Link Gene Expression To The Physiology Of Sepsis Via Pathway Analysis

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Abstract

Sepsis is one the most challenging conditions in the critical care units of hospitals to diagnose and treat. Proper treatment has to commence as soon as possible to prevent a patient from getting into septic shock and possibly dying. In this project signaling pathways are being studied to form a link between the responsible genes and the physiology of sepsis by using different bioinformatics databases. An earlier diagnosis will be possible by identified patterns of pathways that can indicate the targeted organs and clinical outcomes of sepsis. This report will describe information to healthcare providers to more accurately diagnose and treat sepsis in a timely manner and to reduce the associated costs and patient mortality.
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1. **Objective**

The goal of this project is to link proteins and their representative signature genes to signaling pathways and targeted organs in sepsis. The processes investigated in this report include:

- Pyrogen for fever spikes
- Blood pressure pathways responsible for acute renal failure (ARF)

2. **Introduction**

Sepsis is a serious medical condition in which a body’s immune system’s response to an infection gets out of control and causes inflammation. Inflamed organs eventually shutdown that in turns can cause death if not treated quickly and accurately. Bacteria, viruses, parasites and fungi, termed pathogens, are examples of foreign cells that can invade the human body. According to statistics these pathogens are a major cause of sepsis in emergency rooms of hospitals. Patients who come for surgery or are admitted in a critical care unit for some other disease can get exposed to these pathogens in the hospital and end up spending a much longer time in the emergency room due to contracting sepsis. In case of severe sepsis, septic shock can result, which is almost invariably fatal. In response to an infection, the immune system, as the body’s primary defense mechanism, fights the infection, but unfortunately, can cause enough collateral damage as to be life threatening. The definition of sepsis is that it is a “systemic (total body) inflammatory response to the presence of microorganism in the body” (infection and sepsis, 2006)
Figure 1: (a) Non sepsis system; (b) Inflammatory response in sepsis
(Parham, 2008)

Figure 1, above, shows the difference between the normal and overreacted immune system. Figure 1(a) shows the proper immune response to an infection, but on the other hand Figure 1(b) shows that something is wrong and the immune system is not behaving properly. In the case of sepsis, the inflammatory proteins produced by the macrophages of the liver, known as CD 14 cells, penetrate into the bloodstream and affect many organs systemically. It is not the infectious agent, but the body’s immune system that is harmful to itself. The correct treatment must start within hours to several days because it can be fatal if not treated accurately and in a timely fashion. Much research has been conducted to solve this critical problem, but still there is no diagnostic method with high selectivity and specificity. A simple blood culture usually takes 48 hours, which is a critical initial time period for someone who maybe is suffering from sepsis. Due to the lack of
knowledge about which antibiotic is most appropriate, broad spectrum antibiotics are often used for treatment that may have severe side effects. Gene expression profiling is one of the innovative ways to identify genes that are involved in the development of sepsis. Another diagnostic approach is via protein analysis such as interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-12 (IL-12), tumor necrosis factor alpha (TNF α) and so on. The pathway analysis linking proteins and their respective genes using different bioinformatics databases and tools will be able to identify the most active pathways and their pattern in the inflammatory cascade, suggesting what might be the consequences of sepsis. Once the targeted organ or impact has been identified, appropriate interventions such as which antibiotics are most relevant to administer for the treatment of sepsis can be undertaken. The current diagnostic test of blood culture can take up to 48 hours to confirm an infection. As the onset of sepsis could be as rapid as 6 to 12 hours, this delay is critical for cancer patients and for patients in the intensive care units of hospitals.

3. Hypothesis

A genetic-based pathway analysis can improve sepsis diagnosis and treatment. Involved proteins and genes can help identify the targeted clinical outcome or organ of sepsis so that appropriate interventions and treatment can be made quickly to prevent the targeted organ from failing.
Figure 2: Targeted organs are the bridge between the diagnosis and treatment

The diagram above shows the relationship between signature genes, proteins, and their signaling pathways and how they can help identify the targeted organ, diagnosis and its treatment.

4. Physiology of Sepsis

In response to an infection the body produces pro-inflammatory proteins by the macrophage cells. These proteins include cytokines including IL-1, IL-6, IL12, TNFα and the chemokines including IL-8 are responsible for the signaling cascade that induces other substances and processes to react against the infection. The diagram below shows the cross-talk between the physiology of sepsis and many immune cells. Innate immune cells receive signals from the proteins embedded in the cell wall of bacteria and then in reaction start their defense mechanism to make the environment unfavorable for the foreign cell.
Macrophages and dendritic cells are activated by the ingestion of bacteria and by stimulation through cytokines (e.g., Interferon-gamma) secreted by CD4 T cells. Alternatively, CD4 T cells that have an anti-inflammatory profile (type 2 helper T cells [Th2]) secrete interleukin-10, which suppresses macrophage activation. CD4 T cells become activated by stimulation through macrophages or dendritic cells. For example, macrophages and dendritic cells secrete interleukin-12, which activates CD4 T cells to secrete inflammatory (type 1 helper T-cell [Th1]) cytokines. Depending on numerous factors, for example, the type of organism and the site of infection, macrophages and dendritic cells will respond by inducing either inflammatory or anti-inflammatory cytokines or causing a global reduction in cytokine production (energy). Macrophages or dendritic cells that have previously ingested necrotic cells will induce an inflammatory cytokine profile (Th1). Ingestion of apoptotic cells can induce either an anti-inflammatory cytokine profile or energy. A plus sign in the Figure indicates upregulation, and a minus sign indicates downregulation; in cases where both a plus sign and a minus sign appear, either upregulation or downregulation may occur, depending on a variety of factors. (Hotchkins & Karl, 2003)

Overactivity of the innate immune system can produce inflammation in different organs, eventually causing organs to start to fail. If not stopped quickly, death may occur in a matter of days. The diagram below describes the effects of pro-inflammatory responses on different proteins and their targeted organs in sepsis.
4.1. **Physiological Pathways**

There are many types of physiological pathways as mentioned below:

- **Metabolic**: It includes carbohydrate, energy (ATP), lipid, nucleotide, amino acid and biodegradation.
- **Regulatory**: It includes genetic information processing such as transcription and translation, environmental such as membrane transport and ligand receptor interaction and cellular such as cell motility, growth, death, development and behavior.
- **Signaling**: Signal molecules, receptors, proteins (involved with endocrine, neuronal) are the important components of this pathway.

In this project the focus is on the signaling that causes the body to initiate an immune response that results in inflammation. Inflammation happens when the signaling pathways process gets out of control and excessive antibodies are produced.
4.2. Bioinformatics Tools and Databases

During the twentieth century there has been a wealth of information that has come forward about the human DNA, RNA, protein and their sequences. Due to the emerging technologies and especially the mapping of the human genome, this information has grown and continues to increase. With the help of bioinformatics tools meaningful conclusions can be deduced from the available data.

According to the Bjorn Peters, Ph.D., Assistant Manager, Center for Infectious Diseases, Allergy and Asthma Research, “the key challenge in bioinformatics today is not initial development of new algorithms, but truly understanding the available data” (Peters, 2007). In this project, different publicly available databases and tools will be used to analyze the pathways to test the hypothesis and draw conclusions and results. Some of the databases that have been used in the project are as follows.


b. Uniprot: “The mission of Uniprot is to provide the scientific community with a comprehensive, high-quality and freely accessible resource of protein sequence and functional information.” (Uniprot, 2009)

c. Biocarta: “Online maps depict molecular relationships from areas of active research. In an "open source" approach, this community-fed forum constantly integrates emerging proteomic information from the scientific community. It also catalogs and summarizes important resources providing information for over 120,000 genes from multiple species.” (Biocarta, 2009)
d. Millipore pathways: “Online source for visualizing metabolic and signaling pathways.” (Millipore, 2009)

e. Kyoto Encyclopedia of Genes and Genomes (KEGG):
“KEGG PATHWAY is a collection of manually drawn pathway maps representing our knowledge on the molecular interaction and reaction networks.” (Kegg pathways, 2009)

f. Gene Cards:
Is a searchable, integrated database of human genes that provides concise genomic, proteomic, transcriptomic, genetic and functional information on all known and predicted human genes. Information featured in GeneCards includes orthologies, disease relationships, mutations and SNPs, gene expression, gene function, pathways, protein-protein interactions, related drugs & compounds and direct links to cutting edge research reagents and tools such as antibodies, recombinant proteins, clones, expression assays and RNAi reagents. (Genecards, 2009)

4.3. Stages of sepsis

![Figure 5: Sepsis: a disease continuum (Bone, 1992)](image-url)
As summarized above in the figure, progression of sepsis is usually divided into four major stages. The onset of sepsis is very quick, typically within days, and as the sepsis progresses through these stages chances of survival diminish rapidly.

