THE DISCOVERY OF FUNCTIONAL SINGLE NUCLEOTIDE POLYMORPHISMS AND NOVEL GENE MECHANISMS THAT MAY EXPLAIN MORTALITY AND ORGAN DYSFUNCTION IN SEPTIC SHOCK PATIENTS

by

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Abstract

Septic shock (sepsis accompanied by cardiovascular failure) is an extreme manifestation of the host inflammatory response to severe infection. Tumor necrosis factor alpha (TNFα) super family induced nuclear factor kappa B (NF-κB) signaling pathways play a critical role in the pathophysiology of the disease. A better understanding of how TNFα induced NF-κB signaling influences the pathogenesis of septic shock is imperative.

NF-κB signaling is activated by a canonical pathway or a non-canonical pathway. The canonical NF-κB pathway requires the IκB kinase (IKK) complex comprised of IKKα/β/γ. Activation of the IKK complex in response to inflammatory stimuli, such as TNFα, results in ubiquitin-dependent degradation of IκBα or IκBβ, releasing p50 related dimers to the nucleus. In response to TNFα superfamily induced inflammation in the non-canonical pathway, NF-κB inducing kinase (NIK), a docking molecule, recruits IKKα to p100 thus activating IKKα. This phosphorylates p100, which is then degraded, releasing p52 containing RelB heterodimers to the nucleus.

It has been shown that genetic variation in key inflammatory genes contributes to outcome in sepsis. We hypothesized that genetic variation in genes of TNFα super family induced NF-κB signaling would be associated with mortality in septic shock. Specifically, we first tested the hypothesis that genetic variation within the cytosolic members of the canonical and non-canonical pathway may be associated with mortality in septic shock. We found that the CC genotype of NIK rs7222094 is associated with increased mortality and organ dysfunction in septic shock patients. This is perhaps due to altered regulation of NF-κB pathway genes, including CXCL10. We then tested the hypothesis that genetic variation in genes upregulated by these pathways may be associated with mortality in septic shock. We showed that the G allele of TNFAIP2 rs8126 is associated with increased mortality and organ dysfunction in septic shock patients. We then elucidated a novel biological mechanism whereby TNFAIP2 is a novel inhibitor of Ras, CREB and NF-κB; TNFAIP2 levels are controlled by rs8126; and these by allele differences are reflected in the inhibiton of Ras, CREB and NF-κB.

Preface

Section 1.1.4 of the introduction titled "The Innate Immune Response" includes portions of the manuscript I wrote and the figures I created for the publication (used in this thesis as Figures 1.1 and 1.2) *Russell JA, Boyd JH*, *Nakada TA,* ***Thair SA***, *Walley KR.* Molecular Mechanism of Sepsis. Contrib Microbiol. 2011;17:48-85. Epub 2011 Jun 9. Copyright © [2011] Karger Publishers, Basel, Switzerland.

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Chapter 2 is a copy of the publication ***Thair SA,*** *Walley KR, Nakada TA, McConechy M, Boyd JH*, *Wellman H, Russell JA.* A Single Nucleotide Polymorphism in NF-κB Inducing Kinase is Associated with Mortality in Septic Shock. J Immunol. 2010 Feb 15; 186(4):2321-8. Copyright © [2010] The American Association of Immunologists, Inc.

Chapter 3 is a copy of the manuscript in preparation for publication ***Thair SA,*** *Boyd JH, Nakada TA, Fjell CD, Russell JA and Walley KR.*TNFα induces TNFAIP2 expression which inhibits Ras signaling and contributes to mortality and organ dysfunction in septic shock patients.

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List of Abbreviations

A: adenine
AIDS: acquired immune deficiency syndrome
AP-1: activator protein 1
APCs: antigen presenting cells
APC: activated protein C (Drotrecogin alpha (activated))

BAFF/ TNFSF13B: B-cell activating factor

C: cytosine
cAMP: cyclic adenosine monophosphate
CARS: compensatory anti-inflammatory response syndrome
CBP: CREB binding protein
CD14: cluster of differentiation 14
cDNA: complimentary deoxyribonucleic acid
mCD14: membrane-bound CD14
sCD14: soluble CD14
CD40: cluster of differentiation 40
CD40L: cluster of differentiation 40 ligand
CEPH/ CEU: Cells/ DNA of European Ancestry from the Centre d'Etude du Polymorphisme Humain
CHiBi: UBC Centre for High-Throughput Biology
CHUK: IKKα
CI: confidence interval
CLP: cecal ligation and puncture
CORTICUS: Corticosteroid Therapy of Septic Shock Trial
CpG: cytosine-phosphate-guanine
CR: call rate
CREB: cAMP response element binding protein
CRP: C-reactive protein
CXCL8: (IL-8) a chemokine (8) characterized by separation of one amino acid "X" in between two N-terminal cysteines.
CXCL10/ IP-10: a chemokine (10) characterized by separation of one amino acid "X" in between two N-terminal cysteines/ interferon gamma induced protein-10

DAF: days alive and free
DCs: dendritic cells
DMEM: Dulbecco's modified Eagle medium
DNA: deoxyribonucleic acid

EAE: experimental autoimmune encephalomyelitis
EGF: epidermal growth factor
EGFR: epidermal growth factor receptor
ELISA: enzyme linked immunosorbent assay
ENCODE: Encyclopedia of DNA Elements Consortium

ERK1/2: extracellular signal-regulated kinases 1/2
ESICM: European Society of Intensive Care Medicine

FADD: Fas associated protein with death domain

G3BP1: GTPase activating protein (SH3 domain) binding protein 1
G: guanine
GAPs: GTPase activating proteins
GTP: guanosine triphosphate
GTPase: hydrolyse GTP
GWAS: genome wide association study

HIV: human immunodeficiency virus
HLA-DR: human leukocyte antigen receptors
HR: heart rate
[HR]: hazard ratio

IBD: identical by descent
ICU: intensive care unit
IFNγ: interferon gamma
IGF: insulin-like growth factor
IgG: Immunoglobulin G
IgM: Immunoglobulin M
IKAP: inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase complex-associated protein
IKBKB: inhibitor of kappa light polypeptide gene enhancer in B cells, kinase beta
IκBα/ NFKBIA: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
IKK: IκB kinase
IKKAP1: inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma
IL-1, IL-4, IL-6, IL-8, IL-12: interleukin-1, interleukin-6, interleukin-8, interleukin-12
IRAK: interleukin-1 receptor associated kinase
ITAMs: immunoreceptor tyrosine-based activation motifs

JNK: c-Jun N-terminal kinases

LD: linkage disequilibrium
LIGHT: TNFSF14: tumor necrosis superfamily member 14
LPS: lipopolysaccharide
LT: lymphotoxin
LTβR: lymphotoxin beta receptor

MAF: minor allele frequency
MAPK: mitogen-activated protein kinase (signaling pathway)
MBL: mannan binding lectin
MCP-1: macrophage chemoattractant protein-1

MEK: MAP2K1: mitogen activated kinase kinase 1
MEKK-3: mitogen activated kinase kinase 3
MHC II: major histocompatibility complex class two molecule
MIAME: minimum information about a microarray experiement
miRNA: microribonucleic acid
MRE: miRNA recognition elements
mRNA: messenger ribonucleic acid
MyD88: myeloid differentiation primary response gene 88

NEMO: NF- κB essential modulator
NIK: MAP3K14: NF-κB inducing kinase
NF-κB: nuclear factor kappa B
NFKBIA: IKBα
NFKBIB: IKBβ
NFKBIE: IKBε
NFKB1: p105
NFKB2: p100
NLS: nuclear localization sequence
NK cells: natural killer cells

$PaCO_2$: partial pressure of carbon dioxide in the blood
PAMPs: pathogen associated molecular patterns
PCT: procalcitonin
PMNs: polymorphonuclear neutrophils
PROWESS: the Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis

RA: rheumatoid arthritis
Raf: rapidly accelerated fibrosarcoma
RANKL: Receptor activator of nuclear factor kappa-B ligand
Ras: rat sarcoma
RCT: randomized controlled trial
REL: reticuloendotheliosis
RHD: Rel homology domain
RIP-1: receptor interacting protein
RIPK1: receptor interacting serine/threonine protein kinase 1
RISC: RNA induced silencing complex
RNA: ribonucleic acid
RSV: respiratory syncytial virus

SCCHN: squamous cell carcinoma of the head and neck
SIRS: systemic inflammatory response syndrome
SNPs: single nucleotide polymorphisms
SOS: son of sevenless
SPH: St. Paul's Hospital
SVR: systemic vascular resistance

SvO2: mixed venous oxygen saturation

T: thymine
TAK1: transforming growth factor β activating kinase 1
TBK1: TANK (TRAF family member-associated NFKB activator) binding kinase 1
TCR: T cell receptor
TGFβ: transforming growth factor beta
Th_1: T helper cells 1
Th_2: T helper cells 2
TLR: Toll-like receptor
TGF: transforming growth factor
TIRAP: toll-interleukin 1 receptor (TIR) domain containing adaptor protein
TNFα: tumor necrosis factor alpha
TNFAIP2/ B94: tumor necrosis factor alpha induced protein 2
TNFAIP3/ A20: tumor necrosis factor alpha induced protein 3
TNFR1: tumor necrosis factor receptor
TNT: tunneling nanotubles
TRADD: tumor necrosis factor receptor type-1 associated death domain
TRAIL: TNF related apoptosis inducing ligand
TRAF: tumor necrosis factor receptor associated factor
TSS: transcription start site
TWEAK: TNFSF12: TNF superfamily member 12

U: uracil
UBC: University of British Columbia
UCSC: University of California Santa Cruz
UTR: untranslated region

VASST: Vasopressin and Septic Shock Trial
VEGF: vascular endothelial growth factor
VEGI: TNFSF15: tumor necrosis factor superfamily member 15
WBC: white blood cell

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Chapter 1: Introduction

1.1 Septic Shock

1.1.1 Clinical significance

At the annual congress of the European Society of Intensive Care Medicine (ESICM) in October 2002, what was called the "Barcelona Declaration' was issued by the newly formed Surviving Sepsis Campaign [1]. Surviving Sepsis aims to harness the support of governments, health agencies, the public and other health care professionals to decrease the relative mortality of sepsis [2]. The original goal was a reduction of 25% in mortality over the following 5 years [3-5]. Recent studies from 2013 (11 years later) suggest that reaching these goals will be more difficult than projected [6, 7]. Mortality rates due to severe sepsis and septic shock range world wide from 22% to 76% [8-12]. A recent study attempting to understand this wide span of mortality rates suggested a multitude of reasons including population age, comorbidity patterns, access to care, the number of beds assigned to critical care units relative to population size, and potentially unrecognized influences such as genomic variation [13].

The documented incidence of sepsis worldwide is 1.8 million each year, but there is speculation that the value is confounded by a low diagnostic rate and difficulties tracking sepsis in certain countries. The surviving sepsis campaign has proposed that a more realistic rate would be 3 in 1000 bringing the true number of cases per year world wide up to 18 million. Combined with a mortality rate of approximately 30%, sepsis becomes a leading cause of death worldwide [8]. This is more than the number of deaths due to acquired immune deficiency syndrome (AIDS), breast cancer and prostate cancer combined each year [7]. The incidence is set to rise as the

population ages, the elderly being impacted most significantly [8]. Sepsis costs on average US$22 000 per patient. This cost impacts hospitals' financial resources, with US$16.7 billion spent each year in the USA alone [8, 14]. Until approximately 50 years ago, multiple organ failure due to sepsis and septic shock were rarely seen; critically ill patients could not be kept alive for long enough to develop such conditions. Hence the syndromes resulting are relatively new [15]. A deeper understanding of the processes leading to sepsis and septic shock are necessary for the improvement of interventions available to clinicians.

1.1.2 Clinical characteristics

In order to describe septic shock clinicians had to agree on a definition. A consensus conference in 1992 defined the systemic inflammatory response syndrome (SIRS) in order to differentiate between situations involving infection or not [16]. It was concluded that if 2 of the 4 following conditions were met, a diagnosis of SIRS could be applied: a) a body temperature of >38°C or <36°C, b) a heart rate (HR) of >90beats/min, c) tachnypea: a respiratory rate of >20 breaths/ min or hyperventilation indicated by a $PaCO_2$ (partial pressure of carbon dioxide in the blood) of <32torr and d) a white blood cell (WBC) count of >12,000 cells/mm^3 [16]. Hence, the systemic response to infection including the aforementioned was defined as sepsis [16]. A common result of sepsis is the failure of one or more organs. Severe sepsis was defined at this consensus conference as “sepsis associated with organ dysfunction, hypoperfusion or hypotension” [16]. Finally, septic shock was defined as “sepsis with hypotension despite adequate fluid resuscitation along with the presence of perfusion abnormalities” [16]. This was the first time it was recognized that there was a continuum of severity with increasing risk of mortality and that these definitions would not only assist early recognition and treatment but that standardization of

research in the field would be enhanced by potentially reducing noise with more clearly defined patient groups.

Since this first attempt to clearly and accurately define sepsis, severe sepsis and septic shock, evolution of the definition has occurred in an attempt to find a universal medium. In a survey of 1058 physicians including 529 intensivists, only 17% could agree on one definition in 2004 [17]. Issues with using SIRS to identify those at risk for developing sepsis began to surface as many of the 4 criteria are present in other conditions [18]. Not only was the exceptionally broad term of SIRS a problem in terms of diagnosing patients, but also it allowed for a less than optimally defined phenotype to be included in clinical trials [18]. Furthermore, the issue emerged that the use of SIRS to define septic shock was not creating clear boundaries to separate septic shock from other types of shock as any kind of shock will result in increased white blood cell count as a general stress response, hyperventilation due to acidosis and/ or tachycardia to compensate for decreased stroke volume or to create supranormal cardiac output [18]. Hence in 2001, the definition of sepsis was revised and the resulting list of criteria are described in **Table 1.1** as reported by Vincent [18]. No consensus has been reached regarding the importance of each individual criterion; hence, does not yield a specific score, therefore current clinical trials use the original 1992 definition.

Table 1.1 Evolving definitions of sepsis

General signs and symptoms	Fever/ hypothermia
	Tachypnea/ respiratory alkalosis
	Positive fluid balance/ edema
General inflammatory reaction	Altered white blood cell count
	Increased biomarker concentration (C-reactive protein (CRP), IL-6, procalcitonin (PCT))
Hemodynamic alterations	Arterial hypotension
	Tachycardia
	Increased cardiac output/ low systemic vascular resistance (SVR)/high mixed venous oxygen saturation (SvO2)
	Altered skin perfusion
	Decreased urine output
	Hyperlactatemia (increased base deficit)
Signs of organ dysfunction	Hypoxemia
	Coagulation abnormalities
	Altered mental status
	Hyperglycemia
	Thrombocytopenia, disseminated intravascular coagulation
	Altered liver function (hyperbilirubinemia)
	Intolerance to feeding (altered gastrointestinal motility)

Despite the evolution of more clinically defined patient subsets, clinical trials have yet to elucidate effective drug treatments. This has led to the emerging concept of classification of patients by molecular markers such as single nucleotide polymorphisms (SNPs), transcriptional profiles and or cytokine profiles that will be necessary to personalize treatments [19-28].

1.1.3 Current treatment

A "one treatment for all" approach is difficult because septic patients are a heterogeneous population having different progressions of organ failure and have a complex variety of pathogens causing both community acquired infection and nosocomial infection. The Surviving Sepsis guidelines recommend care that takes into account three main principles: source control and antibiotics, hemodynamic resuscitation of hypoperfused organs and sustained support of organ system dysfunction [2, 29]. It is currently understood that dealing with the infection immediately is one of the best ways to ensure survival [29]. Additionally, it is widely and extensively reported that cellular and organ injury result from derangement due to inflammation (systemic or localized) and hypoperfusion [30]. Swift stabilization of a patient's hemodynamic status via fluid resuscitation potentially combined with the use of vasopressor drugs is used as a way to mitigate further organ damage [30]. Sustained support of organs is a complex process. It includes but is not limited to the use of glucocorticoids and, until October 2011 recombinant human activated protein C (APC, Drotrecogin alpha (activated)). Glucocorticoids are targeted at restoring the action of natural or exogenous catecholamines and APC was used as an anticoagulant and anti-inflammatory therapy [29]. Furthermore, the use of mechanical ventilation and renal replacement therapy is possible [29].

A recent randomized double-blind, placebo controlled trial of Corticosteroid Therapy of Septic Shock (CORTICUS) found that the use of low-dose hydrocortisone had no significant effect on 28-day mortality, regardless of the patients' adrenal responsiveness to corticosteroids. However, the duration of time until the reversal of shock was shorter in those treated with hydrocortisone, but the proportions of patients whose shock was reversed was not significantly different between the hydrocortisone and placebo groups [31]. Hence, it would appear that if the patients' condition is going to improve, hydrocortisone will accelerate this process, however the treatment itself may not alter the outcome for those whose condition will not reverse. Previous studies had shown that hydrocortisone was beneficial [32-37], hence these results are somewhat, but not entirely unexpected as it is true that treatment with corticosteroids has been controversial for many years because of conflicting reports [38-40]. The authors point out several key differences between their study and the study by Annane *et al.* [35] that may explain conflicting results such as dissimilar patient populations, different time frames for inclusion criteria to be met, an increased incidence of superinfection and new septic episodes. It is important to note that the CORTICUS study was most likely underpowered; the study was terminated after recruitment of 500 patients instead of the intended 800. Nevertheless, the use of corticosteroids for treatment of sepsis, severe sepsis, and septic shock is declining [5].

It is known that corticosteroids affect inflammatory pathways, as there is an increase in proinflammatory mediators and hemodynamic deterioration after abrupt cessation of treatment [36]. Glucocorticoids (GCs) (a type of corticosteroid such as the hydrocortisone administered for the CORTICUS trial) create their effects by binding to the glucocorticoid receptor (GR) [41, 42]. The ligand bound receptor complex translocates into the cell nucleus where it binds to

glucocorticoid response elements (GRE) in the promoter region of the target genes: a process referred to as transactivation [41, 42]. This has been shown to upregulate the expression of anti-inflammatory proteins in the nucleus. Transrepression also occurs whereby the ligand bound receptor complex interacts with specific transcription factors (such as AP-1 and NF-κB) and prevents the transcription of targeted genes such as interleukins IL-1β, IL-4, IL-5 and appears to prevent translation of tumor necrosis factor alpha (TNFα) [41]. The findings of the CORTICUS trial in light of other conflicting data suggests that further molecular studies regarding the specific effect of GCs on key inflammatory pathways known to be involved in sepsis are needed in concert with extremely uniform inclusion and dosage criteria across trials.

It was announced in October 2011 that Eli Lilly and company (Indianapolis, IN, USA) would be withdrawing APC, the only drug specifically approved for sepsis, from the market. This was due to a study that did not confirm the efficacy found in the original clinical trial [43, 44]. Clinicians now have antibiotics and vasopressors, but not specific drugs. Some authors argue that the reason for the lack of a statistically significant effect is again due to improper clinical enrollment based on the fact that enrollment is not complemented by information about biomarkers that may better identify patients who will or will not benefit from a selected treatment [43]. This raises two issues: what are these markers and important biological processes, and how do we better identify patient groups? Genotyping prior to treatment is one of many ways to further characterize a patient to define appropriateness (efficacy) and dose range (safety). For instance genotyping prior to administration of warfarin and clopidogrel is recommended on the FDA drug label [45]. To study single nucleotide polymorphisms associated with disease (in this case septic

shock) is a direct way to study which markers and patient groups are important. To capitalize on these markers we, must first understand the biology that they influence.

1.1.4 The innate immune response

Dysregulation of the immune response to infection is acknowledged to contribute to the pathogenesis of the disease. Production of both proinflammatory and immunosuppressive cytokines is observed from the very first hours following diagnosis of sepsis and septic shock. The first line of defense against infection in humans is the epithelium [46]. Once breached, the innate immune system is in action within seconds. The innate immune system is the first on the scene, giving the adaptive immune response a chance to develop over a few days. Here we review the innate and adaptive immune systems in relation to sepsis.

Innate immune response

The innate immune system is multifaceted and tightly regulated, allowing for maximum chances of survival. One of the immediate responses is the complement system: a heat-labile component of plasma that augments the opsonization of bacteria as well as facilitating the death of bacteria using antibodies alone. It is also part of the adaptive immune response **(Figure 1.1).** The complement system is composed of a distinct number of proteins and can be activated through three pathways[47]. The earliest complement response, referred to as the alternate pathway involves the spontaneous activation of complement component C3 which binds directly to the surface of a pathogen [47]. A second pathway, the mannan-binding lectin (MBL) pathway, is instigated by the binding of serum lectin (an acute phase liver protein) to mannose containing carbohydrates on the bacteria or virus [47]. The classical pathway is activated by antibodies

such as IgM and IgG binding to an antigen. Activation of these three pathways results in enzymatic reactions to generate the complement cascade of effector molecules. These molecules facilitate opsonization of the pathogen (C3b, C4b), recruitment of inflammatory cells (C3a, C5a), and direct killing of pathogens (C5b, C6, C7, C8, C9) [48].

Another facet of the innate immune system is the phagocytic antigen presenting cells (APCs). "Professional" APCs include dendritic cells (DCs), neutrophils and macrophages and are considered professional because they always express MHC class II molecules on the cell surface; thus they can activate CD4+ naive T cells as well as CD8+ cytotoxic T cells. There are other cell types that are able to present foreign antigens to CD8+ T cells via the MHC I molecules such as fibroblasts, epithelial and endothelial cells and are usually referred to as "non-professional" APCs. We will focus on the "professional" class for simplicity. Foreign substances below the epithelium are immediately recognized by macrophages and migrating neutrophils and are engulfed, degraded and exocytosed on the surface of the APCs in a MHC II molecule. The phagocyte simultaneously releases cytokines (tumor necrosis factor alpha (TNFα), interleukin-1 (IL-1), IL-6, IL-8, IL-12) to recruit additional phagocytic cells and effector molecules to the site of the invasion [49, 50]. As well, cytokines and chemokines are released such as IL-8 (CXCL8) and MCP-1 (macrophage chemoattractant protein-1), attracting leukocytes such as neutrophils and monocytes respectively from the blood [51]. Chemokines facilitate the rolling to firm adhesion of leukocytes on endothelial cells and disrupt tight junctions to allow for passage into the surrounding tissue and then act as a guide to the site of infection along a chemotactic gradient [52]. Neutrophils tend to be the first to infiltrate the site of infection and are usually the predominant cell type followed by monocytes [53]. Neutrophils are capable of eliminating a

large amount of pathogens by phagocytosis alone by directly binding bacterial wall components or uptake of pathogens or microbes coated in the complement component C3b [54]. Natural killer (NK) cells are a type of accessory effector cell to professional APCs [46]. While neutrophils and macrophages ingest pathogens to kill them, NKs (as well as eosinophils, basophils and mast cells) are triggered to release cytoplasmic granules containing perforin and granzymes when an antibody binds the Fc cell surface receptor [55].

From the innate to the adaptive immune system.

While the innate immune system mounts the initial attack on invading foreign bodies, the adaptive immune system is often required for complete clearance and future protection. APCs that have phagocytosed foreign bodies then migrate to the lymph nodes. Naïve $CD4^+$ T cells in the lymph nodes monitor antigen presentation. The binding of the APC ligand to the T cell receptor (TCR) phosphorylates immunoreceptor tyrosine-based activation motifs (ITAMs) via receptor associated kinases. Depending on the overall cytokine profile present which is generated by the initial response to a pathogen and the nature of the antigen presented, T cells differentiate into either $CD4^+$ Th_1, $CD4^+$ Th_2 or CD4+ Th17 cell [56, 57].

Specifically, IL-12 and IFNγ favour Th_1 differentiation and inhibit Th_2 differentiation; whereas IL-4 achieves the opposite [56, 57]. IL-6 induces the generation of Th17 cells from naive T cells together with TGFβ and inhibits TGFβ induced Treg differentiation; Treg cells are commonly believed to be involved in the suppression of autoimmune responses[58]. Th_1 differentiation results in a cell mediated response via further activation of macrophages, neutrophils, CD8+, and NK cells. Th_2 differentiation results in a humoral response, stimulating B cells into antibody

production. This drives the complement system. Th17 cells have been implicated in the induction of autoimmune diseases such as rheumatoid arthritis (RA) and experimental autoimmune encephalomyelitis (EAE), and allergen specific responses and interestingly, are crucial for defense against fungi and extracellular bacteria [58]. In the end it is the adaptive response that clears the pathogen completely from the body.

SIRS (Systemic Inflammatory Response Syndrome) and CARS (Compensatory Anti-Inflammatory Response Syndrome)- when your own immune system hurts you.

The systemic inflammatory response of hyperthermia, tachycardia, tachypnea and leukocytosis is most commonly triggered by infection; however, it has been recognized that these symptoms also manifest after trauma, burn injury, surgery and other insults [46]. As a result, in 1991, the American College of Chest Physicians and Society of Critical Care medicine convened to define the systemic response to sepsis and thus defined the criteria for SIRS exclusive of evidence of infection [15, 59]. Sepsis was hence the SIRS response with evidence of infection. Severe sepsis is defined as sepsis with evidence of organ failure and septic shock is defined as sepsis plus cardiovascular dysfunction.

CARS

In humans, the MHC Class II molecules are referred to as human leukocyte antigen receptors (HLA-DR). Immunoparalysis has been previously defined in patients with monocytic HLA-DR expression of less than 30% for two consecutive days [60]. This group of patients experiencing immunoparalysis are usually a sub-group of the population of patients with sepsis or septic shock characterized by low levels of HLA-DR expression for a number of days [60, 61]. In fact, it has

been reported that monocyte inactivation for more than two days was associated with 58% mortality, which then rises to 88% if the inactivation is present for more than 5 days [60]. This anti-inflammatory response is characterized by an impaired ability to mount a further inflammatory response. Inadequate IFN-γ production may contribute to CARS[60-62]. The Th1 fraction of lymphocytes that produce IFN-γ is diminished in septic patients, but Th2 lymphocytes, such as those cells producing IL-4 and IL-10, are increased [62]. Sepsis impairs the response of NK cells and T lymphocytes to a subsequent bacterial infection. IFN-γ production by these two cell populations is decreased and is associated with diminished bacterial clearance in a cecal ligation and puncture (CLP) model of sepsis in mice [63]. Similarly, IFN-γ production is impaired in patients having sepsis[64]. Thus it appears that lower levels of IFN-γ and subsequent impaired foreign antigen presentation due to lower expression of HLA-DR molecules leads to the inability of the immune system to mount an appropriate response to infection, leading to mortality.

TLRS: Pathogen recognition and Toll-like Receptors (TLRs)

In order to recognize foreign pathogens, the pattern-recognition receptors (such as the Toll-like receptor family (TLRs)) bind to highly conserved molecules called pathogen-associated molecular patterns (PAMPs). TLR1 binds peptidoglycans and triacyl lipoproteins of Gram positive bacteria in a heterodimer with TLR2. TLR2 can also act alone, recognizing lipotechoic acid of Gram positive bacteria; whereas, TLR4 binds lipopolysaccharide of Gram negative bacilli. CD14 interacts with TLR4 in response to Gram negative infection and lipopolysaccharide (LPS) [65]. TLR3 recognizes RNA released from necrotic cells as well as viral infection [66]. TLR5 recognizes flagellin and TLR9 binds unmethylated CpG dinucleotides (i.e. CpG sequences

of DNA); CpG sites are rare in human DNA but common in viral and bacterial DNA. Their location reflects the function. TLRs that recognize components of the bacterial cell wall are positioned to respond to ligands outside of the cytoplasmic membrane; while TLRs that recognize nucleic acids are located within the host cell endosome.

Toll-like receptors signal to the nucleus by first recruiting adaptor proteins such as MyD88 that then activate a relay of kinases (such as IRAK1, IRAK4, TBK1 and IKKi). Examples of the nuclear transcription factors activated by the TLR signaling pathways include NF-κB, activator protein-1 (AP-1), and interferon regulatory factor 3 (IRF-3). These transcription factors (and many others) facilitate the decision making process of the cell: rapidly undergo apoptosis if overwhelmingly infected, fight the infection with inflammatory molecules and proliferation, and/ or balance the inflammatory reaction if no further danger is detected.

TLR dysfunction and sepsis

In a recent review of TLRs and their role in sepsis, it was found that in both animal models and human sepsis, TLRs are highly expressed on monocytes/macrophages. The author postulates that increased TLR expression may not be simply driven by their ligands but that the expression is also driven by feedback loops created by the inflammatory response among different cell types. Both processes contribute to the organ dysfunction and mortality that occurs in sepsis [67]. In fact, in a murine cardiomyocyte model, it was shown that TLR2, TLR4 and TLR5 signal via NF-κB, resulting in decreased contractility and a concerted inflammatory response [68].

Macrophage dysfunction in sepsis

While it is healthy and necessary to produce cytokines in response to infection, excessive amounts of pro-inflammatory molecules produced largely by activated macrophages is believed by some to be the cause of inflammation and septic shock [46]. There is accumulating evidence in humans and in experimental sepsis models that intense activation of the complement system occurs alongside the innate immune system. A recent study showed that the complement activation product, C5a, appears in the plasma of rodents following cecal ligation and puncture (CLP) [69]. C5a interacts with its receptors (C5aR, C5L2) on phagocytes (polymorphonuclear neutrophils (PMNs) and macrophages); it is believed to paralyze the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway of the mitogen-activated protein kinase signaling pathway [70]. Interaction of C5a with these receptors results in diminished innate immunity, defined as intense suppression of phagocytosis, chemotaxis and the respiratory burst. Meanwhile, macrophages are primed and produce large amounts of cytokines/chemokines [71]. Work has been done to inactivate macrophages in the hopes of dampening the cytokine storm with limited success [72]. Despite that, in a recent review of current treatments for sepsis looking at clinical research published between 2008 and 2009, Leone *et al*. found a large portion of clinical research was devoted to the modulation of monocytic HLA-DR [73]. Four years later, we have yet to see this translate into changes in patient care.

Neutrophil dysfunction in sepsis

Neutrophil migration is a co-coordinated process that is highly regulated. Neutrophils are usually the first to arrive at the scene of infection and do a great amount of phagocytosis. Neutrophil migration has been shown to be dysregulated in sepsis. A recent review of the

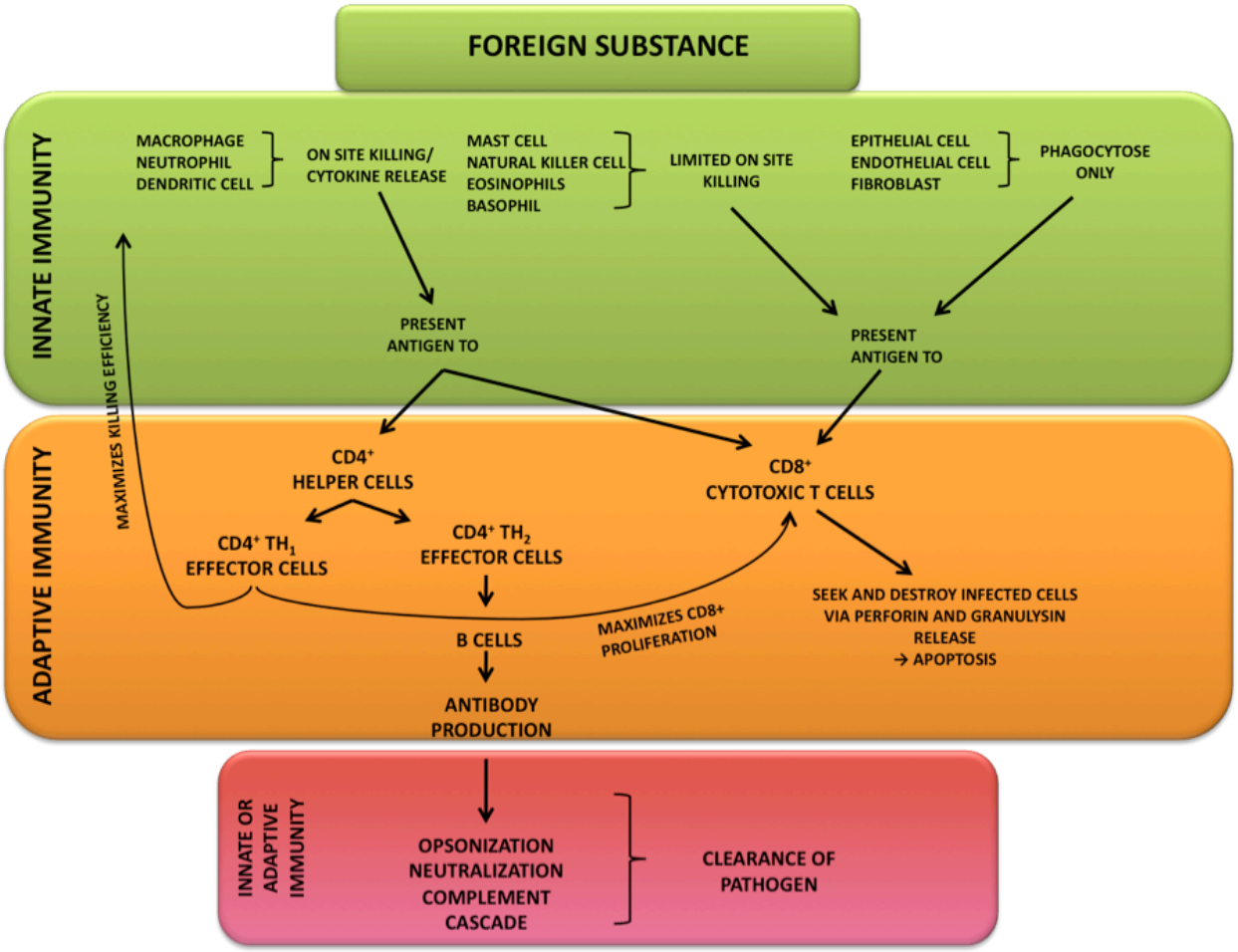
possible mechanisms of dysregulation include impaired chemotactic responses as a result of dysregulated PMN Toll-like receptor signaling, inhibition of migration due to exposure to high temperatures, activation of the anti-inflammatory nuclear transcription factor peroxisome proliferator-activated receptor-gamma, and nitric oxide and metabolite induced suppression of PMN-endothelial interactions [74].

Dendritic cell (DC) dysfunction in sepsis

Dendtritic cells are a type of "professional" APC that can interact with T cells in the lymph nodes. Since DCs are one of the key cell types that communicate with the adaptive immune response, it is interesting to note that circulating numbers of DCs are decreased in patients with sepsis. This suggests less phagocytosis of microorganisms and subsequently less antigen presentation to mount an adaptive response [75]. As well, DCs in septic patients are reprogrammed to produce less IL-12 and more IL-10, perhaps tipping the scales toward immunoparalysis [76, 77].

Figure 1.1 The innate and adaptive immune response

Foreign substances below the epithelium are immediately recognized by macrophages and migrating neutrophils and are engulfed, degraded and presented on the surface of the APCs in a major histocompatibility complex II (MHC II) molecule. The phagocyte simultaneously releases cytokines to recruit additional phagocytic cells and effector molecules to the site of invasion. As well as cytokines, chemokines are released attracting leukocytes from the blood. NKs (as well as eosinophils, basophils and mast cells) are triggered to release cytoplasmic granules containing perforin and granzymes when an antibody binds the Fc cell surface receptor. Occasionally, epithelial cells, endothelial cells or fibroblasts are able to phagocytose foreign substances and present them to circulating CD8+ cells. APCs that have phagocytosed foreign bodies then migrate to the lymph nodes. Depending on the overall cytokine profile present generated by the initial response to a pathogen and the nature of the antigen presented, T cells will differentiate into either CD4+ Th1 or CD4+ Th2 effector cells. Th1 differentiation results in a cell mediated response via further activation of macrophages, neutrophils, CD8+ and NK cells. Th2 differentiation results in a humoral response, stimulating B cells into antibody production, which drives the complement system. In the end, it is the adaptive response that clears the pathogen completely from the body.


