA COMMONWEALTH UNIVERSITY

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List of Abbreviations

\( \alpha \)  \hspace{1cm} \text{Alpha} \\
\( b \)  \hspace{1cm} \text{Beta} \\
\( \Delta \)  \hspace{1cm} \text{Delta} \\
\( \varepsilon \)  \hspace{1cm} \text{Epsilon} \\
\( \gamma \)  \hspace{1cm} \text{Gamma} \\
\( \kappa \)  \hspace{1cm} \text{Kappa} \\
\( \mu \)  \hspace{1cm} \text{Micro} \\
\( \omega \)  \hspace{1cm} \text{Omega} \\
\( \tau \)  \hspace{1cm} \text{Tau} \\
BMDM  \hspace{1cm} \text{Bone-Marrow Derived Macrophage} \\
CARS  \hspace{1cm} \text{Compensatory Anti-inflammatory Response Syndrome} \\
cDNA  \hspace{1cm} \text{Complementary DNA} \\
CO2  \hspace{1cm} \text{Carbon dioxide}
Cy3  Cyanine 3
Cy5  Cyanine 5
dATP  Deoxyadenosine triphosphate
dCTP  Deoxycytidine triphosphate
dGTP  Deoxyguanosine triphosphate
DMEM  Dulbecco's Modified Eagle Medium
DMSO  Dimethyl Sulfoxide
DNA  Deoxyribonucleic acid
DTT  Dithiothreitol
dTTP  Deoxythymidine triphosphate
dUTP  Deoxyuridine triphosphate
EDTA  Ethylenediaminetetraacetic acid
ELISA  Enzyme-linked Immunosorbent Assay
FDR  False Discovery Rate
G  Gauge
g  Gram
GIPLs  Glycoinositolphospholipids
GPI-anchors  Glycosylphosphatidylinositol Anchors
h  Hour
HAT  Histone Acetyl Transferase
HDAC  Histone Deacetylase
HDACi  Inhibitor of HDAC Activity
HRP  Horseradish peroxidase
HSV  Herpes Simplex Virus
IFN  Interferon
IFNAR  Type I Interferon Receptor
IL  Interleukin
IP  Intraperitoneal
IRAK  Interleukin-1 Receptor-associated Kinase
IRF  Interferon Regulatory Factor
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>IRG</td>
<td>Interferon Regulated Gene</td>
</tr>
<tr>
<td>ISRE</td>
<td>Interferon-Stimulated Response Element</td>
</tr>
<tr>
<td>ISGF3</td>
<td>Interferon-Stimulated Gene Factor 3</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus Kinase</td>
</tr>
<tr>
<td>K</td>
<td>Lysine</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram</td>
</tr>
<tr>
<td>KO</td>
<td>Knock Out</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccaride</td>
</tr>
<tr>
<td>LRR</td>
<td>Leucine Rich Repeats</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
</tr>
<tr>
<td>MD-2</td>
<td>Myeloid Differentiation Protein 2</td>
</tr>
<tr>
<td>MEEBO</td>
<td>Mouse Exonic Evidence-Based Oligonucleotide</td>
</tr>
<tr>
<td>Microarray</td>
<td></td>
</tr>
<tr>
<td>ml</td>
<td>Milli-Liter</td>
</tr>
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</table>

xii
mg  milligram

MMTV  Mouse Mammary Tumor Virus

MyD88  Myeloid Differentiation Factor 88

NaB  Sodium Butyrate

NaOH  Sodium Hydroxide

ng  Nanogram

NF-kB  Nuclear Factor Kappa B

NLR  NOD-like receptor

NuRD  Nucleosome Remodeling and Deacetylating

OD  Optical Density

PAMP  Pathogen Associated Molecular Pattern

PBS  Phosphate Buffered Saline

PCR  Polymerase Chain Reaction

pg  Picogram

pH  Power of Hydrogen
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pol II</td>
<td>RNA polymerase II</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern Recognition Receptors</td>
</tr>
<tr>
<td>R</td>
<td>Arginine</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated upon Activation, Normal T-cell Expressed, and Secreted</td>
</tr>
<tr>
<td>RLR</td>
<td>Retinoic Acid-inducible Gene-I-like Receptor</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory Syncytial Virus</td>
</tr>
<tr>
<td>S</td>
<td>Serine</td>
</tr>
<tr>
<td>SAHA</td>
<td>Suberoylanilide Hydroxamic Acid</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodiumdodecyl sulfate</td>
</tr>
<tr>
<td>siRNA</td>
<td>Short Interfering Ribonucleotide</td>
</tr>
<tr>
<td>SSC</td>
<td>Saline-Sodium Citrate</td>
</tr>
<tr>
<td>ssRNA</td>
<td>Single Stranded Ribonucleotide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name/Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducers and Activators of Transcription</td>
</tr>
<tr>
<td>SUIDs</td>
<td>Stanford Unique Identifiers</td>
</tr>
<tr>
<td>T</td>
<td>Threonine</td>
</tr>
<tr>
<td>TAK1</td>
<td>Growth factor-P-activated Protein Kinase 1</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll-IL-1 Receptor</td>
</tr>
<tr>
<td>TIRAP</td>
<td>TIR-domain Associated Protein</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like Receptor</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3,5,5'-tetramethylbenzidine</td>
</tr>
<tr>
<td>TRAF6</td>
<td>TNF Receptor Associated Factor 6</td>
</tr>
<tr>
<td>TRAM</td>
<td>TRIF-related Adaptor Molecule</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR-domain Containing Adaptor Inducing IFN-b</td>
</tr>
<tr>
<td>TSA</td>
<td>Trichostatin A</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer Ribonucleotide</td>
</tr>
<tr>
<td>Tyk2</td>
<td>Tyrosine Kinase 2</td>
</tr>
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Abstract

Histone Deacetylase Inhibitors and Innate Immunity in Septic Shock

By: Joseph Bertsche

A dissertation submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2009

Major Director: Paul M. Fawcett, Ph.D.
Associate Professor, Department of Internal Medicine and Director of Research Resources, Massey Cancer Center at Virginia Commonwealth University

Innate immunity depends on pattern recognition receptors, which recognize pathogen associated molecular patterns (PAMPS), such as Toll-like receptor 4 (TLR4), which detects the gram-negative bacterial toxin, lipopolysaccharide. Engagement of TLR4 by LPS sets off a cascade ending in the activation of pro-inflammatory cytokines and interferon-β (IFN-β) which alerts the host to the infection. However, these responses can be mal-adaptive, especially in the context of bacterial sepsis, where a "cytokine storm" results in death of the host. Pharmacological modulation of these responses may therefore be a promising treatment modality. Inhibition of classic pro-inflammatory cytokines such as IL-1β and TNF-α has been the (largely unfruitful) focus of much research. However it has recently emerged that mice with
defects in type I IFN signaling are also substantially resistant to challenge with endotoxin. We therefore wish to investigate pharmacological inhibition of IFN signaling as a potential means to control sepsis. We analyzed the effects of Trichostatin A (TSA) and Suberoylanilide hydroxamic acid (SAHA) (both broad spectrum HDAC inhibitors) and ST-2-92 (HDAC6 specific inhibitor) on IFN regulation and endotoxic shock. We created an in vivo mouse model for this treatment with TSA and SAHA (which are well tolerated in mouse and human) to look for possible alteration in the survival rate following endotoxin challenge. We as well as others found that treatment with SAHA (50mg/kg) significantly improves survival rate. We also characterized in-vitro modulation of IFN responses through SAHA by mouse DNA microarray. We noticed a decreased expression of many innate immune regulated genes in the SAHA and LPS treated condition compared to the LPS treatment alone. Additionally we observed a decrease in protein levels of IFN-β IL-1β, IL-6, IL-12p40, RANTES and TNF-α in cell culture supernatants treated with SAHA or ST-2-92 and LPS compared to LPS only treatment.

These results show the ability of broad spectrum HDACi through SAHA to increase mouse survival following LPS challenge as well as modulate the induction of innate immune responsive genes in vitro. Furthermore we have shown that HDAC specific inhibition through ST-2-92 can decrease pro-inflammatory transcript as well as protein levels.
Chapter 1 Septic Shock and Innate Immunity

1.1 Sepsis and Septic Shock

Septic shock occurs as a result of a bacterial infection of the blood stream, leading to a gross overproduction of cytokines, which cause deleterious effects on the host. These effects include low blood pressure, coma, systemic organ failure, and possibly death of the host.\textsuperscript{1} It is important to note that septic shock has a 50\% mortality rate and represents the most common cause of death in intensive care units.\textsuperscript{1} More importantly, recent analysis has shown that severe sepsis and septic shock account for 9.3\% of all death in the United States, making this the 10\textsuperscript{th} leading cause of death.\textsuperscript{2} The annual cost of care for patients with sepsis is estimated to be near a $17 billion, and this annual cost is expected to rise due to the quickly rising age of the population, as well as the increase in anti-biotic resistant bacteria.\textsuperscript{2}

Patients with septic shock have an immunological response characterized by a powerful inflammatory response, followed by immunosupression.\textsuperscript{3} Roger Bone has proposed that this bi-phasic response actually occurs in 5 stages. Stage 1 involves a localized pro-inflammatory response to infection. Stage 2 includes the movement of the proinflammatory mediators to a more systemic scale. Stage 3 occurs when there is a loss over the control of the proinflammatory
mediators, resulting in a full blown systemic inflammatory response syndrome (SIRS). Stage 4 is where there is an anti-inflammatory reaction, resulting in immunosuppression, which is termed the compensatory antiinflammatory response syndrome (CARS). Finally, stage 5 is an inappropriate response to this immunosuppressive state that results in multiorgan dysfunction syndrome (MODS). The initial local and systemic inflammatory cascade is started by the release of bacteria, toxins, or other various inflammatory mediators. Most famous of these is lipopolysaccaride (LPS), also known as endotoxin, which is a cell-wall component of gram negative bacteria, and will be discussed later in detail. This stimulates the release of cytokines from many cell types including dendritic cells and macrophages that act to greatly amplify the inflammatory response. In some cases, however, it is known that reaction to LPS is required for containment of certain gram-negative bacteria at an early stage and has a protective effect on the host. The immunodepression phase response during septic shock is mediated by anti-inflammatory substances such as corticosteroids and catecholamines. Additionally, interleukin 10 (IL-10), interleukin 4 (IL-4), and soluble tumor necrosis factor (TNF) receptors are released as anti-inflammatory mediators.