Stage 1: Systemic inflammatory response syndrome (SIRS) is considered as a first stage of sepsis. Although, at this stage there is no confirmation of infection and it may or may not develop into sepsis.

Systemic inflammatory response syndrome (SIRS) describes a systemic inflammation resulting from any major insult to the body, such as trauma, burns, or myocardial infarction, in which two or more of the following are present:

* A temperature higher than 38°C (100.4°F) or lower than 36°C (96.8°F)
* A heart rate greater than 90 beats per minute
* A respiratory rate greater than 20 breaths per minute or a partial pressure of carbon dioxide (PCO2) less than 32 mmHg
* A white blood cell (WBC) count higher than 12 × 10³/mm³, lower than 4 × 10³/mm³, or with more than 10% band cells

Patients with SIRS can be routinely cared for on the medical–surgical floor, but should be closely monitored for signs and symptoms of sepsis.

(Nelson et al, 2009)

Stage 2:

Sepsis is identified by the presence of two of the SIRS criteria along with a known or suspected infection. However, in many cases of sepsis, the actual cause of infection is never identified. Martin and colleagues found that “specific organism causing sepsis were recorded in 51% of all discharge records” from the 22 year periods they studied.
The delay in waiting for confirmation of infection can slow the treatment of sepsis; the most effective course of action once SIRS is identified and infection is suspected is to treat the infection and monitor the patient for signs and symptoms of organ failure, which will indicate that the condition has progressed to severe sepsis. (Nelson et al, 2009).

The table below has a list of some of the affected organs and their symptoms. These are only physical symptoms and it is too late to prevent the organ from failing or avoiding severe damage. Presently there is no biomarker that can indicate the organ that is being attacked before the body shows the physical symptoms.

<table>
<thead>
<tr>
<th>No</th>
<th>Organ</th>
<th>Signs and symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pyrogen</td>
<td>• Fever</td>
</tr>
<tr>
<td>2</td>
<td>Cardiovascular</td>
<td>• Systolic blood pressure (BP) &lt; 90mmHg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Mean arterial pressure &lt; 65mmHg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• A drop from baseline pressure &gt; 40 mmHg</td>
</tr>
<tr>
<td>3</td>
<td>Hematologic (Coagulation cascade)</td>
<td>• Platelets &lt; 100 X 10^7/mm³</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Platelets decreased by 50% over three days</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Acutely abnormal prothrombin time or partial thromboplastin time (PTT) without anticoagulation therapy (international normalized ratio &gt;1.5; PPT &gt;60 seconds)</td>
</tr>
<tr>
<td>4</td>
<td>Hepatic</td>
<td>• Bilirubin level &gt; 2 mg/dL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Alkaline phosphatase level &gt; 100 units per liter</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Aspartate aminotransferase level &gt; 100 units per liter</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Alanine aminotransferase level &gt; 100 unites per liter</td>
</tr>
<tr>
<td>5</td>
<td>Metabolic</td>
<td>• Serum lactate levels &gt; 2 mmol/L (most sources, such as the Surviving Sepsis Campaign guidelines, agree on a serum lactate level &gt; 4mmol/L, but some hospital data strongly indicates an increase risk of death with serum lactate levels &gt; 2mmol/L)</td>
</tr>
<tr>
<td>6</td>
<td>Neurologic</td>
<td>• Mental state acutely altered from baseline</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• A Glasgow Coma Scale score of &lt; 15</td>
</tr>
<tr>
<td>7</td>
<td>Pulmonary</td>
<td>• Respiratory rate &gt; 24 breaths per minute</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Oxygen saturation level &lt; 92 % with patient on oxygen at 6 L/min using face mask</td>
</tr>
<tr>
<td>8</td>
<td>Renal</td>
<td>• Urine output &lt; 0.5 mL/Kg/hr despite volume resuscitation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Increase in the creatinine level of 0.5 mg/dL</td>
</tr>
</tbody>
</table>

Table 1: “Signs and Symptoms of organ failure.” (Nelson et al, 2009)

Stage 3:

“Severe sepsis occurs when a patient who meets the sepsis criteria shows one of the signs
and symptoms of organ failure detailed in table 1. Once severe sepsis is suspected, the
patient requires aggressive treatment in a critical care area.” (Nelson et al, 2009).

Stage 4:
Septic shock is defined as severe sepsis plus hypotension (a systolic blood pressure below
90 mmHg) that doesn’t respond to fluid resuscitation. Septic shock is associated with a
high mortality rate. The patient’s chances of recovery are significantly reduced if, by this
stage of sepsis, she or he hasn’t already been transferred to the ICU. (Nelson et al, 2009).

5. Pathway Analysis

“At this point in time, the literature richly illustrates that no single mediator / system /
pathway drives the pathophysiology of sepsis.” (Remick, 2007) Study of the patterns of
pathway involved in different stages and different organs will help in earlier diagnosis.

5.1. Pyrogen for fever spikes

Fever is one of the responses by our immune system to an infection. Increased body
temperature makes the host environment unfavorable for a pathogen. Fever is also
known to induce some of the anti-inflammatory proteins due to stress that has some
protective effects in sepsis, but sustained high body temperatures breakdown proteins and
can result in severe consequences such as heat stroke and brain damage.

Induction and regulation of fever results from complex bilateral communications that
involves both chemical and neural pathways. Researchers have come up with many
theories and pathways for this complex reaction of the body.
5.1.1. Pyrogen

A pyrogen is a substance that induces fever. These can be either internal (endogenous) or external (exogenous) to the body. (Fever, 2009)

5.1.2. Exogenous Pyrogen

The body’s response to an infection varies depending on the type of pathogen that is involved. There are two broader classes of bacteria, one being gram-positive bacteria and another being the gram-negative bacteria. (Fever, 2009)

The bacterial substance lipopolysaccharide (LPS), present in the cell wall of some the gram-negative bacteria, is an example of an exogenous pyrogen. This is one of the most investigated bacterial substances in the research related to sepsis. LTA and PGN are the examples of gram-positive alternatives of the exogenous pyrogen that are found to be involved in initiation of the body’s inflammatory response in sepsis. Sepsis pathways may vary depending on the type of exogenous pyrogen or exotoxin. (Fever, 2009)

5.1.3. Endogenous Pyrogen

Cytokines (especially interleukin 1) are a part of the innate immune system, and are produced by phagocytic cells, causing an increase in the thermoregulatory set-point in the hypothalamus. Other examples of endogenous pyrogens are interleukin 6 (IL-6), and tumor necrosis factor-alpha. (Fever, 2009)
5.2. Overview of the fever pathway

Figure 6: Overview of the fever response. (Leon, 2002)

After contact with an infectious or inflammatory stimulus, cytokines are produced by macrophages and other immune cells. Fever is stimulated by endogenous pyrogens [interleukin (IL)-1, IL-6, IL-8, macrophage-inflammatory protein-1 (MIP-1), and interferon-γ (IFN-γ)] and inhibited by endogenous antipyretics [IL-10, arginine vasopressin (AVP), tumor necrosis factor-α (TNF-α), and glucocorticoids]. It is the sum of the interactions of endogenous pyrogens and antipyretics with one another that is responsible for the ultimate height and magnitude of fever. Cytokines signal the
hypothalamus to increase the thermal set point. This results in the initiation of a number of behavioral and physiological mechanisms that increase heat production and decrease heat loss to ultimately produce fever. (Leon, 2002).