FOREIGN SUBSTANCE
INNATE IMMUNITY
MACROPHAGE
NEUTROPHIL
DENDRITIC CELL
ON SITE KILLING/
CYTOKINE RELEASE
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EOSINOPHILS
BASOPHIL
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KILLING
EPITHELIAL CELL
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EFFECTOR CELLS
CD4+ TH2
EFFECTOR CELLS
MAXIMIZES CD8+
PROLIFERATION
SEEK AND DESTROY INFECTED CELLS
VIA PERFORIN AND GRANULYSIN
RELEASE
→ APOPTOSIS
B CELLS
ANTIBODY
PRODUCTION
INNATE OR
ADAPTIVE
IMMUNITY
OPSONIZATION
NEUTRALIZATION
COMPLEMENT
CASCADE
CLEARANCE OF
PATHOGEN

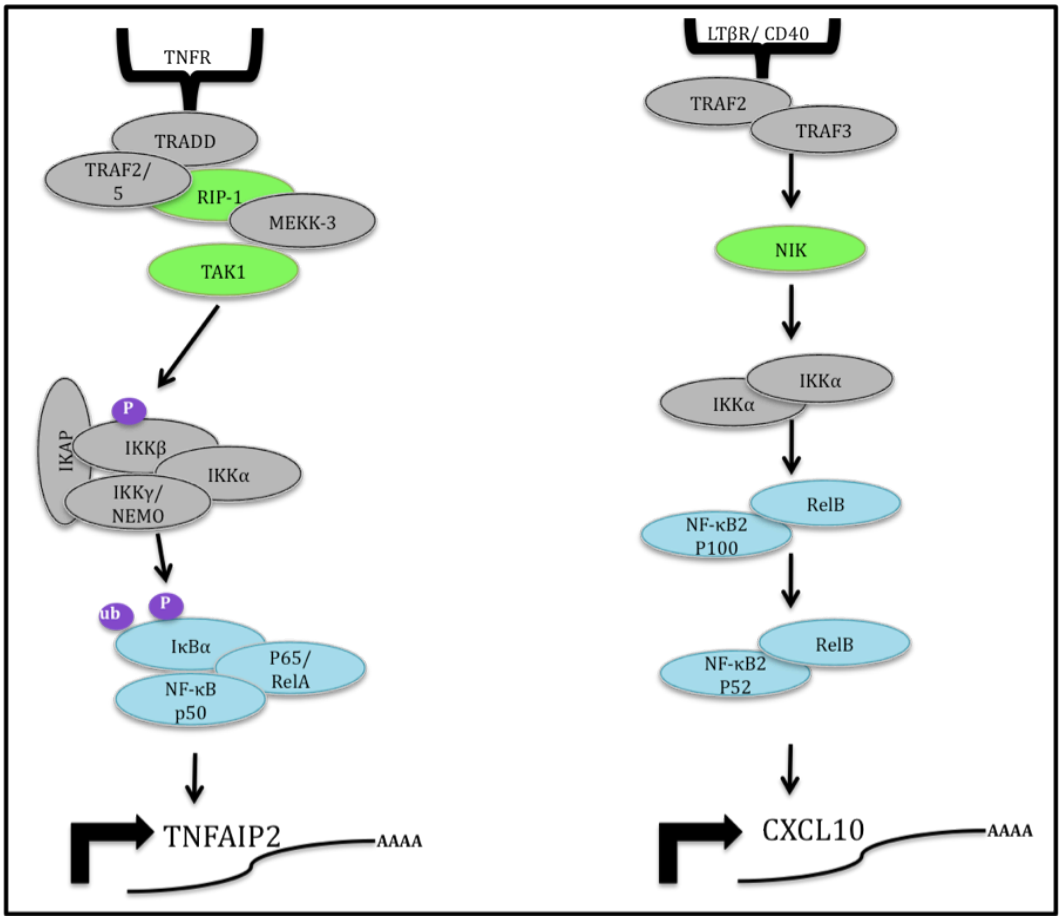
1.2 TNFα Superfamily Induced Nuclear Factor kappa B (NF-κB) Signaling in Septic Shock

It is known that tumor necrosis factor alpha (TNFα) is involved in the pathophysiology of septic shock [78]. Shortly after the cloning and characterization of TNFα [79], experiments in baboons immunized with anti-TNFα demonstrated protection from hypotension followed by lethal renal and pulmonary failure due to bacteraemic shock; this suggested TNFα as the root of sepsis and anti-TNFα the cure [80, 81].

The existence of a TNFα superfamily was first realized during the processes of elucidating the true nature of lymphotoxin (LT) and TNFα. Using hundreds of liters of culture medium from a lymphotoxic cell line, human LT was the first cytotoxic factor to be isolated and characterized from a B-lymphoblastoid cell line [82, 83]. Up to this point, there was evidence of its anti-tumor activity but due to the small amount prepared, purification for characterization was a limiting factor. As the actions of LT and TNFα were similar, experiments done using the neutralization of LT with specific antibodies resulted in the isolation of a second cytotoxic factor, TNFα [79]. Further characterization of the action of these two ligands indicated a common receptor [84]; hence the beginnings of a super family which today encompasses 19 ligands acting on 29 receptors (as reviewed in [85]). These ligands and their receptors activate various signaling pathways including NF-κB [85]. Depending on the TNFα superfamily ligand present, this can activate NF-κB via the canonical or non-canonical pathways [85]. **(Figure 1.2)**

Figure 1.2 Canonical and non-canonical signaling pathways

A model depicting the two signaling pathways. On the left is the classical pathway mediated by the IκBα phosphorylation and degradation, releasing RelA containing heterodimers to the nucleus. On the right is the NIK mediated activation of IKKα and processing of p100 to release RelB: p52 heterodimers to the nucleus. The arrow between TRAF2/3 and NIK represent the stabilization and activation of NIK, whereas all other arrows represent phosphorylation events of the kinase cascade until the heterodimers reach the nucleus and act as transcription factors for the transcription of genes such as TNFAIP2 (canonical signaling) and CXCL10 (non-canonical signaling).



1.2.1 Canonical signaling

Canonical NF-κB signaling can be induced by a plethora of ligands binding to receptors including, but not limited to, cytokine induced signaling (eg. IFNγ), toll-like receptors (TLRs) or the TNFα superfamily receptors [87]. All of these events are important in the pathophysiology of septic shock: here we focus on the events following TNFα induced NF-κB signaling.

Following binding of TNFα, tumor necrosis factor receptor associated factor (TRAF2) is recruited to the tumor necrosis factor receptor (TNFR1) through its interaction with tumor necrosis factor receptor type-1 associated death domain (TRADD) [88]. Interestingly, experiments demonstrated that TRAF2-deficient cells have relatively intact TNF signaling to NF-κB [89]. Simultaneously, evidence of an interaction of TRAF5 with TNFR1 signaling complex was uncovered, yet TRAF5 knockouts could still activate NF-κB [90]. It was then shown that TRAF2/5 double knockout cells, however, were unable to activate IκB kinase (IKK) [89-91]. Therefore, current consensus is that TRAF2 and TRAF5 are together required for NF-κB activation by TNFR1. TRAF2 and 5 form a complex with TRADD and receptor interacting protein (RIP-1). At this point TRAF2 is released and RIP-1 recruits mitogen activated kinase kinase (MEKK-3) and transforming growth factor β activating kinase 1 (TAK1) [92]. This leads to the phosphorylation of the IKKs (IKKα and IKKβ) beginning with TAK1 phosphorylation of the β subunit of the IKK multicomponent complex termed the signalsome [93-95]. The most common IKKα/β heterodimeric complex also includes NF- κB essential modulator (NEMO)/IKKγ/inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma (IKKAP1) and a scaffolding protein called inhibitor of kappa light polypeptide gene enhancer in B-cells kinase complex-associated protein (IKAP) [96]. IKBα, which serves to sequester NF-κB

homo and heterodimers in the cytoplasm by binding the Rel homology domain (RHD) to interfere with its nuclear localization sequence (NLS) [97-99], is phosphorylated by the IKKs, ubiquitinated and sent for proteosomal degradation, thus releasing the dimers for translocation to the nucleus [100]. The signalsome is where many of the different stimuli induced paths merge and the steps between this axis point and the nucleus that follow are considered the "canonical pathway" [85].

Overall, the NF-κB family consists of five key molecules: NF-κB1 (p105/ p50), NF-κB2 (p100/p52), RelA (p65), RelB and c-Rel [87]. p105 and p100 are processed via the proteosome to produce p50 and p52 respectively [87]. Various homo or heterodimer combinations of the five molecules allows for differential transcription depending on the signal induction [87]. NF-κB dimers are retained in the cytoplasm by IκBs, the aforementioned degradation of IκBs reveals the NLS and the dimers can translocate to the nucleus. Phosphorylated NF-κB dimers bind to κB DNA elements and induce transcription of target genes. **(Figure 1.2)**

1.2.2 Non-canonical signaling

Until approximately 1997, the dogma of NF-κB signaling was that all signals converged at the IKKs and IκBα and proceeded through to the nucleus from there. At this time, Malinin *et al.* discovered a novel TRAF2 interacting protein that appeared to be a potent stimulator of NF-κB signaling in response to TNF and IL-1 called MAP3K14 or NF-κB inducing kinase (NIK) [101]. Concurrently, it was shown that NIK was capable of activating IKKα by phosphorylation at Ser 176 [102]. Scrutiny of this novel NIK protein in relation to the canonical pathway and its molecular mechanism revealed several key aspects: that there appeared to be negative regulation

[103] and that it was a docking molecule for p100, and this docking lead to its activation and proteasomal processing [104, 105]. Further studies elucidated the mechanism of regulation whereby NIK is constitutively transcribed, translated and degraded through and interaction with TRAF3 [106, 107]. Simultaneously, through attempts to deepen the knowledge that Malinin described regarding degradation of IκBα and canonical signaling, Senftleben *et al*. found IKKα preferentially phosphrorylates NF-κB2 (p100), a mechanism requiring upstream kinases, specifically NIK, suggesting that NIK is not involved in IKBα degradation but that a second evolutionarily conserved pathway had been discovered [108]. Further, it was shown that NIK -/- mice exhibited normal canonical NF-κB activity upon stimulation with TNF and IL-1 with no changes in NIK transcription, but that LTβ stimulation resulted in increased NIK transcription. This added support to the evidence of a novel separate pathway based on regulated NF-κB processing rather than IκB degradation [109]. Taken together, the unique characteristics of this non-canonical pathway include stimulation of receptors with TNFα superfamily ligands such as CD40L, LT/ LTβ and BAFF [110]. Degradation of TRAF3 stabilizes NIK protein, allowing it to act as a docking molecule for p100, phosphorylating p100 at Ser 866/870, which recruits IKKα homodimers to NIK [104]. Subsequently p100:RelB associated heterodimers are processed to p52:RelB heterodimers for translocation to the nucleus [87, 104]. This is in contrast to canonical signaling translocation of RelA, c-Rel and/or p50 dimers [87].

1.2.3 Cross talk between canonical and non-canonical signaling

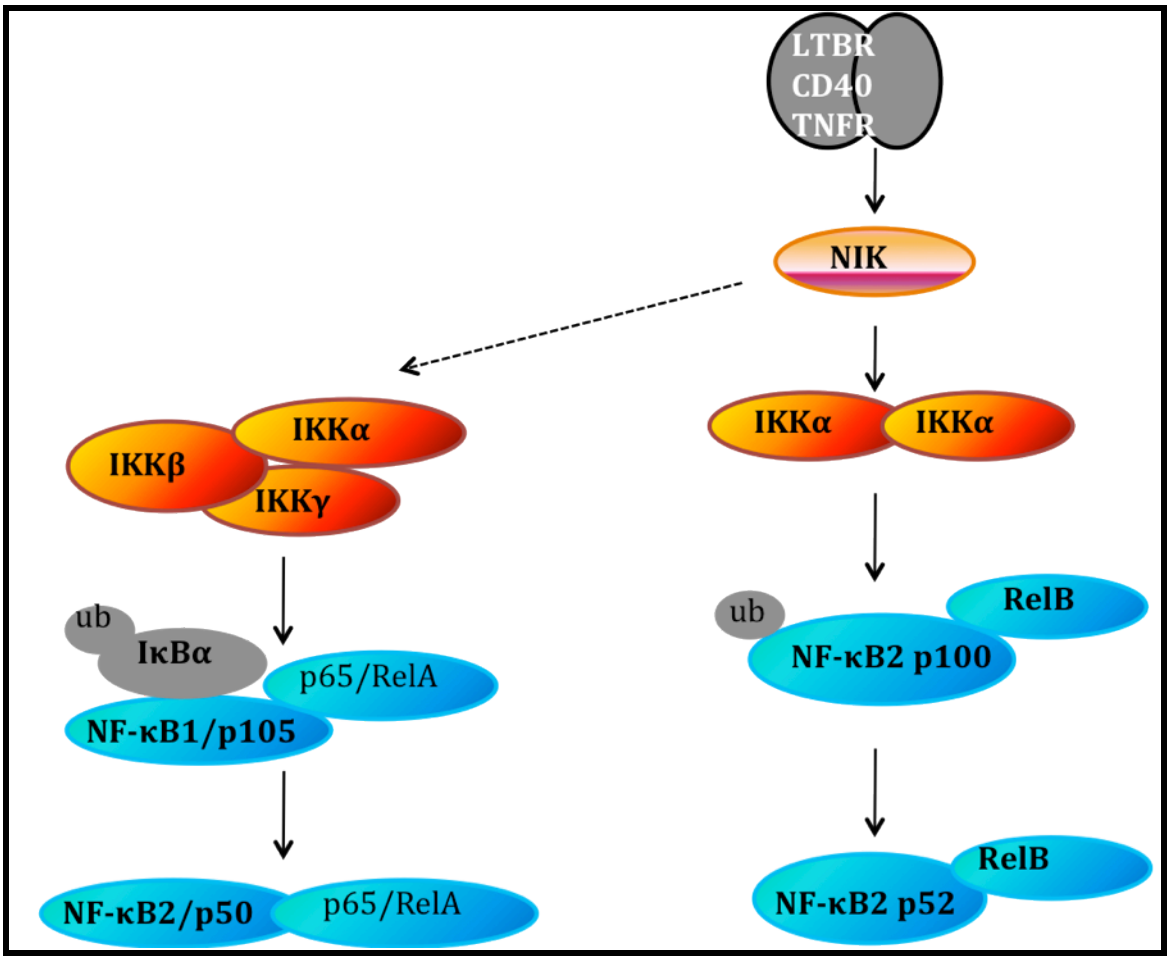
The discovery of two distinct and separate pathways further explains more of the incredibly diverse and elegant signaling orchestrated by NF-κB. As the mechanism of the non-canonical pathway was being distilled, sophisticated studies to further understand the mechanism of the

signalsome led to the discovery of a directional activation of the heterodimeric IKKα/β complex, proposed to be the pivot point for cross talk between the two pathways [111]. Recent evidence suggests that the IKK-NIK axis may be a pivot point for control of both non-canonical and canonical signaling [110-112]. **(Figure 1.3)**

In a study investigating the role of TRAF3 in canonical signaling, Zarnegar *et al.* demonstrated that while TRAF3 inhibits NIK and non-canonical signaling, it also inhibits canonical signaling [112]. Furthermore, in TRAF3 deficient mice, canonical signaling is dysregulated in concert with an accumulation of NIK, suggesting TRAF3 controls levels of NIK that facilitate cross talk between the two pathways [112]. Inspection of the LTβR associated signaling complexes showed that over expression of TRAF3 increased TRAF3 accumulation with LTβR complexes and decreased IκBα phosphorylation, hence inhibiting canonical signaling [113]. Conversely, knock down of TRAF3 increased the presence of non-canonical pathway members such as p100, RelB and NIK, as well as increased processing of p100 to p52 [113]. Upon examination of timing of experimental procedures, it is interesting to note that many studies that separated the two pathways and excluded NIK from canonical signaling focused on early events (from minutes to less than 2 hours) [108, 109]. However, many studies that collect data at much later time points (several hours to days) find NIK plays a pivotal role in canonical signaling [101, 111, 112].

Figure 1.3 NIK is a pivot point for canonical or non-canonical signaling

NF-κB activity can be a result of a canonical (or classical) pathway and by a non-canonical (or alternative) pathway involving multiple genes. In the non-canonical pathway (right of diagram) NF-κB-inducing kinase (NIK), a docking molecule recruits IKKα to p100, activating IKKα. IKKα phosphorylates p100 resulting in phosphorylation, ubiquitination and proteosomal degradation of p100, resulting in nuclear translocation of p52 containing RelB heterodimers. The canonical NF-κB pathway (left of diagram) requires the IκB kinase (IKK) complex comprised of IKKα/β/γ. Activation of the IKK complex results in phosphorylation and ubiquitin-dependent degradation of IκBα or IκBβ allowing translocation of p50 related dimers into the nucleus. The arrow between the receptor and NIK represent the stabilization and activation of NIK; whereas, all other arrows represent phosphorylation events of the kinase cascade.



This is not the only point at which evidence of cross talk has been reported. The abundance of RelB, c-Rel, p50 and p52 is dependent on RelA (more commonly associated with canonical signaling) dimer mediated transcription and translation [114]. It has also been shown that in the absence of p105 (*nfkb*1-/-) p52 (the processed product of non-canonical p100) compensates for the lack of p50 (the processed product of canonical p105) partners for dimerization, thus p100 stores are depleted, reducing noncanonical signal responsiveness [115]. Hence, changes in canonical signaling can affect the noncanonical pathway, a situation that may occur in disease states.

Furthermore, while IKBα, β and γ play major roles in the regulation of RelA, c-Rel and p50 based canonical signaling, they have not been found to have any influence over RelB/p52 regulation [114]. Instead, the C terminus of p100 inhibits RelB/p52 transcriptional activity until its proteasomal processing releases it. Therefore, it was deemed IKBδ [116, 117]. Interestingly, in cells lacking all other IKBs, RelA:p50 was found associated with IKBδ and NIK/IKKα dependent degradation of IkBδ not only releases RelB:p52 dimers but eventually RelA:p50 dimers, suggesting that IKBδ is also capable of sequestering canonical pathway mediators [116, 117]. In light of this, the inflammatory (RelA in response to TNFα) induction of p100 expression could mediate the inhibition of RelA:p50 complexes via its IKBδ activity, increasing the pool of RelA:p50 complexes sequestered via p100's IKBδ activity [116]. Despite ongoing canonical signaling (for example exposure to TNF), p100 would not be degraded until traditionally considered developmental signals such as LTβ were present, and the accumulation would amplify such actions as lymph node development or B cell maturation, acting as a cross talk toggle between innate and adaptive immunity [115, 118]. In fact, it has been shown that basal RelA activity was required for LTβ induction of RelB:p52 dimer generation [118].

1.2.4 Negative regulation of NF-κB

NF-κB initiates the signalling for the inflammatory response whether pro-inflammatory or anti-inflammatory. It is critical for the body to be able to terminate these responses when no longer needed and abberations of these processes is the root of a plethora of diseases including sepsis and septic shock [119]. Termination is accomplished via a wide array of evolutionarily conserved members including: decoy receptors, ubiquitinases, de-ubiquitinases, transcriptional

regulators, splice variants, microRNAs and direct binding inhibitors [120]. The negative regulation of toll-like receptor (TLR) induced NF-κB signaling has been heavily studied, suggesting a complex system of tightly control processes under normal circumstances [121]. One of the most famous examples of TNFα induced NF-κB signaling is A20 (TNFAIP3). Initially discovered as a TNF inducible gene in human umbilical vein endothelial cells [122] A20 has shown to play an essential role in regulating the NF-κB driven inflammatory response [123-125]. A20 expression is upregulated in response to NF-κB signaling [126]. A20 is unique in that it can behave as a ubiquitinizing and de-ubiquitinizing protein of many members of NF-κB signaling, resulting in a negative feedback loop upon it's expression [127]. For example, during TNFα-induced signaling, A20 acts as a de-ubiquitinase that removes K63 linked polyubiquitin chains from RIP1 preventing interaction with NEMO, then A20's E3 ligase activity then results in K48 linked poly-ubiquitination, leading to the proteasomal degradation of RIP1, thus terminating the signaling [128].

There is less known about the negative regulation of the non-canonical pathway as the focus has largely been in the shifts in the signaling that occur as the pools of non-canonical and canonical NF-κB homo and heterodimers change over time depending on stimuli as described above. It has been shown that lupus patients have abnormal CD40 induced NF- κB signaling [129], however instead of seeing constitutive translocation of p52/RelB in response to excess CD40 and its ligand CD154, Zhang *et al.* found that there was increased IKBα degradation and increased canonical signaling via p50, RelA and c-Rel, but not RelB and p52 [129]. Again this suggests that a shift from non-canonical signaling to canonical over time acts as the "inhibition" of the

non-canonical signaling, and it is yet to be determined if there are direct inhibitors specifically designed to shut it off.

1.3 Mitogen Activated Protien Kinase (MAPK) signaling in septic shock

While NF-κB signaling has been central to much of the research for septic shock due to its well known role in transcriptional responses to inflammation, research is revealing that MAPK signaling is an important pathway in inflammation. MAPK signaling is heavily studied in the setting of cancer with estimates that 80% of cancers are due to mutations in various members of this pathway [130-133]. As well, the evasion of the immune system by tumors is heavily studied [134]. In cancer, therapies can be targeted at 6 pathways simultaneously, including MAPK [132]. It is conceivable that sepsis and septic shock will need to be treated in the same way [135]. Here we discuss MAPK signaling in relation to TNFα superfamily induced signaling and emerging evidence for cross talk between MAPK and NF-κB signaling.

1.3.1 MAPK signaling via Ras

TNF superfamily signaling mediates a wide array of activities such as apoptosis (via TNF, LT, CD95L, TRAIL, VEGI, TWEAK and LIGHT) [85]. For example, TNF binding to the TNF receptor (TNFR1) activates the recruitment of TRAF2 and RIP to the TNFR via their binding to the adaptor protein TRADD. The latter also recruits FADD which initiates the activation of a caspase cascade that leads to apoptosis [136]. Conversely, a large portion of the superfamily members activate survival (RANKL and BAFF), or proliferation/ differentiation via TNF, RANKL, CD27L, BAFF and CD40L to name a few [85]. Alternatively, TNF can activate NF-κB to invoke pro- or anti-apoptotic events, or induce hundreds or thousands of transcripts

including but not limited to cytokines, chemokines, immunoreceptors, cell adhesion molecules, acute phase proteins, stress response genes, cell surface receptors, apoptosis effector molecules, transcription factors and enzymes [137].

The Ras super family consists of over 150 small guanosine triphosphate (GTPases) including the most studied p21 Ras proteins: H-Ras, K-Ras and N-Ras, considered to be the classical proteins [130]. The family of p21 Ras proteins exhibit 85% sequence homology and are famous for their relationship with the Raf/ MEK/ ERK (extracellular-signal regulated kinases) signaling pathway [130]. Binding of extracellular ligands such as growth factors, cytokines, chemokines, extracellular matrix and hormones to their cell-surface receptors activates Ras; whereby. SOS removes the GDP bound to the inactive Ras, allowing Ras to associate with the abundant GTP available in the cytoplasm thus initiating Raf [138]. Raf in turn activates the protein kinases MEK1 and MEK2 (mitogen activated protein kinase (MAPK) /extracellular signal-regulated kinase (ERK)) which then phosphorylate and activate MAPK/ERK proteins ERK1 and ERK2 [138]. This pathway mediates diverse biological functions such as cell growth, survival and differentiation in a plethora of cell types, predominantly through the regulation of transcription, cytokine production, metabolism and cytoskeletal rearrangements [130, 138]. GTPase activating proteins (GAPs) catalyze the hydrolysis of GTP to GDP, rendering the Ras protein inactive again [139]. While intensely studied in relation to cancer, the MAPK signaling pathway is a hub of cellular control and directly affects proper functioning of cell growth, proliferation and anti-apoptosis as well as cytokine and chemokine transcription, and is known to play a role in many other types of disease, especially inflammation via the induction of cyclic adenosine monophosphate (cAMP) response binding protein (CREB) [140, 141]. This

Ras/Raf/MEK/ERK/CREB cascade will simply be referred to as the classical MAPK pathway from here on in this body of work.

1.3.2 CREB and NF-κB

NF-κB has been well studied in relation to sepsis and septic shock [87, 142, 143] however, despite evidence that cyclic AMP response element (CRE)- binding protein (CREB) is an important transcription factor for inflammatory genes, it has not been studied in relation to sepsis as extensively as NF-κB has [141]. Not only is there evidence that CREB is directly involved in transcription of genes such as IL-6, IL-10 and TNFα, studies are emerging which show potential mediation via an interaction with S276 of the RelA subunit of NF-κB with the CREB co-activators CREB binding protein (CBP) or p300 [141, 144-147]. It is conceivable that if there is evidence of cross talk at the level of transcription between NF-κB and MAPK pathways, then cellular mechanisms to facilitate cross talk between the two pathways may exist at other points. [130].

1.3.3 Cross talk between NF-κB and MAPK

Based on the fact that TNFα superfamily signaling has so many different outcomes, it would be expected that "the right hand would know what the left hand is doing" through elegant systems of cross talk. The balance of pro and anti-apoptotic signals resulting from TNF stimulation dictates the fate of the cell [85].

Specifically, it has been shown that TNF induces cell death via cleavage of caspase-8 and caspase-3 [148] while also promoting cell survival via NF-κB [100]. In colonic epithelial cells,

the balance between death and life under the umbrella of TNFα has been exemplified in the development of inflammatory bowel disease, a condition characterized by epithelial cell death [149]; whereas, the growth factors epidermal growth factor (EGF) and insulin-like growth factor (IGF), which induce MAPK signaling, have been associated with mucosal repair [150, 151]. In septic shock, TNFα is well studied in its detrimental role and evidence is mounting for a protective effect induced by growth factors, for example IGF. Interestingly, a SNP rs17513961 located 5' of the IGF gene met the threshold for genome wide significance in a GWAS of the the Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis (PROWESS) study, a clinical trial for APC in septic shock [152]. It has been shown that IGF is downregulated in sepsis and treatment with IGF in a murine model was protective [153, 154]. The IGF1- binding protein complex modulates the sepsis-induced inhibition of protein synthesis in skeletal muscle [155] and circulating IGF1-binding protein increases significantly and selectively in response to systemic endotoxin in an ovine model of sepsis [156]. Based on the premise that the pro-apoptotic functions of TNF are antagonized by cytokines such as IGF, insulin and EGF [157], an elegant study done in 2006 by Janes *et al.* was designed to illuminate the cross talk that TNFα induced NF-κB activates. The group revealed a multistep time-varying "autocrine cascade" whereby TNFα induced release of transforming growth factor alpha (TGFα) completes a circuit via the epidermal growth factor receptor (EGFR)/MEK/ERK signaling pathway and its subsequent cellular processes [158].

Cross talk has also been observed at the level of receptor density of TLRs. A recent study found that mRNA expression of TLRs was independent of NF-κB signaling yet dependent on MAPK pathway components such as ERK, c-Jun N-terminal kinases (JNK) and p38 [159]. MAPK up

or downregulation in response to the TNF signals could very well affect TLR density on APCs. This would affect the APCs' ability to recognize pathogens to dampen the immune reponse once the infection is under control but when dysregulated in extreme circumstance such as septic shock, may be a detriment to the patient.

1.4 Single Nucleotide Polymorphisms (SNPs)

1.4.1 Genomic structure

Single nucleotide polymorphisms are genetic variations that occur due to de novo mutations at a single nucleotide. If not corrected immediately by cellular machinery designed to proof read DNA and/ or selected against over time, they become incorporated into the genetics of the population. Hence SNPs are a stable form of nucleotide variation within the population at a particular site; whereas, de novo mutations that change one nucleotide to another may not persist over time. The ancestral nucleotide and the new stabilized mutation (now called a SNP) represent two possible alleles for this locus. The frequency of alleles for a given polymorphism vary within the population and if biallelic create a spectrum of three genotypes depending on which alleles are respresented on the cell's chromosomes. Linkage disequilibrium (LD) is the non-random association of alleles at adjacent loci [160]. It is created when a new mutation occurs on or near to a polymorphic locuson the same chromosome, an association which degrades over time due to recombination [160]. Independent segregation creates a situation whereby no alleles associate with each other. LD is the opposite. A particular allele at one locus is found on the same chromosome as a particular allele of a second locus more often than would

be predicted if independent segregation was occurring [160]. This can be due to recombinantion hot spots [161, 162].

One of the earliest measurements of LD is D, defined as the difference between the observed association of two alleles compared to the expected association if there was random segregation [163]. However, the most common measures of LD in the current field are D' and even more popular, r^2. D' is more useful than D in that the absolute value of D' is calculated by dividing D by its maximum possible value for the allele frequencies at the two loci; hence, D'=1 is complete LD. The problem is that when D' <1, the relative magnitude is difficult to clearly interpret and therefore not as informative [164]. r^2 is the correlation of alleles at two loci whereby r^2=1 is complete LD [165, 166]. Values between 0-1 essentially embody how much information could be drawn about one locus by inspecting another [165]. These relationships are the major influence on the design of genetic association studies, from SNP selection on genome wide association study (GWAS) chips to imputation of missing data.

When sequencing of the human genome (the Human Genome Project) was completed, the International SNP Map Working Group had identified approximately 1.4 million SNPs in the human genome [167]. Currently, more than 17 million SNPs in the human genome have been catalogued in the SNP Database (dbSNP; http://www. ncbi.nlm.nih.gov/projects/SNP/). SNPs are the most abundant type of genetic variation in the human genome; there is approximately one SNP to every 1kb of DNA sequence can be expected throughout the genome if comparing two unrelated individuals [167].

In order to avoid genotyping millions of polymorphisms for association with disease, an indirect approach is dependent on surrogate markers (tagSNPs) to locate disease variants through use of the knowledge of the LD of two or more SNPs with each other [168]. tagSNPs represent a "bin" of SNPs that are in LD with the tagSNP and hence the principle is that once the genotype of the tagSNP is known, then the genotypes of the SNPs in LD with that tagSNP can be inferred or imputed. The current consensus is that to genotype approximately 500,000 SNPs would capture most of the variation mapped by the HapMap project [169-172]. tagSNPs may be a functional SNP or they may be representing a potentially functional SNP that is in LD with it. Further functional studies are required to determine if the SNP is in fact functional and if so the molecular mechanism involved.

Most recently, the 1000 Genomes Project (initiated in 2008) has completed its goal of sequencing the genomes of at least 1,000 individuals from different populations around the world (http://www.1000genomes.org/). This has added approximately 15 million new SNPs to the human genome data set, hence 17 million in dbSNP[173]. The association of rare polymorphisms with disease outcome that have a large effect size with a disease are becoming a reality [174]. Until technology becomes so affordable that all samples could be sequenced, the reality lies within genotyping tagSNPs. The technology is such that that is not an issue; the issue is determining which SNP in the "bin" of SNPs represented by the tagSNP is actually having a biological effect.

1.4.2 3’ untranslated region (UTR) SNPs

Initial focus on promoter regions and how SNPs in transcription binding sites can alter mRNA expression levels has resulted in a greater understanding of allele specific expression [175-177]. This can result in individuals that are homozygous for one allele to have different levels of mRNA and potentially protein compared to the homozygous individual for the alternate allele. In an additive model, this results in the heterozygous individuals falling in between the two extremes. Intronic or intergenically located SNPs can be especially difficult, yet not impossible to elucidate impact on gene expression [178]. It is known that DNA is not linear, it exists in a dynamic state of condensed and unwound chromatin [179, 180]. The looping back of DNA on itself and the subsequent interactions via cellular machinery such as enhancers or silencers is somewhat understood, yet the events are difficult to capture *in vitro* and *in vivo*; hence, the focus on regions where regulatory elements may bind controling gene transcription rate or splicing [180]. With the advent of micro-ribonucleic acid RNA (miRNA) discovery came a renewed focus on the 3’UTR as researchers unveiled the transcriptional regulation these molecules possessed by binding to regulatory regions in the 3’ UTR.

miRNAs were discovered in *C. elegans* in 1993 [181] and are evolutionarily conserved, endogenous single stranded RNAs between 18-25 nucleotides in length [182]. Since their discovery, over 100 miRNAs have been characterized in invertebrates and over 800 in mammals [183]. miRNAs bind miRNA recognition elements (MRE) [184, 185]. Bioinformatics predicted that nucleotides 2-7 named the “seed site” are the most critical for binding [186-188]. This is accepted as the current dogma in light of the fact that the 5’ end of the miRNA sequence is the most conserved [189]. Binding of the miRNA to its MRE in the 3’ UTR of mRNA recruits the

RNA induced silencing complex (RISC) resulting in inhibition of translation either via targeting for degradation or interference with initiation of translation [184].

Not surprisingly, studies have now uncovered disruption of binding due to SNPs in the seed sites of miRNA binding domains [190-192]. Logically it would follow that a growing number of SNPs in microRNA sites are being associated with human diseases [193] including obesity [194], cornonary heart disease [195], cancers [196-198] and suceptibility to tuburculosis [199] to name several. To date, it appears that no studies have looked at the influence of polymorphims affecting miRNAs in sepsis; however, miRNAs are emerging as potential biomarkers which could be affected by 3' UTR SNPs residing in these MREs [200-204] and have been screened in relation to NF-κB signaling [205].

1.4.3 SNPs and septic shock

The current research being done to associate SNPs with septic shock is based on evidence that the severity of outcome in infections is heritable. The proportion of phenotypic variance that can be explained by the genetic variance is the definition of heritability [206]. There are two main definitions of heritability; H^2 is referred to as broad sense heritability, whereas h^2 is narrow sense. H^2 represents the amount of phenotypic variation that can be explained by genetic variation and this can be estimated, for example, if genetically identical individuals are exposed to different environment [206]. H^2 includes the additive genetic value, dominance and epistatic components of the individuals genotypes and can be mathematically described as $H^2 = V_G/V_P$, where V_G is the variance due to genetic differences within a population and V_P is the variance in phenotype [206]. In order to determine H^2 twin studies have been employed under the

assumption that monozygotic (MZ) twins are identical in their DNA, whereas dizygotic (DZ) twins are 50% identical. The expectation is that disease concordance will be higher in those identical than those not. Narrow sense heritability (h^2) is a measure of the additive or average genetic effects of a single allele (V_A) and can be mathematically described as $h^2 = V_A / V_P$ [206].

Interestingly, twin studies of severity of infection support a genetic component that influences outcome. In a study of 12,346 twin pairs born in Denmark between 1994 and 2003 it was shown that the severity illness due to respiratory syncytial virus infection had a significantly greater concordance rate in MZ twins than in DZ twins (concordance rate: 0.66 vs. 0.53 p=0.01) [207]. When comparing the severity of the immune response to Chlamydia trachomatis in MZ and DZ twins, Bailey et al found the heritability due to genetic factors to be 39% [208]. There may also be a heritable component to bacterial colonization as a recent study showed that the concordance rates for the presence of tongue malodor (caused mainly by facultative bacteria on the tongue that produce volatile organic compounds) among identical and fraternal twins were 67% and 11%, respectively [209]. Intermediate phenotypes commonly explored in infectious settings such as infection associated mannan-binding lectin levels and mannan-binding lectin associated serine protease 2 (MASP-2) activity have estimated heritability of 77% and 75% respectively [210]. Furthermore, in a longitudinal study to assess genetic and environmental influences on adult mortality, Sorensen and colleagues followed 960 families that included who were placed early in life with adoptive parents unrelated to them [211]. They evaluated the risks of the adopted child dying when a biologic or adoptive parent died of the same cause and compared these risks with the adoptees' risk of dying from the same cause when either the biologic or adoptive parents were still alive at the ages of 50 and 70 [211]. The death of a biologic parent

before the age of 50 from infection resulted in relative risks of death in the adoptees of 5.81 (2.47 to 13.7), compared to 1.19 (0.16 to 8.99) for cancers. The death of an adoptive parent resulted in relative risks of death in the adoptees that were not significantly different between causes [211].