Initially, investigations into treatment of septic shock involved the use of anti-inflammatory therapies, largely the use of corticosteroids. The use of antibodies to LPS itself have also been evaluated. In clinical trial however, these efforts showed no benefit to patients. Additionally, many anti-cytokine therapies have been evaluated, but ultimately showed no ability to improve survival in patients. Many of these trials involved the use of antagonists to cytokines tumor necrosis factor and interleukin 1 (IL-1), as well as the use of antibodies to tumor necrosis
There are several explanations for the failure of the previously attempted treatment modalities for septic shock, as described by Astiz and Rackow. The first of these is that septic shock is a very complex syndrome that is not clearly understood. Given this inherent complexity, developing a single therapeutic agent may or may not be a viable solution to the problem. The second possible explanation for the failure of these initial studies is that optimal timing and doses of the agents still remain uncertain. Finally, given the bi-phasic nature of the immunological response to septic shock, the therapeutic agents that down-regulate the immune system allow for greater damage caused by the immunosuppressive proteins released during the second phase of the septic shock response.

1.2 Innate Immunity

Given that septic shock often results from a vast up regulation of cytokines during innate immune response pathways, it is necessary to understand innate immunity. Mammalian immunity consists of two branches; the well-characterized adaptive immunity and the more recently appreciated innate immunity. Adaptive responses require days to become active and rely heavily on innate immune responses for activation, while innate immune responses initiate within minutes infection. An additional distinction lies in the specificities of the two immune branches. Adaptive immunity relies on very specific antigen antibody interactions, through the use of T-cells and B-cells. Innate immunity however involves the use of pattern recognition receptors (PRR), with a classic example being the evolutionarily conserved toll-like receptor
(TLR) family, which recognizes a much more broad class of antigen known as pathogen associated molecular patterns (PAMPs). Other well studied examples of innate immune receptors include retinoic acid-inducible gene-I-like receptors (RLRs) which are anti-viral and NOD-like receptors (NLRs) which detect bacteria.

Innate immunity can be further broken down into humoral innate immunity and cellular innate immunity. Humoral innate immunity is comprised of the cell-less responses to microbes, including physical barriers, complement cascade, antimicrobial peptides, and acute phase response mechanisms. The acute-phase response mechanisms are used to prevent infection from expanding and include fever, iron depletion, and the production of fibrinogen.\textsuperscript{11} Although humoral immunity does not require the use of cells to function, there are examples of interaction with other cell based systems, such as the classical complement system. Cellular innate immunity is essential for the innate immunity system and utilizes phagocytes such as neutrophils and macrophages. Macrophages in particular play a very important role in the innate immune response to septic shock. These phagocytes are the first cells of the innate immune system to encounter the invading pathogens. They either directly kill the invading pathogens through phagocytosis or induce production of cytokines. The quick responses of the innate immune system direct the induction and development of the longer lasting, much more specific response of the adaptive immunity branch, with one of the mechanisms being the use of the TLRs.

1.2.1 Toll-Like Receptors

Toll-like receptors are a family of mammalian evolutionarily conserved innate immune
receptors that are expressed in various host cells. The toll protein was originally discovered in fruitflies (*Drosophila*) and was found to be involved in embryonic development. Other studies revealed that *Drosophila* toll protein showed similarity to the mammalian type I interleukin 1 receptor (TIR domain). Further studies were able to show that the toll protein plays an important role in mediating immune responses. It was shown that *Drosophila* carrying toll-deficient mutations were much more susceptible to infection by the fungus *Aspergillus fumigates*. Later, a human homologue of *Drosophila* toll protein was cloned and subsequently identified as an initiator of innate immune responses. A mouse homolog to the human toll protein has also been identified and characterized.

Thus far, there have been 13 members of the TLR family identified in mice and 10 identified in humans. TLRs are type I integral membrane glycoproteins, with the extracellular N-terminal domain consisting of leucine rich repeats (LRRs). The LRR motifs are characterized by 22-29 amino acid repeats that have seemingly random hydrophobic leucine residues separated by hydrophilic residues (LxxLxLxxN) and are evolutionarily conserved, being found in archea viruses, bacteria, plants, and animals. The C-terminal domain, known as Toll-IL-1 receptor (TIR), shows homology to the IL-1 receptor and is also shown to be conserved throughout evolution. The TIR domain is critical for the recruitment of adaptor molecules to carry out the downstream signaling pathways.

TLRs are expressed both as cell surface receptors (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11) and in intracellular vesicles (TLR3, TLR7, TLR8 and TLR9). Upon homo- or hetero-dimerization, TLRs are able to detect various PAMPs of a bacterial, viral, and protozoan
origin. As previously mentioned, LPS from gram negative bacteria is recognized by TLR4, with the help of extracellular proteins myeloid differentiation protein 2 (MD-2) and CD 14. Peptidoglycan from gram positive bacteria is recognized by TLR2. Flagellan from flagellated bacteria is recognized by TLR5. Unmethylated CpG genomic DNA from bacteria is recognized by TLR 9. TLRs are also able to detect PAMPs of a viral origin. Envelope protein from respiratory syncytial virus (RSV) and mouse mammary tumor virus (MMTV) have been shown to be recognized by TLR 2, 4, and 6. The genomes of some viruses such as herpes simplex virus (HSV) contain unmethylated CpG DNA which is detected by TLR 9 as previously mentioned. In addition, the genome of RNA viruses consists of ssRNA which is detected by TLR7 and TLR8. TLRs are also able to recognize PAMPs of fungal origin. Studies have shown that phospholipomannans and b-glucans are able to be recognized by TLR2 and glucuronoxylomannans are recognized by TLR4. Finally, TLRs are able to recognize protozoal PAMPs. TLR2 and TLR4 are able to recognize Glycoinositolphospholipids (GIPLs) and glycosylphosphatidylinositol anchors (GPI-anchors) from Trypanosoma species, *P. falciparum* and *T. gondii*. Genomic DNA from *Trypanosoma* is able to be recognized by TLR9.

**1.2.2 TLR4**

In the case of septic shock caused by gram negative bacterial infection, LPS engages TLR4. Therefore, it is necessary to further investigate TLR4 signaling and downstream pathways in response to LPS. LPS contains two main moieties; a polysaccharide and lipid A portion, with much of LPS responses being mediated by the lipid A portion. Upon LPS engagement, there is homodimerization of the TLR4. Activation of the TLR4 receptor complex
activates multiple signaling pathways.

### 1.2.3 MyD88-Dependant Signaling

The myeloid differentiation factor 88 (MyD88)-dependant pathway is utilized by all of the TLRs with the exception of TLR3. TLR1, TLR2, TLR4 and TLR6 recruit the additional adaptor TIR-domain Associated Protein (TIRAP), which is used to link the TIR domain with MyD88. In the case of TLR4, as well as numerous other TLRs, engagement of the receptor by LPS leads to the recruitment of MyD88 and the Interleukin-1 receptor-associated Kinase (IRAK) family of protein kinases (Figure 1). This initial step then leads to the activation of TNF receptor associated factor 6 (TRAF6) by IRAK. TRAF6 is then able to lead to activation of growth factor-P-activated protein kinase 1 (TAK1) through polyubiquitination. TAK1 is then able to activate the inhibitor of kappa-B Kinase (IKK) complex, which subsequently activates nuclear factor kappa B (NF-κB). NF-κB is then free to translocate to the nucleus where it acts as a transcription factor for important inflammatory cytokines such as Interleukin 1-β (IL1-β) and IL-6. TAK1 is additionally able to activate the mitogen activated protein kinase (MAPK), which leads to the induction of more pro-inflammatory cytokines. There has been a great emphasis on modulating this pathway in the context of septic shock in an effort to reduce the resulting pro-inflammatory cytokines produced, but so far this effort has remained fruitless.

### 1.2.4 MyD88-Independent Signaling

The failure of modulating only the MyD88-dependent arm of the response to LPS to reduce the resulting pro-inflammatory cytokines likely lies in the fact that, in the case of TLR4,
there is activation of not only the MyD88-dependent pathway, but also the MyD88-independent, or TIR-domain containing adaptor inducing IFN-β (TRIF) dependant pathway, upon LPS stimulation (Figure 1).  In order for initiation of the TRIF-dependant arm to occur, TLR4 requires the additional linker adaptor TRIF-related adaptor molecule (TRAM), which links the TIR domain of TLR4 with TRIF. Once activated, the N-terminal domain of TRIF interacts with TRAF6, and the C-terminal domain interacts with RIP1. Together these interactions are able to activate TAK1, which previously mentioned activates both NFκB and the MAPK pathway. Additionally, TRAF3 acts as a linker between TRIF and the TBK1/IKKe. This complex phosphorylates and activates IRF3 and IRF7, which translocate to the nucleus and act as transcription factors for the type I interferon (IFN) response. The most notable type I IFN activated through this pathway is IFN-β

1.3 Type I Interferon Response

The type I IFNs are an integral part of TLR responses. They are an evolutionarily conserved family of cytokines in vertebrates and are the most diverse of all of the cytokine families. Type I IFNs include multiple IFNα subtypes, β,ε,κ,ω,δ,μ and τ. IFN-β will be the main focus of this section because it is largely the type I IFN activated in the case of septic shock.