### 5.2.1. Classical Pathway

![Figure 7: Classical pathways of producing fever](image)

Pathogen-associated molecular patterns (PAMPs) produced by cell wall component of Gram-negative bacteria (LPS) are recognized by TLR4; it involves the binding of LPS to a serum protein, lipopolysaccharide-binding protein (LBP), which in turn binds to membrane CD14, which transfers the complex to TLR4 in an MD-2 dependent manner. Sequential, intracellular signaling steps are consequently initiated that lead to the transcription of genes encoding soluble innate immune protein, including pyrogenic cytokines. Most prominent among these are tumor necrosis factor (TNF)-α, interleukin
(IL)-1β, IL-6 and interferon (IFN)-α; the latter is produced predominantly in response to viral infection. These cytokines are not stored as preformed molecules (except TNF-α and IL-6, present to a small extent in mast cells), but synthesized and secreted after stimulation. They are then released into the bloodstream and transported to sites in or close to the POA that reacts to their stimulation by selectively expressing COX-2 and mPGES-1. Both isoenzymes are transcriptionally regulated by NF-kB and have been demonstrated to be specifically implicated in the febrile response. PGE2, thus induced by these cytokines, is considered to be the proximal, final fever mediator in the POA. (Blatteis, 2005)

Once PGE2 reaches the brain it induces fever through neural pathways as compared in the figures below for the normal and infected conditions:

Figure 8: Neural pathways of PGE2 (Nakamura, 2005)
Under PGE2-free condition (Normal), neurons in the DMH and rRPa are tonically inhibited by inputs from EP3 receptor-expressing GABAergic neurons in the POA. It is possible that the DMH neurons receive excitatory inputs from unknown regions, but cannot be activated due to stronger inhibition from the POA neurons. After infection (Infection), PGE2, which is produced in brain vasculature in response to immune signals, suppresses the tonic firing of the POA neurons by activating the EP3 receptor, and thereby, the DMH and rRPa neurons are released from the tonic inhibition. Excitation of the DMH neurons, which can be triggered by the excitatory inputs from the unknown regions, activates sympathetic premotor neurons in the rRPa, which in turn stimulate the sympathetic output system and finally develop fever. Blue, red, and black circles denote cell bodies of activated inhibitory neurons, activated excitatory neurons, and suppressed neurons, respectively. IML, intermediolateral cell column; SPN, sympathetic preganglionic neuron. (Nakamura et al, 2005)

There have been numerous studies conducted about the cytokines’ response and production of fever by PGE2 mediation that point out the weaknesses as follows:

1. “The classical model of the initiation of fever is predicated on the arrival of blood-borne cytokines at their brain site of action.” (Blatteis, 2005)

According to the studies “ TNF α, the first of the cytokines to appear in the blood of i.v. LPS-challenged conscious human, rats and guinea pigs, is not detectable until at least 30 minutes after its injection, whereas Tc rises within 10-12 minutes.” (Blatteis, 2005) This is one of the reasons classical pathways now have been supplemented by alternative pathways to explain the initial response of the rise in temperature.
Moreover in this context, the systemic administration of antibodies against particular cytokines predominantly reduces the second rather than the first phase of LPS fever. Natural infectious fever also is not consistently associated with detectable levels of blood-borne cytokines, and such fevers frequently abate before the disappearance of blood-borne cytokines, further suggesting that fever could develop and be maintained independently of cytokines activity. (Blatteis, 2005)

2. Cytokines are relatively larger molecule, thus they do not have the ability to cross the blood-brain barrier (BBB). The BBB is a metabolic or cellular structure in the central nervous system (CNS) that restricts the passage of various chemical substances and microscopic objects (e.g. bacteria) between bloodstream and the neural tissues itself, while still allowing passage of substances essential to metabolic function (e.g. oxygen). (Blood-brain barrier, 2009)

To explain this problem “two alternative mechanisms of cytokine signal transfer were therefore proposed: (1) cytokines that have been permitted into the perivascular clefts of the OVLT could activate local sensory neurons projecting to the POA, thereby transmitting their pyrogenic message inward, or (2) they could induce there a secondary lipophilic messenger that, in turn, could convey the original message to the POA.” (Blatteis, 2005)

There is one more alternative that has been studied by many scientists that perhaps cytokines do not cross the BBB at all, “but rather that they could exert their febrigenic effects through PGE2 released abluminally into the brain at all, but the brain parenchyma and diffusing to receive POA neurons. This
interpretation, although prevalent, however does not overcome the obstacle that, in the absence of circulating cytokines, these could not be the signals for the presumptive, very prompt induction of preoptic PGE2 and, hence fever after i.v. LPS or after high doses of i.p. LPs.” (Blatteis, 2005)

5.2.2. Initial peripheral Fever: Direct signaling of LPS

“Circulating LPS could itself be the direct stimulus that evokes the expression of COX-2 and mPGES-1.” (Blatteis, 2005) These enzymes play a main role in production of PGE2. The concern is that LPS is not a strong trigger for COX and mPGES-1 release, and, according to some studies, LPS may require up to 1 hour to release these enzymes in vivo, suggesting that there must be some other alternative pathways for the quick rise in temperature. According to one study of an animal model:

The i.v. administration of LPS triggers within 2 min the C cascade via the alternative pathway, resulting in the production in blood of C4a, C3a and C5a and of surface-bound and fluid-phase C3b and iC3b [100]. Kc express the receptors for these C components, and it has been demonstrated in vitro that the production of PGE2 by Kc and other phagocytes is initiated within minutes after their addition, whereas C depletion limits this release [14, 101–105]. PGE2, under these conditions, could be generated via the hydrolysis of membrane-associated phosphoinositide (PI, which has high arachydonoyl chain content) by PI-specific phospholipase C (PI-PLC); indeed, arachidonic acid (AA) liberation by PI-PLC is 10-fold more rapid (within seconds) than that mediated by cPLA2. Moreover, PI- PLAC is reportedly activated by C, but not by LPS or IL-1β, and the subsequent conversion of this AA to PGE2 is catalyzed by COX-1. Hence, we hypothesized
that the initial peripheral fever trigger could indeed be PGE2 released by Kc stimulated by LPS-activated C components and binding to PGE2 (EP) receptors on vagal afferents. (Blatteis, 2005)

There are many theories that try to explain fever pathways via using animal models. In summary, initial peripheral pathways are considered to be responsible for the early phase febrile response in LPS induced fever. At the same time classical pathways are also considered responsible for the second phase prolonged febrile response.

It is shown in classical pathways that macrophages are responsible for the production of the antipyretic proteins (IL10, heat shock proteins (HSPs), and so on) to regulate the anti-inflammatory response to keep the balance in the febrile response. In sepsis however this balance is uncontrolled and the pro-inflammatory proteins affect the entire body.
5.3. Antipyretic Pathways

5.3.1. IL10 Pathways

The Figure above describes a summary of fever data obtained with gene knockout mice. Solid arrows represent stimulatory pathways; dashed arrows represent inhibitory pathways. A: IL-6 and IL-10 were shown in gene knockout mice to be essential for fevers to a low dose of lipopolysaccharide (LPS). IL-6 knockout mice do not develop fever in response to a low dose of LPS, thus implicating IL-6 as an essential pyrogen in this fever model. IL-10 knockout mice respond with exacerbated fever that correlates with enhanced plasma IL-6 levels. Thus, IL-10 functions as an endogenous antipyretic in this fever model through the inhibition of IL-6 levels. IL-1, IL-1 type I receptor (IL-1r1), and TNF p55 and p75 receptor (TNFR) knockout mice developed fever in a way that is
virtually identical to wild-type mice, implicating these cytokines as nonessential mediators of fever to a low dose of LPS. B: a high dose of LPS induces the release of IL-1, IL-6, and TNF independently. IL-1 interacts with IL-1r1 to induce IL-6 to produce fever and function as an endogenous pyrogen in this model of fever. In some instances, LPS can produce fever independently of IL-1 by directly stimulating the production of IL-6. TNF interacts with its p55/p75 receptors to inhibit fever and act as an endogenous antipyretic. Turpentine induces a serial pathway of fever regulation. IL-1 interacts with the IL-1r1 to induce IL-6 and produce fever. IL-1 and IL-6 are essential in this fever model. IL-1 knockout mice do not develop fever or a rise in plasma IL-6 to turpentine. IL-1r1 and IL-6 knockout mice also do not develop fever in response to turpentine. TNF and IL-10 are not involved in turpentine fever. (Leon, 2002)

Following is the IL 10 Pathway retrieved from Biocarta:

Figure 11: Fever inhibitory pathways of IL-10 (Biocarta, 2009)
IL-10 is a cytokine with potent anti-inflammatory properties, repressing the expression of inflammatory cytokines such as TNF-alpha, IL-6 and IL-1 by activated macrophages. The IL-10 receptor is in the JAK/STAT class of receptors, but activation of the JAK/STAT pathways by IL-10 does not appear, on its own, to be responsible for the anti-inflammatory properties of this cytokine. The anti-inflammatory actions of IL-10 appear to require induction of the enzyme heme-oxygenase-1 (HO-1) through a MAP kinase pathway involving the p38 kinases. HO-1 is involved in the biosynthesis of heme, and catalyzes a reaction producing carbon monoxide, free iron, and the heme precursor biliverdin. HO-1 is induced by IL-10 and is also induced by oxidative stress. Blocking HO-1 with inhibitors or antisense blocks the anti-inflammatory actions of IL-10. The anti-inflammatory actions of HO-1 appear to be the result of signaling by carbon monoxide it produces since removal of CO blocks the anti-inflammatory action of IL-10 and HO-1. The anti-inflammatory actions of IL-10 may be therapeutically useful either directly or through modulation of HO-1 activity. (Biocarta, 2009)

5.3.2. HSP inhibitory pathways

There is strong evidence that HSPs have anti-inflammatory roles. In vitro studies have shown that the heat shock response reduces levels of TNF-α, IL-1, IL-6 and IL-10. Inducible HSPs exist in the cytosol, bound to proteins called heat shock factors (HSFs). A stress causes HSPs to dissociate from HSFs and the HSFs are the phosphorylated. NF-KB is also resident in the cytosol of the cell, bound to an inhibitory protein called I-KB. During the inflammatory process, NF-KB is activated, uncovering the nuclear translocation signal on the NF-KB dimer. Unbound NF-KB is then able to serve its role as a DNA promoter to enhance the transcription of mRNA, which codes for the
inflammatory molecules.