Heritability does not provide information about the underlying genetic architecture that leads to the resultant phenotype. For example, a trait with high heritability can have hundreds of contributing loci, or conversely a trait with low heritability could be explained by a single locus [212, 213]. Based on the evidence that there is a genetic component to the severity of infection, followed by the fact that infection can be the precipitating factor leading to septic shock, it is logical to infer that septic shock is heritable. Since the Sorensen study, researchers have attempted to determine the genetic components that are responsible for these observations. The result is a large body of work associating polymorphisms with outcome or various intermediate phenotypes has been done, and while associations have been made, many reports are conflicting.

1.4.4 Previous evidence and trials

1.4.4.1 Candidate gene studies

Candidate gene studies were the first to take advantage of this new found relationship between genetics and infectious disease. Based on hypotheses generated from previous knowledge of biological plausibility, there are many advantages to the candidate gene approach; they can be done quickly, are generally inexpensive and can identify variation within genes with small effects. A large body of work has been done in sepsis, septic shock and other infectious diseases and many associations have been found. At the centre of this body of work, a theme emerges of

candidate genes that have been looked at in many ways (including varying outcomes or phenotypes, different time points, *in vivo* studies of humans, *ex vivo* studies of various cell types removed from either humans with sepsis, severe sepsis or septics shock or from healthy volunteers to be stimulated with various pro-inflammatory cytokines, to name a few) **(Table 1.2).**

There are some studies that report conflicting results, but over the course of the last 20 years or so several key aspects have been identified that may explain these issues. A good example of this progression is the TNFα promoter polymorphism commonly referred to as -308 or G-308A. Previous work showed that the polymorphism at this location directly affects TNFα expression, an exciting finding due to the expectation that if genotyped in patients, this could explain differences in outcome due to differences in levels of circulating TNFα [214]. Hence, in 1999, Mira *et al.* genotyped this polymorphism in 89 Caucasian septic shock pateints and 87 controls and found an association of the A allele (sometimes referred to as TNF2) with septic shock susceptibility and mortality [215]. This TNF G-308A polymorphism had also been associated with increased risk of cerebral malaria in Gambian children (n=376)[216] and with an increased risk of severe disease in cases of meningococcal children (n=98) [217], suggesting a biological influence. A prospective study in 2004 with 213 septic shock patients and 354 healthy controls was unable to find an association with this polymorphism and susceptibility to sepsis, illness severity or outcome [218]. A power calculation done prior to the study showed that 200 pateints or more were needed to detect a difference between cases and controls in adult Caucasians, suggesting that the findings in this study were perhaps different than the Mira study due to size. Since then, a number of studies also looked at this TNF G-308A polymorphism (see Table 1.2).

For illustration we will discuss 3 subsequent studies. In 2001 Waterer *et al.* published a study looking at the TNF G-308A polymorphism specifically in respect to community aquired pneumonia and found no association however had included several ethnicities. The allele frequencies of TNF G-308A in all patients did not differ significantly suggesting the inclusion of multiple ethnicities was a non-confounding factor; and while the size of the study was larger (n=280) and the phenotype was very clear, in retrospect there is a possibility that since it would appear that the TNF G-308A polymorphism is not associated, it could be a different polymorphisms that does have population stratification across ethnicities [219]. Jessen *et al* studied a septic population (n=319) described as Danish (2007) and also found no association of TNF G-308A with disease severity (from sepsis to severe sepsis to septic shock), suggesting there is no association. However, the authors do not mention if all Danish participants were Caucasian [220]. Furthermore, a study in 2009 published by Henckaerts *et al.* of a large cohort (n=774) critically ill ICU patients found no association of TNF G-308A with susceptibilty to bacteremia or mortality but the authors admit is it possible that the lack of positive results are due to two confounders: this was all comers to the ICU rather than a defined phenotype, as well as mixed ethnic backgrounds [221]. The authors found no significant differences between genotype frequency when the critically ill were compared to Danish or Belgian healthy control groups; however, the issue of the ethinic diversity (or similarity) within the control groups still stands. Hence when considering results from association studies, it is very important to consider at least four aspects of the study: the size of the study for statistical power to detect differences; whether or not multiple ethnicities are included, asthis can lead to spurious associations (or lack of associations) due to population stratification; the age of the population (pediatric sepsis versus adult) and the level of consistency across populations in terms of the exact phenotype studied.

For consistency, Table 1.2 while extensive, may not be exhaustive and only includes studies in adult humans with sepsis, severe sepsis and/or septic shock, found a statistically significant association, and had more than 100 participants (cases and controls combined). It is important to note that many of these polymorphisms have been associated with pediatric sepsis and other infectious diseases. Of the 35 studies are reported in this table, notably, only 4 of these studies report corrected p values, therefore raw p values are reported.

Table 1.2. Candidate gene association studies of polymorphisms or haplotypes studied in adult sepsis, severe sepsis or septic shock.

Criteria: Adult, positive association, n> 100 combined cases and controls.

All ethnicities= ALL, Caucasian only = CAUC

Odds ratio (OR) (if not reported then these studies are association with alleles and outcome for sepsis, severe sepsis or septic shock)

(d)= derivation cohort (r)= replication cohort

GENE NAME	Ethnicity	n cases	n control	OR (95%CI)	p value	Ref
Apolipoprotein E APOepsilon3 allele	All ethnicities (ALL)	34	309	0.28 (0.18-0.64)	0.007	[222]
Cluster of differentiation 14 (CD14) 159C	ALL	252	0	na	0.02	[23]
CD14 159C	ALL	228	0	1.7 (1.01-2.76)	0.047	[223]
CD14 159C	CAUC	90	122	5.3 (1.2-22.5)	0.02	[224]
Chemokine C-X-C motif ligand 2 (CXCL2) -665 (AC)n 24 +/- repeat alleles	ALL	178	357	3.65 (1.41-9.43)	0.0006	[225]
Factor V Leiden R506Q	ALL	3894	0	0.82 (0.57-1.17)	0.05	[226]
Fibrinogen β (GAA haplotype of -854, -455, +9006)	CAUC	631	0	0.66 (0.46-0.94)	0.02	[227]
Heat shock protein 70 (HSP70) HSP70-2+1267 AA	ALL	30	313	3.5 (1.8–6.8)	0.0005	[228]
IL-6 -174 G/C	CAUC	326	207	0.11 (0.02-0.57)	0.008	[229]
IL-6 -174 G/C	ALL	112	0	na	0.04	[230]

GENE NAME	Ethnicity	n cases	n control	OR (95%CI)	p value	Ref
IL-6 -174C/1753C/2954G (C/C/G), G/G/G, or G/C/C haplotype clade	CAUC	228	0	na	0.02	[231]
IL-10 -592/ +734/ +3367 CGG haplotype	CAUC	550	0	na	0.007	[232]
IL-10 -1082G	CAUC	61	50	6.1 (1.4–27.2)[233]	0.024	[233]
IL-10 -1082G	ALL	116	141	1.5 (no CI reported)	<0.05	[234]
IL-1RA variable number of tandem repeats of 86bp in intron 2, allele 2	CAUC	78	130	6.47 (1.01–41.47)	0.04	[235]
Interluekin 1 receptor associated kinase 1 (IRAK 1) T1595C	CAUC	155	0	2.9 (1.1-7.7)	0.047	[236]
Lipopolysaccharide (LPS) binding protein (Gly98)	ALL	204	250	na	0.02	[237]
Lymphotoxin alpha (LTA) +A252G	ALL	854	854	na	0.005	[238]
Mannan binding lectin associated serine protease 2 (MASP2) D120G/ V377A haplotype	CAUC	774	652	2.35 (1.38–3.99)	0.002	[221]
Mannose binding lectin (MBL) XAAA/O haplotype	ALL	252	0	na	0.02	[23]
MBL A/O O/O haplotypes	CAUC	174	0	na	0.0001	[239]
MBL A/A haplotype	ALL	250	272	na	0.001	[240]

GENE NAME	Ethnicity	n cases	n control	OR (95%CI)	p value	Ref
nucleotide-binding oligomerization domain containing 2 (NOD2)/ caspase recruitment domain family, member 15 (CARD15) Leu1007fsinsC	ALL	132	0	2.9 (1.5–5.5)	< 0.001	[241]
PAI-1 4G/4G	ALL	50	131	5.9 (1.9-18.0)	0.04	[242]
PAI-1 4G/4G or 4G/5G	CAUC	208	0	2.57 (1.18 - 5.62)	0.018	[243]
Protein C -1641	CAUC	62 (d) 402 (r)	0	na	<0.05 (d) 0.028 (r)	[26]
Protein C 673 T/C (rs2069912)	EAST ASIAN	100	0	na	0.035	[22]
Protein C -1654 C/T (rs1799808) /1641 G/A (rs1799809) haplotype	CHINESE HAN	240	323	1.74 (1.17-2.60)	0.008	[244]
Toll like receptor 1 (TLR1) -7202G (rs5743551)	CAUC	1498	0	3.16 (1.43-6.97)	0.004	[245]
TLR1 -7202G	ALL	1183	167	2.56 (1.28-5.15)	0.008	[246]
TLR 2	ALL	252	0	na	0.07	[23]
TLR4 896G	ALL	228	0	2.9 (1.13-7.66)	0.027	[223]
TLR9 rs187084 rs352162 rs352162	CHINESE HAN	557	0	rs187084 1.36 (1.05-1.75) rs352162 1.38 (1.07-1.76) rs352162 1.4 (1.08-1.80)	rs187084 0.019 rs352162 0.012 rs352162 0.011	[247]

GENE NAME	Ethnicity	n cases	n control	OR (95%CI)	p value	Ref
Tumor necrosis factor alpha (TNFα) Nco1 polymorphism B2 allele	ALL	110	0	3.45 (1.54-7.77)	0.003	[248]
TNF α -238A	ALL	233	0	na	0.0063	[249]
TNF α rs1800629 A	ALL	159	0	7.14 (3.10-16.45)	1.2×10^{6}	[250]

1.4.4.2 Genome wide association studies

The advent of genome wide association studies (GWAS) brought new hope to the field of genetic association studies; to look at all SNPs in relation to a particular phenotype in a biologically unbiased fashion in a large cohort. Scientists hoped that by looking at as many SNPs as possible in a non-biased fashion, novel associations would be found shedding new light on diseases that are still plaguing the population. To date, more than 1500 GWAS (compiled in the NHGRI online GWAS catalog) [251] have been reported, the majority investigating disease susceptibility with a small number of studies investigating drug treatment responses. These reports have been successful in advancing our understanding of the pathophysiology of complex diseases such as Type 2 diabetes [252] and plasma cholesterol levels [178]. In a study summarizing all infectious disease related outcome based studies between 2001 and 2010, of 3730 articles reviewed, only 23 were GWAS, none of which were sepsis related [253]. Since then, one GWAS in septic shock has been published [152].

The progression of sepsis, severe sepsis and septic shock is highly complex involving multiple organs, the cardiovascular system, endocrine system and nervous system, to name a few. Hence,

it it assumed that multiple systems are at play; and furthermore, there are interactions among them. To address this, Man *et al*. used the genome wide data generated from the Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis (PROWESS) trial and conducted analyses using single-marker marginal effects and established a methodology for assessing interactions on a genome-wide scale both for predictive (treatment response) and prognostic (28-day mortality) biomarkers [152]. 1446 patient DNA samples were genotyped for 856 627 SNPs. Interestingly, the single-marker analysis, which utilized traditional association methodology including logistic regression to identify SNP-by-treatment interactions yielded very few markers with a significant signal. As previously mentioned, rs17513961 in an untranslated region of LOC222052 (found near the insulin-like growth factor I (IGF1)-binding protein gene), met the threshold for genome-wide significance ($p=5.0 \times 10^{7}$). The authors then went on to test a subset of patient who only received placebo in order to identify prognostic markers but did not identify anything of significance. Perhaps this was due to small sample size as this was only 700 of the total patients [152]. This negative result highlights the need for very large cohorts in order to overcome the potential noise created by the complexity of the biology at play, multiple genes with small effects acting in concert.

Much of the biology behind septic shock has been delineated using a reductionist approach for this very reason. An interesting GWAS study of a unique biological model relevant to sepsis was recently published [254] following the findings of a candidate gene study reporting that common genetic variants in TLR1 were associated with hyper-responsiveness to Pam_3CSK_4, a synthetic triacylated lipoprotein [246]. The GWAS was performed on whole blood from 360 healthy volunteers exposed to Pam_3CSK_4, using 8 cytokine responses (TNFα, IL-1β, IL-6, IL-8,

IL-10, GCSM, IL-1RA and MCP-1) as the phenotype(s) for association with genotype [254]. Unlike the PROWESS study, this group found 19 loci that reached genome-wide significance within the TLR10/1/6 locus on chromosome 4 [254]. It would appear that modern day genetic studies for complex polygenic diseases are toggling between to two approaches: investigate genome wide in adequately powered cohorts to overcome the noise, or modify and increase the sophistication of candidate gene approaches to include a broader scope of the biology upstream and downstream of the candidate. The work described here highlights the possibilities for advances in medicine in the future based on the discovery of SNPs associated with septic shock and the studies designed to elucidate the biological mechanisms at play.

Chapter 2: A Single Nucleotide Polymorphism in NF-κB Inducing Kinase (NIK) is Associated with Mortality in Septic Shock

2.1 Introduction

Septic shock is an extreme manifestation of the host inflammatory response to severe infection and the NF-κB signaling pathway is one of the most important signaling pathways involved in the pathogenesis of this pathological state [143]. Septic shock is defined as sepsis accompanied by organ failure including cardiovascular failure [78]. In response to infection, NF-κB signaling (among other pathways) leads to the transcription of a wealth of inflammatory mediators which contribute to the development of cardiovascular failure, including cytokines, chemokines, adhesion molecules, and reactive oxygen and nitrogen species, to name a few [87, 255]. The induction of this response is necessary for the resolution of infection however this response, when extreme, leads to organ damage and mortality in many cases. A better understanding of the key pathways and critical molecules involved in the pathogenesis of septic shock is imperative.

NF-κB signaling is activated by a canonical (or classical) pathway and by a non-canonical (or alternative) pathway involving multiple genes (**Figure 2.1**)[142]. Briefly, the canonical NF-κB pathway requires the IκB kinase (IKK) complex comprised of IKKα/β/γ[87]. Activation of the IKK complex in response to inflammatory stimuli results in phosphorylation and ubiquitin-dependent degradation of IκBα or IκBβ and translocation of p50 related dimers into the nucleus [87]. In response to inflammation in the non-canonical pathway, NF-κB inducing kinase (NIK), a docking molecule, recruits IKKα to p100 and then activates IKKα [87, 104]. IKKα

phosphorylates p100 which is subjected to phosphorylation, ubiquitination and proteosomal degradation, resulting in the release and nuclear translocation of p52 containing RelB heterodimers [87, 104].

Genetic variation in key inflammatory genes contributes to outcome in sepsis. [19, 22, 26, 215, 248, 256]. Since NF-κB is a centrally important signaling pathway, we tested the hypothesis that genetic variation in genes involved in the NF-κB pathways would be associated with mortality in septic shock. To accomplish this, we genotyped 56 SNPs in 18 genes in a single centre derivation cohort of septic shock patients. We next tested for replication of an arising SNP association in a second multicentre cohort of septic shock patients. We tested for similar association with organ dysfunction to understand the involved clinical phenotype in more detail. To test for biological plausibility of the observed SNP association we next measured gene expression in genotyped lymphoblastoid cell lines and identified a candidate gene whose expression was associated with the candidate SNP. We then tested for association of this gene product with the candidate SNP both *in vitro* and *in vivo*.

2.2 Methods

Patient cohorts

St. Paul's Hospital Cohort (Discovery Cohort): All patients admitted to the St. Paul's Hospital (SPH) Intensive Care Unit, Vancouver, Canada between July 2000 and January 2004 were screened. Using the current consensus definition (≥2 SIRS criteria, known or suspected infection, hypotension unresponsive to fluid resuscitation alone) 601 patients had septic shock on admission and had DNA available [26, 257]. Twelve patients in this cohort had also been enrolled in the Vasopressin and Septic Shock clinical trial [258] and were therefore excluded. Thus 589 patients in total were included in this analysis. This study was approved by the Institutional Review Board at SPH and the University of British Columbia.

VASST Cohort (Replication Cohort): The Vasopressin and Septic Shock Trial (VASST) was a multicenter, randomized, double blind, controlled trial evaluating the efficacy of vasopressin versus norepinephrine in 779 patients who were diagnosed with septic shock according to the current consensus definition [259]. Clinical phenotyping has been described elsewhere [258]. All patients were enrolled within 24 hours of meeting the definition of septic shock and DNA was available from 616 patients. The research ethics boards of all participating institutions approved this trial and written informed consent was obtained from all patients or their authorized representatives. The research ethics board at the coordinating centre (University of British Columbia) approved the genetic analysis.

SNP selection and genotyping of patient cohorts

We used Ingenuity IPA (Version 8.6 Build 93815 Content 3003) to identify 19 cytosolic genes in the NF-κB canonical and non-canonical pathway that also had dense resequencing data publically available (**Figure 2.1**) (Seattle SNPs Program for Genomic Applications http://pga.mbt.washington.edu/, Cardiogenomics http://cardiogenomics.med.harvard.edu/home, Innate Immunity http://www.pharmgat.org/IIPGA2/index_html, formerly http://innateimmunity.net/, Berkeley University PGA http://pga.jgi-psf.org/ and SouthWestern PGA http://pga.swmed.edu/). tagSNPs were identified for genotyping in patient cohorts using a linkage disequilibrium (LD)-based tag SNP selection method [260], using an r^2 threshold of 0.65for SNPs with a minor allele frequency > 5% yielding 59 SNPs in 20 cytosolic genes; receptor and their ligands as well as downstream gene targets of NF-κB signaling were excluded (**Table 2.1**). DNA was extracted from peripheral blood samples using a QIAamp DNA Blood Midi Kit (QIAgen, Mississauga, ON, Canada) and genotyped using the Illumina Golden Gate Assay at the UBC Centre for Molecular Medicine and Therapeutics genotyping core facility (Luminex Molecular Diagnostics, Toronto, Canada). Primer probe sets are detailed in **Appendix A Table A.4.** All SNPs with minor allele frequencies (MAF) < 0.01 or call rates (CR) < 0.95 were removed from analysis. Additionally, SNPs with MAF < 0.05 were removed if CR < 0.99. Hence, three SNPs were removed from the analysis yielding 56 SNPs in 18 cytosolic genes for analysis. Call rates are reported in **Appendix A Table A.1.**

Clinical phenotype

The primary outcome was 28-day mortality. Secondary outcomes were days alive and free of organ dysfunction during the first 28 days calculated according to the Brussels criteria [259].

Biological plausibility experiments

Microarray mRNA expression analysis *In Vitro*

Lymphoblastoid DNA from the Coriell Institute was genotyped for NIK rs7222094 in 85 CEPH population samples using Sanger sequencing of the region. Sequencing was performed at the McGill University and Génome Québec Innovation Centre, Montréal, Canada. Primers for sequencing the region surrounding rs7222094 are as follows: Forward 5'-GGGTTCCCTATGGAGGAGAG -3' Reverse 5'-CTGTCCAGCTCTCCAGGTTC-3'. These 85 CEPH population lymphoblastoid cell lines of known genotype for NIK rs7222094 were cultured in RPMI 1640 and subsequently stimulated in triplicate by the addition of Cytomix [19, 261-263] (2.5ng/mL of each TNFα, IL-1β, IFNγ [R & D Systems, Mineapolis, MN] and 12.5μM of CpG [Sigma-Aldrich, Oakville, ON, Canada]) for 6 hours. RNA was extracted from the 3 stimulated samples as well as 2 control biological duplicates using the QIAgen RNeasy kit (QIAgen Mississauga, Ontario, Canada) and mRNA expression was measured using the Human WG-6 v.3 expression chip (Illumina, San Diego, CA, USA) (Génome Québec Innovation Centre, Montréal, Canada). Microarray raw data was normalized and fold change was calculated using the Flexarray(v.1.4.1) package available from Genome Quebec (http://genomequebec.mcgill.ca/FlexArray/license.php). MIAME compliant microarray data is publically available at GEO http://www.ncbi.nlm.nih.gov/geo/ (GSE25543).

ELISA protein expression analysis *In Vitro*

26 CEPH population lymphoblastoid cell lines of known genotype for NIK rs7222094 were cultured in RPMI 1640 and subsequently stimulated by the addition of Cytomix [19, 261-263]

(2.5ng/mL of each TNFα, IL-1β, IFNγ [R & D Systems, Mineapolis, MN] and 12.5μM of CpG [Sigma-Aldrich, Oakville, ON, Canada]) for 24 hours. Of the 85 cell lines used for the microarray experiment, 13 were homozygous for the minor allele, thus all 13 were interrogated, matched by a random selection of 13 major allele cell lines. Supernatant was collected and biological duplicate CXCL10 concentrations were measured by ELISA (R & D Systems, Minneapolis, MN, USA).

ELISA protein expression analysis *In Vivo*

Briefly, whole blood samples were drawn into chilled 7mL EDTA vacutainer tubes (BD, Mississauga, ON, Canada), put on ice immediately then spun at 3000 rpm for 15 minutes at which point plasma was collected and stored at -70C until further use [258]. Baseline plasma samples from 60 patients of known genotype for rs7222094 (30 TT and 30 CC) were randomly selected and assayed for CXCL10 concentrations in duplicate by ELISA (R & D Systems Minneapolis, MN, USA).

Statistical analysis

We assessed baseline characteristics using a chi-squared test for categorical data and a Kruskal-Wallis test for continuous data and then reported the median and inter-quartile ranges. We then tested for association between SNP genotype and 28-day mortality in the SPH discovery cohort using an Armitage trend test, as is commonly used for initial discovery surveys in genome-wide association studies (**Table 2.1**). One SNP emerged as statistically significant after a Bonferroni correction for multiple comparisons. We then tested for replication of this finding in the VASST cohort of septic shock patients. To correct for potentially confounding variables, including age,

gender, ancestry, and surgical versus medical diagnostic category, we used Cox regression. We then tested for association between secondary outcome measures of days alive and free of organ failure using Kruskal-Wallis tests in both SPH and VASST cohorts. Vasopressin treatment by genotype (NIK rs7222094 CT) interaction was assessed using logistic regression analysis (interaction statistics): P(Death) ~ vasopressin + genotype + vasopressin x genotype.

A student's t-test was performed to test for differences between genetic groups (rs7222094 CC versus TT) of CXCL10 ELISA concentrations. Analyses used SPSS (version 16; SPSS, Chicago, IL, USA), R statistical software package and GraphPad PRISM (version 5.02; La Jolla, CA, USA).

2.3 Results

28-day mortality in septic shock patients

Of the 56 tagSNPs in 18 genes in the canonical and non-canonical NF-κB pathways, one SNP, rs7222094 in NIK was significantly associated with 28-day mortality in the SPH discovery cohort (uncorrected P=0.00024, Bonferonni corrected P=0.013) (**Figure 2.1 and Table 2.1**).

Figure 2.1 NF-κB signaling pathways as dictated by Ingenuity Pathway Analysis (IPA)

All genes circled in red were genotyped for tag SNPs for a total of 20 genes and SNPs of which 56 SNPs in 18 genes were included in the analysis. (www.Ingenuity.com).

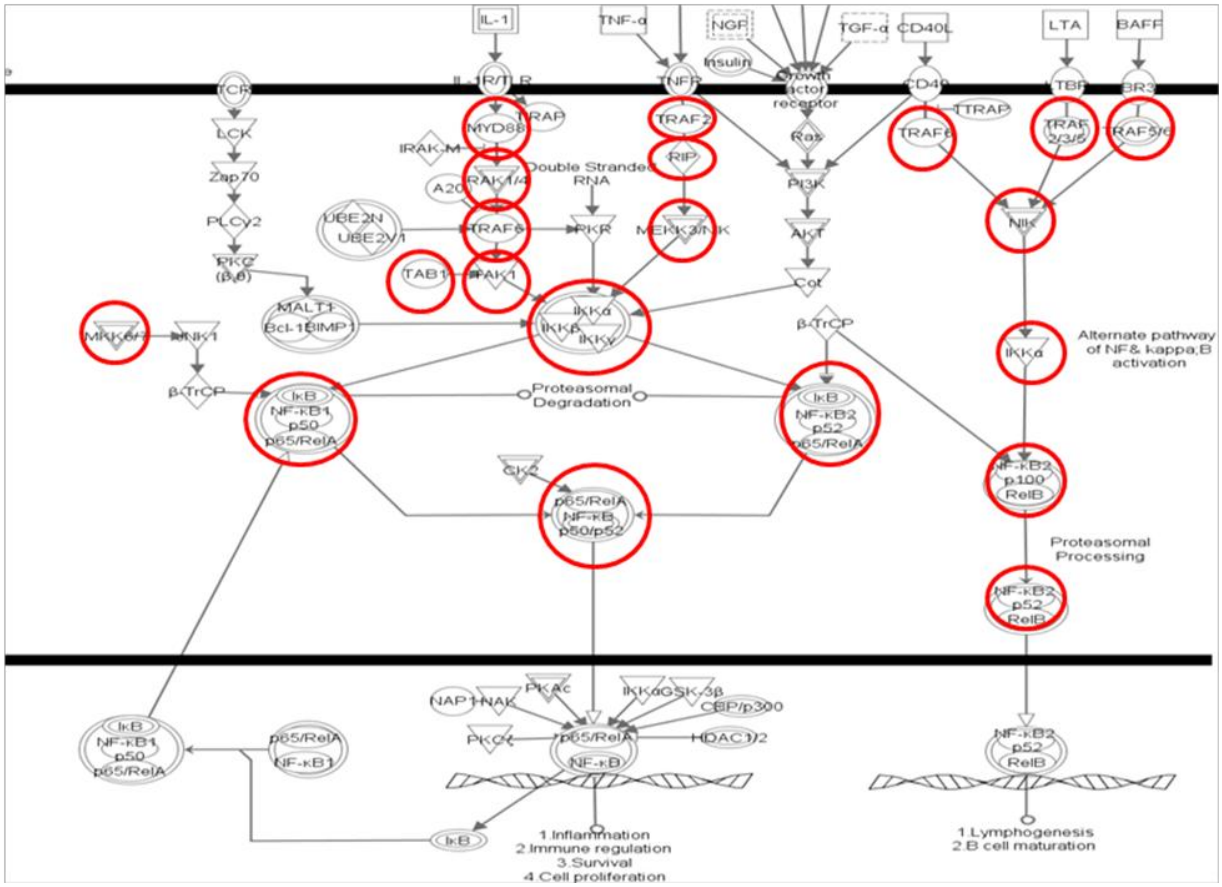


Table 2.1 Armitage trend test on mortality and SNPs of NF-κB pathway genes of patients in the SPH cohort

Canonical Pathway	SNP	Uncorrected p value	Bonferroni Corrected p value
MYD88	**rs2239621**	0.443	1.000
	rs6853	0.489	1.000
	rs7744	0.006	0.336
IRAK4	**rs4251513**	0.839	1.000
	rs4251520	0.969	1.000
TIRAP	**rs1786697**	0.310	1.000
	rs614700	0.342	1.000
	rs646005	0.223	1.000
MAP3K7	**rs1145727**	0.712	1.000
	rs157688	0.946	1.000
	rs205342	0.810	1.000
	rs967779	0.691	1.000
MAP3K7IP1	**rs5750824**	0.117	1.000
	rs6001585	0.039	1.000
	rs739141	0.028	1.000
	rs7949	0.080	1.000
IKBKB	**rs10958715**	0.272	1.000
	rs3747811	0.759	1.000
	rs5029748	0.508	1.000
RIPK1	**rs11242823**	0.693	1.000
	rs2326173	0.430	1.000
	rs4959774	0.455	1.000
MAP2K6	**rs12453226**	0.066	1.000
	rs1548444	0.331	1.000
	rs2586545	0.152	1.000
NFKBIA	**rs1957106**	0.945	1.000
	rs2233415	0.181	1.000
	rs3138055	0.338	1.000
	rs696	0.693	1.000
NFKBIB	**rs10775533**	0.006	0.336
	rs4803006	0.033	1.000
NFKBIE	**rs2282151**	0.362	1.000
	rs3799962	0.650	1.000
	rs730775	0.522	1.000
RELA	**rs1049728**	0.669	1.000
	rs11227247	0.722	1.000
NFKB1	**rs11722146**	0.817	1.000
	rs230521	0.557	1.000

Canonical Pathway	SNP	Uncorrected p value	Bonferroni Corrected p value
	rs230542	0.627	1.000
	rs3774934	0.808	1.000
Non-canonical Pathway			
TRAF2	**rs10283820**	0.349	1.000
	rs10781520	0.661	1.000
	rs2784069	0.402	1.000
TRAF6	**rs2303439**	0.152	1.000
	rs5030411	0.772	1.000
	rs540386	0.683	1.000
NIK (MAP3K14)	**rs2074293**	0.818	1.000
	rs7222094	0.00024	0.013
	rs9972936	0.883	1.000
CHUK	**rs11190421**	0.050	1.000
	rs11591741	0.234	1.000
	rs3818411	0.658	1.000
	rs7903344	0.644	1.000
NFKB2	**rs1409312**	0.442	1.000
	rs3802681	0.867	1.000
	rs7086205	0.226	1.000

Hardy-Weinberg equilibrium (HWE) and minor allele frequencies (MAF) of all SNPs genotyped are presented along with literature based MAF in **Appendix A Table A.1.** Allele frequencies of rs7222094 differed between ethnic groups within the SPH and VASST cohorts **(Appendix A Table A.2)**. Therefore, our primary analysis was limited to Caucasians only (SPH n=453, VASST n=517) and a secondary analysis of all patients included ethnicity as a covariate (SPH n=589, VASST n=616).

To consider and correct for potential confounding variables due to differences at baseline in septic shock patients, we used Cox regression to test an additive model in the SPH septic shock cohort and then used the same analysis in the VASST septic shock cohort. Patients in the SPH cohort who had the CC genotype of NIK rs7222094 had a significantly increased hazard of 28-day mortality compared with patients having the CT or TT genotypes of rs7222094 (Hazard Ratio [HR] 1.35; 95% confidence interval [CI] 1.12-1.64; P=0.002 Caucasian only) (**Table 2.2)**. This finding replicated in the VASST cohort (HR 1.24; CI 1.00-1.52; P= 0.048 Caucasian only) (**Table 2.2)**. The results were similar for all patients with ethnicity included as a covariate in a Cox regression model **(Figure 1 and Table 2.3).**

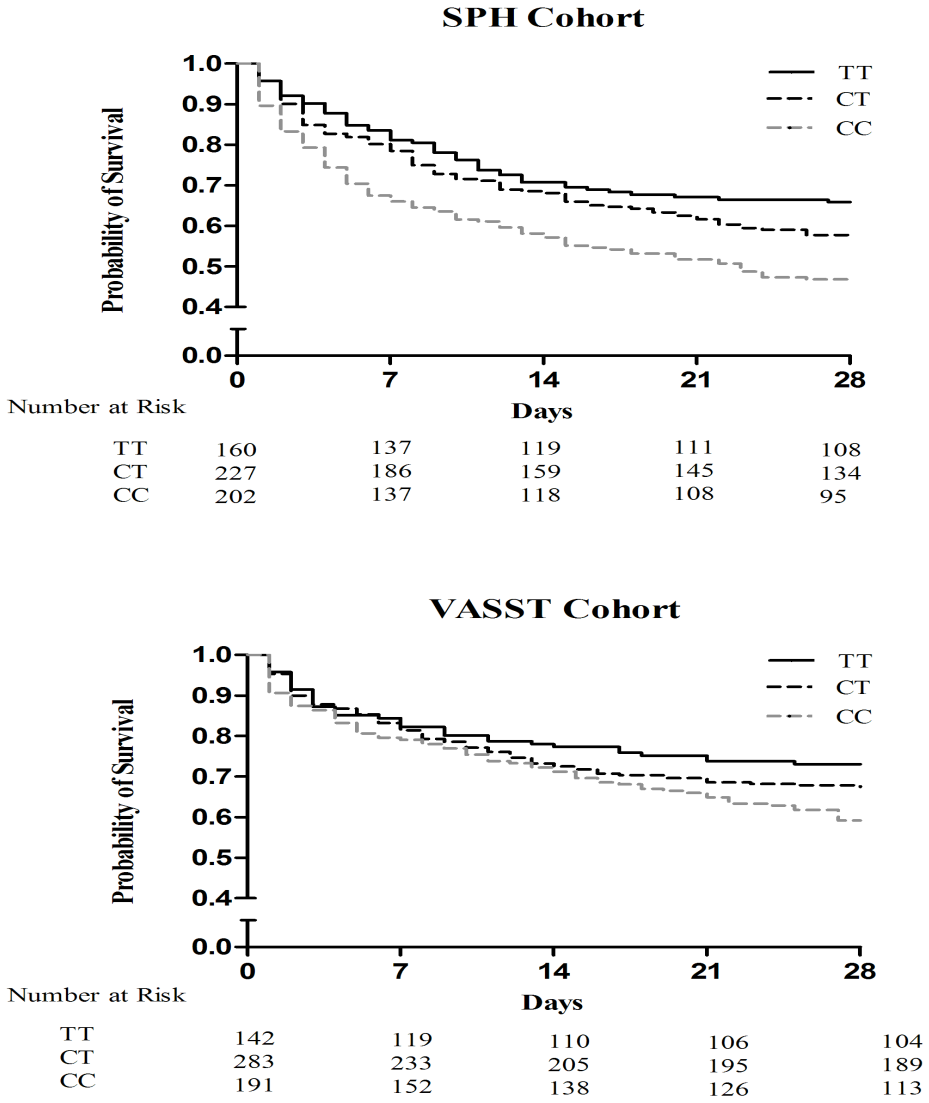
Table 2.2 Hazard ratios of 28-day mortality in caucasian patients with septic shock (Cox regression)

	SPH Cohort (n=453) Hazard Ratio (95% Confidence interval)	P	VASST Cohort (n=517) Hazard Ratio (95% Confidence interval)	P
Age- per year	1.006 (1.003-1.009)	0.0036	1.019 (1.008-1.030)	0.0003
Female	0.9092 (0.670-1.123)	0.530	0.987 (0.717-1.348)	0.930
Surgical Diagnosis	0.8244 (0.600-1.118)	0.220	0.859 (0.583-1.233)	0.420
NIK rs7222094 C allele	1.35 (1.116-1.635)	0.002	1.240 (1.002-1.524)	0.048

Table 2.3 Hazard ratios of 28-day mortality in patients with septic shock (Cox regression)

	SPH Cohort (n=589) Hazard Ratio (95% Confidence interval)	P	VASST Cohort (n=616) Hazard Ratio (95% Confidence interval)	P
Age- per year	1.006 (1.003-1.009)	9.0×10^{-4}	1.017 (1.008-1.026)	2.0×10^{-4}
Female	0.868 (0.668-1.122)	0.28	1.004 (0.756-1.328)	0.98
European Ancestry	0.962 (0.717-1.290)	0.76	0.870 (0.609-1.272)	0.46
Surgical Diagnosis	0.937 (0.712-1.220)	0.63	0.800 (0.557-1.123)	0.20
NIK rs7222094 C allele	1.329 (1.126-1.573)	8.0×10^{-4}	1.250 (1.030-1.521)	0.024

Figure 2.2 Survival curves for patients with septic shock in the St. Paul's Hospital (SPH) and Vasopressin and Septic Shock Trial (VASST) cohorts by NIK genotype of rs7222094. Patients who were homozygous minor (CC) (grey dashed line) experienced increased mortality in both the SPH and VASST cohort.