As previously described, the My-D88 independent pathway induced by LPS engagement of TLR4 leads to induction of IFN-β, which is able to move extracellulary and engage type I interferon receptor (IFNAR), a cell surface receptor to which all type I IFNs bind). This receptor differs from other cytokine receptors because it can recognize more than 15 different ligands.
The IFNAR is comprised of two chains, IFNAR1 and IFNAR2. It has been predicted that the ability for the IFNAR to interact with many ligands lies in the use of different residues on the IFNAR2 chain. IFNAR chains lack intrinsic kinase activity, so like many other receptors, it must rely on associated janus kinases (JAKs) to phosphorylate the signal transducing molecules like signal transducers and activators of transcription (STAT). IFNAR1 is pre-complexed with tyrosine kinase 2.
Figure 1. Classical Paradigm of TLR4 pathway (See text for discussion)
MyD88-dependent

TLR4  MD2  MyD88

CD14  IRAK-4  IRAK-1

TRAF6  TAK1

NF-κB

TNF-α, IL-1β

MyD88-independent

TLR4  MD2

CD14  TRIF

TBK1  IRF3

IFN-β

IRGα

Extracellular Milieu

Cytosol
(Tyk2), which is thought to stabilize the cell surface expression levels of the receptor.\textsuperscript{30, 31} IFNAR2 is pre-associated with JAK1.\textsuperscript{32} STAT1 and STAT2 are known to bind phosphotyrosines on both IFNAR1 and IFNAR2, while STAT3 interacts with phosphosines on IFNAR1.\textsuperscript{33, 34} Once the STATs are phosphorylated, they dissociate from the receptor, dimerize, and then translocate to the nucleus to regulate gene transcription by forming a transcription factor complex.\textsuperscript{28} The transcription factor complex that is formed is the IFN-stimulated gene factor 3 (ISGF3) complex, which is composed of STAT1, STAT2, and interferon regulatory factor (IRF) 9. This complex binds to IFN-stimulated response elements (IRSEs) that are located in the promoter region of IFN-regulated genes (IRGs).\textsuperscript{28} Microarray analysis has shown the large extent of IFN related effects by identifying more than 1000 IRGs, with examples being IFN-β itself, IP-10, IL-12p40, and RANTES.\textsuperscript{35, 36} These IRGs are important to the case of septic shock because they encode proteins that are the effectors of the IFN response, and therefore effectors of the broader inflammatory response.

1.4 Animal Models of Septic Shock

Much of the initial effort in septic shock research dealt with looking into the MyD88-dependant pathway. Much of this research has gone unrewarded however, so researchers have more recently turned their efforts to the MyD88-independent pathway. Many groups have turned to evaluating knockout mice in the LPS induced model of septic shock. Initially, it was found that mice deficient in TLR4 itself were highly resistant to the LPS-induced model of septic shock.\textsuperscript{15, 37} Additionally, mice deficient in various components of the type I interferon response, show a great resistance to the LPS induced model of septic shock, including IFNβ-/-.\textsuperscript{38, 39} Tyk2 -
IRF3-/-, IFNAR1-/-, and STAT1-/- mice. These KOs demonstrate that disruption of the type I interferon response pathway can lead to a reduction in the lethal effects of septic shock. In addition to continuing to evaluate knockout animal models, there needs to be a shift toward pharmacological modulation of the pathway to attempt to recapitulate the results seen in the mouse KO mouse models.

1.5 Histone Modification

Histones are subject to numerous posttranslational modifications such as acetylation and methylation of lysines (K) and argenines (R), phosphorylation of serines (S) and threonines (T), ubiquitination, and sumoylation. These modifications change the availability of the chromatin to DNA-binding transcription factors by modifying the local histones around which the nucleosomal DNA is wound. The acetylation of amino groups of lysine residues on the N-terminal tail of core histone proteins is an extensively studied example where there is a dynamic chromatin state achieved through the competition between two enzymes, histone acetyl transferase (HAT) and histone deacetylase (HDAC). It is becoming more apparent that this acetylation is the most important of the mentioned histone modifications in the context of septic shock.

1.5.1 Histone Acetylation/Deacetylation

Histone acetylation is most commonly associated with an increase in gene expression. Through the acetylation of the lysine residues, the chromatin opens up into a relaxed state and allows for increased transcription to occur. With opposite action, HDACs are traditionally
It is more recently becoming apparent, however, that this is an overly simplistic view, and the actual mechanism by which HATs and HDACs regulate gene expression is very complex. Microarray analysis of gene expression following treatment with HDAC inhibitors shows that only a small number of genes are differentially regulated as a result of the treatment, and the proportion of genes which increased or decreased in expression level was approximately equal.\(^\text{15 Mariadason, J.M. 2000}\)\(^\text{16 Chang, H.M. 2004}\) This interesting result is confirmed by recent results that show HDACs to indeed be activators of transcription.\(^\text{44, 45}\) Moreover, it is also becoming clear that for activation of a small set of genes, both HAT and HDAC activity appears to be required.\(^\text{45, 46}\)

These non-traditional roles of HDACs are significant in the context of innate immunity and septic shock because HDAC activity is required for the activation of select interferon responsive genes. This is accomplished by recruitment of RNA polymerase II (Pol II) to the promoters by HDACs.\(^\text{46}\) It has also been shown that transcription of IFN\(^\text{τ}\) itself requires general histone deacetylase activity.\(^\text{47}\)

### 1.5.2 Histone Deacetylase

There are four classes of mammalian HDACs, based on their similarity to protein initially found in yeast. Class I consists of HDAC 1, 2, 3, and 8 and exhibits similarity to yeast Rpd3.\(^\text{48, 49}\) Class II consists of HDAC 4, 5, 6, 7, 9, and 10 and exhibits similarity to Hda1.\(^\text{50, 51}\) Class III consists of the sirtuins and is homologous to Sir2. Class IV consists of only HDAC 11.\(^\text{52}\) Class I HDACs are thought to be primarily localized in the nucleus, whereas class II HDACs shuttle
between the cytoplasm and the nucleus. Histone deacetylases cannot bind DNA directly; rather they form multiprotein complexes that can regulate gene expression. Two of the more well-characterized complexes that contain class I HDACs are the Sin3 complex and the nucleosome remodeling and deacetylating (NuRD) complex.

### 1.6 Histone Deacetylase Inhibitors

There have been many pharmacological inhibitors of HDAC activity (HDACi) developed. The best characterized of these are the hydroxamic acids trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA), and IFIT2357. Other HDACis include the short chain fatty acids sodium butyrate (NaB) and valproic acid, which is a widely used anti-seizure agent. Since HDAC inhibition has been shown to promote cell-cycle arrest and enhance the activity of pro-apoptotic pathways, HDAC inhibition is currently under evaluation as a novel chemotherapeutic treatment modality. Thus far, the two most widely used are TSA and SAHA. SAHA was approved by the FDA in 2006 for the treatment of cutaneous T-cell lymphoma under the generic name Vorinostat. Both SAHA and TSA are broad spectrum HDACi, which means that they inhibit many HDACs of different classes.

In the context of septic shock, the potential effects of HDACi on additional pro-inflammatory pathways must be taken into consideration. It appears likely that HDACi also affects type II IFN signaling. This is accomplished because HDAC activity is required for gene expression through the STAT1 transcription factor. STAT1 is known to be activated following receptor engagement of both type I IFN and type II IFN (IFN-γ) IFN receptors. Indeed, several
studies have shown that TSA, IFIT2357 and SAHA are all able to inhibit IFN-γ dependant gene expression. This is complicated in the case of septic shock however because in some cases it was found to be protective.

Since the many HDACs are known to be active and involved in various different pathways, it would be beneficial to shift from using the broad spectrum HDACi in favor of ones that act very specifically on a narrow range of HDACs. This makes it an active area of research, and more isospecific HDACi are being developed and evaluated. siRNA has been used to selectively inhibit specific HDACs, but this shows the most promise in continuing to elucidate the roles of the individual HDACs. Evidence has emerged that which indicates that HDAC6 may be important for type I IFN signaling. Nusinzon and Horvath were able to show that siRNA knockdown of HDAC6 leads to a significant decrease in IFN-β transcript levels. Given this result, it is necessary for HDAC6 specific inhibitors to be evaluated in the context of septic shock.

1.6.1 HDAC6 Specific Inhibitors

Early on, during a genetic high throughput screen with a cell-based assay, Haggarty et al. were able to identify Tubacin as an efficient HDAC6 specific inhibitor, which was originally developed to inhibit HDAC6-mediated tubulin deacetylation. This was a promising start, however the in vitro selectivity of tubacin for HDAC6 was later found to be rather moderate. In addition, there is a laborious 27 stage synthesis involved in synthesizing Tubacin, again making it a poor candidate.
There has been a more recent push to develop a so called “second generation” HDAC6 specific inhibitors, in light of the shortcomings of the previous generation HDAC6 specific inhibitors. Some of these new compounds have been described by Kozikowski et al.\textsuperscript{68} These compounds were evaluated for their ability to block pancreatic cancer cell growth and found to be about 10-fold more potent than SAHA, the broad spectrum HDAC inhibitor. The most promising of their candidate compounds shows \textit{in vitro} picomolar concentration activity at HDAC6, while others showed nanomolar concentration activity.\textsuperscript{68} These compounds are very encouraging in the context of preventing septic shock and should be further evaluated.

1.7 Summary

In summary, septic shock is a serious infection condition that currently has a subpar treatment regime. The deleterious effects of septic shock are largely a result of a cytokine storm initiated primarily by signaling by TLR4 through the NFkB and type I IFN pathways. Experiments using KO mice for various components of these pathways have demonstrated a great resistance to lethal LPS challenge, indicating that modulation of these pathways represents a possible treatment modality. The literature has shown that HDAC activity is a required component for activation of the type I IFN response, so the use of HDACi represent a possible option for modulation of the pathway leading to reduced pro-inflammatory cytokine levels and ultimately improved outcome for patients with septic shock.
2.1 Introduction

Studying septic shock in a human model can be difficult for a variety of reasons, including the complex nature of the disease, the heterogeneity of the population, and the lack of clearly defined biomarkers with which to make a diagnosis. In light of this, the use of animal models has been increasingly used as a valuable tool for the study of sepsis and septic shock. Mice and rats are the most commonly used species for animal models of septic shock because of their relatively low cost, ease of breeding, and the fact that they share many common disease pathways with humans. Most animal models of septic shock involve the administration of toxins, most notably LPS, which can be administered through intravenous, intraperitoneal, and intratracheal routes. One possible downside to the use of mice and LPS in an animal model of septic shock is that rodents are known to be particularly resistant to LPS while humans are relatively sensitive to it. In one study, it was found that mice require a 250-fold larger dose administered intraperitoneally to achieve a similar response to intravenous injection in humans.
As previously mentioned, the use of LPS to induce a septic shock model in mice is well documented. There have been numerous examples given of KO mice that show great resistance to challenge with LPS.\textsuperscript{15, 37-39} In order to evaluate pharmacological modulation of the type I IFN pathway in the LPS induced model of septic shock, we performed animal experiments with the use of HDACi. We used both TSA and SAHA at varying concentrations along with LPS challenge of differing concentrations to evaluate this model (Figure 1). All animal experiments were conducted under animal protocols AM10091 and AM10048.