Below is the picture of pathway of stress induction of HSP regulation retrieved from Biocarta pathways.

![Stress induction of HSP regulation](image)

Figure 12: Stress induction of HSP regulation. (Biocarta, 2009)

Mammalian cells can respond to a variety of stresses such as heat, cold, oxidative stress, metabolic disturbance, and environmental toxins through necrotic or apoptotic cell death, while increased expression and phosphorylation of heat shock proteins such as Hsp27 can protect cells against cellular stress. Heat shock proteins commonly exhibit molecular chaperone activity and also interact with a wide variety of proteins to exert specific effects. The small heat shock protein Hsp27 exists as monomers, dimers, and oligomers in the cell, and each form has distinct activities. Oligomers are the main form of Hsp27 with molecular chaperone activity and are disrupted by phosphorylation of Hsp27 to form
dimers and monomers. S-thiolation of Hsp27 on the cysteine residue also dissociates oligomers and may provide another route of regulating the action of Hsp27 in stress. MAP kinase cascades mediate Hsp27 phosphorylation. Heat stress activates the p38 kinase cascade and induces phosphorylation of Hsp27 by the downstream MAP kinases Mapkapk2 and Mapkap3. Cytokines such as TNF and IL-1 can also induce Hsp27 phosphorylation through this MAP kinase cascade, protecting cells in some settings against cytotoxic responses. In stressful conditions, dissociation of oligomeric Hsp27 by phosphorylation may allow lower molecular weight forms to perform other non-chaperone functions.

One action of Hsp27 induced by stress is to protect cells against apoptosis and a common component of apoptotic pathways are the mitochondrial release of cytochrome c. One way that Hsp27 reduces apoptosis is by preventing the release of cytochrome c and by binding to cytochrome c in the cytosol. Downstream, Hsp27 also blocks caspase 9 activation and the subsequent activation of caspase 3, inhibiting the rest of the proteolytic caspase cascade. Yet a further role of Hsp27 in blocking apoptosis is through blocking Fas-induced apoptosis. Fas is a receptor in the TNF receptor gene family that induces apoptosis when stimulated by its cell-bound ligand, Fas-ligand (see the Fas Signaling pathway). Fas induces apoptosis through two pathways, one mediated by the protein Daxx. Phosphorylated Hsp27 dimers block apoptosis by binding with Daxx and preventing downstream activation of the kinase Ask1. The interaction of Hsp27 with actin filaments may also prevent apoptosis triggered by some agents like staurosporine that damage actin. Unphosphorylated Hsp27 monomers regulate actin filament growth by binding to the end of fibers and capping them. Finally, Hsp27 appears to prevent
damage to cells by reactive oxygen species (ROS), by altering the oxidative environment of the cell through induction of glutathione expression, as well as blocking apoptosis induced by ROS. Modulation of Hsp27 expression and phosphorylation may provide a useful means to alter cellular sensitivity to stress.

A detailed literature survey was formed that provided a list of major proteins involved in the regulation of febrile response in sepsis. Further investigation was performed by using different bioinformatics databases (as listed above) to acquire responsible genes and associated signaling pathways. All this information is organized in the Table below that can be used by health care providers and researchers to more accurately diagnose and treat sepsis in a timely manner.