Similarly in an unadjusted univariate analysis, the C allele of rs7222094 TC was associated with mortality in SPH (Mortality (Caucasian only) TT 33.8%, CT 42.4%, CC 53.7% p=0.005; (all ethnicities) TT 33.8%, CT 43.2%, CC 53.0 % p=0.001) and a similar trend was observed in VASST (Mortality (Caucasian only) TT 26.3%, CT 34.7%, CC 38.4% p=0.09; (all ethnicities) TT 27.5%, CT 33.2%, CC 40.8 % p=0.03). Allele frequencies of survivors versus non-survivors in both Caucasian and all ethnicities are reported in **Table 2.4.**

Table 2.4 Allele frequencies for NIK rs7222094 in both SPH and VASST cohorts of survivors and non-survivors

	SPH Cohort		P value	VASST Cohort		P value
	T	C		T	C	
Survivors (Caucasian)	0.57	0.43	0.005	0.52	0.48	0.09
Non- Survivors (Caucasian)	0.45	0.55		0.45	0.55	
Survivors (All ethnicities)	0.52	0.48	0.001	0.49	0.51	0.03
Non- Survivors (All ethnicities)	0.40	0.60		0.41	0.59	

In the SPH Caucasian cohort, patients having the CC genotype of rs7222094 had greater baseline creatinine concentrations (P=0.04) and significantly higher PaO_2/FIO_2 at baseline (P= 0.002) than patients having the CT or TT genotypes of rs7222094 (**Table 2.5**). The only difference at baseline among VASST Caucasian patients was that patients having the CC genotype of rs7222094 had significantly lower platelet counts than patients having the CT or TT genotypes of rs7222094 (P= 0.02) (**Table 2.5**). Since the VASST cohort was a clinical trial comparing efficacy of vasopressin versus norepinephrine in septic shock, in a secondary analysis we tested for an interaction by logistic regression between NIK rs7222094 and vasopressin treatment in Caucasian patients. We found no significant interaction (Interaction statistic P=0.462).

Table 2.5 Baseline characteristics by NIK genotype rs7222094 for Caucasian patients with septic shock in both SPH and VASST cohorts.

Values shown are median and inter-quartile range.	SPH COHORT				VASST COHORT			
	TT (N=139)	CT (N=191)	CC (N=123)	P VALUE	TT (N=133)	CT (N=251)	CC (N=133)	P value
AGE –YR	59 (46-71)	62 (48-73)	61 (49-73)	0.34	64 (51-74)	63 (51-72)	63 (50-73)	0.77
GENDER % MALE	66.2	69.1	57.7	0.12	54.1	58.6	72.2	0.0052
APACHE II	25 (20-30)	26 (20-31)	26 (20-33)	0.61	26 (22-32)	26 (22-31)	27(22-32)	0.76
SURGICAL %	30.2	29.8	33.3	0.79	19.6	22.7	20.3	0.73
PRE-EXISTING CONDITIONS (%)								
CHRONIC HEART FAILURE	5.8	7.9	4.9	0.54	9.8	8.0	6.0	0.52
CHRONIC PULMONARY DISEASE	17.3	19.9	12.2	0.19	21.8	18.7	14.3	0.27
CHRONIC LIVER DISEASE	11.5	10.0	8.1	0.66	9.0	9.6	12.0	0.68
CHRONIC RENAL FAILURE	3.6	3.1	4.9	0.74	10.5	10.0	11.3	0.92
CHRONIC CORTICOSTEROID USE	5.8	6.8	4.9	0.77	19.6	22.3	18.8	0.67
CARDIOVASCULAR VARIABLES -DAY 1								
HEART RATE- BPM	115 (95-130)	114 (95-130)	115 (95-131)	0.86	124 (112-135)	129 (108-140)	126 (108-140)	0.95
MEAN ARTERIAL PRESSURE- MMHG	55 (50-60)	55 (50-58)	54 (49-59)	0.41	55 (48-61)	55 (50-61)	56 (51-62)	0.44
CENTRAL VENOUS PRESSURE- MMHG	12 (8-15)	11 (6-15)	11 (8-15)	0.71	14 (11-17)	14 (12-18)	14 (10-17)	0.11
LABORATORY VARIABLES- DAY 1								
WHITE BLOOD CELL COUNT- 103/MM3	13.7 (9.0-19.0)	15.0 (11.1-21.0)	14.0 (9.3-20.0)	0.055	14.6 (9.0-23.7)	13.8 (7.3-20.6)	11.8 (7.0-19.4)	0.07

Values shown are median and inter-quartile range.	SPH COHORT				VASST COHORT			
	TT (N=139)	CT (N=191)	CC (N=123)	P VALUE	TT (N=133)	CT (N=251)	CC (N=133)	P value
PLATELET COUNT-103/MM3	157 (84-234)	178 (103-245)	144 (76-255)	0.13	168 (75-256)	174 (93-276)	136 (81-199)	0.02
PAO2/FIO2- TORR	125 (80-194)	138 (84-196)	170 (109-232)	0.0015	193 (150-255)	197 (140-261)	185 (126-254)	0.70
CREATININE-UMOL/L	125 (80-219)	148 (91-245)	180 (94-329)	0.04	141 (83-269)	150 (90-238)	167 (94-294)	0.35
LACTATE -MMOL/L	1.4 (0.0-2.9)	1.3 (0.0-3.0)	1.8 (0.0-4.9)	0.09	1.7 (0.8-3.6)	1.7 (1.0-3.3)	2.1 (1.2-4.3)	0.17

Organ Failure in Septic Shock Patients

Patients homozygous for the C allele of rs7222094 in both the SPH and VASST Caucasian cohorts had more organ dysfunction as defined by Brussels criteria compared with patients having the CT or TT genotypes of rs7222094 [259]. SPH patients with rs7222094 CC genotype had significantly fewer days alive and free of renal dysfunction (P= 0.003) and fewer days alive and free of acute renal replacement therapy (P=0.008) as well as fewer days alive and free of hematological dysfunction (P= 0.011) during the 28-day study period than patients having the CT or TT genotypes of rs7222094 (**Table 2.6**). Patients of the VASST cohort with the rs7222094 CC genotype show the same trend toward more renal dysfunction (**Table 2.6**). The number of patients affected by each type of organ dysfunction by genotypic group is outlined in **Table 2.7**.

In the SPH cohort, patients with the rs7222094 CC genotype also had significantly fewer days alive and free of cardiovascular dysfunction (P= 0.008), with correspondingly fewer days alive and free of vasopressor support (P=0.01), which was also seen as a trend in the VASST cohort (**Table 2.6**). SPH cohort patients also experienced more hepatic and neurologic dysfunction (P= 0.007 and P=0.006 respectively) (**Table 2.6**).

Table 2.6 Days alive and free of organ dysfunction in Caucasian septic shock patients according to NIK rs7222094 genotype

Values shown are median and inter-quartile range.

	SPH Cohort				VASST Cohort			
	TT (n=139)	CT (n=191)	CC (n=123)	P value	TT (n=133)	CT (n=251)	CC (n=133)	P value
Organ Dysfunction								
Cardiovascular	15 (13-16)	13 (12-15)	11 (9-13)	0.008	15 (14-17)	14 (12-15)	13 (11-15)	0.227
Respiratory	12 (10-14)	11 (10-13)	10 (8-12)	0.320	9 (7-10)	8 (7-9)	7 (6-9)	0.554
Renal	17 (15-19)	16 (14-17)	13 (11-15)	0.003	19 (17-21)	17 (16-18)	15 (14-17)	0.067
Hematologic	18 (16-20)	18 (17-20)	15 (13-17)	0.011	19 (18-21)	19 (17-20)	18 (16-20)	0.391
Hepatic	19 (17-21)	17 (16-19)	14 (12-17)	0.007	19 (17-21)	18 (17-20)	18 (16-20)	0.856
Neurologic	19 (17-20)	18 (16-19)	15 (13-17)	0.006	15 (13-17)	14 (13-15)	13 (11-15)	0.203
Artificial Organ Support								
Vasopressor	17 (15-19)	16 (14-17)	13 (11-15)	0.010	15 (14-17)	14 (12-15)	14 (12-15)	0.253
Ventilator	12 (10-14)	10 (9-12)	9 (7-11)	0.230	12 (10-13)	10 (9-12)	10 (8-12)	0.328
Renal replacement therapy	18 (16-20)	17 (15-18)	14 (12-16)	0.008	20 (18-22)	18 (17-19)	18 (16-20)	0.215
Any Organ Dysfunction and/ or Support	0 (0-0)	0 (0-0)	0 (0-2)	0.084	0 (0-9)	0 (0-12)	0 (0-4)	0.062

Table 2.7 Septic shock patients affected by organ dysfunction in Caucasian according to NIK rs7222094 genotype

	SPH Cohort				VASST Cohort			
	TT (n=139) N (%)	CT (n=191) N (%)	CC (n=123) N (%)	P value	TT (n=133) N (%)	CT (n=251) N (%)	CC (n=133) N (%)	P value
Organ Dysfunction								
Cardiovascular	139 (100)	191 (100)	123 (100)	NA	133 (100)	251 (100)	133 (100)	NA
Respiratory	132 (95.0)	183 (95.8)	116 (94.3)	0.830	130 (97.7)	246 (98.0)	133 (100)	0.240
Renal	81 (58.3)	133 (69.6)	97 (78.9)	0.002	76 (57.1)	160 (63.7)	92 (69.2)	0.124
Hematologic	71 (51.1)	119 (62.3)	86 (69.9)	0.007	74 (55.6)	139 (55.4)	87 (65.4)	0.134
Hepatic	65 (46.8)	108 (56.5)	81 (65.9)	0.008	71 (53.4)	139 (55.4)	72 (54.1)	0.927
Neurologic	95 (68.3)	139 (72.8)	96 (78.0)	0.212	110 (82.7)	217 (86.5)	123 (92.5)	0.060
Artificial Organ Support								
Vasopressor	125 (89.9)	178 (93.2)	114 (92.7)	0.531	133 (100)	251 (100)	133 (100)	NA
Ventilator	134 (96.4)	185 (96.9)	116 (94.3)	0.509	128 (96.2)	241 (96.0)	128 (96.2)	0.991
Renal replacement therapy	62 (44.6)	112 (58.6)	83 (67.5)	0.0007	59 (44.4)	134 (53.4)	75 (56.4)	0.115
Any Organ Dysfunction and/ or Support	139 (100)	191 (100)	123 (100)	NA	133 (100)	251 (100)	133 (100)	NA

CXCL10 mRNA production by lymphoblastoid cell lines *In Vitro*

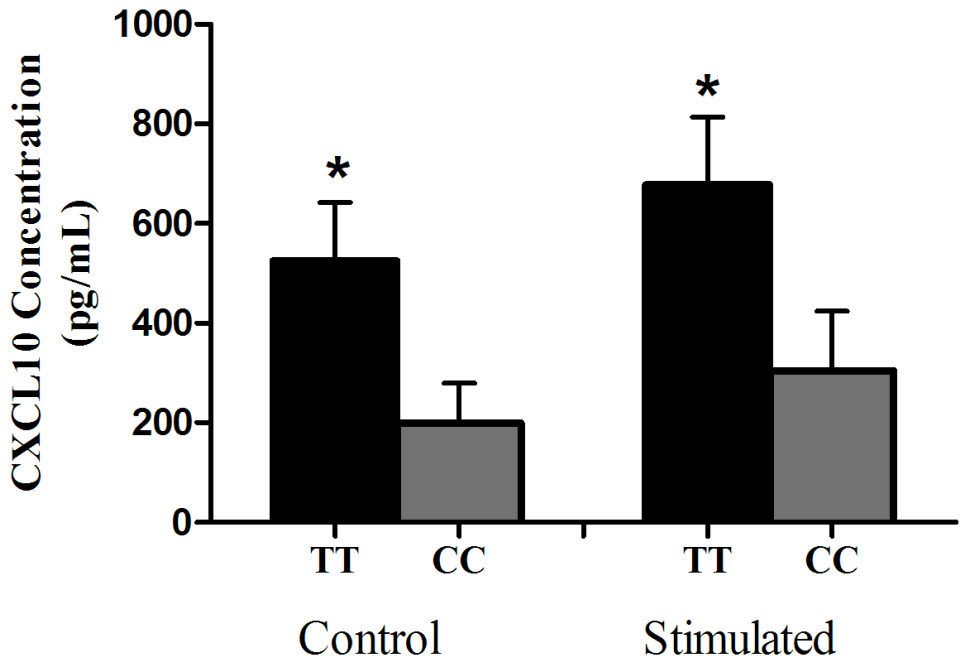
The gene with the greatest difference (delta) in fold change between major (TT) and minor (CC) genotypes was CXCL10 (Delta fold change 0.67, uncorrected student's t test between groups P=0.055) suggesting lower mRNA expression of CXCL10 for the CC genotype compared to TT or TC. **(Appendix A Table A.3).**

CXCL10 protein production by lymphoblastoid cell lines *In Vitro*

Protein levels of CXCL10 were measured in 26 cell lines of known genotype for rs7222094. Cell lines homozygous for the C allele of rs7222094 produced less CXCL10 at both baseline and after inflammatory stimulation than cell lines homozygous for the T allele (P= 0.032 and P=0.050 respectively) (**Figure 2.3**).

Figure 2.3 CXCL10 protein concentrations in lymphoblastoid cell lines.

26 lymphoblastoid cell lines of known genotype (13 TT and 13 CC) for rs7222094 were cultured and stimulated with Cytomix (2.5ng/mL of each TNFα, IL-1β, IFNγ and 12.5uM of CpG) for 24 hours. Supernatant was collected and CXCL10 concentrations were measured by ELISA. Cell lines homozygous for the C allele produce significantly less CXCL10 at both baseline and under stimulation conditions (P=0.032 and P=0.050 respectively).

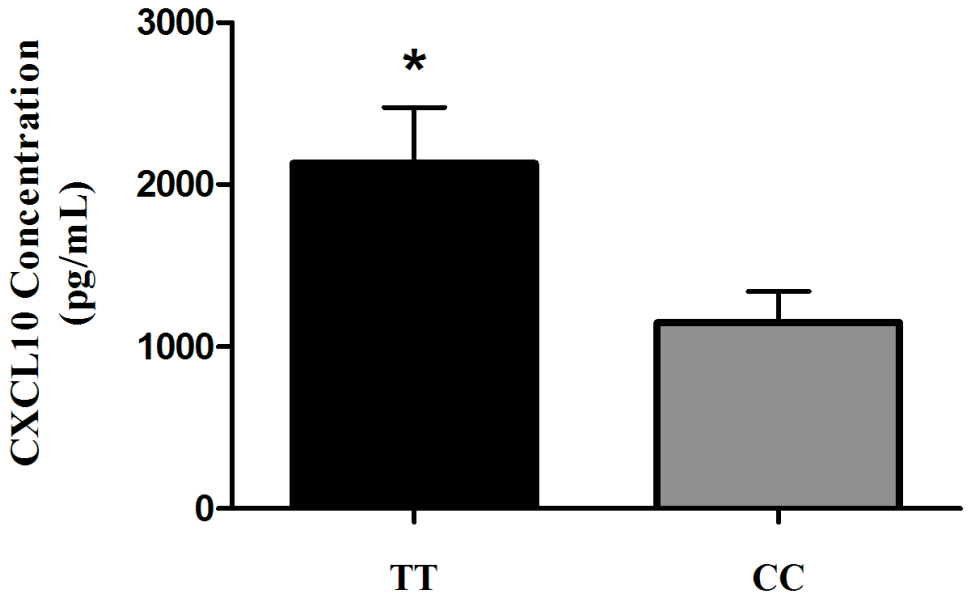


CXCL10 protein production in VASST plasma samples.

Baseline plasma specimens of a random sample of patients with septic shock from the VASST cohort were assayed in duplicate for CXCL10 by ELISA and as was found in both the control and stimulated lymphoblastoid cell lines, patients of the CC genotype had significantly lower CXCL10 than those of the TT genotype (P=0.017) (**Figure 2.4**).

Figure 2.4 CXCL10 protein concentrations in septic shock patients of the VASST replication cohort.

Baseline plasma samples from 60 VASST septic shock patients of known genotype (30 TT and 30 CC) for rs7222094 were randomly selected and assayed for CXCL10 concentrations by ELISA. Patients homozygous for the C allele had significantly lower concentrations of CXCL10 than patients who were homozygous for the T allele (P=0.017).



2.4 Discussion

We found that patients of the CC genotype of NIK rs7222094 had significantly increased mortality compared with patients having the CT or TT genotypes of rs7222094 in two cohorts of patients who had septic shock. Specifically, Caucasian patients in the SPH cohort who had the CC genotype of NIK rs7222094 experienced a significant increase in the hazard of death over the 28-days (HR 1.35; 95% confidence interval [CI] 1.12-1.64; P=0.002). This effect was also found in the VASST Caucasian cohort (HR 1.24; CI 1.00-1.52; P= 0.048). The results were similar for all patients with ethnicity included as a covariate in a Cox regression model. Also, patients of the CC genotype of rs7222094 of SPH experienced more renal and hematological dysfunction compared with patients having the CT or TT genotypes of rs7222094 (P= 0.003 and P= 0.011 respectively). Patients of the VASST cohort with the rs7222094 CC genotype showed the same trend toward more renal dysfunction as found in the SPH cohort.

NIK was first discovered in human B cells and hence we chose lymphoblastoid cell lines for this phase of our study [101]. CXCL10 is a chemokine transcribed in response to NF-κB during inflammation and is generally thought to signal in response to canonical pathway stimuli [264, 265]. In a recent study by Zarnegar *et al.* [112] CXCL10 levels were dependent on NIK suggesting non-canonical signaling. CXCL10 is transcribed in response to NF-κB activation and has been shown to be downregulated upon inhibition of NIK [112]. The CC genotype of NIK rs7222094 was associated with significantly decreased levels of CXCL10 in supernatant of lymphoblastoid cell lines at baseline and following cytomix stimulation (p= 0.032 and p=0.050).

Similarly, CXCL10 levels were significantly lower in septic shock patients of the CC genotype (P=0.017), suggesting a biologically plausible explanation for our observations.

It was interesting to find that the patients with the NIK rs7222094 CC genotype experienced increased mortality while supernatant from cell lines of the same genotype had decreased levels of CXCL10. This effect replicated when CXCL10 concentration was measured in VASST patient samples. As mentioned previously, CXCL10 is a pro-inflammatory chemokine released during inflammatory states such as allograph rejection and infection [266]. It is plausible that pro-inflammatory molecules are necessary to mount an effective immune response during septic shock. CXCL10 is a chemokine that instigates chemotaxis of activated T cells and natural killer cells [266]. We speculate that without enough CXCL10 to drive recruitment of inflammatory cells, it is possible that these patients (who had the CC genotype of NIK rs7222094) have an immunological disadvantage and so have increased mortality of septic shock.

The original characterization of NIK indicated that NIK was a powerful effector of canonical NF-κB signaling under TNFα and IL-1β stimulation [101]. Later studies were unable to confirm this mechanism and instead the discovery of the non-canonical pathway emerged as subsequent research appeared to distill the two pathways into distinct yet separate processes [104, 105, 108, 109]. Evidence is building suggesting that the IKK-NIK axis is a pivot point for control of both non-canonical and canonical signaling [110-112]. It is possible that the timing of experimental procedures is critical to our understanding of NIK since many studies that separated the two pathways and excluded NIK from canonical signaling focused on early events (from minutes to less than 2 hours) [108, 109]. In contrast, many studies find NIK plays a pivotal role in

canonical signaling when evaluating effects at time points of several hours to days [101, 111, 112]. The current understanding of the regulation of NIK is that NIK is constitutively transcribed, translated and degraded via its interaction with TRAF3. However, following degradation of TRAF3 after appropriate stimuli, NIK recruits IKKα to p100, activating IKKα thereby initiating proteasomal degradation of p100 to p52 and consequently translocation of heterodimers to the nucleus [104, 107]. It is conceivable that accumulation of NIK over time is a key facet to its mechanism.

Since NIK has been implicated in the host response to infection, we speculate that NIK could modulate the immune response during septic shock. NIK is important in the host response to numerous infections including respiratory syncytial virus (with evidence to suggest activation of both non-canonical and canonical signaling [267]), HIV [268], hepatitis B [269], E. coli [270], as well as the response to LPS [271].

Several lines of evidence suggest why polymorphisms of NIK may be predictors of outcome in septic shock [143]. First, NIK stimulates inflammation by up regulating the non-canonical (and possibly the canonical) pathway of NF-κB activation. Second, NIK is required for optimal IgG production by lymphocytes [272]. Third, NIK may modulate blood pressure: NIK has a role in the mechanism of action of the calcium channel blocker nifedipine [273] and angiotensin II induces inflammation through NIK activation of the non-canonical NF-κB pathway [274]. However, polymorphisms of NIK have not been widely studied. In a survey of 181 SNPs of 17 genes in the NF-κB pathway, polymorphisms of NIK were not associated with rheumatoid arthritis susceptibility [275]. Interestingly, rs4792847 of NIK was found to be significantly

associated with response to anti-TNF treatment in rheumatoid arthritis. Patients with GG genotype had the greatest improvement at the 6 month mark in a discovery cohort; however this was effect was not found in a replication cohort [276]. The GG genotype of rs4792847 (patients who may have had a more favourable response to anti-TNF) is in high linkage disequilibrium with the TT genotype of rs7222094 (r^2=1.0). This is consistent with our observation of a protective effect of the TT genotype, that is, lower mortality and higher CXCL10 production, seen in our study of patients who had septic shock and replicated in our *in vitro* experiments.

To our knowledge, NIK has not been implicated in septic shock to date. However, NIK is a drug target for other diseases [142]. Inhibitors of NIK have been synthesized for diseases such as multiple myeloma and other cancers, as anti-inflammatory agents for inflammatory diseases [277] and as a vaccine adjuvant [278]. Our data suggests that rs7222094 may be of interest in randomized controlled trials of therapies for septic shock by defining risk categories of patients and perhaps defining response to anti-inflammatory agents.

This study has several limitations. The analysis of the SPH and VASST cohorts was performed retrospectively. The association of rs7222094 with mortality, organ dysfunction and CXCL10 levels does not prove a causal link. Furthermore, CXCL10 was used as a marker for differences in NIK induced NF-κB signaling. We do not currently know of, nor did we test for the influence of CXCL10 itself on organ dysfunction or mortality. In view of the large number of genes connected in some way to NF-κB signaling, we chose to limit our analysis to genes of the cytosolic members of the NF-κB pathway, excluding receptors and downstream targets.

Therefore this analysis does not include all potentially functional variants, in particular those recently published after the design of this study [246, 279, 280].

In this study, 16/56 SNPs are not in HWE (**Appendix A Table A.1),** despite the call rates being >95%. We inspected raw genotype data and did not find evidence of technical errors. An alternate explanation for this finding is the presence of population stratification [281]. Although our analysis was limited to those of Caucasian ethnicity, this was self-reported. Hence we calculated λ for this population, a method of genomic control developed by Devlin and Roeder [282]. This compares the sample median of test statistics to the chi-square median value for an estimation of inflated false positive test values by computing a χ^2 value for all markers; determining the median χ^2 value and dividing that by 0.456 [282]. This value is referred to as λ which can be used to divide all raw test statistics by [282]. Values close to 1 suggest population stratification is not inflating the test values [282]. Using PLINK to calculate λ, genomic inflation factor based on median χ^2, for 1536 SNPs genotyped in all Caucasian patients of the SPHICU cohort we found a λ value of 1.1. It is possible that some of these SNPs may also be associated with the phenotype of 28-day mortality; hence, λ may be biased to a slightly higher value, however Devlin and Roeder have shown that this will not influence the calculation of the median [282]. This suggests that population stratification is not influencing our finding; supporting the potential biological significance of rs7222094 (raw p value= 0.00024). The departure of HWE may be due to an over representation of the associated allele in the study population. There were no controls recruited for this study, which would be informative to have for comparison of HWE in the general population. It is plausible that over representation of associated alleles in genes known to be essential in the NF-κB signaling pathway genes that are

imperative to the proper functioning of the immune system, as patients of genotypes that protect against the development of sepsis are not admitted to the ICU and hence are not enrolled in these studies, skewing HWE.

In conclusion, we found that NIK rs7222094 was consistently and significantly associated with mortality in two independent cohorts of patients who had septic shock. Patients homozygous for the CC genotype of rs7222094 had increased mortality and also experienced more renal and hematological failure in the SPH cohort of Caucasian patients with a similar trend in VASST Caucasian patients. Furthermore, we found that lymphoblastoid cell lines homozygous for CC of rs7222094 produced less CXCL10 *in vitro* at baseline and following Cytomix stimulation than cell lines having the TT genotype of NIK rs7222094 (P=0.032 and P=0.050 respectively). As well, patients who had septic shock in the VASST cohort who were NIK rs7222094 CC genotype had significantly lower plasma levels of CXCL10 than those of the TT genotype (P=0.017). We speculate that polymorphisms of NIK could be used to predict risk of death from septic shock and to predict response to anti-inflammatory treatment such as inhibitors of NIK

Chapter 3: TNFα Induces Tumor Necrosis Factor Alpha Induced Protein 2 (TNFAIP2) Expression Which Inhibits Ras and NF-κB Signaling and Contributes to Mortality and Organ Dysfunction in Septic Shock Patients

3.1 Introduction

Tumor necrosis factor alpha (TNFα) is involved in the early inflammatory response of septic shock and induces a plethora of genes and signaling pathways. However, the modifying role of highly transcribed genes induced by TNFα stimulation during septic shock is incompletely understood. We reasoned that highly regulated genes induced by TNFα stimulation may play a clinically important modulatory role. We further reasoned that if these genes had clinically important modulatory effects then genetic variants of these genes may be associated with clinical outcome in septic shock. To identify robust signals in genes reliably induced by TNFα in HeLa cells we first used microarray expression measurements to compile a list of gene fold changes in response to TNFα stimulation and searched for replication of genes with a fold change greater than 2 in methodologically comparable publicly available data sets. Next, in genes reliably induced by TNFα we genotyped single nucleotide polymorphisms (SNPs) that tagged all groups of SNPs in linkage disequilibrium within that gene, and tested for association between these tagSNPs and mortality in a septic shock cohort of patients from the Vasopressin and Septic Shock (VASST) trial. This approach identified a gene, TNFAIP2, and genetic variant, rs8126, which allowed us to gain insight into the potential mechanism of its modulatory effect. Specifically, we found that TNFAIP2 binds GTPase activating protein (SH3 domain) binding protein 1 (G3BP1) and inhibits Ras and downstream MAPK signaling leading to decreased

CREB activity. We also found that similar to A20, TNFAIP2 inhibits NF-κB and finally that the extent of Ras, CREB and NF-κB inhibition is associated with rs8126 genotype.

3.2 Methods

Gene expression microarray measurements

To illuminate transcripts that are highly expressed and hence represent key genes in the response to TNFα, gene expression microarrays were performed on mRNA collected from HeLa cells stimulated with TNFα and compared to control. HeLa cells were cultured in DMEM with 10 % FBS and were treated with or without 10 ng/mL of TNFα for 4 hours in biological quadruplicate. RNA was harvested and gene expression was interrogated using the Illumina human HT-12 (v.4) expression beadchip at the McGill University and Genome Quebec innovation Centre (Montreal, Quebec). Data was normalized and fold change calculated using the FlexArray package (1.4.1). MIAME compliant data from the HT-12 Illumina gene expression microarray is available at GEO (accession # pending). We then performed a literature search for replication in publically available data sets using the following criteria in the PubMed search engine: TNF and HELA and MICROARRAY and HUMAN **(Appendix B Table B.1)**. We then inspected the methods of the papers to determine the following criteria: cell type= HeLa; stimulation = human recombinant TNFα; stimulation time= 3-5hrs; data files not encrypted when downloaded from GEO. This resulted in 2 papers [283, 284]. All genes with a fold change greater than 2 from these two data sets as well as our data set were then cross-referenced for common genes.

VASST cohort of septic shock patients

The Vasopressin and Septic Shock Trial (VASST) was a multicenter, randomized, double-blind, controlled trial evaluating the efficacy of vasopressin versus norepinephrine in 779 patients who were diagnosed with septic shock according to the current consensus definition [259]. Clinical phenotyping has been described previously [258]. All patients were enrolled within 24 hours of meeting the definition of septic shock, and DNA was extracted from 632 patients. Written informed consent was obtained from all patients or their authorized representatives and the trial was approved by all ethics boards of the participating institutions. The research ethics board at the coordinating center (University of British Columbia) approved the genetic analysis.

tagSNPs in genes identified in the above microarray studies were selected using a linkage disequilibrium based method using an r^2 threshold of 0.65 for SNPs with a MAF of >5% and were genotyped in all patients of the VASST cohort. DNA was extracted from peripheral blood samples using a QIAamp DNA Blood Midi Kit (QIAgen, Mississauga, ON, Canada) and genotyped using the Illumina Golden Gate Assay at the UBC Centre for Molecular Medicine and Therapeutics genotyping core facility (Luminex Molecular Diagnostics, Toronto, Canada). [260]. We then obtained the genotypes of 68 SNPs in the region +/- 50,000bp of TNFAIP2 in 530 Caucasian Vasopressin and Septic Shock Trial (VASST) patients from the Human Illumina Duo 1.2M SNP chip [v.3] as it is known that associations of phenotypes with tagSNPs may represent signals from functional SNPs within the linkage disequilibrium (LD) block that the tagSNP represents [260].

Quality control for genotyping data (tagSNPs)

All SNPs with minor allele frequencies (MAF) < 0.01 or call rates (CR) < 0.95 were removed from analysis. Additionally, SNPs with MAF < 0.05 were removed if CR < 0.99.

Quality control for genotyping data (GWAS)

Sample data from Illumina *GenomeStudio* was imported into Golden Helix SVS software vs. 7.6.4. Genotype data generated with the HapCluster algorithm was selected for analysis [285]. All of the following procedures were performed in SVS unless indicated otherwise.

Call rate

The Illumina 1M genotyping array includes markers for copy number variation, hence the data for these markers was removed and the subject genotyping call rates were calculated. One sample was detected as a low outlier and removed from the dataset. All other samples had call rates greater than 0.95.

Cryptic relatedness

All pairs of samples were analyzed for evidence of relatedness using the Purcell allele sharing algorithm as implemented in SVS [286]. Identical by decent (IBD) estimation was performed using a set of 36,163 SNPs with the criteria of MAF > 0.05, call rate > 0.95, HWE P-value > 1e-5, and pair-wise LD R^2 <0.1. This analysis identified one subject with elevated relatedness to almost all other subjects. This is possibly due to sample contamination (contaminated samples have an increased number of heterozygous genotypes, which increases observed allele sharing with unrelated samples) and was removed.

Gender verification

Reported gender in the provided phenotype data was verified by analysis of X chromosome heterozygosity rates for each subject. This method assumes that males should have a heterozygosity rate near zero as they have only one copy of the X chromosome, and females should have a heterozygosity rate greater than zero. This method identified two samples that had mismatches between reported gender and calculated gender. Both of which were previously identified by Sirius Genomics as having XXY karyotypes. These samples were retained for subsequent analysis.

Population structure

Genotypes from 269 HapMap samples from the Illumina 1M Duo were used as a reference to verify the reported ethnicity of the samples via principal components analysis (PCA). Principal component calculations were based on the same 36,163 SNPs used for IBD testing. The HapMap samples were from four distinct ethnic groups: Caucasian (CEU), Chinese (CHB), Japanese (JPT), and Yoruban-African (YRI). PCA confirmed the expected diverse ethnic background of the subjects. It was decided to include all subjects in subsequent analysis and to apply a correction factor of 0.015 to define Caucasian based on PCA. It should be noted that calculating principal components with HapMap reference samples included is primarily for the purpose of confirming the reported ethnicity of the subjects.

SNP quality control

All remaining SNPs within this dataset were filtered based on minor allele frequency (MAF) and call rate (CR) to select SNPs for inclusion in association tests. All SNPs with MAF < 0.01 or CR

< 0.95 were removed from analysis. Additionally, SNPs with MAF < 0.05 were removed if CR < 0.99. This process resulted in a filtered list of 950,354 autosomal markers for analysis.

Co-immunoprecipitation of endogenous TNFAIP2

HeLa cells were cultured as above and were treated with or without 10ng/mL of TNFα for 30 minutes or 4 hours. Cell lysate was collected as per UBC Centre for High-Throughput Biology (CHiBi) protocols. Co-immunoprecipitation was performed using agarose beads conjugated to either TNFAIP2 or IgG control (Santa Cruz Biotech) according to manufacturers recommendations. Co-immunoprecipitation complexes we eluted from the beads and sent for analysis by mass spectrometry at the UBC CHiBi. The experiment was repeated in house and lysate was western blotted for G3BP1 and TNFAIP2 (Santa Cruz Biotechnology).

Association of TNFAIP2 with changes in phosphorylation

HeLa cells were cultured as above and transfected with either a TNFAIP2 complete cDNA over expression or control vector for 48 hours (Origene custom plasmid). Cell lysate was collected as per the KINEXUS protocol and samples were sent to KINEXUS (Vancouver, British Columbia) for analysis using the KPSS 1.3 Kinetworks Assay (n=1). Differences in phosphorylation were calculated by KINEXUS as "Percent (%) Change from Control" by subtracting the signal intensity of the control sample from the treated, and dividing the result by the control signal intensity. %CFC = (Treated-Control)/Control*100%.

TNFAIP2 protein levels

Clones of the complete cDNA for TNFAIP2 over expression vector expressing either the A or G allele of TNFAIP2 rs8126 were assayed for protein levels of TNFAIP2 at 48 hours by western blot (Origene custom plasmids: detailed vector information for custom plasmids CW100407 and CW101532 is available at www.origene.com) (biological replicates, n=5).

Prior to any experimentation, clones were sequenced to confirm TNFAIP2 gene cDNA orientation, sequence and allele present using the following primers:

Forward:

5'-TGA CCT TAC GGG ACT TTC CT -3'

5'-GTG TTC TGC GTC TTC ACC AA-3'

5'-CCC CAA TGA CAT CAT CAA CA-3'

5'-TGG AGA CCC TGG AAA ACA TC-3'

5'-TCA TGA CTG TCC TCC TCG TG-3'

5'-AGT CCT GCA CTC CTT CTG GA-3'

5'-TGG GCT CAA GGG ATC TTG TA-3'

Reverse:

5'-GGG CTG ACC CTT CTT CTT CT-3'

5'-AGT GCA GAA AGA CGC TCT CG-3'

5'-CAG TGG CTT CAG GTC CTC AT-3'

5'-GAA CCA GCG CTG CTC TTA AC-3'

5'-AAA CCT GTC ACG GCA ATA GG-3'

5'-ACC TGG GTT CTC TGG GTA GG-3'

5'-CCT GGA TCC TGT TCA ACA TAC-3'

TNFAIP2 levels were normalized to β-actin as a loading control and densiometry was calculated using ImageJ (v. 1.46r). A student's t test was used to determine significance between alleles.

Ras activation assay

HeLa cells were cultured as above and transfected with either TNFAIP2 G allele or A allele over expression vector for 48 hours (Origene site directed mutagenesis of rs8126). Cell lysate was collected and assayed using the Millipore Ras Activation Assay Kit as per the manufacturer (biological replicates, n=5). A student's t test was used to determine significance between alleles.