### 2.2 Methods and Materials

**TSA**

Female C57Bl/6 mice (4-6 weeks) weighing 18-23g were purchased from Jackson Labs (Bar Harbor, ME). All animals were housed in plastic cages and had access to chow and water during the course of experimentation. These animals were kept at room temperature and exposed to light cycles of 12 h light and darkness. The mice were randomly divided into two groups (n=8): LPS control and TSA + LPS. TSA was dissolved in a DMSO carrier, and LPS was dissolved in a sterile PBS carrier. For 7 days prior to LPS challenge, the mice in the TSA + LPS group were given daily subcutaneous injections of either 2.5 mg/kg or 7.5 mg/kg TSA (Upstate Biotechnology, Lake Placid, NY), while the LPS control mice were only given subcutaneous injections of DMSO carrier. On day 7, immediately following the final TSA and DMSO injections, all mice were injected intraperitoneally with either 20 mg/kg, 30 mg/kg, or 50 mg/kg LPS (from *S. typhimurium*), purchased from Sigma Chemical Co (St. Louis, MO). Survival rate of the mice was then monitored for a period of 96 hours following LPS challenge. The endpoint of these experiments was considered signs of significant respiratory distress and or loss of ambulation, at
which point the mice were euthanized.

**SAHA**

For the initial SAHA experiments, female C57Bl/6 mice (4-6 weeks) weighing 18-22g were used, and for the final SAHA experiment, male C57Bl/6J (6-8 weeks) weighing 19-23g were used. All mice were purchased from Jackson Labs (Bar Harbor, ME). All animals were housed in plastic cages and had access to chow and water during the course of experimentation. These animals were kept at room temperature and exposed to light cycles of 12 h light and darkness. The mice were randomly divided into two groups (n=8): LPS control, and SAHA + LPS. SAHA was dissolved in minimal DMSO and then further diluted with sterile PBS and LPS was again dissolved in sterile PBS.

For the initial SAHA experiments, at 72 hours, 48 hours, 24 hours, and immediately before LPS challenge, the SAHA + LPS group mice were injected subcutaneously with doses 50 mg/kg or 100 mg/kg with SAHA purchased from Cayman Chemical. Mice in the control group were injected subcutaneously with equivalent volumes of DMSO. Immediately following the final SAHA injections, all mice were given intraperitoneal (IP) injections of 30 mg/kg LPS (*S. typhimurium*). Survival rate was then measured for 96 hours following LPS challenge. Again, the endpoint of these experiments was considered signs of significant respiratory distress and or loss of ambulation, at which point the mice were euthanized.

For the final SAHA experiment, SAHA (50 mg/kg) was given intraperitoneally 2 hours prior to, and immediately after LPS challenge. Mice in the control group received DMSO (1 µl/g body weight) intraperitoneally. All mice were given IP injections with 20 mg/kg LPS (*S. typhosa*). Survival rate was then observed for 96 hours. Again, the endpoint of these experiments was considered signs of significant respiratory distress and or loss of ambulation, at which point the mice were euthanized.

**2.3 Results**
For the initial experiment we chose a TSA concentration of 2.5 mg/kg and an LPS dose of 50 mg/kg based on dosages previously described in the literature (Figure 1A). In this experiment, all mice reached endpoint within 20 hours, indicating too high a dose of LPS. There did seem to be a minimal delayed effect of the LPS in the SAHA treated mice. This effect could be better seen with a lower dose of LPS, so for the next experiment we chose a TSA dose of 7.5mg/kg and a LPS dose of 20mg/kg (Figure 1B). In this experiment, all TSA treated and control mice survived, demonstrating that a non-lethal dose of LPS was used. This is useful because it provides a low range of dosing for the LPS type used in this experiment. For the final TSA animal experiment we used a dose TSA of 7.5 mg/kg and an LPS dose of 30 mg/kg (Figure 1C). This experiment showed no significant difference in survival rates between TSA treated and control mice. 50% of TSA treated mice survived past 72 hours whereas as 37.5% of control mice survived. Additionally, there was no observable difference in the onset of symptoms and time to endpoint between the two groups. This shows that a higher dose of TSA might be needed to have an effect, but the dose of 7.5 mg/kg was at the high range of what was shown to be acceptable in the literature. As a result, we chose to evaluate SAHA in the same type of experiment.

We began with a SAHA dose of 0.05 mg/kg based on previously described literature values, and maintained the LPS dose of 30 mg/kg (Figure 1D). Here, 12.5% of SAHA treated mice survived whereas all control mice reached endpoint within 54 hours. There appeared to be no significant difference between the two groups, suggesting that a higher dose of SAHA should be used. Next, we chose a SAHA dose of 0.1 mg/kg and the same LPS dose of 30 mg/kg (Figure
This experiment seemed to show some delayed effects of the LPS challenge in the SAHA treated mice. The control mice all reached endpoint in 42 hours whereas the SAHA-treated mice took 48 hours to reach endpoint. If indeed there is a difference being created by the SAHA treatment, a higher SAHA dose would be required to determine the effect.

Before we had a chance to perform another mouse experiment with a greatly increased SAHA dose, evidence was published that SAHA indeed has a protective effect against LPS induced septic shock in rodents. The experimental design was to give IP SAHA injections (50mg/kg) to experimental mice 2 hours before LPS challenge (20mg/kg) and then another SAHA injection immediately following challenge. They monitored mouse survival over the next several days and found that the SAHA treated mice showed a significantly higher survival rate (87.5%) than the control mice (0%). This was a promising result, so we decided to use the same experimental design to confirm these results. Indeed, we found that in this case the SAHA was able to greatly improve survival rates of experimental mice (78%) compared to control (0%), with a significant p-value of 0.0143.

2.4 Discussion

There were several experimental design differences between our initial SAHA experimental design and the published successful attempt that could account for the difference in results. These include the use of a much higher dose of SAHA, the use of a different LPS isolate, and the use of male instead of female mice. Regardless, this result is very promising in terms of being able to use a pharmacological compound to ameliorate lethal effects associated
with septic shock and improve survival rate.

These are promising initial results for the use of SAHA in treatment of septic shock, but further analysis is needed however to provide a more optimal dosing regime. Different doses and routes of administration need to be studied in an attempt to fine the lowest effective dose. Experiments should also be performed where SAHA is administered at time points following LPS challenge to evaluate increased survivability, as this applies more to a clinical setting. Additionally, the use of HDAC6 specific inhibitors should be evaluated as their more specific effect should prove beneficial in this setting.
**Figure 2. Effect of LPS on SAHA or TSA treated versus wild type mice**

**A.** C57Bl/6 mice were given either TSA (2.5mg/kg) or DMSO control subcutaneously 1 shot per day for 7 days, and then on day 7 all mice were challenged with LPS (50mg/kg) IP. Survival rate was observed for 24 hours.

**B.** C57Bl/6 mice were given either TSA (7.5mg/kg) or DMSO control subcutaneously 1 shot per day for 7 days, and then on day 7 all mice were challenged with LPS (20mg/kg) IP. Survival rate was observed for 72 hours.

**C.** C57Bl/6 mice were given either TSA (7.5mg/kg) or DMSO control subcutaneously 1 shot per day for 7 days, and then on day 7 all mice were challenged with LPS (30mg/kg) IP. Survival rate was observed for 72 hours.

**D.** C57Bl/6 mice were given either SAHA (0.05mg/kg) or DMSO control subcutaneously 1 shot per day for 3 days, and then on day 3 all mice were challenged with LPS (30mg/kg) IP. Survival rate was observed for 54 hours.

**E.** C57Bl/6 mice were given either SAHA (0.1mg/kg) or DMSO control subcutaneously 1 shot per day for 3 days, and then on day 3 all mice were challenged with LPS (30mg/kg) IP. Survival rate was observed for 54 hours.

**F.** C57Bl/6 mice were given either SAHA (50mg/kg) or DMSO control 1 shot IP, then 2 hours later all mice were challenged with LPS (30mg/kg) IP. Immediately after LPS challenge, a second IP dose of SAHA (50mg.kg) was given. Survival rate was observed for 96 hours.
Effect of LPS (50 mg/kg) on TSA (2.5 mg/kg) treated vs. wild type mice
Effect of LPS (30 mg/kg) on TSA (7.5 mg/kg) treated vs. wild type mice
Effect of 30 mg/kg LPS dose on SAHA treated (0.05 mg/kg) vs. wild type mice
Effect of 30 (mg/kg) LPS on SAHA (0.1mg/kg) treated vs. wild type mice
Effect of LPS (20mg/kg) on SAHA (50mg/kg) treated vs. wild type mice
Chapter 3 Microarray Analysis of Global Response to SAHA and LPS

3.1 Introduction

As mentioned, HDACi have been a focus of cancer research for their potential ability to regulate cell cycle, differentiation and apoptosis in cancer cells. Because of this, microarray analysis has been done using various HDACi. For instance, microarray analysis has been used to help elucidate the antitumor effects observed with the HDACi FK228. Additionally, microarray analysis of TSA treatment on human MOLT-4 and LNCaP human prostate cancer cells showed that roughly similar numbers of genes were up and down regulated following treatment, and this was used to elucidate the mechanism of apoptosis induced by TSA.

There was some promising unpublished preliminary RT-PCR data in our lab that suggested that HDACi could decrease otherwise high transcript levels of IFN-β subsequent to stimulation with LPS in BMDMs. To our knowledge, HDACi treatment in an LPS induced model of septic shock has never been evaluated on a genome wide scale, so we performed microarray analysis to ascertain the effects of SAHA treatment on LPS treated cells in terms of innate immune responses (Figure 3).
3.2 Materials and Methods

The use of microarrays was an essential component of this thesis. We performed in-house fabrication of mouse whole genome 70-mer oligonucleotide arrays using a custom high-speed contact printer. The oligonucleotide features that were spotted on the arrays were based on the "Mouse Exonic Evidence Based Oligonucleotide Chip" or MEEBO Chip. The MEEBO Chip reference design contains 38,467 features, with a goal of providing at least one oligonucleotide corresponding to an expressed exon for each gene in the mouse.