<table>
<thead>
<tr>
<th>No</th>
<th>Protein</th>
<th>Gene</th>
<th>Pathway</th>
<th>Database</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LBP (lipopolysaccharide-binding protein)</td>
<td>LBP Synonyms: MGC22233, NP 004130.2</td>
<td>Toll-like receptor signaling</td>
<td>KEGG</td>
</tr>
<tr>
<td>2</td>
<td>IL1 Beta, Catabolin</td>
<td>IL1B Synonyms: IL1F2, IL-1, IL1-BETA, NP_000567.1</td>
<td>Toll-like receptor signaling</td>
<td>KEGG</td>
</tr>
</tbody>
</table>
| 3  | IL6 (B-cell stimulatory factor 2) (BSF-2) (Interferon beta-2) (Hybridoma growth factor). It is produced at the sites of acute and chronic inflammation, where it is secreted into the serum and induces a transcriptional inflammatory response through IL66 receptor, alpha. (Unigene) | IL 6: Synonyms: IFNB2, HGF, HSF, BSF2, NP_000591.1 | 1. Toll-like receptor signaling pathway  
2. Cytokine and inflammatory response | KEGG  
Biocarta |
| 4  | Tumor necrosis factor (TNF-alpha) (Tumor necrosis factor ligand super family member 2) (Cachectin) Mainly secreted by macrophages. | TNF Synonyms: TNFA, DIF, TNFSF2, TNF-alpha, NP_000585.2 | Toll-like receptor Signaling pathways | KEGG       |
| 5  | Interleukin-8 (IL8) Alternative names: C-X-C motif   | IL8: Synonyms: NAF, GCP1, LECT, LUCT, CXCL8, GCP-1, LYNAP, MDNCF, MONAP, NAP-1, NP 000575.1 | Toll-like receptor signaling  
Cytokine Network  
Cytokine and inflammatory response | KEGG  
Biocarta  
Biocarta |
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<tbody>
<tr>
<td>6</td>
<td>Interleukin-2 (IL-2) (Aldesleukin). Important for the proliferation of T and B lymphocytes.</td>
<td>IL2 Synonyms: IL-2, TCGF, lymphokine, NP_000577.2</td>
<td>Toll-like receptor signaling</td>
<td>Biocarta</td>
<td>KEGG</td>
</tr>
<tr>
<td>7</td>
<td>Monocyte differentiation antigen CD14 (Myeloid cell-specific leucine-rich glycoprotein) (CD antigen CD14) Cooperates with MD-2 and TLR4 to mediate the innate immune response to bacterial lipopolysaccharide (LPS). Acts via MyD88, TIRAP and TRAF6, leading to NF-kappa-B activation, cytokine secretion and the inflammatory response. Up-regulates cell surface molecules, including adhesion molecules. (Uniprot)</td>
<td>CD14 NP_000582.1</td>
<td>Toll-like receptor signaling</td>
<td>Biocarta</td>
<td>KEGG</td>
</tr>
<tr>
<td>8</td>
<td>Toll-like receptor 4 (hToll) (CD antigen CD 284) Member of TLR family, which plays a fundamental role in pathogen recognition and activation of innate immunity. (Unipote)</td>
<td>TLR4: Toll-like receptor 4. Synonyms: TOLL, CD284, hToll, ARMD10, NP_612564.1</td>
<td>Toll like receptor signaling</td>
<td>Biocarta</td>
<td>KEGG</td>
</tr>
<tr>
<td>11</td>
<td>TNRIA: Tumor necrosis factor receptor superfamily member 1A (p60) (TNF-R1) (TNF-R1) (TNFR-1) (p55) (CD antigen CD120a)</td>
<td>TNFRSF1A Synonyms: FPS, P55, p60, TBP1, TNF-R, TNFAR, TNFR1, p55-R, CD 120a, TNFR55, p55-R,</td>
<td>1. TNF/stress related signaling 2. TNFR1 signaling</td>
<td>Biocarta</td>
<td>Biocarta</td>
</tr>
<tr>
<td>ID</td>
<td>Gene Name</td>
<td>Synonyms</td>
<td>Notes</td>
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<tr>
<td>12</td>
<td>Interleukin-10 (IL-10)</td>
<td>TNFR60, TNF-R-1, TNF-R55, MGC19588, TNFRSF1A, NP_001056.1</td>
<td>Cytokine network</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Tumor necrosis factor receptor superfamily member 1B (Tnfrsf1b)</td>
<td>IL10, Synonyms: CSIF, TGIF, IL10A, MGC126450, MGC126451, IL-10, NP_000563.1</td>
<td>Cytokine and inflammatory response</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Interleukin-1 receptor antagonist protein (IL-1ra)</td>
<td>TNFRSF1B, Synonyms: p75, TBPII, TNFRB, TNFR2, CD120b, TNFR1B, TNFR80, TNF-R75, p75TNFR, TNF-R-II, TNFRSF1B, NP_001057.1</td>
<td>IL10 anti-inflammatory signaling</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Heme oxygenase 1 (HO-1)</td>
<td>IL1RN: Interleukin 1 receptor antagonist isoform 2, Synonyms: IRAP, IL1F3, IL1RA, IL-1ra3, ICIL-1RA, MGC10430, IL1RN, NP_776213.1</td>
<td>JAK-STAT signaling pathway</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Calcium-independent phospholipase A2:</td>
<td>PLA2G6, Synonyms: GVI, PLA2, INAD1, iPLA2, PARK14, PNPLA9, Cal-PLA2, PLA2-VIA, PLA2G6, NP_003551.2</td>
<td>Cytokine-Cytokine receptor interaction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Corticoprotein-releasing hormone,</td>
<td>CRH, Synonyms: CRF</td>
<td>Mitogen-Activated Protein Kinase signaling</td>
<td></td>
<td></td>
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<tr>
<td>Pathway ID</td>
<td>Pathway Name</td>
<td>Description</td>
<td></td>
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<tr>
<td>18</td>
<td>NF-κB1: P105 → P50</td>
<td>105KD protein undergoes cotranslational processing by the 26S proteasome to produce a 50 KD protein. 105 KD is a Rel protein-specific transcription inhibitor and the 50 KD is a DNA binding subunit of the NF-KB protein complex. (Unigene)</td>
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<tr>
<td>19</td>
<td>RelA: P65</td>
<td>V-rel reticulon endotheliosis viral oncogene homology A (avian) RELA: NP_068810.2 (Unigene)</td>
<td></td>
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<tr>
<td>20</td>
<td>c-Rel</td>
<td>It is transcription factor that is a member of the Rel/NFKB family. (Unigene)</td>
<td></td>
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</tr>
<tr>
<td>21</td>
<td>IκB alpha: NF-kappa-B inhibitor alpha</td>
<td>NFKBIA Synonyms: IKBA, MAD-3, NFKB, NP_065390.1</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>22</td>
<td>Inhibitor of nuclear factor kappa-B kinase subunit alpha.</td>
<td>CHUK: Conserved helix-loop-helix ubiquitous kinase Synonyms: IKK1, IKKA, IKBKα, TCF16, NFKB1, IKK-alpha,</td>
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</tr>
<tr>
<td>22</td>
<td>Inhibitor of nuclear factor kappa-B kinase subunit beta</td>
<td>IKBKB</td>
<td>1. MAPK/Erk signaling 2. NF-KB signaling 3. TNF/stress related signaling 4. Signal transduction through IL1R</td>
<td>Biocarta</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Synonyms: IKKB, IKK2, NFKB1KB, FL40509, IKB-beta, MGC131801, NP_001547.1</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>23</td>
<td>NEMO (IKB kinase subunit gamma)</td>
<td>NEMO</td>
<td>1. WNT signaling Pathway 2. ALK in cardiac myocytes</td>
<td>Biocarta</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Synonyms: FIP3, IP, IP1, IP2, IPD2, FIP-3, Fip3p, AMCBX1, Ikkgamma, IKBKG</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>24</td>
<td>C5a</td>
<td>C5: CPAMD4, FIJ17816, FIJ17822, MGC142298, NP_001726.2</td>
<td>1. Rho cell motility signaling 2. Role of P13K subunit P85 in regulation of actin organization and cell migration 3. Y branching of actin filament</td>
<td>Biocarta</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Synonyms: CPAMD4, FIJ17816, FIJ17822, MGC142298, NP_001726.2</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Synonyms: ASP, ARMD9, CPAMD1, NP_000055.2</td>
<td></td>
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</tr>
<tr>
<td>26</td>
<td>C-C motif chemokine 5</td>
<td>CCL5: chemokine (C-C Immune response CCR3</td>
<td>Millipore</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2: List of proteins, genes and their respective pathways.

<table>
<thead>
<tr>
<th>(Small-inducible cytokine A5) (T-cell-specific protein RANTES) (SIS-delta) (T cell-specific protein P228) (TCP228) (Eosinophil-chemotactic cytokine) (EoCP) [Cleaved into: RANTES(3-68); RANTES(4-68)]</th>
<th>motif) ligand 5. Synonyms: SISd, D17S136E, SCYA5, TANTES, TCP228, MGC17164, NP_002976.2</th>
<th>signaling in eosinophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-C chemokine receptor type 1 (C-C CKR-1) (CC-CKR-1) (CCR-1) (CCR1) (Macrophage inflammatory protein 1-alpha receptor) (MIP-1alpha-R) (RANTES-R) (HM145) (LD78 receptor) (CD antigen CD191)</td>
<td>CCR1: Chemokine (C-C motif) receptor 1. Synonyms: CMKBR1, CMKR1, SCYAR1, CKR1, CD191, CCR-1, HM145, NP_0012861</td>
<td>Selective expression of chemokine receptors during T-cell polarization</td>
</tr>
<tr>
<td>CCL4: C-C motif chemokines 4 (Small-inducible cytokine A4) (Macrophage inflammatory protein 1-beta) (MIP-1-beta) (MIP-1-beta(1-69)) (T-cell activation protein 2) (ACT-2) (PAT 744) (Protein H400) (SIS-gamma) (Lymphocyte activation gene 1 protein) (LAG-1) (HC21) (G-26 T-lymphocyte-secreted protein)</td>
<td>CCL4: Chemokine (C-C motif) ligand 4 Synonyms: ACT2, G-26, LAG1, LAG1, MIP1B, SCYA4, SCYA2, AT744.1, MGC126025, MGC126026, NP_001133310.1</td>
<td>1. Pertussis toxin-intensive CCR5 signaling in Macrophage 2. Selective expression of chemokines receptors during T-cell polarization</td>
</tr>
<tr>
<td>Prostaglandin –endoperoxide synthase 2 (Cyclooxygenase) PGE2 biosynthesis</td>
<td>PTGS2 Synonyms: COX2, COX-2, PHS-2, PGG/HS, PGHS-2, hCox-2, GRIPGHS</td>
<td>Mechanism of genes regulation by peroxisome proliferators via PPARa (alpha)</td>
</tr>
</tbody>
</table>

The table consists of the major proteins that have been studied in human or in animal models, but there are many more genes and proteins involved in the fever response.

Listed pathways involve all the major genes that are the blueprint of the protein that will be expressed in sepsis. In a medical setting, gene expression profiling cannot be done on
each and every patient. However, a simple blood test or assay can identify the over or under expressed proteins. There have been 37 differentially expressed known pathways identified in this project that are associated with the fever response in sepsis. Among all of them TLR and NF-KB signaling pathways are the most critical as they are the first to interact with and response to the foreign cells. There are different TLR receptors and depending on the type of pathogen it interacts with it initiates different pathways.
5.3.3. Toll-Like Receptor Pathway

Figure 13: Toll-like receptor pathways. (Retrieved from KEGG pathways)

The innate immune response responds in a general manner to factors present in invading pathogens. Bacterial factors such as lipopolysaccharide (LPS, endotoxin), bacterial lipoproteins, peptidoglycans, as well as CpG nucleic acids activate innate immunity as well as stimulating the antigen-specific immune response and triggering the inflammatory response. Members of the toll-like receptor (TLR) gene family convey signals stimulated by these factors, activating signal transduction pathways that result in transcriptional regulation and stimulate immune function. TLR2 is activated by bacterial lipoproteins,
TLR4 is activated by LPS, and TLR9 is activated by CpG DNA; peptidoglycan recognition protein (PGRP) is activated by peptidoglycan (PGN). The downstream signaling pathways used by these receptors are similar to that used by the IL-1 receptor, activating the IL-1 receptor associated kinase (IRAK) through the MyD88 adaptor protein, and signaling through TRAF-6 and protein kinase cascades to activate NF-KB and Jun. NF-KB and c-Jun activate transcription of genes such as the pro-inflammatory cytokines IL-1 and IL-12. Several recent reports have suggested that the functional outcomes of signaling via TLR2, TLR4 and PGRP are not equivalent. For example, while the LPS-induced, p38-dependent response was dependent upon PU.1 binding, the PGN-induced, p38 response was not. The intracellular receptor for PGN, PGRP is conserved from insects to mammals. In insects, PGRP activates prophenoloxidase cascade, a part of the insect antimicrobial defense system. Because mammals do not have the prophenoloxidase cascade, its function in mammals is unknown. However, it was suggested that an identical protein Tag7 was a tumor necrosis factor-like (TNF-like) cytokine. PGRP/Tag7 possesses cytotoxicity and triggers intranucleosomal DNA fragmentation in target cells in the same way as many known members of the TNF family. Fragmentation of DNA is one of the characteristics of apoptosis. The possibility that in another system, PGRP/Tag7 would induce NF-kB activation, as observed for TRAIL (TNF-related apoptosis-inducing ligand) receptors cannot be ruled out. (Croston, 2009)
5.3.4. NF-KB signaling Pathway