CREB luciferase assay

HeLa cells were cultured as above. Cells we co-transfected with 0.7ug CREB luciferase reporter construct (pGL4.29[luc2P/CRE/Hygro], Promega) with 0.2ug of the renilla control plasmid (pRL-TK, Promega) as well as 0.5ug of one of the following constructs: TNFAIP2 rs8126 A allele, TNFAIP2 G allele or control plasmid (Origene) for a total of 1.4ug of DNA per 400,000 cells. The pGL4.29[*luc2P*/CRE/Hygro] vector contains a cAMP response element (CRE) that transcribes luciferase reporter gene *luc2P* (*Photinus pyralis*) when bound by the transcription factor CREB. The pRL-TK vector is a wild type *Renilla* luciferase control vector that provides constitutive expression of the *Renilla* luciferase (cloned from *Renilla reniformis*) that can be used as an internal control value for which to normalize the CREB luciferase expression values. The HSV-thymidine kinase promoter (pRL-TK) is a weak promoter, particularly useful in providing low constitutive expression of the *Renilla* luciferase control reporter. This was

performed in biological triplicate with technical duplicates. Luciferase activity was measured according to manufacturers protocol using the Dual Glo Luciferase Assay (Promega). A student's t test was used to test for differences between alleles.

NF-κB luciferase assay

HeLa cells were cultured as above. Cells we co-transfected with 0.7ug pNF-κB luciferase reporter construct (pNFkB-Luc, Clontech) with 0.2ug of the renilla control plasmid (pRL-TK, Promega) as well as 0.5ug of one of the following constructs: TNFAIP2 rs8126 A allele, TNFAIP2 G allele or control plasmid (Origene) for a total of 1.4ug of DNA per 400,000 cells. The pNFkB-Luc is designed with four tandem repeats of the NF-κB consensus sequence followed by a TATA-like promoter (pTAL) from the herpes simplex virus thymidine kinase (HSV-TK) promoter. These elements are followed by the firefly luciferase gene. The pRL-TK *Renilla* control plasmid is as described in the previous assay. This was performed in biological triplicate with technical duplicates. Luciferase activity was measured according to manufacturers protocol using the Dual Glo Luciferase Assay (Promega). A student's t test was used to test for differences between alleles.

Statistical analysis

We used a Kaplan-Meier test to test for association between tagSNPs and septic shock mortality. Nyholt correction was applied to correct for multiple tests [287]. In order to prevent spurious associations due to population stratification, all subsequent analysis was limited to Caucasian patients. We then used an Armitage trend test to test for association of the 68 SNPs in TNFAIP2 and septic shock mortality. Upon determining an association of rs8126 with mortality, we used

Cox regression analysis in the VASST cohort patients to correct for potentially confounding variables, including age, gender, ancestry, and surgical versus medical diagnostic category. We used Kaplan-Meier analysis to test an additive model in the VASST cohort. We then tested for association between secondary outcome measures of days alive and free of organ failure using the Kruskal-Wallis test. We assessed baseline characteristics using a χ^2 test for categorical data and a Kruskal–Wallis test for continuous data and then reported the median and interquartile ranges. Analyses used SPSS (version 16; SPSS, Chicago, IL), R statistical software package, and GraphPad Prism (version 5.02; GraphPad, La Jolla, CA).

3.3 Results

TNFAIP2 Discovery Using Microarrays and tagSNP Genotyping

Details of TNFα-induced gene expression that we observed and that are reported in the literature are complied in **Appendix B Table B.2**. Three genes with a greater than 2 fold change were common to all lists: TNFAIP2 (B94), NFKBIA (IκBα) and NFKB1 (p105) **(Appendix B Figure B.1).** Of 13 tagSNPs in these three genes, one SNP rs8176373 was significantly associated with mortality in the VASST cohort of patients (n=624 (all ethnicities) p=0.0019, 0.018 (Nyholt correction) and n=517 (Caucasian only) p=0.0072, 0.063 (Nyholt correction)**(Table 3.1).**

Table 3.1 Log rank analysis on 13 tagSNPs in 3 genes found by microarray

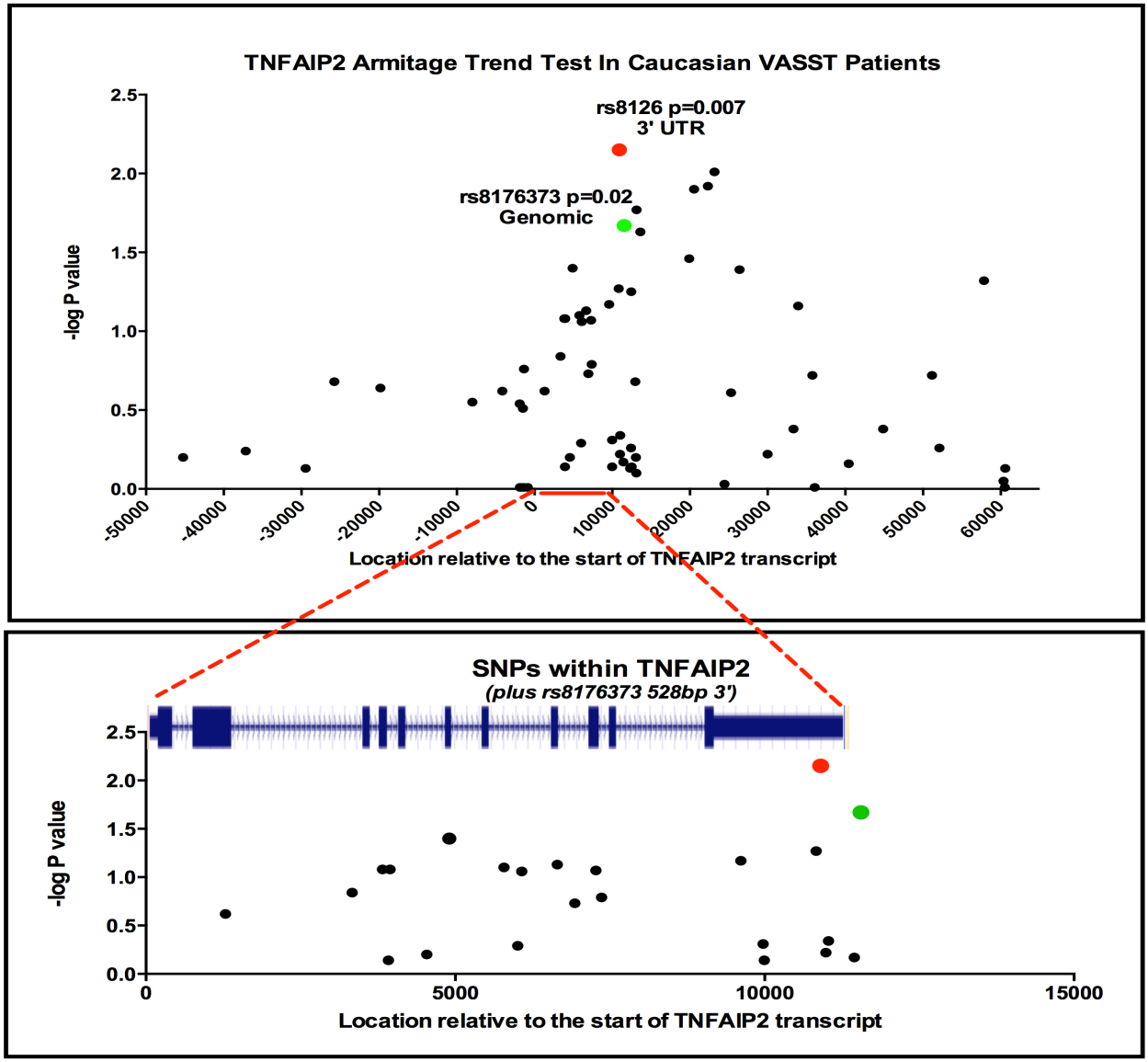
VASST		(All Ethnicities) n=616			(Caucasian Only) n=517		
GENE	SNP	P value	P value (Bonferroni)	P value (HWE)	P value	P value (Bonferroni)	P value (HWE)
TNFAIP2	rs8176373	0.0019	0.018	0.326	0.0072	0.063	0.676
TNFAIP2	rs749206	0.005	0.050	0.978	0.024	0.211	0.959
TNFAIP2	rs710100	0.082	0.803	0.380	0.194	1.000	0.173
TNFAIP2	rs3178152	0.221	1.000	0.731	0.303	1.000	0.606
NFKBIA	rs696	0.223	1.000	0.947	0.326	1.000	0.905
NFKBIA	rs3138055	0.395	1.000	0.915	0.258	1.000	0.584
NFKBIA	rs2233514	0.585	1.000	0.102	0.436	1.000	0.053
NFKBIA	rs1957106	0.759	1.000	0.055	0.887	1.000	0.210
NFKB1	rs1599961	0.138	1.000	0.066	0.240	1.000	0.035
NFKB1	rs230521	0.156	1.000	0.329	0.172	1.000	0.102
NFKB1	rs3774934	0.201	1.000	0.299	0.953	1.000	0.569
NFKB1	rs11722146	0.239	1.000	0.489	0.210	1.000	0.171
NFKB1	rs230542	0.397	1.000	0.157	0.395	1.000	0.039

Dense Genotyping of +/- 50,000bp surrounding TNFAIP2 identifies rs8126

Of the 68 SNPs interrogated in the region by an Armitage trend test, rs8126 was the most significantly associated with mortality in Caucasian patients of the VASST cohort (n=519, p=0.007). **(Figure 3.1).** rs8126 is 3' untranslated region (UTR) SNP, a region known for it's involvement in regulation of translation and miRNA binding [184].

Figure 3.1 Armitage trend test of 68 SNPs +/- 50,000bp of TNFAIP2.

Of the 68 SNPs interrogated, rs8126 was the most significantly associated with 28-day mortality in the VASST cohort (Caucasian only p=0.007). Green depicts the original discovery tagSNP rs8176373 and red depicts rs8126. Note that 0 on "the location relative to the start of transcription" axis is the TSS.



28-day mortality and organ failure in septic shock patients

Patients of the GG genotype of TNFAIP2 rs8126 had significantly increased 28-day mortality compared to that of patients with the AA genotype (log rank p=0.0043) **(Figure 3.2).** Visual inspection of Kaplan-Meier curves suggests a G dominant model whereby those of the AA genotype could be compared to all GG/AG genotypes and all subsequent analysis was performed using this genetic model. Patients who had the GG/AG genotype (TNFAIP2 rs8126) had significantly increased hazard of 28-day mortality compared with that of patients having the AA genotypes hazard ratio [HR] 1.46; 95% confidence interval [CI] (1.08-1.96) p=0.013) **(Table 3.2).** There were no significant differences in baseline characteristics of the patients in the VASST cohort when compared by AA versus GG/AG genotype **(Table 3.3).** Patients of the rs8126 GG/AG genotypes experienced significantly fewer days alive and free (therefore more organ dysfunction, worse outcome) of respiratory (p=0.008) and renal failure (p=0.001) as well as renal replacement therapy (p=0.021) than those of the AA genotype **(Table 3.4).**

Figure 3.2 Kaplan-Meier survival curves for Caucasian patients with septic shock in the Vasopressin and Septic Shock Trial (VASST) cohort by TNFAIP2 rs8126.

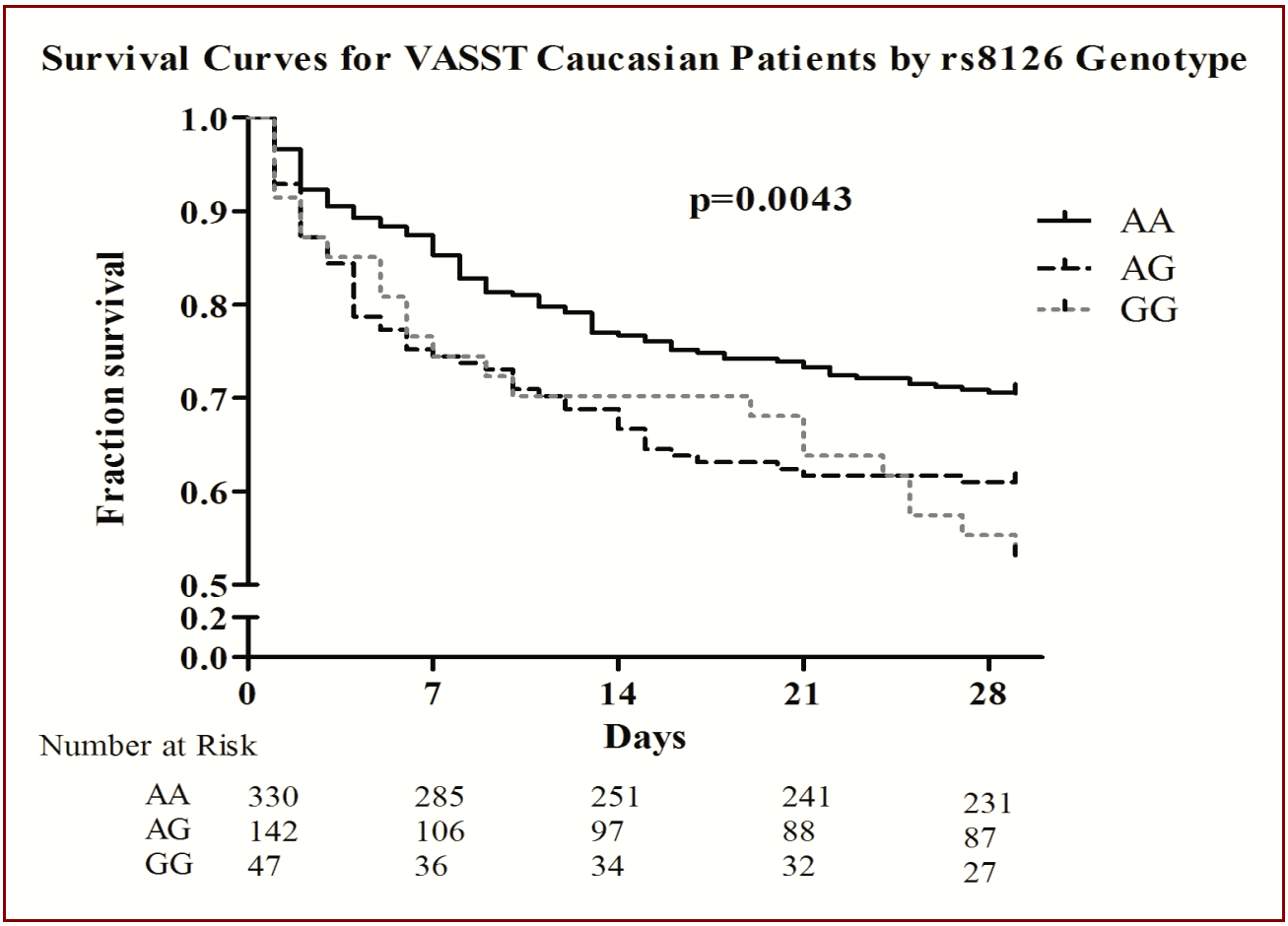


Table 3.2 28-day mortality hazard ratios in VASST Caucasian patients with septic shock (Cox regression).

Cox regression analysis was performed to correct for age, gender and surgical versus medical diagnosis using a major allele model.

	VASST Cohort (n=519) Hazard Ratio (95% Confidence interval)	P
Age- per year	1.019 (1.008-1.030)	2.0×10^{-4}
Female	1.030 (0.760-1.391)	0.85
Surgical Diagnosis	0.853 (0.584-1.213)	0.38
TNFAIP2 rs8126 G allele	1.460 (1.083-1.961)	0.013

Table 3.3 Baseline characteristics in VASST Caucasian septic shock patients by TNFAIP2 rs8126 genotype.

Data reported as median and interquartile range

	AA (n=330)	GG/AG (n=189)	P value
Age –yr	63 (53-72)	64 (50-74)	0.42
Gender % Male	62.4	61.9	0.23
APACHE II	27 (22-32)	26 (21-32)	0.38
Surgical %	22.8	21.2	0.50
Pre-Existing Conditions (%)			
Chronic Heart Failure	10.6	7.0	0.37
Chronic Pulmonary Disease	18.5	17.0	0.66
Chronic Liver Disease	9.5	11.2	0.82
Chronic Renal Failure	9.5	10.9	0.27
Chronic Corticosteroid Use	20.6	20.3	0.86
Cardiovascular Variables -Day 1			
Heart Rate- bpm	125 (108-135)	128 (112-141)	0.094
Mean Arterial Pressure- mmHg	55 (49-61)	56 (50-62)	0.53
Central Venous Pressure- mmHg	15 (12-18)	14 (11-17)	0.20
Laboratory Variables- Day 1			
White Blood Cell Count- 103/mm3	14.1 (8.4-22.4)	13.7 (7.4-20.5)	0.36
Platelet count- 103/mm3	179 (96-272)	152 (87-242)	0.31
PaO2/FiO2- torr	182 (136-246)	197 (143-256)	0.17
Creatinine-umol/L	170 (93-262)	141 (89-248)	0.27
Lactate -mmol/L	1.9 (1.1-3.8)	1.7 (0.8-3.3)	0.25

Table 3.4 Days alive and free of organ dysfunction and artificial support in VASST Caucasian septic shock patients by TNFAIP2 rs8126 genotype.

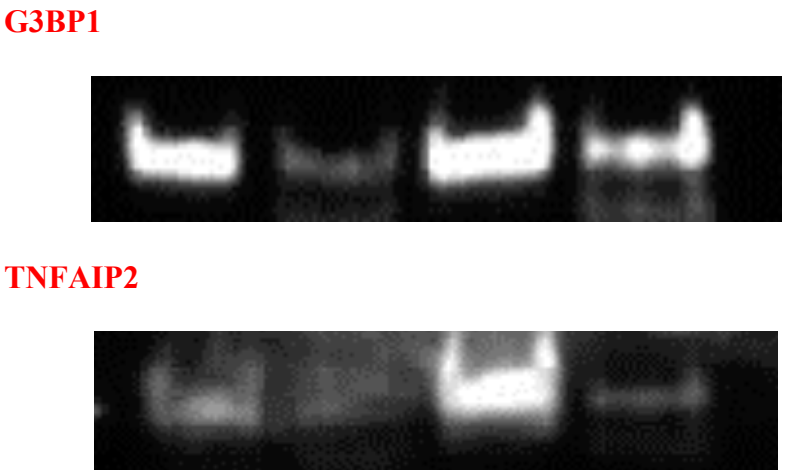
Parameter	GG/AG (n=189)	AA (n=330)	P value
Organ Dysfunction			
Cardiovascular	13 (0-24)	20 (0-24)	0.06
Respiratory	2 (0-14)	4 (0-16)	0.008
Renal	13 (1-28)	24 (7-28)	0.001
Hematologic	24 (3-28)	25 (8-28)	0.39
Hepatic	23 (3-28)	27 (8-28)	0.06
Neurologic	14 (0-23)	17 (0-25)	0.07
Artificial Organ Support			
Vasopressor	16 (0-24)	20 (0-24)	0.07
Ventilator	6 (0-19)	10 (0-22)	0.08
Renal replacement therapy	20 (4-28)	28 (9-28)	0.021
Any Organ Dysfunction and/ or Support	0 (0-4)	0 (0-11)	0.012

Co-immunoprecipitation of endogenous TNFAIP2 identifies a novel interaction with G3BP1

At the time of the study, the protein-protein interactions of TNFAIP2 that may give clues about the function of the protein were not known. We chose to do a screen of potential interactions using co-immunoprecipitation of endogenous TNFAIP2 with mass spectrometry. The results from the screen revealed a list of potential members, which included RasGAP associated protein G3PB1 **(Appendix B Table B.3).** G3BP1 was first reported in 1996 when Parker *et al.* identified and cloned a molecule capable of interacting with the SH3 domain of RasGAP, a modulator of Ras signaling in the mitogen activated protein kinase (MAPK) pathway [288]. To validate these findings, the experiment was repeated and lysate was western blotted for G3BP1 and TNFAIP2 to confirm. G3BP1 is present when TNFAIP2 is present suggesting a physical interaction between the two proteins **(Figure 3.3).**

Figure 3.3 G3BP1 is found to co-immunoprecipitate with endogenous TNFAIP2.

HeLa cells were stimulated with TNFα for either 30 minutes or 4 hours and then co-immunoprecipitated with TNFAIP2 antibody or IgG control. 68kDa G3BP1 (upper panel) is present when TNFAIP2 is pulled down by co-immunoprecipitation. 74kDa TNFAIP2 (lower panel) is confirmed to be present on the same blot and only a small amount is present in the IgG control.



TNFα stimulation (10ng/mL)	30 min	30 min	4 hours	4 hours
Immunoprecipitation Antibody	TNFAIP2	IgG control	TNFAIP2	IgG control

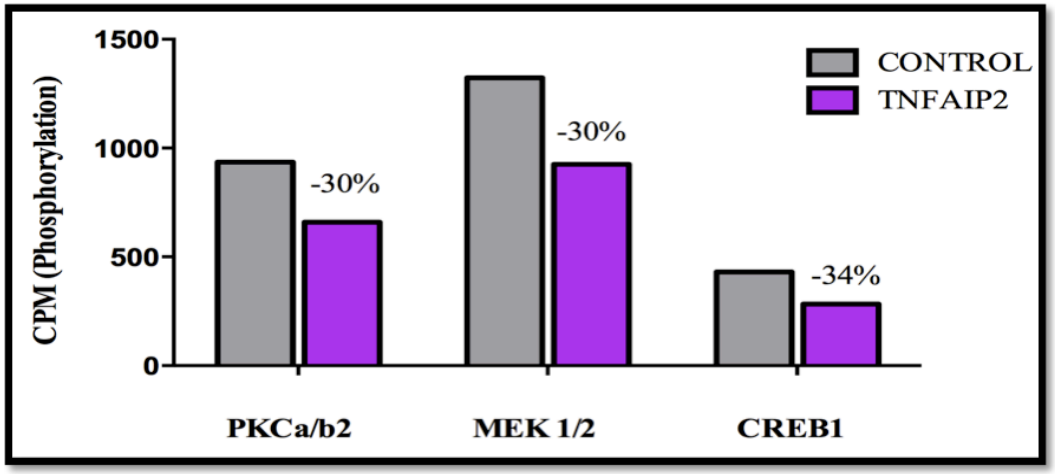
Over expression of TNFAIP2 is associated with a decrease in phosphorylation of Mitogen Activated Protein Kinase (MAPK) signaling pathway members

In order to determine the action of TNFAIP2 after translation, a screen of phosphorylated proteins was performed at KINEXUS. Of 29 epitopes on 25 proteins measured, 8 epitopes showed a greater than 25% +/- change in HeLa cells transfected with a TNFAIP2 over-expression vector for 48 hours when compared to control. **(Appendix B Table B.4).** We found that of those 8, 3 members of the MAPK signaling pathway, specifically, PKCα/β2 T638/T641 (-30%), MEK1/2 S218 +S222 (-30%) and CREB1 S133 (-34%) were all dephosphorylated at sites known to activate these kinases and hence suggest that over expression of TNFAIP2 inhibits the MAPK pathway. **(Figure 3.4).**

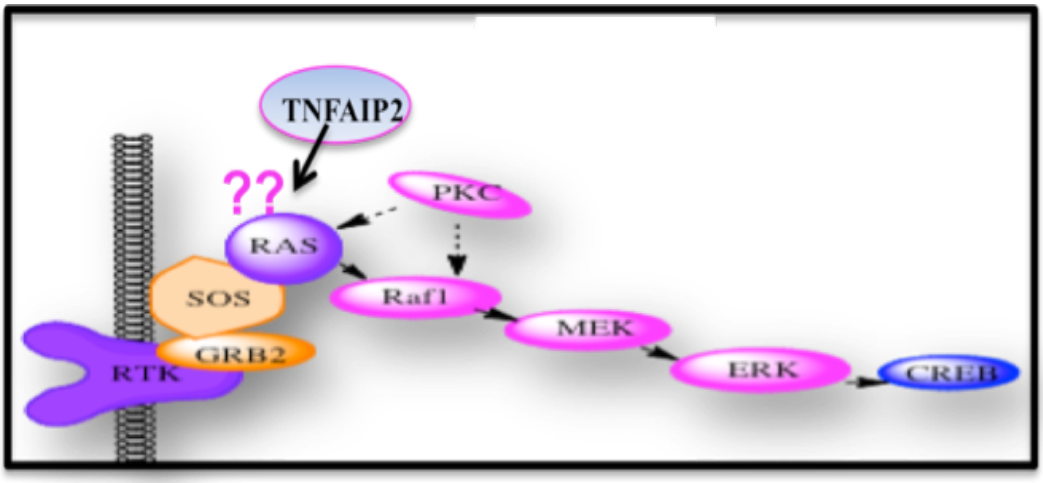
Figure 3.4 Over expression of TNFAIP2 is associated with a decrease in phosphorylation of MAPK signaling pathway members

A. Over expression of TNFAIP2 is associated with decreased phosphorylation of activation epitopes on 3 key members of MAPK signaling. B. A proposed schematic of TNFAIP2 in relation to the aforementioned signaling.

A.



B.



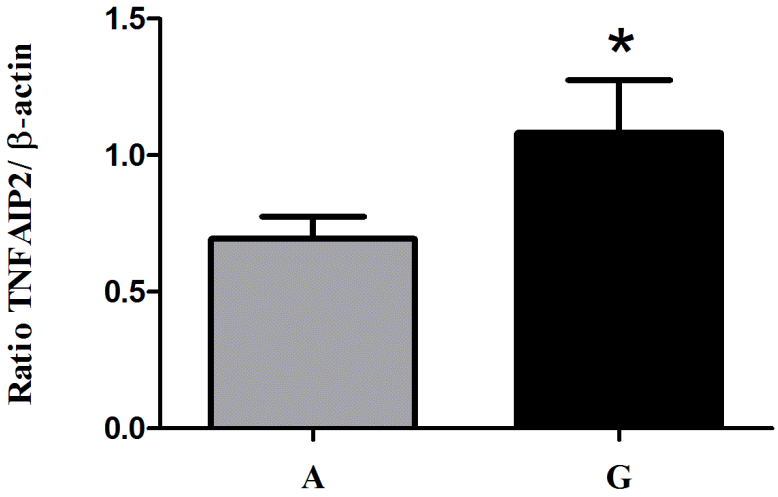
Genetic regulation of TNFAIP2 protein levels by rs8126

To further understand the potential mechanistic data revealed by the KINEXUS screen and co-immunoprecipitation data and to determine if rs8126 is a functional SNP that controls levels of TNFAIP2 protein, clones of the TNFAIP2 over expression vector expressing either the A or G allele of TNFAIP2 rs8126 were transfected into HeLa cells for 48 hours and cell lysates were assayed by western blot for TNFAIP2. We found that the ratio of the G allele to β-actin was 1.1, whereas the A allele had a ratio of 0.7, a fold difference of 1.6 suggesting that TNFAIP2 levels are higher when the G allele (minor) is present. (biological replicates, n=5, p=0.045) **(Figure 3.5).**

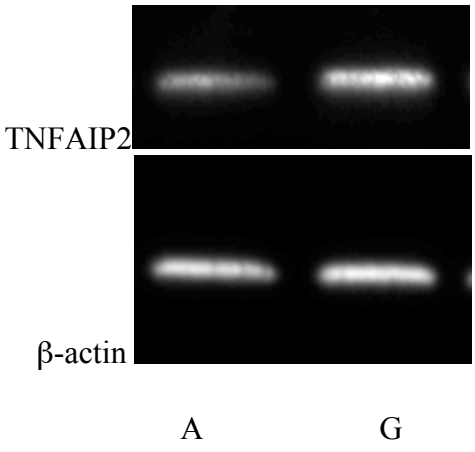
Figure 3.5 TNFAIP2 levels are associated with TNFAIP2 rs8126 genotypes.

A. Over expression of TNFAIP2 clones representing rs8126 A allele and rs8126 G allele in HeLa cells. 1.6 fold difference greater protein levels of TNFAIP2 are present for rs8126 G allele (Ratio of TNFAIP2/β-actin 1.1) then when compared to the A allele (Ratio of TNFAIP2/β-actin 0.7). (n=5, p=0.045). B. Representative image of TNFAIP2 and β-actin western blots.

A.



B.



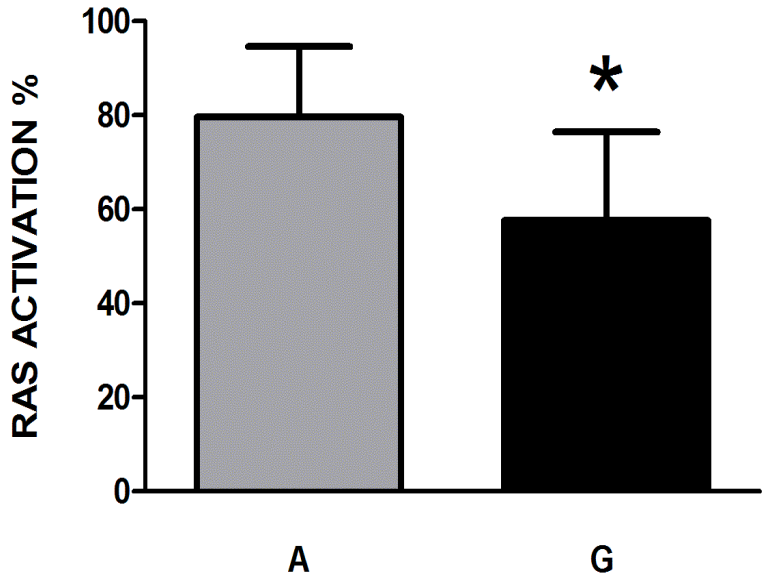
TNFAIP2 inhibits Ras activation according to rs8126 allele

Based on the finding that TNFAIP2 may inhibit MAPK signaling and that there appears to be genetic regulation of TNFAIP2 levels according to rs8126 allele (G allele yields more TNFAIP2 than A), clones of the TNFAIP2 over expression vector expressing either the A or G allele of TNFAIP2 rs8126 were assayed for % Ras activation compared to the positive control. Both alleles inhibited Ras activity and interestingly, the G allele (minor) exhibited significantly lower amounts of Ras activation (58%) compared to the A allele (80%) (biological replicates, n=5, p=0.026) **(Figure 3.6).** This suggests that the increased levels of TNFAIP2 present when the G allele is expressed lead to a greater inhibitory effect of TNFAIP2 on Ras.

Figure 3.6 Inhibition of Ras signaling by TNFAIP2 rs8126

A. Ras activation compared to positive control after over expression of TNFAIP2 clones representing rs8126 A allele and rs8126 G allele in HeLa cells. Presence of the A allele is associated with a decrease in Ras activation to 80%, there was a greater decrease to 58% when the G allele is present and the difference between the two alleles is statistically significant (n=5, p=0.026). B. Representative image of Ras activation assay blots.

A.



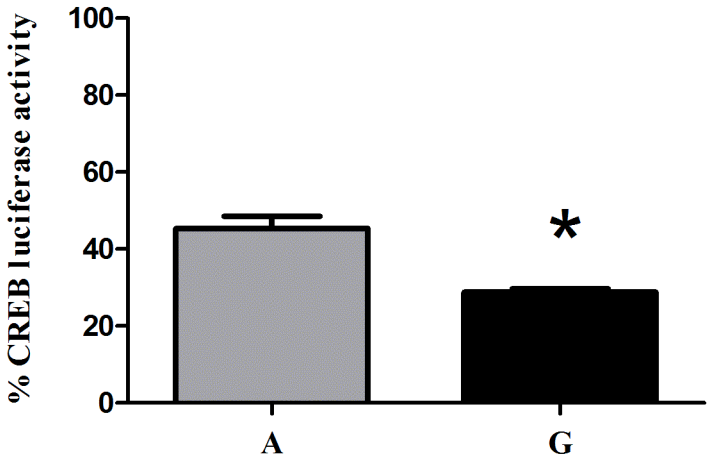
B.

TNFAIP2 inhibits CREB luciferase activity according to rs8126 Allele

It was shown in the phosphorylation assay that overexpression of TNFAIP2 reduced the phosphorylation of CREB (Figure 3.4). In order to further examine the effect of TNFAIP2 on the MAPK pathway and search for more evidence that rs8126 is in fact a functional SNP, we chose to look downstream at CREB activity. Cells we co-transfected with a CREB luciferase reporter construct, as well as of one of the following constructs: TNFAIP2 rs8126 A allele, TNFAIP2 G allele or control plasmid. When co-transfected with the A allele, CREB activity is inhibited to 45% relative to the control vector ($p_{(A \text{ to control})}$ =0.0009), even further inhibited to 29% when co-transfected with the G allele ($p_{(G \text{ to control})}$ =0.0002), and there is a statistically significant difference between the two alleles (biological replicates, n=3, $p_{(A \text{ to } G)}$ = 0.026) **(Figure 3.7).** This validates the TNFAIP2 associated inhibition seen at the level of CREB during the phosphorylation screen, supports the by allele differences of inhibition at the level of Ras and provides further evidence that not only does TNFAIP2 inhibit MAPK signaling overall, but that there is an allele specific response that consistently affects signaling from the cell membrane to the nucleus.

Figure 3.7 TNFAIP2 inhibits CREB luciferase activity by rs8126 allele.

When co-transfected with the A allele, CREB activity is reduced to 45% compared to the control vector ($p_{(A \text{ to control})}$ =0.0009), is even further reduced to 29% ($p_{(G \text{ to control})}$ =0.0002) when the G allele is expressed and there is a statistically significant difference between the two alleles (n=3, ***$p_{(A \text{ to } G)}$ = 0.026).

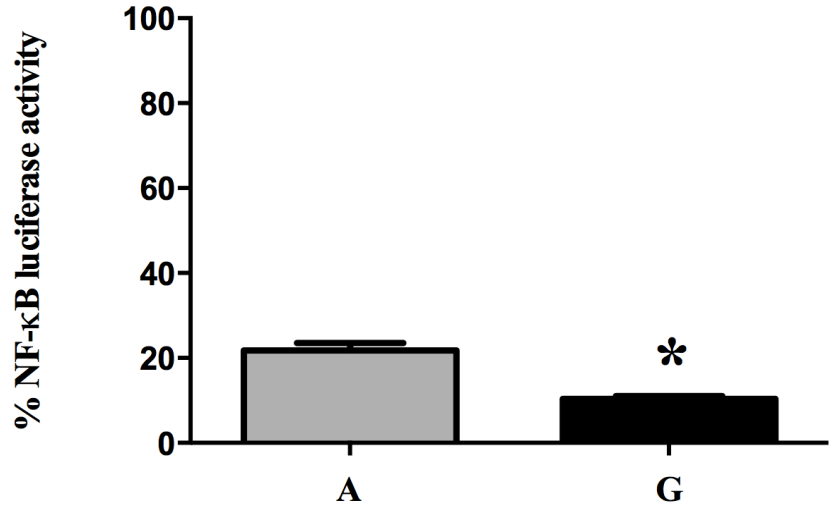


NF-κB luciferase assay

It is known that TNFAIP3 (A20) is upregulated in response to NF-κB signaling in order to negatively regulate NF-κB [126, 128]. In light of the inhibitory effects of TNFAIP2 on CREB, we repeated the CREB co-transfection with TNFAIP2 rs8126 A or G experiment but this time used a luciferase reporter for NF-κB activity. Again, cells were co-transfected with the NF-κB luciferase reporter and either allele of TNFAIP2 rs8126 constructs as well as the NF-κB reporter. Both alleles of TNFAIP2 significantly inhibit NF-κB compared to control, specifically the A allele inhibits NF-κB activity to 22% ($p_{(A \text{ to control})}$ =0.01) and the G allele inhibits NF-κB activity to 10% ($p_{(G \text{ to control})}$ =0.009), as well there is a significant difference between the two alleles (biological replicates, n=3, $p_{(A \text{ to } G)}$ = 0.013) **(Figure 3.8).** This suggests that TNFAIP2 also inhibits NF-κB signaling and may be a pleiotropic molecule in the regulation of cellular pathways.

Figure 3.8 TNFAIP2 inhibits NF-κB luciferase activity by rs8126 allele.