In order to compare the expression patterns of SAHA treated and SAHA treated LPS infected versus untreated control, total RNA was collected as previously described. It was then reverse transcribed in a two-step reaction using Superscript® II Reverse Transcriptase from Invitrogen (Carlsbad, CA). In the initial step, 35 ng of total RNA samples were primed at 70°C with 2μg of oligo dT primer (5'-TTT TTT TTT TTT TTT TTV N-3'). Then, cDNA was synthesized in a 30 uL reaction containing 50mM Tris-HCl pH 8.5, 75mM KC1, 3mM MgCl2, 0.01 M DTT, 5mM dATP, 5mM dCTP, 5mM dGTP, 3mM dTTP, 2 mM aminoallyl dUTP and 100 units of Reverse Transcriptase at 42°C for 2 hours. Leftover RNA was then hydrolyzed by addition of 100mM NaOH and 10mM EDTA and incubation at 65°C for 10 minutes. The hydrolysis reaction was then neutralized by the addition of 500 mM HEPES pH 7. cDNA was then transferred to a Microcon YM-30 column (Fisher Scientific, Hanover Park, IL), and spun at full speed for 6 minutes. cDNA was then washed with MiliQ H2O and spun at full speed for 6 minutes, to remove the hydrolyzed RNA, NaOH and buffer components. This wash step was repeated, and then column was flipped into a new microtube, and spun for 1 minute at full speed to recover cDNA. cDNA samples were coupled by linkage of primary amine group on newly synthesized cDNA strands to N-hydroxysuccinimidyl ester group attached to either Cyanine 3 (for untreated control sample) or Cyanine 5 (for treatment sample) dye. This dye-coupling reaction was carried out in 100mM sodium bicarbonate.
buffer at a pH of 9.0 for 3 hours in the dark. Uncoupled dye was removed using the Qiaquick PCR purification system from Qiagen (Valencia, CA). The labeled cDNA samples were then combined with their proper counterpart. 20μg of mouse cot-1 DNA, 20μg poly-A RNA and 20μg of yeast tRNA were added to combined labeled samples. The final hybridization samples contained labeled cDNA, 3.4X SSC and 0.3% SDS in TE buffer. Probes were then denatured at 100°C for 2 minutes and spun down. Probes were then placed on microarrays and covered with an appropriate sized cover glass. Hybridizations were allowed to continue for 14-16 hours at 63°C in a custom microarray chamber with humidity maintained by drops of 3X SSC on the cover glass. Microarrays were then washed 3 times with increasingly reduced stringency solutions starting at 2X SSC and 0.3% SDS to 1% SSC to 0.2X SSC. Slides were dried by centrifugation and scanned using Genetix 4200A microarray scanner. Array images were quantized with the GenePix 5.1 package (Axon), and submitted to the Ramhorn Array database (http://ramhorn.csbc.vcu.edu). Ramhorn is maintained by the Fawcett lab and is a sophisticated microarray analysis platform that is a VCU-specific implementation of the open-sourced Longhorn Array Database. 80

3.3 Results/Discussion

Wild-type BMDMs were treated with 1μM SAHA or DMSO control and then immediately treated with 10ng/ml LPS. Total RNA samples were collected at 0, 4, and 8 hours post treatment. Labeled RNA samples from experimentally treated conditions were hybridized onto the same microarray with their untreated mock control counterparts. Our microarrays were produced locally in our lab at VCU, but the platform was based on an open-sourced spotted oligonucleotide microarray standard called the "Mouse Exonic Evidenced-Based Oligonucleotide" (MEEBO) which has been widely adopted.
Our experimental design used a “Type I” approach where each experimental condition was hybridized against an untreated control. The microarrays were scanned and all data was then uploaded to the VCU Ramhorn database for analysis. To obtain the clustergram displayed in figure 3, we performed the following analysis. For each array, the Ramhorn was used to select for spots with hybridization intensities more than 1.8 fold above the background in both channels, with more than 50% good data across the array set were retrieved. To then determine which genes were altered in their expression in a treatment-dependent manner, we then used the Significance Analysis of Microarrays (SAM) algorithm to perform a series of two-class unpaired analyses between data from LPS versus SAHA+LPS conditions at 4 and 8 hours post-treatment. The SAM analysis was done using 1000 permutations and we controlled the False Discovery Rate (FDR) of each analysis by choosing the lowest delta value resulting in a median FDR of 5% or less. This results in reporting of genes with qvalues (the multiple-comparison adjusted analog of the p-value employed by SAM) <0.05. The Stanford Unique Identifiers (SUIDs) representing each significantly regulated gene were then retrieved and this pool of SUIDs was used to reacquire the data from Ramhorn. The average was calculated across all the biological replicates of each condition and time point. Then the data was filtered again to select SUIDs that showed at least two-fold difference in expression. SUIDs that passed this filtering were considered “selected” and were collected in a master spreadsheet. Duplicate SUIDs were removed and only non-redundant SUIDs were used for the final retrieval of data from Ramhorn database using all arrays from all treatment regimes and time points. Genes with 50% good data across the entire sets of arrays using the initial conditions were used for the final data retrieval. Therefore, under this scheme, designation of "selected" for a gene for any one condition or time
point was sufficient to include the gene in the final cluster and display all of its associated data whether significant in its own right or not. Mean averages of all biological replicates were then calculated for all treatment conditions and time points. The Cluster program \(^2\) was used to perform hierarchical clustering of the dataset using the average linkage clustering method, and exported to the Treeview \(^3\) program for visualization.

**Upon preliminary examination of figure 3, which represents 1712 genes, certain striking aspects are immediately apparent. It is immediately apparent that roughly equal numbers of genes are up or down regulated by SAHA. Additionally it is apparent that only a smaller subset of these genes is activated by LPS with the rest either showing no regulation or down regulation in the LPS treatment condition. Finally, it seems that nearly all genes showing strong up regulation in the LPS treatment condition are up regulated in the SAHA+LPS treatment condition at both 4 and 8 hour time points. To elucidate more subtle differences between the condition points in the data, closer examination of the clustergram is needed. To help accomplish this, we have highlighted 5 main clusters which seem to represent significant aspects of the data.**

**Cluster 1a** broadly represents the genes that are mildly up or down regulated by SAHA alone or LPS alone but are strongly down regulated in the SAHA+LPS condition. This appears equally evident at both the 4 and 8 hour time point. This result could indicate that these are genes which are not clearly regulated by either SAHA or LPS independently, but upon combined treatment there is a strong repression of the genes.
Cluster Ib broadly represents the genes that are strongly down regulated in the SAHA and SAHA+LPS treatment conditions, but either not differentially regulated or mildly up regulated in the LPS condition. These results are consistent across the time course, with possible increased repression occurring at time 8 hours. This indicates that these genes are likely ones which are down regulated by SAHA treatment.

Cluster II represents genes whose expression SAHA alone has very little effect on, but LPS causes a large activation. Given this, it is likely that these genes play a large role in the case of septic shock. This is important because many of these genes that are so highly up regulated upon treatment with LPS are not nearly as highly expressed under the SAHA+LPS treatment condition. This presents promising evidence that treatment with SAHA following LPS treatment can alter the gene expression patterns of innate immune regulated genes that play an important role in the course of septic shock. Interestingly, there is only a very small subset of these genes that show strong up regulation under LPS as well as SAHA+LPS conditions, indicating that SAHA has an inhibitory impact on most genes that are strongly activated by LPS. Further analysis of this cluster reveals that it is enriched for innate immune regulated genes.

Cluster IVa broadly represents genes that are activated in the SAHA and SAHA+LPS conditions and slightly repressed in the LPS alone condition. This data suggests that the genes in this cluster are not differentially expressed as a result of LPS treatment, but are activated by the presence of SAHA.
**Cluster IVb** represents, in similar fashion to cluster IVb, genes that are activated in both SAHA and SAHA+LPS conditions and strongly repressed in LPS alone condition. This data suggests that these genes are, much like cluster IVa, activated by the presence of SAHA, albeit to an apparently lesser extent. However, unlike cluster IVa, these genes appear to be strongly repressed in the presence of LPS. This is a very important finding because while it is expected that genes will be activated by SAHA, it is interesting that these activated genes are also repressed during LPS treatment.

**Clusters III and V** contain seemingly random genes that contain no obvious set pattern of expression. The genes in this cluster show mild up regulation in the SAHA treated condition at time 4 and 8 hours. They show mild down regulation upon LPS treatment at 4 hour, but at 8 hours under the same condition show both up regulation and down regulation. Additionally, in the SAHA+LPS treatment condition, they show a near equal split of up regulation and down regulation at 4 hours as well as 8 hours post treatment.
Figure 3. Clustergram of transcriptional responses in BMDMs treated with SAHA alone, LPS alone or LPS and SAHA

Wild-type BMDMs were treated with 1µM SAHA or DMSO control and then immediately treated with 10ng/ml LPS. Total RNA samples were collected at 0, 4, and 8 hours post treatment. RNA samples from experimentally treated conditions were labeled with Cyanine-5 (red) while RNA samples from untreated mock control were labeled with Cyanine 3 (green). Labeled RNA samples from experimentally treated conditions were hybridized onto the same microarray with their untreated mock control counterparts. Genes were selected using the procedure described in the text. All 3 experimental conditions are shown; SAHA, LPS, and SAHA+LPS. The DIF column represents SAHA+LPS condition values subtracted from LPS alone condition values. Each column under the different treatment conditions represents incubation times of 4 and 8 hours respectively. Data is colorimetrically represented such that increasing red intensity reflects increasing up-regulation as compared to time zero, while green reflects down-regulation.
Collectively these microarray results reveal various effects of SAHA treatment in the LPS treatment model. It is apparent that roughly equal numbers of genes are activated or repressed upon treatment with SAHA. It is also apparent that LPS stimulation strongly activates only a small subset of genes, which are to a large extent innate immune regulated. Furthermore we found that the LPS stimulated activation of many of these genes is modulated to varying extents through LPS treatment.