Nuclear factor kappaB (NF-kB) is a nuclear transcription factor that regulates expression of a large number of genes that are critical for the regulation of apoptosis, viral replication, tumorigenesis, inflammation, and various autoimmune diseases. The activation of NF-kB is thought to be part of a stress response as it is activated by a variety of stimuli that include growth factors, cytokines, lymphokines, UV, pharmacological agents, and stress. In its inactive form, NF-kB is sequestered in the cytoplasm, bound by members of the IkB family of inhibitor proteins, which include IkBa, IkBb, IkBg, and IkBe. The various stimuli that activate NF-kB cause phosphorylation of IkB, which is followed by its ubiquitination and subsequent degradation. This results in the exposure of the nuclear localization signals (NLS) on NF-kB subunits and the subsequent translocation of the molecule to the nucleus. In the nucleus, NF-kB binds with a
consensus sequence (5’GGGACTTTCC-3’) of various genes and thus activates their transcription. IkB proteins are phosphorylated by IkB kinase complex consisting of at least three proteins; IKK1/IKKa, IKK2/IKKB, and IKK3/IKKg. These enzymes phosphorylate IkB leading to its ubiquitination and degradation. Tumor necrosis factor (TNF), which is the best-studied activator, binds to its receptor and recruits a protein called TNF receptor death domain (TRADD). TRADD binds to the TNF receptor-associated factor 2 (TRAF-2) that recruits NF-kB-inducible kinase (NIK). Both IKK1 and IKK2 have canonical sequences that can be phosphorylated by the MAP kinase NIK/MEKK1 and both kinases can independently phosphorylate IkBa or IkBb. TRAF-2 also interacts with A20, a zinc finger protein whose expression is induced by agents that activate NF-kB. A20 functions to block TRAF2-mediated NF-kB activation. A20 also inhibits TNF and IL-1 induced activation of NF-kB suggesting that it may act as a general inhibitor of NF-kB activation. (Wu & Ponnappan, 2009) Several other known pathways retrieved from KEGG and Biocarta as well as their legends are provided in the appendix section of this report.

5.4. Acute Renal Failure (ARF)

“Acute renal failure is defined as a significant acute decline in Glomerular filtration rate.” (Oppert et al, 2007) Where GRF represents the flow rate of filtered liquids through the kidney. Presently there are two criteria for ARF identification.

1. Rise in creatinine above twice the upper limit of normal
2. Drop in urine output to <0.5 ml/kg body weight for at least 4 hours despite fluid resuscitation. (Oppert et al, 2007)
“The risk of mortality of patients with ARF is more then twice as high as in septic patients without ARF.” (Oppert et al, 2007)

5.4.1. Sepsis induced ARF pathway

Acute renal failure occurs in patients with approximately 19 % in moderate sepsis, 23 % in severe sepsis and 51 % in septic shock. (Schrier & Wang, 2004)

Figure 15: Arterial vasodilatation and renal vasoconstriction in patients with sepsis (Schrier & Wang, 2004)

Figure 15 shows the physiologic pathway by which endotoxemia stimulates the induction of nitric oxide synthesis, which leads to nitric oxide mediated vasodilatation. The resultant arterial under-filling is sensed by the baroreceptors of the heart and results in an increase in sympathetic tone, which in turn, results in an increased outflow release of arginine vasopressin from the central nervous system (CNS), thus activating the rennin-
angiotensin-aldosterone system (RAAS). These increases in renal sympathetic and angiotensin activities lead to severe renal vasoconstriction with sodium and water retention and a predisposition to acute renal failure. (Schrier & Wang, 2004)

5.4.2. Myeloid differentiation factor 88 (MyD88) dependent Acute Renal Failure Pathway

According to the study done by Dear et al, “Sepsis induced organ failure is mediated by different pathways in the kidney and liver: Acute renal failure is dependent on MYD88 but not renal cell apoptosis.” (2006)

In the study that was conducted both the CLP mice (lacking TLR4) and the wild type mice, developed ARF, but they found that:

MyD88/−/− mice did not develop ARF compared to wild-type controls. In contrast, MyD88/−/− mice developed liver injury comparable to wild-type. After CLP, MyD88/−/− mice had significantly reduced apoptosis in the spleen compared with wild-type. Apoptosis was not detected in the kidney of wild-type or MyD88/−/− mice after CLP.

Myeloid differentiation factor 88 (MyD88) is a central adaptor protein for the majority of toll-like receptors, acting as a link between the receptors and downstream kinases [2]. These MyD88-dependent pathways lead to activation of the transcription factor NF-kB and production of cytokines such as TNF-α. (Dear et al, 2006)

5.4.3. TNF mediates LPS-induced ARF
In another study conducted by Cunningham et al, “Acute renal failure endotoximia is caused by TNF acting directly on TNF receptor-1 in kidney.” (2002)

Mice deficient in TNFR1 (TNFR1+/+) were resistant to LPS-induced renal failure. Compared with TNFR1+/+ controls, TNFR1-/- mice had less apoptosis in renal cells and fewer neutrophils infiltrating the kidney following LPS administration, supporting these as mediators of ARF. TNFR1+/+ kidneys transplanted into TNFR1-/- mice sustained severe ARF after LPS injection, which was not the case with TNFR1-/- kidneys transplanted into TNFR1+/+ mice. Therefore, TNF is a key mediator of LPS-induced ARF, acting through its receptor TNFR1 in the kidney.

\[
\text{LPS} \quad \text{TNFR1 } +/- \rightarrow \text{ Resistant to ARF} \\
\text{TNFR1 } +/- \rightarrow \text{ Not resistant}
\]

Figure 16: TNFR1 mediated ARF pathway

5.4.4. Inhibitory Pathways
Effects of \( \beta_2 - \)AR inhibition on LPS induced Renal CD14-TLR4-TNF\( \alpha \) Pathway:
Figure 17: Conceptual relationships among CD14, TLR-4, TNFα and β2 –AR, in LPS-induced ARF. (Nakamura, et al, 2008)

LPS initiates intracellular signaling events via CD14 and TLR-4 in the kidney. The activation of CD14 and TLR-4 complexes could result in the stimulation of renal TNF-α production. On the other hand, β2 –ARs couple to Gsα to raise intracellular level of camp, which inhibits the CD14-TLR4-TNF-α signaling cascades. Conversely, β2 –AR antagonist 2 (ICI118,551) activates CD14 and TLR4 complexes with subsequent TNF-α stimulation.

High levels of TNF-α induces systemic hypotension which can lead to renal hypoperfusion; they directly influence renal hemodynamics by damaging glomerular endothelial, and mesangial cells; they may induce the production of endothelin, prostaglandin, and NO; finally, they may play a role in the initiation and progression of certain risk factors for ARF. In sepsis, cytokine release results in leukocyte activation along with the expression of adhesion molecules, oxygen free radicals, arachidonic acid metabolites, platelet-activating factor, NO, endothelins, and heat shock proteins. Thus it
is this cytokine cascade that contributes to endothelial cell damage of the renal vasculature, leading to the development of ARF. The activation of the cAMP signaling pathway by means of β2-AR agonists down-regulated TNF-alpha gene expression using renal resident macrophage cells exposed to endotoxin. Consequently, the renal β2-AR system is able to modify the inflammatory responses initiated in renal disease and injury through the inhibition of renal TNF-α generation via camp-PKA activation.