When co-transfected with the A allele, NF-κB activity is reduced to 22% compared to the control vector ($p_{(A \text{ to control})}$ =0.01), is even further reduced to 10% when the G allele is expressed ($p_{(G \text{ to control})}$ =0.009) and there is a statistically significant difference between the two alleles (n=3, $p_{(A \text{ to } G)}$ = 0.013).



3.4 Discussion

We have shown that TNFAIP2, a highly upregulated gene following exposure to TNFα, is a negative regulator of Ras, CREB and NF-κB. As well, we discovered that the 3'UTR SNP rs8126 regulates TNFAIP2 and is associated with mortality in septic shock. We have found biological plausibility for our clinical genetics association study: we discovered TNFAIP2 rs8126 allelic differences in the negative regulation of Ras, CREB and NF-κB. Using gene expression data that replicated in published literature we found three genes with a fold change greater than 2 in HeLa cells stimulated with TNFα that were common to all data sets (TNFAIP2, NFKBIA and NFKB1). Previous work in our lab and others has shown that genetic variation within inflammatory genes is associated with outcome in sepsis and septic shock [19-23, 26, 246, 279, 280] as well as with levels of cytokines [25]. Hence we hypothesized that the genes implicated in the microarray would have SNPs associated with outcome in septic shock and that these SNPs would functionally alter signaling. tagSNP genotyping of 13 tagSNPs within the 3 genes showed that rs8176373 of TNFAIP2 was associated with 28-day mortality in the VASST cohort of septic shock patients (p=0.0019, Bonferroni corrected p=0.025). In order to elucidate the source of the tagSNP signal we densely genotyped +/- 50,000bp upstream and downstream of TNFAIP2 and performed an Armitage trend test that identified rs8126 as the SNP most significantly associated with 28-day mortality. Interestingly, rs8176373 is 528 basepairs 3' of the gene,whereas rs8126 is located in the 3' UTR, a region commonly found to regulate mRNA expression and stability [184].

Based on the discovery of rs8126, we analyzed the Caucasian patients of the VASST cohort by this SNP. Kaplan-Meier analysis showed a statistically significant difference in 28-day mortality

whereby those of the GG/AG genotype had increased mortality and suggested a dominant G allele model (p= 0.0043). This model would imply that either one or two copies of the G allele are detrimental. To test for potentially confounding variables such as age, gender and surgical diagnosis we performed Cox regression using a major allele model and found that patients who had the GG/ AG genotype of TNFAIP2 rs8126 had a significantly increased hazard of 28-day mortality compared with patients having the AA genotype (Hazard Ratio [HR] 1.46; 95% confidence interval [CI] 1.08-1.96; p=0.013 Caucasian only). We also found that patients of the GG/AG genotype had fewer days alive and free of organ dysfunction than those of the AA genotype, specifically respiratory and renal dysfunction (p=0.008 and p=0.001 respectively), as well as more renal replacement therapy (p=0.021). Combined this data suggests that the G allele may be detrimental, the patients carrying one or two of these alleles have more organ dysfunction and increased mortality.

TNFAIP2 (B94) was discovered as a TNFα inducible gene, is regulated by NF-κB under most conditions, induced by retinoic acid in acute promyelocytic leukemia and has been associated with various carcinomas, however little is known about the action of TNFAIP2 at the molecular level [283, 289-294]. In order to determine potential protein protein interactions we first conducted a co-immunoprecipitation for TNFAIP2 and queried the proteins associated using mass spectrometry. One of the proteins returned from the screen was G3BP1, which was validated using western blots of the co-immunoprecipitation. GAP SH3 Binding Protein 1 (G3BP1) was first reported in 1996 when Parker *et al.* identified and cloned a molecule capable of interacting with the SH3 domain of RasGAP [288]. RasGAP is responsible for the hydrolysis of the Ras associated GTP to GDP in order to inhibit the MAPK pathway [288]. G3BP1 has

been shown to co-immunoprecipitate with RasGAP in proliferating cells, hence the authors hypothesized that it sequesters and inhibits RasGAP in order to allow for growth factor signaling induced proliferation [288].

This finding was replicated in at least two other studies as well as the observation that G3BP1 could co-immunoprecipitate with mRNAs and to bind the 3' untranslated region of *c-myc* mRNA and cleave it[295-297]. Specifically, in proliferating cells G3BP1 has a hypo-phosphorylated status and looses its ability to cleave mRNA; conversely when hyper-phosphorylated in quiescent cells, regains the ability to cleave mRNAs [295]. These latter functions led to the discovery of the role of G3BP1 in the formation of stress granules [298]. Stress granules are found in the cytoplasm to store mRNAs during stress conditions in order to re-asses the progression to translation or degradation based on the changed cellular conditions [298].

Until recently it was accepted that G3BP1 was pleiotropic, playing roles in Ras signaling and stress granule formation, however a study by Annibaldi *et al*. in 2011 was unable to replicate the interaction between G3BP1 and RasGAP [299]. We have shown that G3BP1 co-immunoprecipitates with endogenous TNFAIP2 in HeLa cells and that there is an association between TNFAIP2 and Ras signaling, suggesting that G3BP1 may play a role in Ras signaling under certain conditions. Specifically, we hypothesize that TNFAIP2 may inhibit G3BP1 allowing RasGAP to hydrolyze GTP to GDP and inhibit MAPK signaling, which would explain the inhibition of Ras and CREB observed in HeLa cells with over expression of TNFAIP2.

In light of this potential interaction of G3BP1 and the Ras pathway, we over expressed TNFAIP2 for 48 hours in HeLa cells and performed the KPSS 1.3 phosphorylation array at KINEXUS. Within the classical MAPK pathway we found 3 members with significant decreases in phosphorylation of activation sites when compared to control (PKC: -42%, MEK1/2: -43%, CREB1: -52%). Further evidence of an influence over MAPK signaling via endogenous or over expressed TNFAIP2.

TNFAIP2 is upregulated by TNFα and LPS and has been associated with a number of cancers, in particular head and neck as well as nasopharyngeal cancers [289, 290]. These cancers specifically involve Ras/Raf/MEK/ERK and a phase II trial of an ERK inhibitor showed moderate effects in nasopharyngeal carcinoma suggesting response due to inhibition of this pathway is helpful [300]. Despite attempts to modulate many different points in the Ras pathway from inhibiting farnesylation to prevent plasma membrane localization necessary for induction of the Raf/MEK/ ERK effectors, to attempting to inhibit effectors of that and concurrent pathways, researchers have yet to find an effective way to shut down the most commonly mutated pathway in cancer [301].

We have reported evidence of an inhibition of Ras based MAPK signaling including inhibition of CREB in response to over expression of TNFAIP2. Specifically a Ras activation assay by allele showed that Ras activation was inhibited (80% relative to control) for a A allele of rs8126 compared an even further reduction of 58% relative to control activation for the G allele, suggesting that increased levels of TNFAIP2 inhibit Ras activation (n=5, p=0.026). To further

support this novel interaction, we demonstrated that CREB luciferase activity was also inhibited by over expression of the A allele and again, further inhibited by the G allele (n=3, p=0.026).

We hypothesize that TNFAIP2 is upregulated in cellular response settings to negatively regulate the MAPK pathway and its downstream signaling, specifically that of the transcription factor CREB. In light of the increased TNFAIP2 present when the G allele is expressed in HeLa cells, the increased transcription of TNFAIP2 in cancer [289] and the association of that allele with a poor outcome both in septic shock and in cancer [290], these series of observations may reflect a type of "maximum response". This may not be enough to dampen the constitutive MAPK signaling due to mutations in cancer, but conversely may lead to too much inhibition of MAPK or NF-κB signaling in septic shock, creating a state of immunosuppression due to lower transcription of cytokines, or lack of proliferation of immune cells. There is evidence that CREB is directly involved in transcription of genes such as IL-6, IL-10 and TNFα and studies are emerging which show potential competition for transcription via an interaction with S276 of the RelA subunit of NF-κB with the CREB co-activators CBP or p300 [141, 144-147]. It is conceivable that if there is evidence of cross talk at the level of transcription, that mechanisms to negatively and positively regulate the two pathways could be instigated by a pleiotropic molecule such as TNFAIP2. It is interesting to note that A20 (TNFAIP3) also discovered as a TNFα inducible gene, is well know for its up regulation in response to NF-κB signaling in order to negatively regulate the NF-κB pathway itself. A20 deubiquitinates RIP at Lys63 and then acts as an ubiquitin ligase to target RIP for degradation [302, 303]. We have shown that TNFAIP2 inhibits NF-κB luciferase activity compared to control regardless of allele expressed and that

there is a statistically significant difference in the amount of inhibition when comparing rs8126 A (22%) to rs8126 G (10%) (n=3, p=0.013).

Some of our data conflicts with a recent publication whereby patients of the CC (GG) genotype of TNFAIP2 rs8126 have increased risk of squamous cell carcinoma of the head and neck (SCCHN) (odds ratio of 1.48, [CI] 1.06-2.05) [290], which is similar to our cohorts in that the patients of the GG genotype have increased mortality (Hazard Ratio [HR] 1.46; 95% confidence interval [CI] 1.08-1.96; P=0.013) and organ dysfunction, however Lui *et al.* presents luciferase reporter constructs of just the 3' UTR representing both alleles of rs8126 where the C (G) allele makes less TNFAIP2, the opposite of our observations, a result that could be explained by the differences in the constructs, we use the full cDNA, whereas they were only expressing the 3' UTR.

Taken together, our data suggest that the genotype of rs8126 influences the level of inhibition of Ras; that those of the G allele of rs8126 make more TNFAIP2 (G allele to A allele fold change 1.6, n=5, p=0.045) and so have increased dampening of the Ras pathway compared to those of the A allele, an effect we confirmed through the modulation of the transcription factors CREB and NF-κB. This suggests that these pathways can be inhibited by TNFAIP2 and that levels of TNFAIP2 influence the signaling in a dose dependent manner, for example we speculate that as a therapeutic for cancer at high doses, it would be enough to effectively dampen the pathway and restore balance in the cell, whereas inhibition of TNFAIP2 in sepsis using something such as small molecule inhibitors may relieve the inhibition.

This study has several limitations. The analysis of the VASST cohort was performed post hoc and despite a similar genetic model in another septic shock cohort, we do not have statistically significant data in a replication cohort of patients who had septic shock. TNFAIP2 rs8126 is being genotyped in a large collaborative study focused on acute lung injury in critically ill patients for future analysis of rs8126 by 28-day mortality, but at the time of preparation this data was not available. We have not yet measured levels of TNFAIP2 in our septic shock cohort, however the evidence in the current study may warrant doing so in a future study. It would be interesting to see the effect of siRNA on Ras activation and/or NF-κB activation however this was not included in the scope of this publication. It is interesting to note that Chen *et al* [289] found that silencing TNFAIP2 reduced the migration and invasion of nasopharyngeal carcinoma HK1 cells, however knockdown did not affect VEGF and hence the migration and metastasis of the carcinomas mediated by VEGF could be through a separate mechanism suggesting that TNFAIP2 may be pleiotropic in action. The group did not over express TNFAIP2. As well, a future study including these experiments performed in HUVECs or a renal derived cell line could enhance the understanding of the tissue specific action of this mechanism. As well, this data supports potential future studies to determine if TNFAIP2 and G3BP1 are present in a complex with Ras itself, work not included in the scope of this project.

In conclusion, we have discovered an association of the G allele of TNFAIP2 rs8126 with increased 28-day mortality and organ dysfunction in septic shock patients. Co-immunoprecipitation of G3BP1 with endogenous TNFAIP2 indicated a role of TNFAIP2 in MAPK signaling, which was also observed in a screen of phosphorylation sites. Investigation of upstream Ras activation and downstream CREB luciferase activity created a platform to further

investigate the genetic regulation of TNFAIP2. We found levels of TNFAIP2 protein differ by genotype and this is reflected in the Ras activation and CREB driven luciferase activity suggesting that increased TNFAIP2 levels of the rs8126 G allele, inhibit Ras and CREB. Similar to the known role of A20 in negative regulation of NF-κB signaling, over expression of TNFAIP2 inhibits NF-κB driven luciferase activity with significant differences by rs8126 allele. The changes may reflect feed back mechanisms in TNFα super family induced NF-κB signaling.

Chapter 4: Conclusion

Science and medicine have yet to distill the complex biology at play in a patient suffering from septic shock. Advances describing portions of the biology have not yet translated to changes in patient care with targeted therapeutics. A large body of work supports a role for the genetics and personalized medicine, yet candidate gene/ single nucleotide polymorphism approaches have been confounded in the past by poorly defined phenotypes and small cohort sizes. Furthermore, to date there has only been one genome wide study published is sepsis. Considering the current classification/ diagnosis of septic shock patients and combining that with the advances in genomics and economic feasibility compared to 5 years ago pose the question: should we be grouping by clinical characteristics or molecular signatures? Or both? And if so, in what combination? This elusive algorithm could be the answer to capitalizing on the influence of complex genetics in a complex disease state. For clearly defined phenotypes this may be found at the level of GWAS, for complex biology in a heterogeneous population such as sepsis this may be found at an intermediate level between the candidate SNP study and the GWAS.

This body of work is not genome wide, nor is it a monogenic candidate gene study. Using two different approaches we have discovered one potentially functional SNP and one plausibly functional SNP. It must be recognized that there are many other SNPs in genes involved in the direct signaling and/ or cross talk that influences these biological processes that may or may not have functional properties of a large enough effect to explain some of the effect we have reported. Future studies that include an assessment of all of these factors in concert would be ideal to determine the proportion of these contributions to the whole.

4.1 Role of NIK in Septic Shock

4.1.1 Brief summary of findings

We presented data whereby patients of the CC genotype of NIK rs7222094 had significantly increased mortality compared with patients having the CT or TT genotypes of rs7222094 in two cohorts of patients who had septic shock. Specifically, Caucasian patients in the SPH cohort who had the CC genotype of NIK rs7222094 experienced a significant increase in the hazard of death over the 28-days (HR 1.35; 95% confidence interval [CI] 1.12-1.64; P=0.002), an effect that replicated in the VASST Caucasian cohort (HR 1.24; CI 1.00-1.52; P= 0.048). Also, organ failure was stratified by rs7222094 genotype; patients of the CC genotype of rs7222094 of SPH experienced more renal and hematological dysfunction compared with patients having the CT or TT genotypes of rs7222094 (P= 0.003 and P= 0.011 respectively). While not replicated significantly, rs7222094 CC genotype showed the same trend toward more renal dysfunction in VASST cohort. In order to determine a biologically plausible output for functional studies, genome wide gene expression microarray data was generated from B lymphoblastoid cell lines of known genotype for rs7222094 stimulated with "cytomix" (TNFα, IL-1β, IFNγ and CpG). Cytomix compared to control. Fold change was calculated for each gene and compared for the greatest difference between major (TT) and minor (CC) genotypes. Interestingly, the greatest difference was in CXCL10. Biological plausibility studies found that the CC genotype was associated with significantly lower levels of CXCL10 in both B lymphoblastoid cell lines and VASST septic shock patients, as observed in the mRNA. CXCL10 has been shown to be regulated by NIK and is known for its role in T cell recruitment [112, 266]. We hypothesize that

in patients of the CC genotype, decreased mortality may be influenced by a lack of the full innate immune response due to less chemokine signaling for T cell recruitment in the areas of infection.

4.1.2 Functional plausibility of NIK rs7222094 in septic shock

rs7222094 is a non-coding SNP located in intron 2 of NIK (MAP3K14). We have shown an association of this SNP with outcome in septic shock in 2 cohorts as well as significantly different levels of CXCL10 [25]. Further studies are warranted to determine the molecular action of the SNPs. The genomic DNA representing NIK is 53,911bp creating a significant technical challenge for cloning, mutating rs7222094 and over expressing the entire gene in order to capture the potential function of rs7222094. Intron 2 is 1184bp, which could easily be cloned but the translation of the information found using a small piece to represent the whole of such a massive gene may present more questions than answers. At the time of this study, a search of HapMap, Perlegen and SeattleSNPs data using the SeattleSNPs genome browser for SNPs in LD (a generous cut off of r^2=0.4) with rs7222094 returned a list of intronic SNPs only **(Table 4.1)**. Interestingly, a promoter SNPs rs4792814 had an r^2 value of 0.74, but was located ~10,000bp from TSS, posing significant technical difficulties for cloning. Since then the 1000 Genomes Project has been published and a recent search using HaploReg v.2 publically available from the Broad Institute reveals an extended list of SNPs with r^2>0.8 with rs7222094 **(Table 4.1) [304].** Interestingly, all of these SNPs are found in intron 1 and inspection of UCSC Genome Browser ENCODE CHiPseq data for intron 1 in relation to the location of these SNPs shows dense regions of transcription factor binding. Future biological plausibility studies may be designed to test the functionality of these SNPs.

Table 4.1 SNPs in LD with rs7222094

Data available from SeattleSNPs genome browser 2010			
SNP rs ID	**r^2 with rs7222094**	**Promoter/ Exon/ Intron**	**Position Chr. 17**
rs2867316	0.53	intronic	43367447
rs721579	0.42	intronic	43370481
rs2867316	0.53	intronic	43376447
rs9908330	0.89	intronic	43383100
rs4328483	0.74	promoter	43401540
Data available from HaploReg v2 (Broad Institute) 2013			
SNP rs ID	**r^2 with rs7222094**	**Promoter/ Exon/ Intron**	**Position Chr. 17**
rs7222094	1.0	intron 2	43367653
rs9903009	0.89	intron 1	43378213
rs7221403	0.97	intron 1	43380666
rs9908330	0.91	intron 1	43383100
rs4792847	0.98	intron 1	43383484
rs1476532	0.91	intron 1	43383596
rs12449740	0.89	intron 1	43385349
rs11869999	0.94	intron 1	43389736
rs7216796	0.94	intron 1	43392208

One of the aforementioned SNPs rs4792847 which is in perfect LD with rs7222094 (r^2=0.98) was associated with rheumatoid arthritis susceptibility, specifically the patients of GG genotype (linked to the TT genotype of rs7222094) had a more favourable response to anti-TNF treatment and greater improvement at the 6 month mark [275, 276], in line with our findings of a potentially protective effect of the TT genotype of rs7222094 including decreased mortality and higher CXCL10 levels [25]. The rheumatoid finding was not replicated in a second cohort however [276]. A recent study assessed several SNPs including rs7222094 in relation to

allograft rejection in Hispanic renal transplant recipients, but it was not significantly associated [305].

Alternative to studies search for exonic non-synonymous SNPs in LD with rs7222094, as technology advances and global collaborations take place, the function of previously assumed "functionless" SNPs in genomic regions of gene deserts and intronic regions are proving to have more influence on biological function than previously expected [178]. Not only potential binding sites for enhancers and repressors, it is apparent that genetic variation can disrupt normal splicing events in disease, whether due to *de novo* mutations or stabilized SNPs [180]. To date NIK has five known splice variants for coding protein that vary significantly and hence the profile of the various transcript pools in relation to genotype between diseased and control states would also be worthy of investigation.

4.1.3 Potential impact of NIK (rs7222094) on treatment of septic shock

We presented data whereby levels of CXCL10 vary by genotype of NIK rs7222094, and it is known that knockdown of NIK reduced CXCL10 [112]. CXCL10 is a key player in the recruitment of T cells to sites of inflammation [266], which is consistent with several studies linking NIK to the host response to infection such as respiratory syncytial virus (RSV) [267], human immunodeficiency virus (HIV) [268], hepatitis B [269], E. coli [270], as well as the response to LPS [271], hence supporting a role for NIK in septic shock.

In order to fully understand the impact of the shift in NF-κB signaling from non-canonical at early time points to influencing canonical at later time points [101, 108, 109, 111, 112] would

warrant a temporal study at multiple time points from 30 minutes to 3 days minimum. Ideally to monitor canonical and non-canonical outputs as well as the overall level of NIK at these time points may highlight an aberration in the septic process similar to lupus [129]. The advancement of microarrays over the last two years to accurately interrogate the population of splice variants of NIK present by rs7222094 genotype at these time points is much more of a feasible project for future studies than at the time the initial study was performed. While inhibitors of NIK have been synthesized for treatment of constitutive non-canonical signaling contributing to multiple myeloma [306-309], we saw that patients with less CXCL10 did worse. Hence for the treatment of septic shock it may be that first patients at risk must be identified by NIK genotype in order to personalize their treatment in a way that would actually up regulate NIK orchestrated signaling to enhance their inflammatory response [310, 311]. This idea is somewhat counter intuitive to the current dogma that inflammation in septic shock must be suppressed in order for the patient to survive. This is not the first argument for treatment to reverse immunosuppressive states [60, 61, 64] and hence argues the case for personalized medicine that considers time point, genotype and downstream effects, as further suppression of CXCL10 production in a patient that is already at the low end of the concentration spectrum may actually put them at increased risk.

4.2 Role of TNFAIP2 in Septic Shock

4.2.1 Brief summary of findings

To identify genes highly regulated in response to TNFα we first used microarray measurements to identify high levels of gene expression up regulation that replicated in publically available data sets. We found 3 genes common to all data sets: TNFAIP2, NFKBIA and NFKB1. We then

hypothesized that single nucleotide polymorphisms (SNPs) within these TNFα induced genes would be associated with mortality in septic shock. Of the 13 tag SNPs in these three genes only one, rs8176373 of TNFAIP2 was significantly associated with 28-day mortality in the Vasopressin and Septic Shock Trial (VASST) cohort of septic shock patients. An Armitage trend test of the region found the G allele of TNFAIP2 rs8126, a 3'UTR SNP to be most highly associated with increased 28-day mortality in septic shock as well as increased organ dysfunction. We also found that TNFAIP2 binds G3BP1and inhibits Ras and downstream MAPK signaling leading to decreased CREB activity. We also found that similar to A20, TNFAIP2 inhibits NF-κB and finally that the extent of Ras, CREB and NF-κB inhibition is associated with rs8126 allele.

4.2.2 Role of TNFAIP2 rs8126 in septic shock

It was established early on that TNFα was involved in the pathophysiology of septic shock when experiments in baboons immunized with anti-TNFα demonstrated protection from hypotension followed by lethal renal and pulmonary failure due to bacteraemic shock [80, 81]. Years later researchers have yet to elucidate an effective intervention for septic shock in relation to TNFα [312]. It is clear that there are multiple systems at play rather than a single cytokine. It is not just TNFα itself but the gene expression it induces [85]. The intense systemic response to TNFα is driven by nuclear factor kappa B (NF-κB)[85]. However, the role of highly transcribed genes as a result of TNFα via NF-κB during septic shock is unclear.

TNFAIP2 (B94) was discovered as a TNFα inducible gene and has been reported to be involved in endothelial capillary tube formation/ angiogenesis [292-294]. It is transcribed via NF-κB

under most conditions [283], upregulated in response to LPS [291, 313], induced by retinoic acid in acute promyelocytic leukemia [314] and after infection with *Yersinia pestis* [315] and has been associated with the migration and metastasis of nasopharyngeal carcinomas [289]. Despite all of these associations, little is known about the molecular action of TNFAIP2 in cellular pathways related to human septic shock. We have reported the discovery of the novel function of TNFAIP2, a gene transcribed by TNFα induced NF-κB signaling and the novel finding of its inhibition of Ras based MAPK signaling, inhibition of NF-κB similar to TNFAIP3 (A20), as well as its genetic regulation via functional studies of TNFAIP2 rs8126.

4.2.3 Novel mechanism of TNFAIP2 protein and discovery of functional SNP rs8126

We have shown that increased TNFAIP2 (via over expression) inhibits MAPK signaling, suggesting a novel function for TNFAIP2 and a novel cross talk discovery whereby a gene transcribed in response to TNFα stimulation goes on to not only impact its own production by inhibiting NF-κB, but also the inhibition of MAPK signaling. Previous work has shown other examples of the cross talk between TNFα signaling and MAPK signaling where by TNF activates and early phase TGFα autocrine circuit to cross talk through the EGFR/MEK/ERK signaling pathway as a cellular mechanism to balance inflammation [158]. Specifically, the balance between TNFα induced cell death and MAPK growth factor induced cell survival. Although most studies only look at one cell type at a time, there is evidence that abnormal levels of cell death is a common characteristic of patients with septic shock [135]. Since increased levels of TNFAIP2 leads to inhibition of MAPK signalling, we would expect that patients with increased TNFAIP2 would have increased cell death, which may explain the patients who have at least one G allele experiencing increased organ dysfunction and mortality.

An alternative (or potentially parallel) impact of this cross talk is that it has been shown that MAPK signaling is responsible for TLR mRNA expression [159]. Again increased levels of TNFAIP2 resulting in inhibition of MAPK signaling would inhibit TLR expression, impairing the cell's ability to respond to TLR ligands. This supports future studies that focus on understanding the balance between these pathways in order to restore it.

As it becomes evident that TNFAIP2 is a pleiotropic protein, a search for other functional studies of TNFAIP2 recently uncovered an interesting study. TNFAIP2 has a Sec-6 homology domain suggesting that the protein could be involved in exocytosis. In order to understand the formation of tunneling nanotubules (TNTs) and their role in intercellular transport of cellular components, a study done in mice searching for molecules expressed by M cells and not intestinal epithelial cells determined Tnfaip2 *(Mus muculus)* to fill the criteria and went on to determine this mechanism of action [316-318]. The group found that over expression of Tnfaip2 increased the development of TNTs in co-operation with RalA and the exocyst complex [316]. However due to the knowledge that the nanotube formation they were witnessing was largely due to actin remodeling and the fact that research has shown that these processes involve the small GTPases of the Rho family the group focused on that and did not look at any other effectors such as Ras. Furthermore, based on bioinformatic sequence alignments, to our knowledge, the region of DNA surrounding rs8126 is not present in the mouse cDNA FASTA sequence and hence the effect of this functional SNP would not be present in the mouse studies. Of the 29 epitopes on 25 proteins measured by KINEXUS, we only found members of the MAPK pathway to be affected and hence did not explore the same interactions as described in these publications.

Interestingly, many cancers can be traced back to mutations in the MAPK pathway, leading to uninhibited signaling and unchecked cell growth [131]. Clinical trials still struggle to develop effective inhibitors for this pathway [319]. It is possible that the knowledge of the role of TNFAIP2 not only in MAPK but also NF-κB may lead to more informed research for the control of these signaling pathways.

We have reported differences in inhibition of NF-κB, Ras and CREB by rs8126 allele. Recently Lui *et al.* described the role of rs8126 in squamous cell carcinoma of the head and neck (SCCHN) due to the potential disruption of a purported miRNA binding site for miR-184 [290]. microRNAs (miRs) are small 20-24 nucleotide non- coding RNAs that mediate translational repression by binding to miR recognition elements (MREs) found in the 3'UTR of their mRNA targets [184]. Luciferase reporter constructs of just the 3' UTR representing both alleles of rs8126 showed that the C (G) allele makes less TNFAIP2, the opposite of our observations [290]. It is important to note that despite the differences in mRNA/ protein expression profiles, the clinical relevance of this SNP still stands, the patients of the GG/AG genotypes in our septic shock cohort have increased mortality (Hazard Ratio [HR] 1.46; 95% confidence interval [CI] 1.08-1.96; P=0.013) and organ dysfunction where similarly Lui *et al.* the patients of the CC (GG) genotype have increased risk of SCCHN (odds ratio of 1.48, [CI] 1.06-2.05).

The Armitage trend test revealed rs8126 as more highly associated with mortality than the originally associated tagSNP rs8176373. Located in the 3' UTR, it may be in a more plausible location for a functional SNP when compared to rs8176373, which is found 528bp 3' of the

TNFAIP2 gene in an intergenic region. While the proposition that rs8126 alters miRNA binding is very plausible, the GC content of the entire TNFAIP2 cDNA transcript is very high, hence it is possible that the results from a clone containing only the 3'UTR could be very different than those of the entire transcript due to secondary structure [320]. Secondary structure of mRNA can dictate the availability of regions to regulatory effector molecules such as miRNA [320, 321]. The results also depend on cell context and they differ in that these constructs were expressed in a head and neck and lung cancer cell line, which while very relevant to their study may highlight the importance of tissue specificity. It is known that the expression of the miRNAs themselves vary from tissue to tissue and hence different regulation has been reported for the exact same miRNA sites, thus explaining the differential regulation across studies [184].

4.2.4 Potential impact on treatment of septic shock

It is known that TNFα activates NF-κB, which results in transcription of TNFAIP2 [283, 284]. Despite numerous associations of TNFAIP2 with various disease states described above and the potential role in TNT formation via an interaction with Ral (GTPase), the molecular action of this protein is unknown. We have shown it can act as negative regulation for at least two pathways (NF-κB and MAPK), therefore a proposed model could be imagined whereby TNF toggles the "on" switch and TNFAIP2 is the "off". As well as growth factors, TNFα itself activates MAPK. Therefore in a scenario where TNFα simultaneously activates NF-κB and MAPK signaling, it would be biologically efficient to have a common protein (such as TNFAIP2) that acts as a negative regulator amidst the cross talk between these two pathways.

In the VASST cohort of septic shock patients, the patients of the AA genotype of TNFAIP2 rs8126 have lower mortality compared to those patients carrying at least one if not 2 copies of the G allele, and based on *in vitro* studies would be expected to have less TNFAIP2. We have shown that TNFAIP2 inhibits Ras, CREB and NF-κB regardless of allele, however there is more marked inhibition by the G allele. If truly an inhibitor then upregulated TNFAIP2 may mark a futile attempt to dampen the unchecked activation of MAPK pathways in cancer [289, 290, 314]. In the case of sepsis however this inhibition may be detrimental, for example, the patients carrying at least one G allele, which according to our *in vitro* studies would have more TNFAIP2 and more inhibition of Ras, CREB and NF-κB. A human cathelicidin peptide LL-37, found in high concentrations in neutrophils, inhibits production of TNFAIP2 and appears to be protective in models of sepsis and septic shock [313]. Taken together this could be interpreted as improvement due to the relief of the over suppression by TNFAIP2 of pathways (NF-κB and MAPK) needed to clear the pathogens. With the difference in overall mortality ranging from 47% for AA genotype to approximately 30% for the AG/GG genotypes, and the statistically significant differences of inhibition found between the A and G allele *in vitro*, a case could be argued for personalized medicine whereby rs8126 could potentially inform a caregiver as to where to start with dosage for drugs, if successfully developed, on TNFAIP2 influenced pathways.

4.3 Final Conclusion

In summary we have described two genes (NIK and TNFAIP2) with polymorphisms associated with outcome in septic shock. From these associations we have attempted to elucidate the biology to further understand the role of genetics in the complex disease state of septic shock.

Interestingly, both of these genes are implicated in cancer as well and much can be learned from the intersection of these diseases and the role of these genes within them. The final frontier is the intertwinement of key aspects such as genotype and molecular mechanisms in a complimentary fashion that can be tailored to the patient depending on the specific pathology that resulted in septic shock to restore balance.

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Appendices

Appendix A Supplemental Tables and Figures for Chapter 2: A Single Nucleotide Polymorphism in NF-κB Inducing Kinase (NIK) is Associated with Mortality in Septic Shock

Table A.1 Hardy-Weinberg equilibrium (HWE) and minor allele frequencies (MAFs) in the SPH cohort Caucasian patients with septic shock and as reported in bioinformatic websites.

Gene	SNP	HWE	SPH minor allele	SPH major allele	SPH MAF	Call rate assay	Reported minor allele	Reported major allele	Reported MAF	Source
MYD88	**rs2239621**	0.25	T	C	0.26	99	T	C	0.27	Hapmap CEU
MYD88	**rs6853**	0.3	G	A	0.12	98	G	A	0.12	Hapmap CEU
MYD88	**rs7744**	0.26	G	A	0.18	98	G	A	0.15	Hapmap CEU
IRAK4	**rs4251520**	0.88	C	T	0.11	100	C	T	0.1	Hapmap CEU
IRAK4	**rs4251513**	0.49	C	G	0.53	99	G	C	0.5	Hapmap CEU
TIRAP	**rs1786697**	0.05	G	C	0.22	99	G	C	0.26	PGA-UW-FHCRC European
TIRAP	**rs614700**	0.02	A	G	0.25	96	A	G	0.24	PGA-UW-FHCRC European
TIRAP	**rs646005**	<0.001	G	A	0.39	99	G	A	0.37	PGA-UW-FHCRC European
MAP3K7	**rs1145727**	0.59	G	A	0.23	99	G	A	0.24	Hapmap

Gene	SNP	HWE	SPH minor allele	SPH major allele	SPH MAF	Call rate assay	Reported minor allele	Reported major allele	Reported MAF	Source
MAP3K7	**rs157688**	0.97	A	G	0.39	99	T	C	0.36	Hapmap CEU
MAP3K7	**rs205342**	0.93	G	A	0.37	100	C	T	0.27	Hapmap CEU
MAP3K7	**rs967779**	<0.001	G	A	0.35	99	G	A	0.4	Hapmap CEU
MAP3K7IP1	**rs5750824**	<0.001	G	A	0.24	96	G	A	0.18	Hapmap CEU
MAP3K7IP1	**rs6001585**	0.84	C	A	0.19	95	C	A	0.2	Hapmap CEU
MAP3K7IP1	**rs739141**	<0.001	C	T	0.32	96	C	T	0.34	Hapmap CEU
MAP3K7IP1	**rs7949**	<0.001	G	A	0.33	95	G	A	0.31	Hapmap CEU
IKBKB	**rs10958715**	0.02	A	G	0.09	99	A	G	0.06	Hapmap CEU
IKBKB	**rs3747811**	0.26	T	A	0.49	98	A	T	0.5	Hapmap CEU
IKBKB	**rs5029748**	0.13	A	C	0.34	97	T	G	0.28	Hapmap CEU
RIPK1	**rs11242823**	0.12	A	G	0.5	96	A	G	0.47	Hapmap CEU
RIPK1	**rs2326173**	0.75	A	G	0.35	99	A	G	0.33	Hapmap CEU
RIPK1	**rs4959774**	0.64	G	A	0.11	99	G	A	0.13	Hapmap CEU
MAP2K6	**rs12453226**	0.16	C	G	0.42	99	C	G	0.4	Hapmap CEU
MAP2K6	**rs1548444**	0.22	T	G	0.46	98	T	G	0.46	Hapmap CEU

Gene	SNP	HWE	SPH minor allele	SPH major allele	SPH MAF	Call rate assay	Reported minor allele	Reported major allele	Reported MAF	Source
MAP2K6	**rs2586545**	0.02	C	T	0.41	98	C	T	0.5	Complete Genomics CEU
NFKBIA	**rs1957106**	0.05	A	G	0.29	96	A	G	0.3	PGA-UW-FHCRC European
NFKBIA	**rs2233415**	0.06	T	C	0.26	99	T	C	0.28	PGA-UW-FHCRC European
NFKBIA	**rs3138055**	0.33	G	A	0.3	100	C	T	0.27	Hapmap CEU
NFKBIA	**rs696**	0.93	A	G	0.37	99	T	C	0.39	Hapmap CEU
NFKBIB	**rs10775533**	0.16	C	G	0.4	97	C	G	0.44	PGA-UW-FHCRC European
NFKBIB	**rs4803006**	0.01	A	G	0.39	98	A	G	0.38	Hapmap CEU
NFKBIE	**rs2282151**	<0.001	C	T	0.23	96	C	T	0.17	Hapmap CEU
NFKBIE	**rs3799962**	0.82	G	A	0.44	98	G	A	0.39	Hapmap CEU
NFKBIE	**rs730775**	0.4	A	G	0.43	97	G	A	0.41	Hapmap CEU
RELA	**rs1049728**	<0.001	G	C	0.06	99	C	G	0.03	Hapmap CEU
RELA	**rs11227247**	<0.001	G	T	0.2	97	C	A	0.15	Hapmap CEU
NFKB1	**rs11722146**	0.68	A	G	0.32	98	A	G	0.35	Hapmap CEU

Gene	SNP	HWE	SPH minor allele	SPH major allele	SPH MAF	Call rate assay	Reported minor allele	Reported major allele	Reported MAF	Source
NFKB1	**rs230521**	0.01	G	C	0.45	98	C	G	0.47	Hapmap CEU
NFKB1	**rs230542**	0.43	T	C	0.38	99	T	C	0.35	Hapmap CEU
NFKB1	**rs3774934**	0.78	A	G	0.12	98	A	G	0.05	Hapmap CEU
TRAF2	**rs10283820**	0.35	G	A	0.45	96	G	A	0.5	Complete Genomics CEU
TRAF2	**rs10781520**	0.08	G	A	0.26	97	G	A	0.31	Hapmap CEU
TRAF2	**rs2784069**	0.41	G	A	0.36	99	G	A	0.41	PGA-UW-FHCRC European
TRAF6	**rs2303439**	0.54	T	C	0.17	100	T	C	0.13	Hapmap CEU
TRAF6	**rs5030411**	0.08	C	T	0.44	99	G	A	0.4	Hapmap CEU
TRAF6	**rs540386**	0.88	T	C	0.15	99	T	C	0.11	Hapmap CEU
NIK (MAP3K14)	**rs2074293**	0.12	C	T	0.41	97	G	A	0.5	Hapmap CEU
NIK (MAP3K14)	**rs7222094**	<0.001	C	T	0.48	95	C	T	0.48	Perlegen EUR panel
NIK (MAP3K14)	**rs9972936**	0.03	G	A	0.43	98	G	A	0.48	Hapmap CEU
CHUK	**rs11190421**	<0.001	A	G	0.12	96	A	G	0.1	PGA-UW-FHCRC European
CHUK	**rs11591741**	0.06	G	C	0.4	99	C	G	0.42	Hapmap

Gene	SNP	HWE	SPH minor allele	SPH major allele	SPH MAF	Call rate assay	Reported minor allele	Reported major allele	Reported MAF	Source
										CEU
CHUK	**rs3818411**	0.12	T	C	0.5	99	T	C	0.45	Hapmap CEU
CHUK	**rs7903344**	0.41	T	C	0.5	100	T	C	0.45	Hapmap CEU
NFKB2	**rs1409312**	0.06	G	A	0.19	97	C	T	0.22	Hapmap CEU
NFKB2	**rs3802681**	0.66	G	A	0.38	99	C	T	0.43	Hapmap CEU
NFKB2	**rs7086205**	0.39	C	T	0.18	98	C	T	0.15	Hapmap CEU

Table A.2 Genotype and allele frequencies in all populations of SPH and VASST cohorts.