In order to further examine the microarray data we co-clustered our data along with the data of lab mate Nusara Satproedrai used in her dissertation *Adaptor Specificity in the Toll-like Receptor 2 Pathway*, in which she extensively characterized innate immune responses in BMDMs (Figure 4). The genes selected for this cluster were statistically shown to be innate immune regulated and were selected following SAM analysis from any one treatment condition. This means that although each gene selected may not have been found to be regulated by LPS treatment, it was found to be regulated by another innate immune stimulated treatment. Represented in this cluster are averaged replicates for SAHA, LPS, SAHA+LPS, WT LPS, WT PAM. The WT LPS and WT PAM represent array data from Nusara while all other columns represent our array data. WT PAM is a treatment condition where BMDMs were treated with PAM3CSK4, which is the well-known ligand for TLR2. The DIF column represents values from the SAHA+LPS condition subtracted from the LPS treatment condition. Again, each treatment condition has columns representing 4 and 8 hours post-treatment.

Immediately noticeable are some interesting aspects of this cluster. First, it is apparent that there are a substantial number of genes that are down regulated by SAHA following LPS
treatment. Also, it is apparent that our LPS treatment condition dataset largely corresponds to the WT LPS data. It appears however that many genes were activated to a lesser extent in the WT LPS data set than in our LPS treatment condition. This is most likely attributable to either a different lot of LPS used, or the use of frozen BMDMs instead of fresh. Additionally, it is interesting to note that many of these genes are not activated in the WT PAM condition to the extent that they are in the LPS and WT LPS conditions, indicating that these genes are ones which are activated through TLR 4 but not TLR2. There are genes however that are activated in both the LPS and PAM conditions indicating some level of redundancy between TLR2 and TLR4 signaling.

There are 5 distinct clusters apparent in this figure. Cluster I represents genes that are largely unregulated in the SAHA and SAHA+LPS conditions but down-regulated in the LPS, WT LPS and WT PAM treatment conditions. Cluster II represents genes which are down regulated across all treatment conditions. Cluster III is the most important cluster in this figure. This cluster represents genes which are strongly activated by LPS but are much less activated or even repressed in the SAHA+LPS condition. Cluster IV consists of genes that SAHA has no effect on but are activated by LPS and PAM3CSK4. Cluster V consists of genes which are not activated by LPS or PAM3CSK4 but are repressed by SAHA.

Since Cluster III consists of genes that are activated by LPS but are much less activated or even repressed in the SAHA+LPS condition, it is necessary to look at this cluster in more detail. A zoomed-in image of Cluster III was generated using The Cluster program and displayed in Figure 5. It is immediately apparent that indeed Cluster III was enriched for IRGs as
well as other innate immune responsive genes. Clustered together in Figure 5 are IRGs; Interferon-induced protein 44, Interferon-induced protein 75, Interferon-stimulated protein, Interferon regulatory factor 7, 2’-5’ oligoadenylate synthetase 3, Chemokine (C-C motif) ligand 8 and Chemokine (C-C motif) ligand 12.

It is unfortunate however that many other IRGs and innate immune responsive genes clustered further apart from one another. So in order to more fully appreciate the inhibitory effect that SAHA exhibited on the activation of IRGs Table 1 was generated showing the fold changes of some of these genes. Displayed in this table are genes that were down regulated by SAHA treatment following stimulation with LPS. There is a diverse array of genes that SAHA is able to repress. While many of these are simply genes known to be IFN responsive (Ifit2, 3; Ifi35, 44, 75, 203, 205; Isg20; Oas1g, 2, 3, 11, 21; etc.), there are a large number of genes present that are directly involved in various IFN signaling pathways (Irf5, Irf7, Stat1, Stat2, Jak2, MyD88, etc.). This may indicate that SAHA treatment is able to reduce the downstream IRGs by inhibiting activation of the primary genes which activate the IFN response pathways.
Figure 4. Clustergram comparing our data to previously obtained data using LPS treatment in BMDMs
Figure 5. Zoomed-in image of Cluster III from main clustergram showing innate immune regulated genes.
Table 1: List of genes from microarray data down regulated by SAHA

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Chapter 4 Transcript and Protein Levels of Key Pro-inflammatory Mediators

4.1 Introduction

SAHA has been well appreciated in cancer research for some time, but it is now beginning to be studied in the context of septic shock. It has recently been shown that treatment with SAHA is able to reverse an LPS-induced reduction in histone acetylation in vitro in macrophages. Additionally, SAHA and ITF2357 reduced the transcript levels of iNOS, COX-2 and IL1β present in glial cell cultures after LPS insult. Consistent with these results, SAHA and ITF2357 were found to be able to reduce TNF-α protein levels in a dose-dependent manner. ST-2-92 is a relatively new compound that has not been investigated in terms of ability to modulate key inflammatory cytokine expression following stimulation by LPS. However it is an HDAC6 specific inhibitor, with HDAC6 being shown to be important for activation of the type I IFN response.

Our previously presented microarray work showed that SAHA was able to decrease the expression of many innate immune response genes in the context of LPS stimulation. SAHA has
also been shown to decrease serum level of IL-1β and TNF-α in vivo.\(^7\) Additionally, SAHA and ITF2357 were found to be able to reduce TNF-α protein levels in a dose-dependent manner in glial cell culture.\(^8\) As previously mentioned, ST-2-92 is a relatively new compound that has not yet been investigated in terms of ability to modulate key inflammatory cytokine expression following stimulation by LPS. We have established that pre-treatment with SAHA or ST-2-92 has the ability to decrease IFN-β, TNF-α, and other IRGs transcript induction after treatment with LPS through RT-PCR and microarray analysis. As mentioned, others have shown that SAHA decreases TNF-α and IL-1β protein levels; we wanted to confirm these results with SAHA. We also wanted to investigate the effects of ST-2-92 on TNF-α and IL-1β protein levels, as well as other inflammatory cytokine levels.

4.2 Materials and Methods

Bone marrow was harvested from the femurs and tibias of 4-8 week old female wild type C57BL/6 mice (AM10048), and placed in cold DMEM (Invitrogen, Carlsbad, CA) +1% antibiotic-antimycotic (Invitrogen, Carlsbad, CA). The bones were then cut open at both ends and marrows was eluted into DMEM + 1% antibiotic-antimycotic using 26G needle attached to a 10 ml syringe. Bone marrows were broken up by repeated pipetting until homogenous. Cell suspensions were spun at 500 rpm for 1 minute to remove unwanted residuals. Cell suspensions were transferred to a new collection tube and spun down to collect all cells at 2000 rpm for 10 minutes. Supernatant was aspirated off and washed one time with cold DMEM + 1% antibiotic-antimycotic. Cells were pelleted by spinning at 2000 rpm for 10 minutes, and the supernatant was aspirated off. Fresh bone marrow-macrophage (BMM) media were added (DMEM + 20%...
heat-inactivated FBS (Hyclone, Logan, UT) + 30% L-cell condition media + 1% L-Glutamine (Invitrogen, Carlsbad, CA) + 1% antibiotic-antimycotic + 0.1% 2-mercaptoethanol (Invitrogen, Carlsbad, CA). This growth media is well known to promote the differentiation of the bone marrow cell population into primary macrophages. Cell pellet was broken up by aspirating up and down several times. Cells were then spun down at 2000 rpm for 10 minutes. Supernatant was removed and new BMM media were added. Cells were re-suspended by pipetting up and down several times until homogenized. The bone marrow cells were then plated on 150mm² x 25mm deep tissue culture plates at a concentration of 1.5x10⁷ cells per plate, in a bone marrow macrophage (BMM) media. Cells were incubated in 5% CO₂, 37°C. The cells were allowed to grow for 3 days. On day 3, the cells were fed with 30 mL of the BMM media. On day 6, cell morphology was examined to check for the characteristic macrophage appearance. 10 ml of cold PBS pH 7.0 was then added to the plate and was incubated at 4°C for 15 minutes, casing the macrophages to release from the plate. Macrophages were then collected in a 50 ml tube and spun down at 1200 rpm for 5 minutes. Macrophages were re-suspended in fresh BMM media without antibiotic-antimycotic. Cells were then counted in New Bauer chamber and plated at a desired concentration.

Cell treatment conditions

For treatment with SAHA infections, roughly 16 hours prior to infection, 1.5x10⁷ bone marrow derived macrophages were plated per 3x150 mm tissue culture plate using the BMM media previously described, and incubated overnight at 37°C and 5% CO₂. The next morning, appropriate experimental BMDM were treated with SAHA at a concentration of 1 µM
immediately before infection with LPS from *Escherichia coli* 055:B5 (Invitrogen) at a concentration of 10 µg/mL. For treatment with KB-5-2 and ST-2-92, 6X10^5 bone marrow derived macrophages were plated into 6-well plates. Appropriate experimental BMDM were treated with either 1 µM KB-5-2 or ST-2-92 2 hours prior to infection with LPS from *Escherichia coli* 055:B5 (Invitrogen) at a concentration of 10 µg/mL. Every condition and time point had a mock infection control conducted in parallel. At time 1 hour post infection, gentamycin was added at a concentration of 50 µg/mL to all plates, to kill any extracellular bacteria in the media. At times 0, 4, 8 hours post infection, cells were lysed in the tissue culture plates, and RNA was isolated from the lysate using PureLink Micro to Midi kits (Invitrogen, Carlsbad, CA)

**Quantitative real-time RT-PCR**

Total RNA samples purified from BMDM treated with various conditions as described above were diluted in RNase-free water to the concentration of 20 ng/ml. TaqMan® quantitative real-time RT-PCR experiments were performed in the ABI Prism® 7900 Sequence Detection System (Applied Biosystems, Foster City, CA) using the TaqMan® One Step PCR Master Mix Reagents Kit (P/N: 4309169). Standard curve for mouse IFN-β and TNF-α was constructed using a series of 5-fold dilutions from 100 ng/ml down to 6.4 pg/ml. The TaqMan probes and primers were designed using Primer Express® 3.0 version. The probes were labelled in the 5' end with FAM (6-carboxyfluoresceine) and in the 3' end with TAMRA (6-carboxytetramethylRhodamine). Sequence of primers and probe are listed in Table 2. The
reactions and the synthesis of probes and primers were performed in the VCU Nucleic Acid Research Facilities. The samples were tested in triplicate under conditions recommended by the manufacturer. The comparative CT method (Applied Biosystems, Foster City, CA) \textsuperscript{89} was used to calculate fold relative increase of IFN-\(\beta\), TNF-\(\alpha\) expression from treated BMDM, versus their completely untreated counterparts. All data was normalized with 18s.
<table>
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<th>Sequence</th>
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</table>
ELISA

BMDM were seeded at 6x10^5 cells with 2 ml BMM media in a 6-well flat bottom cell culture plate and incubated in 37°C, 5% CO2, 80% humidity overnight. The next day, appropriate BMDM were pre-treated with SAHA, KB-5-2, and ST-2-92, all at a concentration of 1μM. Appropriate cells were then treated with LPS 10 ng/ml.