The question arose as to the elevation of renal TNF alpha protein synthesis was due to direct effect of β2-AR antagonist in normal tissue. The present study indicated that the serum levels of CRP as a marker of systemic inflammation were not influenced by the co-administration of β2-AR antagonist. Importantly, serum noradrenaline, TNF alpha, IL6, IL10, and NO levels were not increased with the circulating level of endotoxin. Therefore, there is a possibility that administration of β2-AR antagonist directly stimulates the LPS-induced internal CD14 and TLR4 protein synthesis, leading to an increase in the production of TNF alpha in the kidney. In turn, this internal TNF alpha acts through renal TNFR1 to cause ARF, through a variety of mechanisms that may involve renal neutrophil infiltration and renal inflammation. However, the possibility remains that anti-inflammatory effects of β2-AR in other organs could modify renal function through CD14-TLR4- TNF-α signaling pathway. (Nakamura, et al, 2008)

There are several other inhibitory and pro-inflammatory pathways in sepsis-induced ARF. Table 3 has a list of all the major proteins associated with ARF that have been inferred from the literature survey. Different bioinformatics databases have been used to investigate the associated genes and their pathways.

<table>
<thead>
<tr>
<th>NO</th>
<th>Protein</th>
<th>Gene</th>
<th>Pathway</th>
<th>Suggested Treatment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Toll-like receptor 4</td>
<td>TLR4: Toll-like</td>
<td>1. NF-KB signaling</td>
<td>Following CLP,</td>
<td>(Dear et al,</td>
</tr>
<tr>
<td>ID</td>
<td>Gene Name</td>
<td>Function</td>
<td>Synonyms</td>
<td>Additional Information</td>
<td></td>
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<td>------------------------</td>
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</tr>
<tr>
<td>1</td>
<td>(hToll)(CD antigen CD284)</td>
<td>Toll-like receptor 4. Synonyms: TOLL, CD284, hToll, ARMD10, NP_612564.1</td>
<td>2. Toll like receptor mice lacking TLR4 and wild-type mice both developed comparable ARF.</td>
<td>2006) KEGG, Biocarta</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>ProteinC: Cleaved to its activated form on endothelial cells by the thrombin-thrombomodulin complex. Inactivator of coagulation factor Va and VIIIa.</td>
<td>Nitric oxide synthase 3 Synonyms: NOS3 ECNOS, eNOS</td>
<td>1. Corticosteroids and cardio protection. 2. Hypoxia-inducible factor in the cardiovascular system. 3. Ion channel and their functional role in vascular endothelium. 4. VEGF, hypoxia, and angiogenesis</td>
<td>Simvastatin: It’s a powerful lipid-lowering drug, but have proposed for inhibitory effect on macrophages. (Wikipedia, 2009)</td>
<td>(Yasuda, et al, 2006) Biocarta</td>
</tr>
<tr>
<td>6</td>
<td>Endothelial NO synthase (eNO) Function: Vasodilation. Contribute to transmission from one neuron to another, to the immune system.</td>
<td>VEGF Synonyms: VPF VEGF, MVD C1, VEGF-A, MGC70609</td>
<td>1. Hypoxia Inducible factor in the cardiovascular system 2. VEGF, Hypoxia and Angiogenesis.</td>
<td>Simvastatin: Cause: Vascular permeability</td>
<td>(Yasuda et al, 2006) Biocarta</td>
</tr>
<tr>
<td>7</td>
<td>Matrix metalloproteinase 9, GilainaseB, 92kDa</td>
<td>GelB, CLG4B, MMP-9, MMP9</td>
<td>Inhibition of matrix metalloproteinases</td>
<td>Simvastatin: Cause: Vascular permeability</td>
<td>(Yasuda et al, 2006) Biocarta</td>
</tr>
<tr>
<td>9</td>
<td>Tumor necrosis factor ligand superfamily member 2. TNF α</td>
<td>TNF</td>
<td>Synonyms: TNFA, DIF, TNFSF2, TNF-alpha, NP_000585.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1. CD40L Signaling</td>
<td></td>
<td>Simvastatin Cause: Vascular permeability</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2. Cytokine network</td>
<td></td>
<td>(Yasuda et al, 2006) Biocarta</td>
<td></td>
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<tr>
<td></td>
<td>3. IL10 Anti-inflammatory</td>
<td></td>
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<td></td>
<td>4. NF-KB</td>
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<td></td>
<td>5. Signal transduction through IL1R</td>
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<td></td>
<td>6. Stress induction of HSP regulation</td>
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<td></td>
<td>7. TNF/stress Related signaling</td>
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<td></td>
<td>8. TNFR1 signaling</td>
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<tr>
<td></td>
<td>9. TNFR2 signaling</td>
<td></td>
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<tr>
<td>10</td>
<td>Tumor necrosis factor receptor 1</td>
<td>TNFRSF1A</td>
<td>Synonyms: FPS, P55, p60, TBP1, TNF-R, TNFAR, TNFR1, p55-R, CD 120α, TNFR55, TNFR60, TNF-R-1, TNF-R55, MGC19588, TNFRSF1A, NP_001056.1</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>3. TNF/stress related signaling TNFR1 signaling</td>
<td></td>
<td>Prevention of renal failure in septic patients receiving anti-TNF Ab has been observed. But no effect on over all mortality. Outcome: ARF</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>(Yasuda et al, 2006) Biocarta</td>
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<tr>
<td>11</td>
<td>iNO: Nitric oxide synthase 2, inducible. upregulated in Kidney. (has a little role in induced ARF) Immune defense against pathogen.</td>
<td>NOS, INOS, NOS2A, HEP-NOS, NOS2</td>
<td></td>
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<tr>
<td></td>
<td>1. Mechanism of gene regulation by peroxisome proliferators via PPARα (alpha)</td>
<td></td>
<td>iNOS iNOS-/- mice still susceptible to LPS- induced ARF. Oral supplement or intravenous infusion of L-arginine supports a protective effect of NO on renal function in a number of nephrotoxic models.</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>2. NO2-dependent IL-12 pathway in NK cells</td>
<td></td>
<td>(Cunningham, et al, 2002) Biocarta</td>
<td></td>
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</tr>
<tr>
<td>12</td>
<td>Caspase-1: Apoptosis related cysteine peptidase (activates ILβ &amp; IL18) (CASP-1) (EC3.4.22.36) (IL1β converting enzyme)</td>
<td>CASP1</td>
<td>Synonyms: IL1BC, ICE, p45</td>
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<tr>
<td></td>
<td>2. D4-GDI signaling Pathway.</td>
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<tr>
<td></td>
<td>3. FAS signaling pathway(CD95)</td>
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<td></td>
<td>4. IL-18 signaling pathway</td>
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<td></td>
<td>5. Induction of apoptosis through DR3 and</td>
<td></td>
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<tr>
<td>13</td>
<td>Cyclophilin</td>
<td>DR4/5 death receptors</td>
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<tr>
<td></td>
<td>Cyclophilin A: Synonyms: CYPA, CYPH, MGC12404, MGC23397, MGC117158, PPIA</td>
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<td></td>
<td>2. CYPB, SCYLP, CYP-S1, MGC2224, MGC14109, PPIB</td>
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<tr>
<td>14</td>
<td>BSG basigin: CD147 signaling receptor for extracellular cyclophilin (its inhibition less effect on liver)</td>
<td>Anti CD147 antibody Prevent ARF.</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>BSG basigin Synonyms: M6, OK, SF7, TCSF, CD147, EMPRIN</td>
<td>(Dear, et al, 2007) Biocarta</td>
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<tr>
<td></td>
<td>1. CCR3 signaling in Eosinophils</td>
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<td></td>
<td>2. CXCR4 signaling pathway</td>
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<td>3. Growth hormone signaling pathway</td>
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<td></td>
<td>4. IL-5 signaling pathway</td>
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<td></td>
<td>5. IL12 and Tat4 dependent signaling pathway in Th1 development</td>
<td></td>
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<td></td>
<td>6. IL-2 receptor beta chain in T cell activation</td>
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<td></td>
<td>7. IL22 soluble receptor signaling pathway</td>
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<td></td>
<td>8. Msp/Ron receptor signaling pathway</td>
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<td></td>
<td>9. NO2-dependent IL12 pathway in NK cells</td>
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<td></td>
<td>10. Pertussis toxin-insensitive CCR5 signaling in macrophage</td>
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<td></td>
<td>11. Selective expression of chemokines receptors during T-cell polarization</td>
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<td></td>
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<tr>
<td></td>
<td>12. Signaling of hepatocyte growth factor receptor</td>
<td></td>
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</tr>
<tr>
<td>15</td>
<td>Serum amylase: AMY2A amylase, alpha 2A (pancreatic) (increased)</td>
<td>No known pathways were found</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Brush border enzyme (especially meprin-1-alpha) MEPIA meprin A, alpha (PABA peptide hydrolase)</td>
<td>Actinonin to inhibit and reduce renal injury</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>MEPIA Synonyms: PPHA</td>
<td>(Dear, et al, 2007) Biocarta</td>
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<td>-------------------------------------------------------------------------</td>
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<tr>
<td>17</td>
<td>ICAM-1 Upregulated by LPS. Believe to be facilitate ARF. Intracellular adhesion molecule 1(major group rhinovirus receptor) (CD antigen CD54)</td>
<td>ICAM1</td>
<td>T Helper cell surface molecule</td>
<td></td>
<td>(Wu, et al, 2007) Biocarta</td>
</tr>
<tr>
<td>18</td>
<td>Protein Wnt-4 Wingless-type MMTV integration site family, member4</td>
<td>Wnt-4 expressed in mesonephric duct in the embryonic development. Synonyms: SERKAL, WNT4</td>
<td>Wnt-β-catenin pathways</td>
<td></td>
<td>(Terada, et al, 2005) Biocarta</td>
</tr>
<tr>
<td>20</td>
<td>Protein C-ets-1 (p54): Transcription factor. May play a key role in dedifferentiation and regeneration of the renal tubular cells after ARF.</td>
<td>ETS1</td>
<td>ETS-1 pathways: Regulate the transcription of cyclin D1 and control the regeneration of renal tubules in ARF. Over expression of Ets-1 promoted the cell cycle and increased the promoter activity and protein expression of cyclin D1 in LLC-PK1 cells.</td>
<td></td>
<td>(Terada et al, 2005) Biocarta</td>
</tr>
<tr>
<td>21</td>
<td>VCAM: Vascular cell adhesion protein. VCAM 1, INCAM-100, CD antigen CD106: increases vessel permeability, which leads to contraction</td>
<td>VCAM1</td>
<td>1. Adhesion and diapedesis of lymphocytes 2. Cells and molecule involved in local acute inflammatory response</td>
<td></td>
<td>(Tu et al, 2001) Biocarta</td>
</tr>
</tbody>
</table>
| 23 | MCP-1 | C-C motif chemokine 2, Monocyte chemoattractant protein 1, monocyte chemotactic and activating factor (MCAF), monocyte secretary protein JE. | CCL2 chemokine (C-C motif) ligand 2. Synonyms: HC11, MCAF, MCP1, MCP-1, SCYAC, GDCF-2, SMC-CF, HSMCR30, MGC9434, CCL2 | 1. Msp/Ron receptor signaling pathway  
2. Pertussis toxin-insensitive CCR5 signaling in macrophages | (Schor, 2002) Biocarta |
| 24 | NF-κB1: P105 → P50 | 105KD protein undergoes cotranslational processing by the 26S proteasome to produce a 50 KD protein. 105 KD is a Rel protein-specific transcription inhibitor and the 50 KD is a DNA binding subunit of the NF-κB protein complex. (Unigene) | NFKB1 Synonyms: p50, KBF1, p105, EBP-1, MGC54151, NFkB-p50, NFkB-p50, NFkB-p105, NF-kappa-B, DKFZp686C01211, NP_003989.2 | 1. Mitogen-activated protein kinase signaling  
2. Toll-like receptor  
3. Signal Transduction IL1R | Glucocorticoids, cyclosporine, angiotensin-converting enzyme (ACEI), lovastatin-like drugs. (Block NF-KB activation) (Schore, 2002) Biocarta |
| 26 | LTs: they are highly potent vasoconstrictor eicosanoid. And are also implicated in the pathophysiology of sepsis-induced ARF. LTC₄: | LTC₄S: MGC33147 | 1. 5-lipoxygenase enzyme pathway (are related to slow-reacting substances of anaphylaxis. LT antagonists minimize the effects of intravenous LPS administration, including acute renal dysfunction. | (Schore, 2002) Millipore |
| 27 | LTD₄: Cysteinyll leukotriene receptor 1 (cysLTR1) | CYSLTR1 Synonyms: HG55, CYSLTR1, CYSLTR, HMTMF81, | 1. 5-lipoxygenase enzyme pathway  
<table>
<thead>
<tr>
<th>Pathway</th>
<th>Description</th>
<th>Genes and Synonyms</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTE4: Cysteinyl leukotriene receptor 1</td>
<td></td>
<td>Cysltr1 Synonyms: GPCR, HG57, CYSLT@, HPN321, CYCLT2R, KPG_011, bGPCR21, PSEC0146</td>
<td>1. 5-lipoxygenase enzyme pathway 2. Neuropeptidase signaling through G-protein alpha-i- &amp; G-protein alpha-q</td>
</tr>
<tr>
<td>Insulin-like growth factor-I(IGF-I) Increased RPF and GRF</td>
<td></td>
<td>IGF1 Synonyms: IGF1A, IGF1</td>
<td>1. Control of skeletal myogenesis by HDAC &amp; calcium/calmodulin-dependent kinase (CaMK) 2. ErK1/Erk2 Mapk signaling pathway 3. IGF-1 signaling pathway 4. Multiple antiapoptotic pathways from IGF-1R signaling lead to BAD phosphorylation 5. Regulation of BAD phosphorylation 6. Skeletal muscle hypertrophy is regulated via AKT/mTOR pathway</td>
</tr>
</tbody>
</table>