Ethnicity	SPH Cohort rs7222094 (n=589) N (%)	TT	CT	CC	T	C	VASST Cohort rs7222094 (n=616) N (%)	TT	CT	CC	T	C
Caucasian	453 (76.9)	139	191	123	0.52	0.48	517 (83.9)	133	251	133	0.50	0.50
East Asian	72 (12.2)	4	15	53	0.16	0.84	42 (6.8)	1	9	32	0.13	0.87
South Asian	33 (5.6)	13	10	10	0.55	0.45	15 (2.4)	3	9	3	0.50	0.50
Aboriginal	21 (3.6)	2	6	13	0.24	0.76	15 (2.4)	1	3	11	0.17	0.83
Latino	5 (0.9)	1	2	2	0.40	0.60	6 (1.0)	0	4	2	0.33	0.67
African	3 (0.5)	1	1	1	0.50	0.50	14 (2.3)	3	4	7	0.36	0.64
Unknown	0 (0)	0	0	0	0	0	7 (1.14)	1	3	3	0.36	0.64

Table A.3 Top 10 genes (of all genes onWG-6 gene expression microarray) with the greatest difference (delta) in fold change between major (TT) and minor (CC) genotypes of rs7222094

This is fold change (FC) as calculated by Flexigene, then calculate delta FC of TT-CC for rs7222094 genotype and gate on minimum delta FC of 0.2

Gene	CXCL10	CXCL9	UBD	GBP4	NFKBIZ	ZC3H12A	HS.583806	HS.579631	BCYRN1	GBP5
log2(Fold change),07019	1.6684	1.4461	1.9114	2.1008	1.3063	1.4145	0.2764	0.8796	0.7426	1.4432
log2(Fold change),07022	1.8692	2.7322	2.0071	1.8018	1.7409	1.2819	-0.3753	0.0576	0.1372	1.5591
log2(Fold change),07056	2.3282	3.3913	2.4566	2.5178	1.8282	1.1302	0.0979	0.1534	-0.1068	1.1872
log2(Fold change),10846	3.4572	3.0797	2.2258	2.6902	2.0546	1.6655	-0.1688	0.1234	-0.1432	1.5448
log2(Fold change),10851	2.1176	2.2213	1.3539	1.7820	1.4367	1.0648	0.7837	0.9049	0.3524	0.6189
log2(Fold change),10854	1.0984	0.5374	0.7358	1.6935	0.8411	0.5694	-0.3756	-0.4939	-0.0717	1.0569
log2(Fold change),10855	2.0222	1.5636	1.9528	2.3508	1.9198	1.6153	-0.1731	0.0692	0.2824	1.0181
log2(Fold change),10856	0.1963	0.3478	0.6980	0.1368	0.4565	0.2283	-0.1913	-0.0376	0.1126	0.5870
log2(Fold change),10857	2.1983	2.1931	2.2608	2.1063	2.0282	1.0713	-0.6635	-0.9558	-0.2859	1.3407
log2(Fold change),10861	1.1726	0.7126	1.9736	1.4893	1.0696	0.7129	-0.1399	0.2495	-0.2671	0.8088
log2(Fold change),11829	1.1854	1.3612	1.1862	1.1384	1.8100	1.3056	-0.1870	0.1056	0.0946	0.5529
log2(Fold change),11830	0.4708	0.1216	0.7784	0.7263	0.9357	0.6443	0.8838	0.6074	0.4394	0.2782
log2(Fold change),11832	1.4854	0.8708	1.8411	1.1848	1.2372	0.9331	-0.6959	-0.5795	-0.7584	1.0706
log2(Fold change),11840	1.8502	1.4030	1.6869	1.4863	1.6516	1.4059	-0.6045	-0.7166	-0.2253	1.3522
log2(Fold change),11994	1.3145	1.0581	3.0032	2.2267	1.3779	1.1491	0.2558	0.2898	0.1019	2.0529
log2(Fold change),12004	2.7873	3.4608	2.6984	2.4645	1.8595	1.2904	0.4639	0.4506	0.3854	1.2383
log2(Fold change),12006	0.9495	0.9373	1.4431	0.9691	2.2464	1.7760	-0.2050	0.0292	0.1073	0.5655
log2(Fold change),12043	1.8417	1.5038	2.0542	1.5909	1.4580	1.1823	-0.1525	-0.1367	0.1804	1.1159
log2(Fold change),12044	0.3849	1.6145	1.7548	1.2722	1.7687	1.1414	0.5012	0.3296	0.7295	0.3689
log2(Fold change),12057	2.5515	1.9566	1.5898	2.1400	2.3558	1.5408	-0.1921	-0.0535	0.1563	0.9585
log2(Fold change),12145	2.7414	1.6617	2.5580	1.8461	1.2469	1.1147	-1.4697	-0.7733	-0.5747	0.7975
log2(Fold change),12156	1.5249	0.7942	1.9732	1.6336	2.2115	1.2801	-0.0301	0.1937	0.1665	0.4176

Gene	CXCL10	CXCL9	UBD	GBP4	NFKBIZ	ZC3H12A	HS.583806	HS.579631	BCYRN1	GBP5
log2(Fold change),12234	2.8380	2.0167	2.9481	2.1514	1.6151	1.1546	0.1322	0.9364	0.3330	1.7548
log2(Fold change),12248	1.6421	1.3701	0.9772	1.0445	1.5212	0.8660	0.2267	0.5225	0.6518	0.7505
log2(Fold change),12740	3.0061	2.0076	2.6295	2.5974	1.3418	1.0860	0.0086	0.1427	0.1216	1.2542
log2(Fold change),12750	1.4702	0.7680	1.6426	1.9054	1.6073	1.0970	0.4741	0.5309	0.2336	1.5312
log2(Fold change),12751	3.2747	3.3395	2.7349	2.3414	1.0913	0.8955	-0.2250	-0.0133	0.0481	1.3052
log2(Fold change),12753	2.0457	1.5894	2.1951	1.7619	1.4537	0.8114	0.3574	0.2435	0.7653	0.8268
log2(Fold change),12762	2.2576	2.1509	2.5820	1.7292	1.3328	1.1256	0.3383	0.3370	0.2384	1.9351
log2(Fold change),12763	2.9619	1.3099	2.5987	1.7715	1.4851	1.0808	0.3270	0.2773	0.3855	0.9645
log2(Fold change),12802	1.6929	1.2092	1.9454	1.6640	0.8732	1.2239	-0.1644	-0.0236	0.1055	0.5267
log2(Fold change),12813	0.8435	0.6058	2.0686	2.0927	2.0234	1.4988	0.0860	0.1192	0.1377	1.1703
log2(Fold change),12878	2.3051	2.7640	2.5482	2.9757	2.0667	1.2807	0.0712	0.3757	-0.1739	2.3093
log2(Fold change),12891	3.4863	2.0694	3.0289	3.4278	2.1886	1.8206	0.5560	0.7927	0.3409	1.0572
log2(Fold change),12892	2.4799	2.0687	2.9376	2.8152	2.2804	1.4687	-0.1808	-0.0956	0.3452	1.7413
log2(Fold change),06985	1.8072	1.3592	2.1440	2.0682	1.6017	0.9105	0.4357	0.5386	0.4435	0.9558
log2(Fold change),06991	1.2086	1.6983	1.7894	1.6814	1.0679	0.9326	-0.5871	-0.2575	-0.0727	0.6136
log2(Fold change),06994	2.6066	3.1506	2.6661	1.7028	1.8641	1.2774	0.2195	0.5661	0.4163	1.0016
log2(Fold change),07000	1.2910	1.1050	2.5464	1.8240	1.3786	1.2045	0.4750	0.4972	0.1905	1.0608
log2(Fold change),07029	1.3745	0.2076	1.5435	1.5604	1.9221	1.3547	-0.1230	-0.1056	0.3459	0.8956
log2(Fold change),07034	3.5526	3.4245	2.7235	2.9967	2.1288	1.2260	0.3198	0.5452	0.4393	2.1237
log2(Fold change),07048	2.1159	0.9957	2.1420	2.0558	1.9045	1.7196	-0.1154	0.2462	-0.0103	1.8319
log2(Fold change),07055	1.6644	1.7909	1.2247	1.9293	1.4021	0.9005	-0.3728	-0.1794	-0.1724	0.7561
log2(Fold change),07357	1.5190	1.1338	1.6034	1.6011	1.4467	0.8138	0.2632	0.0516	0.8211	0.4431
log2(Fold change),10831	-2.0372	-1.4807	-0.5220	-0.8117	-1.3452	-0.8409	0.9134	0.1810	0.2075	0.0473
log2(Fold change),10835	1.5640	1.1080	2.1729	2.5393	1.9991	1.1685	0.0856	0.1804	0.3584	1.6643
log2(Fold change),10838	1.8932	1.0789	2.2206	1.3532	1.7319	1.0627	0.4321	0.2645	0.2145	1.3847
log2(Fold change),10860	3.0946	1.4257	2.2544	2.4696	2.4431	1.4908	-0.3799	-0.0505	-0.0048	1.6257
log2(Fold change),11831	1.9020	1.1340	1.8704	1.6970	1.5146	0.9959	-0.2260	0.0064	-0.0487	1.2085
log2(Fold change),11839	0.9115	0.2725	0.9722	1.6410	2.0059	1.1257	-0.6581	-0.2173	-0.2437	1.1635
log2(Fold change),11882	1.9042	2.0167	2.6435	2.5916	2.0706	1.2931	0.6373	0.4830	0.6901	0.9840
log2(Fold change),11992	3.2563	2.4240	2.4802	2.6107	1.8021	1.2486	-0.0893	0.0987	0.0456	2.1047
log2(Fold change),11993	2.6616	2.0054	2.0612	2.1080	1.8756	1.4280	-1.0798	-1.0082	-0.3398	1.2659

Gene	CXCL10	CXCL9	UBD	GBP4	NFKBIZ	ZC3H12A	HS.583806	HS.579631	BCYRN1	GBP5
log2(Fold change),11995	0.5160	0.3717	1.3878	0.8068	0.9960	0.7165	0.8476	1.0818	0.7930	0.3376
log2(Fold change),12005	2.8618	2.1313	2.1939	2.5279	1.8899	1.0204	-0.7327	-0.6652	-0.1532	0.3142
log2(Fold change),12056	1.5674	1.0004	1.6324	1.7602	1.8734	1.6052	0.2322	0.2943	0.1519	0.2755
log2(Fold change),12144	2.3617	2.4608	2.0715	2.1838	1.9447	1.5451	-0.5034	-0.3018	-0.2334	1.6882
log2(Fold change),12146	1.2818	0.4447	2.2260	2.2838	1.4352	1.1090	-0.2042	0.0394	-0.0059	1.6041
log2(Fold change),12154	2.5885	1.5415	2.3558	2.1693	2.2567	1.4757	-0.6386	-0.1644	-0.3942	1.1776
log2(Fold change),12155	1.3771	1.6448	2.5103	1.8515	1.1957	1.2849	0.2975	0.2154	0.1145	1.1902
log2(Fold change),12264	2.0766	1.2380	2.5019	2.3793	1.6817	1.3194	0.0344	0.4003	-0.1090	2.1082
log2(Fold change),12717	0.8703	2.7963	1.3255	2.0457	1.8534	1.2351	0.6139	0.7242	0.9839	0.5648
log2(Fold change),12761	0.5458	0.4444	1.2213	1.1066	2.1171	1.6506	0.9915	1.2787	0.4309	0.8219
log2(Fold change),12801	1.8962	1.9108	1.5440	2.2596	1.8909	1.1331	-0.5329	-0.3326	0.0852	1.0734
log2(Fold change),12812	1.9819	0.5924	2.9264	2.2488	1.6252	1.2282	0.5167	0.2119	0.5079	1.5009
log2(Fold change),12814	1.7591	1.3657	1.9151	1.6653	1.5253	1.3073	-0.0696	-0.3263	0.0643	1.2646
log2(Fold change),12815	3.1930	1.9266	1.9999	2.1783	2.2011	1.2688	-0.5237	-0.0959	-0.2929	0.8092
log2(Fold change),12864	0.8394	0.3747	2.3872	2.4086	1.5863	1.2325	-0.7005	-0.4239	-0.2820	0.9028
log2(Fold change),12865	2.6615	2.2448	1.7002	1.9103	0.9655	0.5743	0.1917	0.3165	0.3041	0.1577
log2(Fold change),12873	2.4705	1.0043	2.1128	2.3516	1.7052	0.9364	0.5969	0.9485	0.4881	2.0822
log2(Fold change),12874	0.6079	0.2431	1.3899	1.7625	2.8985	1.7662	-0.7285	0.0528	-0.5716	1.3045
log2(Fold change),12875	2.9349	2.7047	2.7065	3.6441	1.6330	1.0589	-0.3735	-0.0822	0.3172	1.0914
log2(Fold change),06993	0.7047	0.4403	0.4600	1.6469	0.8997	0.7142	-0.3211	-0.3015	-0.1321	0.3423
log2(Fold change),07345	2.2037	2.2761	2.1667	1.8079	0.8235	1.0247	-0.6415	-0.5937	-0.5530	1.3542
log2(Fold change),07348	2.5347	3.1870	2.0832	2.3496	2.1286	1.1048	-0.7925	-0.1667	-0.2131	1.4882
log2(Fold change),10830	1.3277	0.4112	2.6306	1.6482	2.1627	1.3300	-0.7983	-0.3041	-0.3559	0.2689
log2(Fold change),10847	2.2810	1.5667	1.9622	2.1834	1.6787	1.1945	0.0121	0.0940	0.1356	1.5566
log2(Fold change),10859	1.3222	2.4407	2.2588	1.9676	2.4737	1.5704	-0.1613	0.1447	0.0693	1.0542
log2(Fold change),11881	1.2240	0.9365	2.4372	2.0654	1.3216	0.9636	0.2505	0.6566	-0.1182	1.2620
log2(Fold change),12239	1.0091	0.4244	1.5875	1.8724	1.3747	0.9154	-0.4329	-0.6378	-0.1554	1.1240
log2(Fold change),12249	2.6315	2.9128	2.7973	2.1078	1.8752	1.1272	-0.1109	-0.1264	0.1294	1.2806
log2(Fold change),12707	0.5476	0.2003	0.6358	0.6874	1.0678	0.6106	0.0277	0.2802	0.1991	0.5099
log2(Fold change),12752	-1.2834	-0.6470	-1.1881	-1.0769	-1.0197	-0.6470	-0.0241	-0.0386	0.0948	-0.6130
log2(Fold change),12760	1.3677	0.9810	1.8752	1.4394	0.9918	0.6470	-0.6455	-0.5463	-0.1157	1.0406

Gene	CXCL10	CXCL9	UBD	GBP4	NFKBIZ	ZC3H12A	HS.583806	HS.579631	BCYRN1	GBP5
log2(Fold change),12872	0.4820	0.1475	2.0906	1.8023	1.4096	1.3010	0.2425	0.2407	0.0651	1.1439
Delta FC (TT)- (CC)	0.671	0.489	0.351	0.298	0.270	0.257	0.251	0.238	0.218	0.207
Linear Regression p value	0.056	0.092	0.184	0.411	0.379	0.113	0.189	0.182	0.109	0.343

Table A.4 Illumina Golden Gate primer probe sets.

Illumina Golden Gate Assay Primer Probe Sets

Ilmn ID	Name	oligo1	oligo2	oligo3	IllumiCode	Illumicode Seq	Ilmn Str	SNP	CHR	Ploidy	Species	MapInfo	TopGenomicSeq	CustomerS	Olicode Na	Olicode Sequence
rs8065233-123_B_F_1951671	rs8065233	ACTTCGTCA	GAGTCGAG	CTGTCCTGA	70	AGCGTACTCGACCGATCC	BOT	[T/C]	17	diploid	homo sapiens	21147347	GCATCTTGGAGGG	BOT	70	AGCGTACTCGACCGATCCCTG
rs3774934-121_T_F_1951679	rs3774934	ACTTCGTCA	GAGTCGAG	TTATCTAAA	103	TACACAGCGACCGTACCA	TOP	[A/G]	4	diploid	homo sapiens	1.04E+08	AATTTCTGTACACT	TOP	103	TACACAGCGACCGTACCATCGT
rs4251513-123_B_F_1951683	rs4251513	ACTTCGTCA	GAGTCGAG	ATGGAAGA	111	ATCACTCACGACGGGCCT	BOT	[G/C]	12	diploid	homo sapiens	42460471	ATCCATGAAGGATA	BOT	111	ATCACTCACGACGGGCCTAATC
rs967779-123_T_F_1951692	rs967779	ACTTCGTCA	GAGTCGAG	AAGAAATA	147	GTTGTGAGACGCGCCTC	TOP	[A/G]	6	diploid	homo sapiens	91310307	CCTAAAGGGATAA	TOP	147	GTTGTGAGACGCGCCTCGAAC
rs2784069-120_T_F_1951771	rs2784069	ACTTCGTCA	GAGTCGAG	GCTGTGTG	654	ACATCGCCGGTATGCCTC	TOP	[A/G]	9	diploid	homo sapiens	1.37E+08	CCAGGAGGCAGA	TOP	654	ACATCGCCGGTATGCCTCTACG
rs205342-123_T_F_1951796	rs205342	ACTTCGTCA	GAGTCGAG	AGTTGATAT	747	AGGCGATAGGCTGTACG	TOP	[A/G]	6	diploid	homo sapiens	91286166	GGGCAGGATGAG	TOP	747	AGGCGATAGGCTGTACGAATC
rs739141-123_T_R_1951822	rs739141	ACTTCGTCA	GAGTCGAG	AACCTTCC	859	CACCGACTTAATTCCGAA	TOP	[A/G]	22	diploid	homo sapiens	38148950	AAGCACATTAGCC	BOT	859	CACCGACTTAATTCCGAAGGC
rs614700-121_T_F_1951844	rs614700	ACTTCGTCA	GAGTCGAG	AGAGCTTC	933	GCTCTCAGAACGATTAGC	TOP	[A/G]	11	diploid	homo sapiens	1.26E+08	TAGGAAGGGCTAC	TOP	933	GCTCTCAGAACGATTAGCGCG
rs4251520-123_B_F_1951860	rs4251520	ACTTCGTCA	GAGTCGAG	TTAACTTCT	969	CTAGGCAAGGGCGTCTTA	BOT	[T/C]	12	diploid	homo sapiens	42461605	ACAATGGTAATATT	BOT	969	CTAGGCAAGGGCGTCTTACCG
rs11722146-123_T_F_1951965	rs11722146	ACTTCGTCA	GAGTCGAG	CACCATTGA	1331	CCGACAAGCCGTATGTGC	TOP	[A/G]	4	diploid	homo sapiens	1.04E+08	GTAGCTATGATTTT	TOP	1331	CCGACAAGCCGTATGTGCTGG
rs7222094-123_B_F_1951979	rs7222094	ACTTCGTCA	GAGTCGAG	ACCATCTGT	1377	ACGACTTCGTATAGGCTG	BOT	[T/C]	17	diploid	homo sapiens	40723436	GGAGAGAGGTCT	BOT	1377	ACGACTTCGTATAGGCTGGCA
rs6001585-123_T_F_1952032	rs6001585	ACTTCGTCA	GAGTCGAG	TTAGGGAG	1671	GTGCCGCAACTGATAAG	TOP	[A/C]	22	diploid	homo sapiens	38137486	AGTCGCAATTGCC	TOP	1671	GTGCCGCAACTGATAAGCGTT
rs10775533-121_T_R_1952077	rs10775533	ACTTCGTCA	GAGTCGAG	CAAGCTAC	1799	ATAGACCACACCTACGCC	TOP	[C/G]	19	diploid	homo sapiens	44092015	GGTTACAGTGAGG	BOT	1799	ATAGACCACACCTACGCCGTGT
rs7949-123_T_F_1952081	rs7949	ACTTCGTCA	GAGTCGAG	AGTCAAAG	1824	TCACTCAAGGCCGTTAGC	TOP	[A/G]	22	diploid	homo sapiens	38152053	CAGGACCCCCCGG	TOP	1824	TCACTCAAGGCCGTTAGCTGTC
rs11591741-123_B_R_1952095	rs11591741	ACTTCGTCA	GAGTCGAG	TTACATTTG	1868	GACTCTGGATGAGCGGTA	BOT	[G/C]	10	diploid	homo sapiens	1.02E+08	AGCTCAGGGGTTC	TOP	1868	GACTCTGGATGAGCGGTAACG
rs7903344-123_B_F_1952115	rs7903344	ACTTCGTCA	GAGTCGAG	AAACTAGA	1935	GATCTCAGCATCGCCTAG	BOT	[T/C]	10	diploid	homo sapiens	1.02E+08	AGAAAATGGACAA	BOT	1935	GATCTCAGCATCGCCTAGCAAC
rs6853-106_T_F_1952200	rs6853	ACTTCGTCA	GAGTCGAG	GAGGCATC	2174	ACTGCAATACAGGACCGT	TOP	[A/G]	3	diploid	homo sapiens	38159374	CTTGGGAGGGAAT	TOP	2174	ACTGCAATACAGGACCGTTCG
rs1059701-120_T_R_1952228	rs1059701	ACTTCGTCA	GAGTCGAG	CCTGAATG	2257	CAACTGCTTGATGCCGCA	TOP	[A/G]	X	diploid	homo sapiens	1.53E+08	CATCAAAGGCTGA	BOT	2257	CAACTGCTTGATGCCGCACAC
rs646005-120_T_F_1952234	rs646005	ACTTCGTCA	GAGTCGAG	TTGGAATG	2281	TATAGCGTGGGGCCATTG	TOP	[A/G]	11	diploid	homo sapiens	1.26E+08	CATTAATGCTTTGT	TOP	2281	TATAGCGTGGGGCCATTGAGC
rs4803006-124_B_R_1952252	rs4803006	ACTTCGTCA	GAGTCGAG	GTCTTATCA	2328	GTAGTCTTTGGTCACGCG	BOT	[T/C]	19	diploid	homo sapiens	44083387	TACCACGTTCCAG	TOP	2328	GTAGTCTTTGGTCACGCGGGA
rs230521-123_B_F_1952274	rs230521	ACTTCGTCA	GAGTCGAG	AGAAAATG	2401	GGTTGACGGCCTCCTTAG	BOT	[G/C]	4	diploid	homo sapiens	1.04E+08	AGAACATGTGAAA	BOT	2401	GGTTGACGGCCTCCTTAGAGT
rs11190421-120_T_F_1952308	rs11190421	ACTTCGTCA	GAGTCGAG	AACCTAAA	2630	ACGCGGATTGACTGTCCA	TOP	[A/G]	10	diploid	homo sapiens	1.02E+08	ACAGACACAATCT	TOP	2630	ACGCGGATTGACTGTCCAGAA
rs9972936-123_T_F_1952312	rs9972936	ACTTCGTCA	GAGTCGAG	CTTTTAAA	2661	TGCGTTTCACCCACACCG	TOP	[A/G]	17	diploid	homo sapiens	40709460	GGCCACTCCCACT	TOP	2661	TGCGTTTCACCCACACCGAAG
rs3747811-123_T_F_1952320	rs3747811	ACTTCGTCA	GAGTCGAG	GGAGAGA	2708	ATGGACACGACATCATGC	TOP	[A/T]	8	diploid	homo sapiens	42248662	TAACTCTATAAGCA	TOP	2708	ATGGACACGACATCATGCTGG
rs540386-123_T_R_1952322	rs540386	ACTTCGTCA	GAGTCGAG	CCCAGCTG	2717	TTGCAGGAAGGGCGATC	TOP	[A/G]	11	diploid	homo sapiens	36481869	TCTGAGTATGCCTG	BOT	2717	TTGCAGGAAGGGCGATCCGAA
rs696-121_B_R_1952337	rs696	ACTTCGTCA	GAGTCGAG	CAGTGTGC	2768	GATAGCTTGGGCACAAC	BOT	[T/C]	14	diploid	homo sapiens	34940844	AAGGGGCTGAAA	TOP	2768	GATAGCTTGGGCACAACCCGT
rs2074293-123_B_F_1952399	rs2074293	ACTTCGTCA	GAGTCGAG	CGTACCCA	2927	GGGTGTGTCCTACAAGA	BOT	[T/C]	17	diploid	homo sapiens	40699856	GTTCCAGCTGGAG	BOT	2927	GGGTGTGTCCTACAAGACCGT
rs1049728-120_B_F_1952403	rs1049728	ACTTCGTCA	GAGTCGAG	CTTGCTCTT	2939	CTCCTCAAGGCACTAGCT	BOT	[G/C]	11	diploid	homo sapiens	65177693	CTACCACCAATGTC	BOT	2939	CTCCTCAAGGCACTAGCTCGAT
rs3802681-120_T_F_1952425	rs3802681	ACTTCGTCA	GAGTCGAG	TAAGTTGTC	3001	CAGCGTGAGTATCATTGT	TOP	[A/G]	10	diploid	homo sapiens	1.04E+08	TTCAAGTCTTCCTT	TOP	3001	CAGCGTGAGTATCATTGTGGG
rs12453226-123_T_R_1952429	rs12453226	ACTTCGTCA	GAGTCGAG	CCAAGTTC	3014	GACATGAGTGCATTGCTC	TOP	[C/G]	17	diploid	homo sapiens	64951048	AGGGTCTAACACT	BOT	3014	GACATGAGTGCATTGCTCCGC
rs10283820-120_B_R_1952438	rs10283820	ACTTCGTCA	GAGTCGAG	CACACAAG	3041	CGTTTGAAGCAGTCCACC	BOT	[T/C]	9	diploid	homo sapiens	1.37E+08	GGTCAGCATTGTG	TOP	3041	CGTTTGAAGCAGTCCACGTCG
rs7086205-120_B_F_1952463	rs7086205	ACTTCGTCA	GAGTCGAG	ACAGCTTTC	3101	CACCGTCTCTGCTATCGA	BOT	[T/C]	10	diploid	homo sapiens	1.04E+08	CCAATAAGATATTT	BOT	3101	CACCGTCTCTGCTATCGAGCGA
rs7744-120_B_R_1952465	rs7744	ACTTCGTCA	GAGTCGAG	AGAGGTAT	3105	GGTCCACGACTACTTTGG	BOT	[T/C]	3	diploid	homo sapiens	38159025	GGGAGCCTAACCA	TOP	3105	GGTCCACGACTACTTTGGGGA
rs11242823-120_T_F_1952504	rs11242823	ACTTCGTCA	GAGTCGAG	TGAATGGG	3314	GGAAAGGCTGCTCCATC	TOP	[A/G]	6	diploid	homo sapiens	3026578	CTTATAACTGTTGA	TOP	3314	GGAAAGGCTGCTCCATCGAAC
rs2233415-121_B_F_1952512	rs2233415	ACTTCGTCA	GAGTCGAG	CCCTCACCT	3347	TCATCTCGGATCAGCCCA	BOT	[T/C]	14	diploid	homo sapiens	34942543	GGGGGTGGGGAG	BOT	3347	TCATCTCGGATCAGCCCAGGC
rs230542-123_B_F_1952533	rs230542	ACTTCGTCA	GAGTCGAG	CCATCTCTG	3450	CGATTGTCGAAGGCAGT	BOT	[T/C]	4	diploid	homo sapiens	1.04E+08	AGTCTTGATTTCTG	BOT	3450	CGATTGTCGAAGGCAGTGAGC
rs2239621-123_B_F_1952555	rs2239621	ACTTCGTCA	GAGTCGAG	CCTATGAG	3517	CAGGCGACACTCCTTCCA	BOT	[T/C]	3	diploid	homo sapiens	38148737	GATACACAGTTTTC	BOT	3517	CAGGCGACACTCCTTCCAAGA
rs3818411-120_T_R_1952583	rs3818411	ACTTCGTCA	GAGTCGAG	GTGGGAAA	3676	CGCTTGCACGGTCGAACA	TOP	[A/G]	10	diploid	homo sapiens	1.02E+08	TTCTCTAGGTGGCA	BOT	3676	CGCTTGCACGGTCGAACATATT
rs5750824-123_B_R_1952584	rs5750824	ACTTCGTCA	GAGTCGAG	GTACATGA	3677	GCACTAATCGACCGTGTT	BOT	[T/C]	22	diploid	homo sapiens	38154623	TAGGTCCCAGCCA	TOP	3677	GCACTAATCGACCGTGTTTAGC
rs10958715-123_B_R_1952585	rs10958715	ACTTCGTCA	GAGTCGAG	ATCTAATCT	3686	CTCGAAATAGGCGACTG	BOT	[T/C]	8	diploid	homo sapiens	42305360	TAGAGCTGAGGTT	TOP	3686	CTCGAAATAGGCGACTGCGAA
rs157688-123_T_F_1952586	rs157688	ACTTCGTCA	GAGTCGAG	AAGAAAAC	3690	GTTGCACAACGAGTCCG	TOP	[A/G]	6	diploid	homo sapiens	91317588	TATTCAATCTATTG	TOP	3690	GTTGCACAACGAGTCCGATCT
rs7218577-123_T_R_1952589	rs7218577	ACTTCGTCA	GAGTCGAG	CTTTCCAG	3695	TTTCGACTCCGTAACACG	TOP	[A/G]	17	diploid	homo sapiens	21155025	ACTGCCGGCCGTC	BOT	3695	TTTCGACTCCGTAACACGACTC
rs730775-125_T_F_1952594	rs730775	ACTTCGTCA	GAGTCGAG	ACACGACA	3711	TATCGTAACCTTGCGAAG	TOP	[A/G]	6	diploid	homo sapiens	44340052	AATCCTCAACTGG	TOP	3711	TATCGTAACCTTGCGAAGAGC
rs2326173-121_B_R_1952611	rs2326173	ACTTCGTCA	GAGTCGAG	CCCCAAAC	3783	GTTTCCGAAGCCCAACG	BOT	[T/C]	6	diploid	homo sapiens	3020552	TGGTGTATTAGTCT	TOP	3783	GTTTCCGAAGCCCAACGTAGT
rs1409312-120_T_F_1952675	rs1409312	ACTTCGTCA	GAGTCGAG	AGAAGCAG	3970	ATGCCAAGACGTAGCCA	TOP	[A/G]	10	diploid	homo sapiens	1.04E+08	CCCCTCAATGCCC	TOP	3970	ATGCCAAGACGTAGCCACTTG
rs3138055-121_B_R_1952770	rs3138055	ACTTCGTCA	GAGTCGAG	GAGAGGTC	4162	ACCTTCAAACCGGGTGG	BOT	[T/C]	14	diploid	homo sapiens	34940205	GCTTTGCAGGCAC	TOP	4162	ACCTTCAAACCGGGTGGCATT
rs2282151-125_T_R_1952835	rs2282151	ACTTCGTCA	GAGTCGAG	AGGAAGAA	4292	CAACAGACCATTACTTGC	TOP	[A/G]	6	diploid	homo sapiens	44334173	CTGAGCACGGTAG	BOT	4292	CAACAGACCATTACTTGCGGG
rs1548444-123_T_R_1952838	rs1548444	ACTTCGTCA	GAGTCGAG	CAAATGAA	4300	ATACGCACGTTTTGAGGG	TOP	[A/C]	17	diploid	homo sapiens	64988702	TCTGGGGACAGAT	BOT	4300	ATACGCACGTTTTGAGGGTAG
rs10781520-120_B_R_1952858	rs10781520	ACTTCGTCA	GAGTCGAG	CTCAGTGA	4872	CTCGAATTGGACTCGCAC	BOT	[T/C]	9	diploid	homo sapiens	1.37E+08	CTCAGGACAGCTT	TOP	4872	CTCGAATTGGACTCGCACTTAT
rs11227247-120_T_R_1952931	rs11227247	ACTTCGTCA	GAGTCGAG	GCACACAC	5123	GATGCCAATATGACGATT	TOP	[A/C]	11	diploid	homo sapiens	65179429	GATAAGCTGGACT	BOT	5123	GATGCCAATATGACGATTGCTA
rs3799962-125_T_F_1953000	rs3799962	ACTTCGTCA	GAGTCGAG	CTCAAAGT	5444	CCTCAATCTTATTCGGCA	TOP	[A/G]	6	diploid	homo sapiens	44336430	TCTCTTCTGGGAC	TOP	5444	CCTCAATCTTATTCGGCAGTGT
rs1957106-123_T_F_1953001	rs1957106	ACTTCGTCA	GAGTCGAG	CCAGTAGC	5449	GCTTAATCTCGTGAGGGT	TOP	[A/G]	14	diploid	homo sapiens	34943521	CTTCCAGGGCTCC	TOP	5449	GCTTAATCTCGTGAGGGTAGG
rs5030411-121_T_R_1953017	rs5030411	ACTTCGTCA	GAGTCGAG	GAAGGATG	5546	CATACGCGAATTGATAGA	TOP	[A/G]	11	diploid	homo sapiens	36490329	CAATATAGGAGAG	BOT	5546	CATACGCGAATTGATAGAAGC
rs2586545-123_T_R_1953027	rs2586545	ACTTCGTCA	GAGTCGAG	TTCCGTTTT	5581	TGCCCTATTGCTACTATAC	TOP	[A/G]	17	diploid	homo sapiens	64964613	TTGACCATTTCATG	BOT	5581	TGCCCTATTGCTACTATACGTG
rs4959774-121_T_F_1953122	rs4959774	ACTTCGTCA	GAGTCGAG	GTTCTTTTC	5934	CGTCGGAGGTATTCAGTC	TOP	[A/G]	6	diploid	homo sapiens	3021335	ACCTTCTTTTCTTG	TOP	5934	CGTCGGAGGTATTCAGTCACTA
rs2303439-121_B_F_1953129	rs2303439	ACTTCGTCA	GAGTCGAG	GAATAGGT	5961	GTATCAGGCATTTGTCGT	BOT	[T/C]	11	diploid	homo sapiens	36470866	AATGAATTTTGGA	BOT	5961	GTATCAGGCATTTGTCGTTAGC
rs5029748-123_T_F_1953144	rs5029748	ACTTCGTCA	GAGTCGAG	GAACAAGT	6015	AGTCCGTTCCAGAAGGG	TOP	[A/C]	8	diploid	homo sapiens	42259706	CCCAACCCAGAAT	TOP	6015	AGTCCGTTCCAGAAGGGTCTT
rs1786697-121_B_F_1953174	rs1786697	ACTTCGTCA	GAGTCGAG	TGAGCTGT	6130	TGCTAATACTCTTCTCTCG	BOT	[G/C]	11	diploid	homo sapiens	1.26E+08	GACACTCGAGGAA	BOT	6130	TGCTAATACTCTTCTCTCGACG
rs1145727-123_B_R_1953175	rs1145727	ACTTCGTCA	GAGTCGAG	GGGCATGG	6131	ATAGTCCGAAGGAATGCT	BOT	[T/C]	6	diploid	homo sapiens	91285144	AAAACATAATTAAA	TOP	6131	ATAGTCCGAAGGAATGCTGAC

Appendix B Supplemental Tables and Figures for Chapter 3: TNFα Induces Tumor Necrosis Factor Alpha Induced Protein 2 (TNFAIP2) Expression Which Inhibits Ras and NF-κB Signaling and Contributes to Mortality and Organ Dysfunction in Septic Shock Patients

Table B.1 Results from literature search

1. Analysis of gene expression profiles in HeLa cells in response to overexpression or siRNA-mediated depletion of NASP.

   Alekseev OM, Richardson RT, Alekseev O, O'Rand MG.