Cell culture supernatants were collected at 4 and 8 hours after treatment. IFN-β protein level was determined using Murine IFN-β ELISA kit v 1.4 (Pierce, Rockford, IL). 100 ul of cell culture supernatant from each sample or standard sample was added to each well of a 96-well and incubated for 60 minutes at room temperature for cytokines from the samples binding to antibodies immobilized on the well. The plate was washed 3 times with ELISA wash buffer supplied with the kit. 100 μl of the second antibody solution was then added to each well on the plate, and again incubated for 60 minutes at room temperature, followed by 3 washes. 100 ml of HRP-conjugated secondary antibody was then added to each well and incubated at room temperature for 60 minutes, followed by 3 washes. 100 ul of TMB substrate was added to each well and incubated in a dark, closed chamber at room temperature for 15 minutes. The reaction was stopped by adding stop solution. OD 450 was read within 5 minutes after adding stop solution. A standard curve was plotted using a series of 10-fold dilutions on standard solution supplied with the kit. Cytokine concentrations were determined using a standard curve.

Bioplex Assay
Mouse IL-1β, TNF-α, RANTES, IL-12p40 and IL-6 protein levels were evaluated using the multiplex assay from Bio-Rad Laboratories (Hercules, CA). The bioplex assay is a captured sandwich ELISA assay in which antibodies specific for each cytokine are covalently coupled to color-coded polystyrene beads. The beads were mixed, diluted, and fixed onto a pre-wet membrane on the bottom of a 96-well plate. 50 µl of cell culture supernatant from each treatment condition and standard samples were added to the plate. The plate was then shaken for 30 minutes at room temperature. The plate was then washed and 25 µl of biotinylated detection antibody was added to each well of the plate. The plate was again shaken for 30 minutes at room temperature. Streptavidin-phycoerythrin was then added to each well to bind the detection antibody. The sample in each well was read by the flow-based Bioplex suspension array system (Bio-Rad Laboratories, Hercules, CA) in which a specific reaction for each cytokine was identified and quantified based on the bead's color and fluorescence. Concentrations of each cytokine were quantified using the standard curve constructed from a series of 4-fold dilutions from the stock standard sample supplied with the kit using Bioplex Manager™ software (Biorad Laboratories, Hercules, CA).

4.3 Results/Discussion

Fold changes for the qRT-PCR work are displayed in supplementary table 1 and Protein level values for the ELISA and Bioplex are displayed in supplementary table 2.

It is evident that KB-5-2 treatment had no effect on transcript levels of both IFN-β and TNF-α, but treatment with ST-2-92 was able to reduce both transcript levels significantly (Figure 10). This demonstrates for the first time that treatment with ST-2-92 can reduce transcript levels
of not only primary type I IFN response cytokines, but also important pro-inflammatory cytokines from the MyD88-dependant pathway as well.

As expected, there was a large increase in protein accumulation for all proteins upon LPS stimulation compared to mock control. In the case of INF-β, there was significant reduction of protein levels with pre-treatment of both SAHA and ST-2-92 at 1μM concentration (Figure 11). Upon post-treatment of SAHA and ST-2-92 at 1 μM concentration, there was a slight inhibition of IFN-β. Again, we see significant inhibition of IFN-β protein production following treatment with both SAHA and St-2-92 at 0.1 μM concentration. IFN-β is the main player leading to the second wave of the IFN response, so inhibition of this protein leads to an overall decrease of the pro-inflammatory cytokine response launched through the type I IFN response.

For IL-1β (Figure 12) pre-treatment with high dose 1μM SAHA and ST-2-92 significantly decreased the observed protein levels compared to LPS alone condition. The 4 hour post-treatment of high dose SAHA and ST-2-92 showed minimal decrease on IL-1β protein levels. With pre-treatment of a low dose 0.1μM SAHA and ST-2-92 there was found to be no significant decrease in IL-1β protein levels. IL-1β is expressed through the My-D88-independent pathway upon TLR4 signaling, so HDACi through both SAHA and ST-2-92 is able to not only inhibit proteins expressed as a result of the MyD88-independent pathway, but the MyD88-dependant pathway as well.

For IL-6 (Figure 13), again high dose pre-treatment with both SAHA and ST-2-92 showed a significant decrease in protein level. In the high dose post-treatment conditions, ST-2-
92 continued to show a significant ability to decrease protein levels while SAHA largely lost its effect, showing only minimal inhibition. In the low dose pre-treatment SAHA showed a significant decrease in protein level while ST-2-92 showed only minimal significant inhibition of observed IL-6 protein levels. This is the first instance where we see largely differing results between SAHA and ST-2-92 treatment. Upon post-treatment of HDACi, ST-2-92 is the more potent inhibitor, whereas with pre-treatment at low dose, SAHA is the stronger inhibitor.

With IL-12p40, high dose (1μM) pre-treatment with SAHA and ST-2-92 showed a significant ability to decrease IL-12p40 protein levels (Figure 14). Upon high dose post-treatment with SAHA and ST-2-92, there was mild protein reduction. Finally, with low dose pre-treatment of the HDACis, there was significant reduction in protein level similar to that seen in the high dose pre-treatment condition. This demonstrates that for some proteins of interest, both SAHA and ST-2-92 reduce protein levels not only with standard high dose pre-treatment, but also with treatment after LPS insult, or low dose pre-treatment.

In the case of RANTES (Figure 15), both high dose pre-treatment and post-treatment of SAHA and ST-2-92 showed a significant ability to decrease protein levels. This inhibition was lost, however, with the low dose 0.1μM treatment of both SAHA and ST-2-92. RANTES is a key type I IFN regulated cytokine, so inhibition here is likely to be important in the context of septic shock.

Finally, for TNF-α (Figure 16), there were extremely high levels of protein accumulated after LPS treatment. Upon high dose pre-treatment of SAHA and ST-2-92, there was significant
decrease of protein levels. This effect was largely lost in the high dose post-treatment condition with both drugs. With low dose pre-treatment, however, there was significant reduction observed again in protein levels. TNF-α is a key inflammatory cytokine induced by NFκB in the MyD88-dependant pathway of the TLR4 response to LPS, indicating that HDACi acts on both the type I IFN response and NFκB mediated responses.

Collectively, these results are very important for several reasons. First, it is apparent that both broad spectrum HDACi through SAHA and HDAC6-specific inhibition through ST-2-92 are able to reduce pro-inflammatory cytokine protein levels. The HDAC6-specific inhibitor is preferable to the broad-spectrum inhibitor because it limits the number of pathways that are targeted. Second, it is important to note that in all proteins examined, post-LPS treatment of both HDACis were able to decrease protein levels to a varying extent. If they are to be successful in a clinical setting, these drugs need to be effective after the detrimental effects of LPS have begun to occur. Finally, it is important to note that in all proteins tested, with the exception of IL-1β and RANTES, both SAHA and ST-2-92 inhibit protein secretion at the low dose range of 0.1µM. This will be potentially beneficial in a clinical setting where it is best to use as low a dose as possible.
Figure 10. Effect of KB-5-2 and ST-2-92 on IFN-β and TNF-α transcription in BMDMs

BMDMs were treated with media control, 2 hour pre-treated with 1μM KB-5-2 or ST-2-92, and then treated with 10ng/ml LPS. At 6 hours post LPS treatment, total RNA samples were collected and submitted for real-time RT-PCR. Each bar represents the average of 3 biological replicates ± standard deviation. T-tests were performed for each condition, with p-values being displayed by the letters a (p<0.05 vs. LPS), b (p<0.001 vs. LPS), c (p<0.0005 vs. LPS), or blank (p>0.05) above each bar.
Figure 11. ELISA assay results for IFN-β

IFN-β protein levels upon stimulation with media control or 10ng/ml LPS were measured in SAHA or ST-2-92 pre-treated and post-treated wild-type BMDMs at concentrations of 0.1μM and 1μM. ELISA assay was performed with cell culture supernatant collected at 18 hours post treatment. Each bar represents an average of 3 biological replicates ± standard deviation.
Figure 12. Bioplex assay results for IL-1β

IL-1β protein levels upon stimulation with media control or 10ng/ml LPS were measured in SAHA or ST-2-92 pre-treated and post-treated wild-type BMDMs at concentrations of 0.1μM and 1μM. Bioplex assay was performed with cell culture supernatant collected at 18 hours post treatment. Each bar represents an average of 3 biological replicates ± standard deviation.
Figure 13. Bioplex assay results for IL-6

IL-6 protein levels upon stimulation with media control or 10ng/ml LPS were measured in SAHA or ST-2-92 pre-treated and post-treated wild-type BMDMs at concentrations of 0.1μM and 1μM. Bioplex assay was performed with cell culture supernatant collected at 18 hours post treatment. Each bar represents an average of 3 biological replicates ± standard deviation.
Figure 14. Bioplex assay results for IL-12p40

IL-12p40 protein levels upon stimulation with media control or 10ng/ml LPS were measured in SAHA or ST-2-92 pre-treated and post-treated wild-type BMDMs at concentrations of 0.1μM and 1μM. Bioplex assay was performed with cell culture supernatant collected at 18 hours post treatment. Each bar represents an average of 3 biological replicates ± standard deviation.
Figure 15. Bioplex assay results for RANTES

RANTES protein levels upon stimulation with media control or 10ng/ml LPS were measured in SAHA or ST-2-92 pre-treated and post-treated wild-type BMDMs at concentrations of 0.1μM and 1μM. Bioplex assay was performed with cell culture supernatant collected at 18 hours post treatment. Each bar represents an average of 3 biological replicates ± standard deviation.
Figure 16. Bioplex assay results for TNF-α

TNF-α protein levels upon stimulation with media control or 10ng/ml LPS were measured in SAHA or ST-2-92 pre-treated and post-treated wild-type BMDMs at concentrations of 0.1μM and 1μM. Bioplex assay was performed with cell culture supernatant collected at 18 hours post treatment. Each bar represents an average of 3 biological replicates ± standard deviation.
$a = p<0.05 \text{ vs. LPS}$, $b = p<0.001 \text{ vs. LPS}$, $c = p<0.0005 \text{ vs. LPS}$
In order to compare the protein results for IFN-β, IL-1β, IL-12p40, IL-6, RANTES and TNF-α back to the microarray data, we searched the microarray data for these spots and took the average of all replicates for each condition. Resulting averages were clustered using the Cluster program and displayed in Treeview (Figure 17). Note that these genes were not selected as significant using SAM statistical analysis. Also note that the IFN-β spot is consistently bad on our custom-spotted microarrays, so this gene was omitted from the results.