   Reprod Biol Endocrinol. 2009 May 13;7:45.

   PMID: 19439102 [PubMed - indexed for MEDLINE] **Free PMC Article**

   Free full text Related citations

2. The LIM-homeodomain transcription factor LMX1B regulates expression of NF-kappa B target genes.

   Rascle A, Neumann T, Raschta AS, Neumann A, Heining E, Kastner J, Witzgall R.

   Exp Cell Res. 2009 Jan 1;315(1):76-96. Epub 2008 Oct 28.

   PMID: 18996370 [PubMed - indexed for MEDLINE]

Related citations

3. Epoxyquinol B, a naturally occurring pentaketide dimer, inhibits NF-kappaB signaling by crosslinking TAK1.

   Kamiyama H, Usui T, Sakurai H, Shoji M, Hayashi Y, Kakeya H, Osada H.

   Biosci Biotechnol Biochem. 2008 Jul;72(7):1894-900. Epub 2008 Jul 7.

   PMID: 18603781 [PubMed - indexed for MEDLINE] **Free Article**

   Related citations

4. Alteration in the activation state of new inflammation-associated targets by phospholipase A2-activating protein (PLAA).

   Zhang F, Sha J, Wood TG, Galindo CL, Garner HR, Burkart MF, Suarez G, Sierra JC, Agar SL, Peterson JW, Chopra AK.

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   Free full text Related citations

5. The AP-2alpha transcription factor regulates tumor cell migration and apoptosis.

   Orso F, Fassetta M, Penna E, Solero A, De Filippo K, Sismondi P, De Bortoli M, Taverna D.

Adv Exp Med Biol. 2007;604:87-95.

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6. Small interfering RNAs generated by recombinant dicer induce inflammatory gene expression independent from the TAK1-NFkappaB-MAPK signaling pathways.

Kettner-Buhrow D, Dittrich-Breiholz O, Schneider H, Wolter S, Resch K, Kracht M.

Biochem Biophys Res Commun. 2006 Sep 1;347(3):566-73. Epub 2006 Jun 30.

PMID: 16843436 [PubMed - indexed for MEDLINE]

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7. A TNF-induced gene expression program under oscillatory NF-kappaB control.

Tian B, Nowak DE, Brasier AR.

BMC Genomics. 2005 Sep 28;6:137.

PMID: 16191192 [PubMed - indexed for MEDLINE] **Free PMC Article**

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8. Identification of direct genomic targets downstream of the nuclear factor-kappaB transcription factor mediating tumor necrosis factor signaling.

Tian B, Nowak DE, Jamaluddin M, Wang S, Brasier AR.

J Biol Chem. 2005 Apr 29;280(17):17435-48. Epub 2005 Feb 18.

PMID: 15722553 [PubMed - indexed for MEDLINE] **Free Article**

Related citations

9. Microarray analysis of tumor necrosis factor alpha induced gene expression in U373 human glioblastoma cells.

Schwamborn J, Lindecke A, Elvers M, Horejschi V, Kerick M, Rafigh M, Pfeiffer J, Prüllage M, Kaltschmidt B, Kaltschmidt C.

BMC Genomics. 2003 Nov 25;4(1):46.

PMID: 14641910 [PubMed - indexed for MEDLINE] **Free PMC Article**

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10. TRAF2 exerts its antiapoptotic effect by regulating the expression of Krüppel-like factor LKLF.

Lin Y, Ryan J, Lewis J, Wani MA, Lingrel JB, Liu ZG.

Mol Cell Biol. 2003 Aug;23(16):5849-56.

PMID: 12897154 [PubMed - indexed for MEDLINE] **Free PMC Article**

Free full text Related citations

11. Identification of NF-kappa B-regulated genes induced by TNFalpha utilizing expression profiling and RNA interference.

Zhou A, Scoggin S, Gaynor RB, Williams NS.

Oncogene. 2003 Apr 3;22(13):2054-64. Erratum in: Oncogene. 2004 Dec 16;23(58):9447.

PMID: 12673210 [PubMed - indexed for MEDLINE]

Related citations

12. Chlamydial infection of polarized HeLa cells induces PMN chemotaxis but the cytokine profile varies between disseminating and non-disseminating strains.

Dessus-Babus S, Knight ST, Wyrick PB.

Cell Microbiol. 2000 Aug;2(4):317-27.

PMID: 11207588 [PubMed - indexed for MEDLINE]

Related citations

Table B.2 Cross referenced lists of 2 published microarrays with HT-12 human microarray data

Thair et al.	Fold Change	Tian et al, 2005 JBC	Fold Change	Zhou et al, 2003 Oncogene	Fold Change
OLR1	12.7	Naf-1	46.0	CCL2	5.0
PTGS2	8.8	IL-6	17.3	NFKB1	4.7
TNFAIP3	7.7	IL-8	13.3	KALRN	4.4
SOD2	5.2	TNFAIP3	12.0	PSMB8	4.2
NFKB2	5.1	TRAF-1	11.9	NFKBIA	4.0
BCL3	5.1	NFKB2	9.6	MRPL10	3.9
C1QTNF1	4.9	CCL-20	7.9	CLIC4	3.7
EFNA1	4.8	CD83	7.6	TNFAIP2	3.7
IRF1	4.7	URF-1	7.1	MRPL10	3.6
C8ORF4	4.7	TNFAIP2	5.9	NFKBIA	3.5
CCL20	4.6	CXCL2/ Gro beta	5.8	TSPAN4	3.5
IER3	4.4	RelB	5.7	IL32	3.3
STAT5A	4.3	GTP cyclohydrolase	5.2	TNFAIP2	3.3
MMP12	4.3	Syndecan-4	5.2	RPS8	3.2
NFKBIA	4.0	NFKBIA	5.1	CASP3	3.2
LOC653156	3.9	IkBe	5.0	GDI1	3.0
JUNB	3.8	IL-7R	4.6	MAN2A1	3.0
IRAK2	3.7	Cox-2	4.4	MYL6	3.0
TNF	3.7	CXCL1	4.3	DROSHA	3.0
IL32	3.6	GFPT2	4.3	FBN1	2.9
RELB	3.6	NFKB1	4.1	GINS2	2.9
TNFAIP2	3.5	Polo-like kinase 2	3.2	PTGES	2.9
DRAM1	3.4	TNFRS9	2.8	CMPK1	2.9
BIRC2	3.3	CXCL3/ Gro gamma	2.7	ACLY	2.8
TNIP1	3.3	c-Rel	2.0	CD59	2.8
GFPT2	3.2	TTP/zinc finger 36	2.0	CYR61	2.8

Thair et al.	Fold Change	Tian et al, 2005 JBC	Fold Change	Zhou et al, 2003 Oncogene	Fold Change
MEOX1	3.1	Follistatin	0.6	PLP2	2.8
GRAMD1B	3.0	CTGF	0.3	N/A	2.8
MUC13	3.0			CXCL1	2.8
EIF4A3	3.0			GADD45A	2.8
CFB	2.9			PPP1R10	2.8
HSPB3	2.9			PLP2	2.8
IKBKE	2.8			RPL27A	2.8
PTPN1	2.8			CDC34	2.7
NFKBIE	2.8			SHFM1	2.7
IL27RA	2.8			ITGB1	2.7
LOC441019	2.7			TNIP1	2.7
PIGA	2.7			A1BG	2.7
LOC100129685	2.7			CCL2	2.7
TAP1	2.7			SOD2	2.7
SAMD4A	2.6			SMARCE1	2.7
ITGA5	2.6			PR	2.7
BHLHB2	2.6			N/A	2.6
IL8	2.6			NA	2.6
EXT1	2.6			ELOVL5	2.6
CEBPD	2.6			MYL12B	2.6
NCOA7	2.5			NFKB1	2.6
NFKB1	2.5			NFKBIA	2.6
GPRC5A	2.5			NCL	2.6
STX11	2.5			MRPL11	2.6
STAMBPL1	2.4			C20orf30	2.5
ADM	2.4			HNF1A	2.5
TRIML2	2.4			EBP	2.5
FNDC3B	2.4			N/A	2.5

Thair et al.	Fold Change	Tian et al, 2005 JBC	Fold Change	Zhou et al, 2003 Oncogene	Fold Change
PLA2G4A	2.4			EEF2	2.5
ZFP36	2.4				
ZSWIM4	2.4				
RPL12P6	2.4				
DKK1	2.4				
BIRC3	2.4				
COTL1	2.4				
TNFRSF12A	2.3				
IER5	2.3				
IFNGR1	2.3				
SLC27A2	2.3				
SDC4	2.3				
DUSP16	2.3				
STEAP4	2.3				
ABTB2	2.2				
PTGES	2.2				
SERPINB8	2.2				
EGLN1	2.2				
PHLDA1	2.2				
GABARAPL1	2.2				
KIAA1949	2.2				
BMP2	2.2				
DUSP5	2.2				
GSK3B	2.2				
EHD4	2.1				
LIMA1	2.1				
ECGF1	2.1				
CDH10	2.1				
PGM2L1	2.1				

Thair et al.	Fold Change	Tian et al, 2005 JBC	Fold Change	Zhou et al, 2003 Oncogene	Fold Change
CSRP1	2.1				
HS.579631	2.1				
NNMT	2.1				
LOC100130835	2.1				
CXORF38	2.1				
C3ORF52	2.1				
LOC100133800	2.1				
C21ORF63	2.0				
LOC221710	2.0				
GPX1	2.0				
C12ORF34	2.0				
HDC	2.0				
TESC	2.0				
EFHD2	2.0				
KLRC2	2.0				
ICAM1	2.0				

Figure B.1 Venn diagram of overlapping genes discovered by cross reference of microarray data.

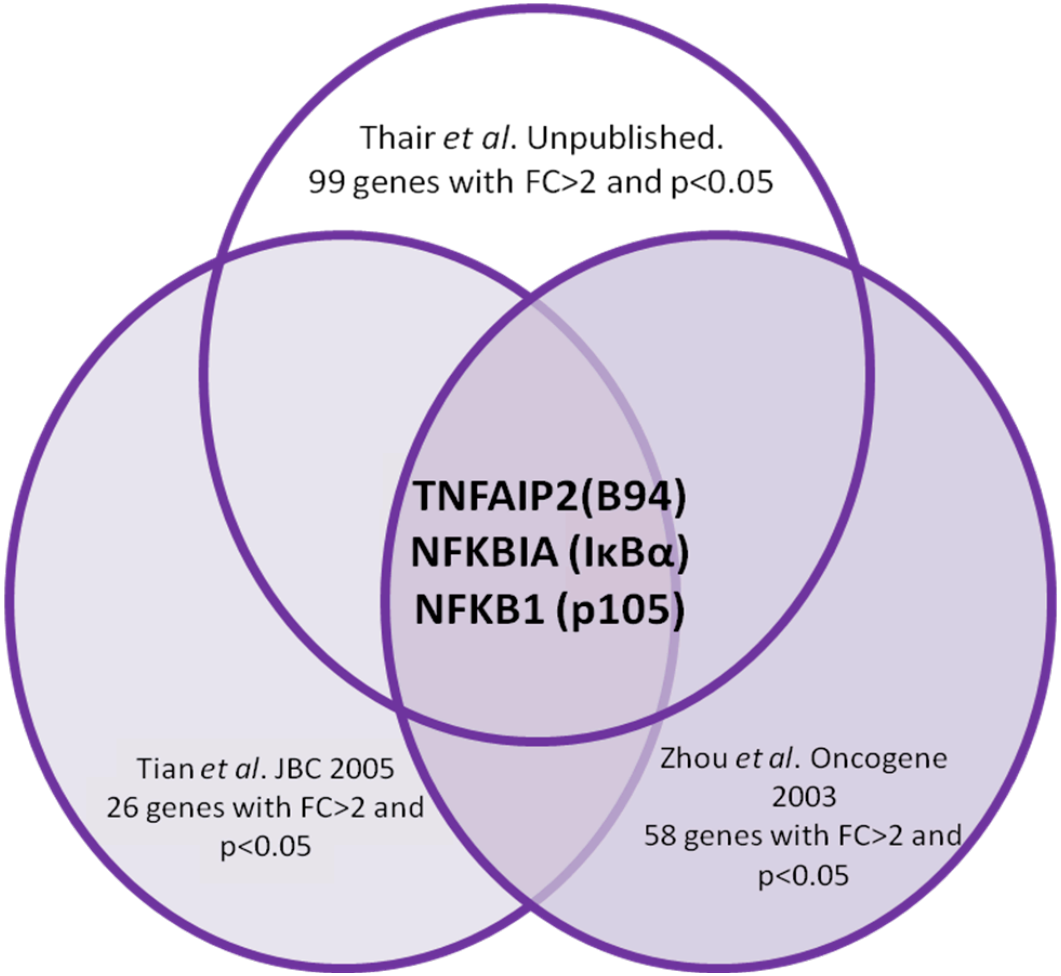

Table B.3 Summary of mass spectrometry findings

Access-ion	# AA	MW kDa	pI	Description	Σ Cover-age	Σ# PSMs	Σ# Pep-tides	A7: Area	4h/ 30 min	4h/ 30 min coun t	Variabili-ty [%]	Score A(3,5)	Cover-age A(3,5)	# PSM A(3,5)	# Pep-tides A(3,5)
B0YJC 4	431	49.6	5.2	Uncharacteri zed protein OS=Homo sapiens GN=VIM PE=3 SV=1 - [B0YJC4_H UMAN]	21.58	9	8	8.45 6E8	0.87 2	6	49.2	929.0 9	21.58	9	8
B1AN R0	615	67.9	9.4	Poly(A) binding protein, cytoplasmic 4 (Inducible form) OS=Homo sapiens GN=PABPC 4 PE=4 SV=1 - [B1ANR0_H UMAN]	9.27	5	4	3.81 4E8	1.03 5	4	12.0	358.2 9	9.27	5	4

Access-ion	# AA	MW kDa	pI	Description	Σ Cover-age	Σ# PSMs	Σ# Pep-tides	A7: Area	4h/ 30 min	4h/ 30 min coun t	Variabili-ty [%]	Score A(3,5)	Cover-age A(3,5)	# PSM A(3,5)	# Pep-tides A(3,5)
B3KX72	750	83.0	8.7	Uncharacterized protein OS=Homo sapiens GN=HNRNPU PE=2 SV=1 - [B3KX72_HUMAN]	8.40	5	4	1.816E8	1.846	3	101.3	376.91	8.40	5	4
B4DR70	429	44.8	9.0	Uncharacterized protein OS=Homo sapiens GN=FUS PE=2 SV=1 - [B4DR70_HUMAN]	10.49	4	3	8.760E7	0.365	2	3.4	369.65	10.49	4	3
B4E3E8	646	71.3	6.6	Uncharacterized protein OS=Homo sapiens GN=DDX3X PE=2 SV=1 - [B4E3E8_HUMAN]	11.92	8	6	8.226E8	0.852	4	23.7	752.20	11.92	8	6

Access-ion	# AA	MW kDa	pI	Description	Σ Cover-age	Σ# PSMs	Σ# Pep-tides	A7: Area	4h/ 30 min	4h/ 30 min coun t	Variabili-ty [%]	Score A(3,5)	Cover-age A(3,5)	# PSM A(3,5)	# Pep-tides A(3,5)
E7EP11	232	25.2	6.9	Uncharacterized protein OS=Homo sapiens GN=HSPA1A PE=3 SV=1 - [E7EP11_HUMAN]	11.64	3	2	8.109E8	0.618	2	1028.1	222.33	11.64	3	2
E7ETL9	544	61.6	8.9	Uncharacterized protein OS=Homo sapiens GN=DDX5 PE=3 SV=1 - [E7ETL9_HUMAN]	14.71	9	7	1.575E9	1.140	6	19.0	780.79	14.71	9	7
E9PCY7	429	47.1	6.3	Uncharacterized protein OS=Homo sapiens GN=HNRNPH1 PE=4 SV=1 - [E9PCY7_HUMAN]	23.31	15	7	6.412E8	0.949	6	13.5	872.61	23.31	15	8

Access-ion	# AA	MW kDa	pI	Description	Σ Cover-age	Σ# PSMs	Σ# Pep-tides	A7: Area	4h/ 30 min	4h/ 30 min coun t	Variabili-ty [%]	Score A(3,5)	Cover-age A(3,5)	# PSM A(3,5)	# Pep-tides A(3,5)
E9PPG 9	217	24.0	6.0	Uncharacteri zed protein OS=Homo sapiens GN=RAE1 PE=4 SV=1 - [E9PPG9_H UMAN]	12.90	2	2	9.56 4E7	1.16 6	1		120.6 0	12.90	2	2
F8VUJ 7	415	46.5	4.9	Uncharacteri zed protein OS=Homo sapiens GN=TUBB PE=3 SV=1 - [F8VUJ7_H UMAN]	6.02	2	2	8.15 9E8	2.05 3	2	9.5	218.2 6	6.02	2	2
F8VZY 5	340	38.1	5.4	Uncharacteri zed protein OS=Homo sapiens GN=KRT7 PE=4 SV=1 - [F8VZY5_H UMAN]	25.59	10	8	7.28 0E8	1.44 2	7	60.1	742.6 4	23.53	10	8

Access-ion	# AA	MW kDa	pI	Description	Σ Cover-age	Σ# PSMs	Σ# Pep-tides	A7: Area	4h/ 30 min	4h/ 30 min coun t	Variabili-ty [%]	Score A(3,5)	Cover-age A(3,5)	# PSM A(3,5)	# Pep-tides A(3,5)
H0YJB9	129	14.7	5.6	Chromosome 14 open reading frame 166 (Fragment) OS=Homo sapiens GN=C14orf166 PE=4 SV=1 - [H0YJB9_HUMAN]	23.26	4	2	3.471E7	1.298	2	77.9	300.59	23.26	4	2
O94979-7	969	105.7	8.4	Isoform 7 of Protein transport protein Sec31A OS=Homo sapiens GN=SEC31A - [SC31A_HUMAN]	3.30	2	2	1.794E8	1.659	2	3.5	185.45	3.30	2	2

Access-ion	# AA	MW kDa	pI	Description	Σ Cover-age	Σ# PSMs	Σ# Pep-tides	A7: Area	4h/ 30 min	4h/ 30 min coun t	Variabili-ty [%]	Score A(3,5)	Cover-age A(3,5)	# PSM A(3,5)	# Pep-tides A(3,5)
O96019-2	387	43.2	6.1	Isoform HArpNbeta-s of Actin-like protein 6A OS=Homo sapiens GN=ACTL6A - [ACL6A_HUMAN]	5.68	2	2	1.372E8	0.117	1		124.50	5.68	2	2
P05783	430	48.0	5.4	Keratin, type I cytoskeletal 18 OS=Homo sapiens GN=KRT18 PE=1 SV=2 - [K1C18_HUMAN]	23.72	13	6	1.060E9	1.858	6	65.6	918.95	23.72	13	6
P09651-3	267	29.4	9.1	Isoform 2 of Heterogeneous nuclear ribonucleoprotein A1 OS=Homo sapiens GN=HNRNPA1 - [ROA1_HU	12.73	2	2	7.967E7	1.615	1		264.47	12.73	2	2

Access-ion	# AA	MW kDa	pI	Description	Σ Cover-age	Σ# PSMs	Σ# Pep-tides	A7: Area	4h/ 30 min	4h/ 30 min coun t	Variabili-ty [%]	Score A(3,5)	Cover-age A(3,5)	# PSM A(3,5)	# Pep-tides A(3,5)
				MAN]											
P11940-2	547	61.1	9.0	Isoform 2 of Polyadenylate-binding protein 1 OS=Homo sapiens GN=PABPC1 - [PABP1_HUMAN]	10.60	5	4	3.151E8	0.955	4	12.0	490.40	10.60	5	4
P22626-2	341	36.0	8.6	Isoform A2 of Heterogeneous nuclear ribonucleoproteins A2/B1 OS=Homo sapiens GN=HNRNPA2B1 -	37.54	11	8	1.343E9	0.639	6	290.4	660.69	37.54	11	8

Access-ion	# AA	MW kDa	pI	Description	Σ Cover-age	Σ# PSMs	Σ# Pep-tides	A7: Area	4h/ 30 min	4h/ 30 min coun t	Variabili-ty [%]	Score A(3,5)	Cover-age A(3,5)	# PSM A(3,5)	# Pep-tides A(3,5)
				[ROA2_HUMAN]											
P23246	707	76.1	9.4	Splicing factor, proline- and glutamine-rich OS=Homo sapiens GN=SFPQ PE=1 SV=2 - [SFPQ_HUMAN]	8.77	8	5	7.74 2E8	0.32 8	3	358.3	890.6 9	7.92	8	5
P55795	449	49.2	6.3	Heterogeneous nuclear ribonucleoprotein H2 OS=Homo sapiens GN=HNRNPH2 PE=1 SV=1 -	15.37	12	5	6.18 6E8	0.85 0	5	25.4	611.1 0	15.37	12	6

Access-ion	# AA	MW kDa	pI	Description	Σ Cover-age	Σ# PSMs	Σ# Pep-tides	A7: Area	4h/ 30 min	4h/ 30 min coun t	Variabili-ty [%]	Score A(3,5)	Cover-age A(3,5)	# PSM A(3,5)	# Pep-tides A(3,5)
				[HNRH2_HUMAN]											
Q08211	1270	140.9	6.8	ATP-dependent RNA helicase A OS=Homo sapiens GN=DHX9 PE=1 SV=4 - [DHX9_HUMAN]	3.94	4	4	6.68 5E8	24.329	3	8.7	410.33	3.46	4	4
Q12905	390	43.0	5.2	Interleukin enhancer-binding factor 2 OS=Homo sapiens GN=ILF2 PE=1 SV=2 - [ILF2_HUMAN]	6.15	2	2	2.30 2E8	1.741	2	37.1	142.86	6.15	2	2

Access-ion	# AA	MW kDa	pI	Description	Σ Cover-age	Σ# PSMs	Σ# Pep-tides	A7: Area	4h/ 30 min	4h/ 30 min coun t	Variabili-ty [%]	Score A(3,5)	Cover-age A(3,5)	# PSM A(3,5)	# Pep-tides A(3,5)
Q13283	**466**	**52.1**	**5.5**	**Ras GTPase-activating protein-binding protein 1 OS=Homo sapiens GN=G3BP1 PE=1 SV=1 - [G3BP1_HUMAN]**	**16.52**	**7**	**5**	**3.859E8**	**0.834**	**4**	**25.6**	**528.08**	**16.52**	**7**	**5**
Q15233	471	54.2	8.9	Non-POU domain-containing octamer-binding protein OS=Homo sapiens GN=NONO PE=1 SV=4 - [NONO_HUMAN]	19.11	18	6	7.743E8	1.015	6	97.5	1657.30	19.11	18	6

Access-ion	# AA	MW kDa	pI	Description	Σ Cover-age	Σ# PSMs	Σ# Pep-tides	A7: Area	4h/ 30 min	4h/ 30 min coun t	Variabili-ty [%]	Score A(3,5)	Cover-age A(3,5)	# PSM A(3,5)	# Pep-tides A(3,5)
Q5T6W2	379	41.8	5.5	Heterogeneous nuclear ribonucleoprotein K (Fragment) OS=Homo sapiens GN=HNRNPK PE=2 SV=1 - [Q5T6W2_HUMAN]	11.87	4	3	1.572E8	1.483	1		252.98	11.87	4	3
Q8WWM7	1075	113.3	8.5	Ataxin-2-like protein OS=Homo sapiens GN=ATXN2L PE=1 SV=2 - [ATX2L_HUMAN]	2.14	2	2	7.258E7	1.338	1		117.24	2.14	2	2

Access-ion	# AA	MW kDa	pI	Description	Σ Cover-age	Σ# PSMs	Σ# Pep-tides	A7: Area	4h/ 30 min	4h/ 30 min coun t	Variabili-ty [%]	Score A(3,5)	Cover-age A(3,5)	# PSM A(3,5)	# Pep-tides A(3,5)
Q92841	650	72.3	8.5	Probable ATP-dependent RNA helicase DDX17 OS=Homo sapiens GN=DDX17 PE=1 SV=1 - [DDX17_HUMAN]	17.08	10	9	1.448E9	0.978	7	29.6	875.24	17.08	10	9
Q96EP5-2	378	40.5	8.2	Isoform 2 of DAZ-associated protein 1 OS=Homo sapiens GN=DAZAP1 - [DAZP1_HUMAN]	8.20	3	2	1.100E8	1.068	1		192.17	8.20	3	2
Q9Y3I0	505	55.2	7.2	UPF0027 protein C22orf28 OS=Homo sapiens GN=C22orf28 PE=1 SV=1	12.08	6	5	6.059E8	0.438	5	231.4	452.37	12.08	6	5

Access-ion	# AA	MW kDa	pI	Description	Σ Cover-age	Σ# PSMs	Σ# Pep-tides	A7: Area	4h/ 30 min	4h/ 30 min coun t	Variabili-ty [%]	Score A(3,5)	Cover-age A(3,5)	# PSM A(3,5)	# Pep-tides A(3,5)
								>2 = enriched in 30 min sample		<2 = enriched in 4hr sample		anything over 40 is within 95%CI			

Table B.4 Kinexus KPSS 1.3 raw data

		Kinexus ID	15350	15351				
		Scan ID	66161	66175				
		Sample Name	Sample 1	Sample 2				
Normalized Data Comparison		Normalize	1	1				
FULL NAME OF PROTEIN	Abreviation	EPITOPE	NORMALIZED C.P.M.		%CFC	INTENSITY	>30% and good intensity	Dephos results in inhibition of MAPK
Adducin alpha (ADD1) [S726]	Adducin a	S726	709	888	-20			
Adducin gamma (ADD3) [S693]	Adducin g	S693	2554	3181	-20			
B23 (nucleophosmin, numatrin, nucleolar protein NO38) [S4]	B23 (NPM)	S4	1722	1714	1			
cAMP response element binding protein 1 [S133]	CREB1	S133	283	431	-34		CREB1	CREB1
Cyclin-dependent protein-serine kinase 1/2 [Y15] (24)	CDK1/2	Y15						
Cyclin-dependent protein-serine kinase 1/2 [Y15] (27)	CDK1/2	Y15	4612	5103	-10			
Double-stranded RNA-dependent protein-serine kinase [T451] (65)	PKR1	T451	237	185	28			
Double-stranded RNA-dependent protein-serine kinase [T451] (73)	PKR1	T451	242	202	20			
Extracellular regulated	Erk1	T202+Y204	223	196	14			

Normalized Data Comparison		Kinexus ID	15350	15351				
		Scan ID	66161	66175				
		Sample Name	Sample 1	Sample 2				
		Normalize	1	1				
FULL NAME OF PROTEIN	Abreviation	EPITOPE	NORMALIZED C.P.M.		%CFC	INTENSITY	>30% and good intensity	Dephos results in inhibition of MAPK
protein-serine kinase 1 (p44 MAP kinase) [T202+Y204]								
Extracellular regulated protein-serine kinase 2 (p42 MAP kinase) [T185+Y187]	Erk2	T185+Y187	122	34	254	low intensity		
Glycogen synthase-serine kinase 3 alpha [S21]	GSK3a	S21	642	530	21			
Glycogen synthase-serine kinase 3 alpha [Y279] (44)	GSK3a	Y279	857	673	27			
Glycogen synthase-serine kinase 3 alpha [Y279] (49)	GSK3a	Y279						
Glycogen synthase-serine kinase 3 beta [S9]	GSK3b	S9	1719	1121	53		GSK3b	
Glycogen synthase-serine kinase 3 beta [Y216] (34)	GSK3b	Y216						
Glycogen synthase-serine kinase 3 beta [Y216] (39)	GSK3b	Y216	870	818	6			
Jun N-terminus protein-serine kinase (stress-activated protein kinase (SAPK)) [T183+Y185] (37)	JNK	T183+Y185						
Jun N-terminus protein-	JNK	T183+Y185						

Normalized Data Comparison		Kinexus ID	15350	15351				
		Scan ID	66161	66175				
		Sample Name	Sample 1	Sample 2				
		Normalize	1	1				
FULL NAME OF PROTEIN	Abreviation	EPITOPE	NORMALIZED C.P.M.		%CFC	INTENSITY	>30% and good intensity	Dephos results in inhibition of MAPK
serine kinase (stress-activated protein kinase (SAPK)) [T183+Y185] (38)								
Jun N-terminus protein-serine kinase (stress-activated protein kinase (SAPK)) [T183+Y185] (44)	JNK	T183+Y185						
Jun N-terminus protein-serine kinase (stress-activated protein kinase (SAPK)) [T183+Y185] (46)	JNK	T183+Y185						
Jun proto-oncogene-encoded AP1 transcription factor [S73] (37)	Jun	S73						
Jun proto-oncogene-encoded AP1 transcription factor [S73] (39)	Jun	S73						
Jun proto-oncogene-encoded AP1 transcription factor [S73] (41)	Jun	S73	341	273	25			
Jun proto-oncogene-encoded AP1 transcription	Jun	S73	374	423	-12			

Normalized Data Comparison		Kinexus ID	15350	15351				
		Scan ID	66161	66175				
		Sample Name	Sample 1	Sample 2				
		Normalize	1	1				
FULL NAME OF PROTEIN	Abreviation	EPITOPE	NORMALIZED C.P.M.		%CFC	INTENSITY	>30% and good intensity	Dephos results in inhibition of MAPK
factor [S73] (43)								
MAPK/ERK protein-serine kinase 1/2 (MKK1/2) [S218+S222]	MEK1/2 (MAP2K1/2)	S218+S222	926	1323	-30		MEK1/2 (MAP2K 1/2)	MEK1/2 (MAP2K1/2)
MAPK/ERK protein-serine kinase 3/6 (MKK3/6) [S218/S207]	MEK3/6 (MAP2K3/6)	S218/S207						
Mitogen & stress-activated protein-serine kinase 1 [S376] (66)	Msk1	S376						
Mitogen & stress-activated protein-serine kinase 1 [S376] (74)	Msk1	S376	116	67	73	low intensity		
Mitogen-activated protein-serine kinase p38 alpha [T180+Y182] (36)	p38a MAPK	T180+Y182						
Mitogen-activated protein-serine kinase p38 alpha [T180+Y182] (40)	p38a MAPK	T180+Y182						
N-methyl-D-aspartate (NMDA) glutamate receptor 1 subunit zeta [S896]	NR1	S896						
Protein-serine kinase B alpha [S473]	PKBa (Akt1)	S473	121	95	27			

Normalized Data Comparison		Kinexus ID	15350	15351				
		Scan ID	66161	66175				
		Sample Name	Sample 1	Sample 2				
		Normalize	1	1				
FULL NAME OF PROTEIN	Abreviation	EPITOPE	NORMALIZED C.P.M.		%CFC	INTENSITY	>30% and good intensity	Dephos results in inhibition of MAPK
Protein-serine kinase B alpha [T308]	PKBa (Akt1)	T308						
Protein-serine kinase C alpha [S657]	PKCa	S657	4443	3223	38		PKCa	
Protein-serine kinase C alpha/beta 2 [T638/T641]	PKCa/b2	T638/T641	659	935	-30		PKCa/b2	PKCa/b2
Protein-serine kinase C delta [T507]	PKCd	T507						
Protein-serine kinase C epsilon [S729]	PKCe	S729						
Raf1 proto-oncogene-encoded protein-serine kinase [S259] (60)	Raf1	S259	784	1820	-57		Raf1	
Raf1 proto-oncogene-encoded protein-serine kinase [S259] (63)	Raf1	S259						
Retinoblastoma-associated protein 1 [S780]	Rb	S780	209	245	-15			
Retinoblastoma-associated protein 1 [S807+S811]	Rb	S807+S811	128	189	-33	low intensity		
Ribosomal protein serine S6 kinase beta 1 [T412] (70)	S6Kb1	T412	347	361	-4			
Ribosomal protein serine S6 kinase beta 1 [T412]	S6Kb1	T412	171	244	-30		S6Kb1	

Normalized Data Comparison		Kinexus ID	15350	15351				
		Scan ID	66161	66175				
		Sample Name	Sample 1	Sample 2				
		Normalize	1	1				
FULL NAME OF PROTEIN	Abreviation	EPITOPE	NORMALIZED C.P.M.		%CFC	INTENSITY	>30% and good intensity	Dephos results in inhibition of MAPK
(85)								
Ribosomal S6 protein-serine kinase 1/3 [T359+S363/T356+S360] (79)	RSK1/3	T359+S363/ T356+S360						
Ribosomal S6 protein-serine kinase 1/3 [T359+S363/T356+S360] (87)	RSK1/3	T359+S363/ T356+S360		80	-100	low intensity		
Signal transducer and activator of transcription 1 alpha [Y701]	STAT1a	Y701						
Signal transducer and activator of transcription 1 beta [Y701]	STAT1b	Y701						
Signal transducer and activator of transcription 3 [S727]	STAT3	S727	629	1058	-41		STAT3	
Signal transducer and activator of transcription 5A [Y694]	STAT5A	Y694						
SMA- and mothers against decapentaplegic homologs 1/5/8 [S463+S465/S463+S465/	Smad1/5/8	S463+S465/ S463+S465/ S465+S467	1240	963	29			

Normalized Data Comparison		Kinexus ID	15350	15351				
		Scan ID	66161	66175				
		Sample Name	Sample 1	Sample 2				
		Normalize	1	1				
FULL NAME OF PROTEIN	Abreviation	EPITOPE	NORMALIZED C.P.M.		%CFC	INTENSITY	>30% and good intensity	Dephos results in inhibition of MAPK
S465+S467]								
Src proto-oncogene-encoded protein-tyrosine kinase [Y419] (44)	Src	Y419						
Src proto-oncogene-encoded protein-tyrosine kinase [Y419] (46)	Src	Y419						
Src proto-oncogene-encoded protein-tyrosine kinase [Y530] (44)	Src	Y530	6244	4871	28			
Src proto-oncogene-encoded protein-tyrosine kinase [Y530] (46)	Src	Y530	2776	2409	15			