It is immediately apparent that these microarray results do not completely correlate to the protein level analysis for these genes. All of the genes with the exception of the interferon beta 1, fibroblast were strongly activated by LPS treatment, with IL-6 showing the strongest activation. Interferon beta 1, fibroblast showed slight activation at time 4 hours following LPS stimulation, but at the 8 hour time point all activation was lost. Tumor necrosis factor also showed slightly less activation than the other genes. The SAHA+LPS treatment condition showed little ability for SAHA to repress the activation of many of the genes. IL-6 was the only gene which SAHA was able to repress upon LPS stimulation. Tumor necrosis factor even showed slight increased activation in the SAHA+LPS treatment condition.

These results are interesting in that at the transcript level SAHA seemingly shows no ability to inhibit the activation of these genes by LPS but at the protein level SAHA shows significant ability to inhibit protein levels of these genes. This indicates that HDACi of these genes may not be lead to a decrease in transcript level but create a decrease in the resulting protein level. The qRT-PCR results refute these microarray results by showing that for IFN-β and TNF-α, HDACi through SAHA is able to reduce transcript levels. These conflicting results
indicate that the microarray results may not be entirely reliable.
Figure 17. Microarray results for key pro-inflammatory cytokines

Averages of microarray replicates each treatment condition for IFN-β, IL-1β, IL-12p40, IL-6, RANTES and TNF-α were clustered using the Cluster program and displayed using Treeview.
Tumor necrosis factor
Interleukin 6
Interleukin 1 beta
Chemokine (C-C motif) ligand 5 (RANTES)
IL12b
Chapter 5 Conclusion and Future Prospects

5.1 Conclusion

Septic shock is a complex disease state which can have severe consequences on the host. Thus far, treatment modalities for this disease are relatively primitive and ineffective; therefore there is a necessity for improvement. This past decade, KO mouse studies have demonstrated that mice deficient in various members of the type I IFN response are highly resistant to the lethal effects of LPS challenge, likely due to their reduced ability to accumulate pro-inflammatory cytokines. Therefore it seems promising that pharmacological inhibition of this type I IFN response through HDACi could recapitulate these KO results. Indeed this initially seems to be the case, as we and others have shown that SAHA treatment in mice can increase survival following LPS challenge. Also, we have shown that both broad spectrum HDACi through SAHA and HDAC6 specific inhibition through ST-2-92 can modulate the pro-inflammatory response to LPS and decrease expression of key inflammatory proteins at the transcript level. SAHA has previously been shown to decrease expression of not only IRGs such as IFN-β and RANTES, but also MyD88-dependant pro-inflammatory cytokines as well. We have shown that ST-2-92 is also able to decrease expression of pro-inflammatory cytokines from
both MtD88 independent as well as MyD88-dependant pathways.

5.2 Future Studies

Though our initial results seem very promising, further studies need to be undertaken to more fully understand and evaluate the use of HDACi in the context of septic shock. In terms of using HDACi in the animal model, different dosing regimes should be evaluated. Animal experiments should be undertaken where increasingly smaller doses of SAHA are used to test for the minimal effective dose. Also, experiments should be done where HDACi is administered at time points after LPS challenge to evaluate its effectiveness in this setting, as this would be more clinically relevant. Furthermore given our data showing the effectiveness of HDAC6 specific inhibitor ST-2-92 at decreasing pro-inflammatory protein levels, animal experiments should be undertaken to evaluate whether this compound can increase survival rate.

There were interesting features of our microarray data that should be further investigated. First, it is a surprise to see that SAHA represses some pro-apoptotic genes, which are inhibitory toward cancer formation, under the conditions of our microarrays. Further analysis of this phenomenon should be undertaken to determine whether it is biologically relevant or just an artifact of the arrays. Additionally, given the promising results with the use of ST-2-92 on transcript and protein levels of pro-inflammatory cytokines; this compound should be used in microarray analysis to get a sense of what it affects on a more global gene expression scale.


### Appendix A

#### Supplementary Table 1 Results of qRT-PCR for KB-5-2 and ST-2-92 treatment

<table>
<thead>
<tr>
<th>Treatment Condition</th>
<th>IFN-β Fold Increase</th>
<th>TNF-α Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS</td>
<td>2630.80 ±478.05</td>
<td>79.92 ±18.99</td>
</tr>
<tr>
<td>KB-5-2</td>
<td>1.26 ±0.66</td>
<td>0.92 ±0.13</td>
</tr>
<tr>
<td>KB-5-2: LPS</td>
<td>3151.99 ±458.17</td>
<td>74.20 ±16.89</td>
</tr>
<tr>
<td>ST-2-92</td>
<td>4.48 ±1.50</td>
<td>0.46 ±0.04</td>
</tr>
<tr>
<td>ST-2-92: LPS</td>
<td>236.17 ±65.26</td>
<td>25.71 ±4.11</td>
</tr>
</tbody>
</table>
## Supplementary Table 2 Results of ELISA and Bioplex Protein Analysis

<table>
<thead>
<tr>
<th>Treatment Condition</th>
<th>IFN-β (pg/ml)</th>
<th>IL-1β (pg/ml)</th>
<th>IL-6 (pg/ml)</th>
<th>IL-12p40 (pg/ml)</th>
<th>RANTES (pg/ml)</th>
<th>TNF-α (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS</td>
<td>570.58±73.20</td>
<td>2186.76±198.81</td>
<td>8389.96±945.81</td>
<td>16907.88±1277.11</td>
<td>16223.95±2682.54</td>
<td>70408.97±10510.33</td>
</tr>
<tr>
<td>SAHA</td>
<td>9.42±1.74</td>
<td>14.18±4.54</td>
<td>4.03±1.66</td>
<td>27.08±18.13</td>
<td>417.05±278.75</td>
<td>25.18±15.09</td>
</tr>
<tr>
<td>ST-2-92</td>
<td>42.31±15.30</td>
<td>13.06±16.97</td>
<td>2.71±1.04</td>
<td>8.49±0.74</td>
<td>309.22±153.65</td>
<td>5.93±32.17</td>
</tr>
<tr>
<td>SAHA (1µM) pre-treatment+LPS</td>
<td>89.15±10.15</td>
<td>623.72±4100.34</td>
<td>463.07±70.65</td>
<td>5582.87±2716.34</td>
<td>7023.84±1901.21</td>
<td>37648.51±3952.121</td>
</tr>
<tr>
<td>ST-2-92 (1µM) pre-treatment+LPS</td>
<td>45.62±45.76</td>
<td>724.16±138.03</td>
<td>366.77±31.12</td>
<td>3535.04±1283.16</td>
<td>9335.93±2063.03</td>
<td>22450.93±15562.58</td>
</tr>
<tr>
<td>SAHA (1µM) post-treatment+LPS</td>
<td>405.95±78.34</td>
<td>1625.89±177.19</td>
<td>5014.17±242.04</td>
<td>9305.92±4562.29</td>
<td>6708.90±1683.12</td>
<td>65700.53±2524.50</td>
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<tr>
<td>ST-2-92 (1µM) post-treatment+LPS</td>
<td>266.13±36.60</td>
<td>1399.51±109.75</td>
<td>1459.79±134.10</td>
<td>9130.51±1171.48</td>
<td>8101.00±917.34</td>
<td>60889.74±13728.81</td>
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<tr>
<td>SAHA (0.1µM)</td>
<td>50.94±10.39</td>
<td>10.91±4.73</td>
<td>3.72±0.76</td>
<td>19.69±9.17</td>
<td>520.52±298.41</td>
<td>32.15±15.80</td>
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<tr>
<td>ST-2-92 (0.1µM)</td>
<td>50.40±47.83</td>
<td>15.01±10.83</td>
<td>2.47±1.88</td>
<td>5.13±3.75</td>
<td>57.41±15.34</td>
<td>5.36±2.99</td>
</tr>
<tr>
<td>SAHA (0.1µM) pre-treatment+LPS</td>
<td>61.50±10.69</td>
<td>1847.54±292.76</td>
<td>478.72±27.22</td>
<td>4089.93±1427.19</td>
<td>9940.00±3544.15</td>
<td>31907.08±7490.40</td>
</tr>
<tr>
<td>ST-2-92 (0.1µM) pre-treatment+LPS</td>
<td>74.30±48.93</td>
<td>1426.71±476.36</td>
<td>4947.03±358.34</td>
<td>4576.69±1054.88</td>
<td>14005.58±2457.65</td>
<td>21444.75±16437.07</td>
</tr>
</tbody>
</table>
VITAE

Joseph Bertsche was born on February 6, 1985 in Newark, Delaware. He graduated from Virginia Commonwealth University, Richmond, Virginia with a Bachelor of Science in Forensic Science in 2007. Following graduation he joined the Molecular Biology and Genetics program in the Department of Microbiology and Immunology of the School of Medicine at Virginia Commonwealth University. During his time in the program he was a teaching assistant for LFSC 101.