Table 3: Pathways to Acute Renal Failure.

There are 58 known pathways in this table that are associated with the genes and their expressed proteins that are specifically responsible for acute renal failure. However on the whole ARF involves not only the pathways in table 3, but also majority of the pathways in table 2.

This report also includes another table (Table 4) consisting of proteins and their time course of appearing in the serum or blood according to various literature papers. The
numbers and timing may vary from paper to paper and just to get a rough idea on what is
going on and at what stage of disease the body is progressing.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Level</th>
<th>Stage (Organs)</th>
<th># of days</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL 10</td>
<td>Higher</td>
<td>3 or more organ failure</td>
<td>Day1: 2,522 +/- 1,365 Day2: 635 +/-198 Day3: 632 +/-271 ng/milligram</td>
<td>(Doughtley et al, 1998)</td>
</tr>
<tr>
<td>IL 10</td>
<td>Higher (but less then in 3 or more organ failure)</td>
<td>Sequential Pulmonary/hepatic/renal failure</td>
<td>Day1: 488 +/- 155 Day2: 255 +/-85</td>
<td>(Doughtley et al, 1998)</td>
</tr>
<tr>
<td>IL 10</td>
<td>Higher (higher then more then 3 organ failure)</td>
<td>Nonsurvival</td>
<td>Day1: 4,701 +/- 3,655 Day2: 991 +/- 401 Day3: 799 +/- 52</td>
<td>(Doughtley et al, 1998)</td>
</tr>
<tr>
<td>IL-10</td>
<td>Higher (but less then even less then 3 organ failure)</td>
<td></td>
<td>Day1: 786 +/- 169 Day2: 287 +/- 73 Day3: 175 +/- 57</td>
<td>(Doughtley et al, 1998)</td>
</tr>
<tr>
<td>IL 8</td>
<td>Very high</td>
<td>High in bronchoalveolar lavage fluid</td>
<td>Higher mortality rates Sepsis induced respiratory syndrome higher mortality rates.</td>
<td>0 h: 1868.2-8425.5 ng/L</td>
</tr>
<tr>
<td>IL6, IL8, IL10, MCP1, TNF alpha</td>
<td>Significantly increased</td>
<td>Severely infected infants with DIC</td>
<td>0 h = 1879.1ng/l 24h = 1064.7</td>
<td>(Yasuda, 2008)</td>
</tr>
<tr>
<td>RANTES</td>
<td>reduced</td>
<td>Severely infected infants with DIC, severe sepsis</td>
<td>3907.</td>
<td>(Yasuda, 2008)</td>
</tr>
<tr>
<td>IL 10, IL6</td>
<td>Higher</td>
<td>With DIC</td>
<td>0h= 4165.8 &amp; 703.2 24h= reduced to 798.5 &amp;80.8</td>
<td>(Yasuda, 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Multiple organ failure</td>
<td>48 hours (Death)</td>
<td></td>
</tr>
<tr>
<td>HSP70</td>
<td>Increased</td>
<td>Severe sepsis</td>
<td>72 hours within</td>
<td>(Adrie et al, 2001)</td>
</tr>
<tr>
<td></td>
<td>Not significant</td>
<td>Severe sepsis</td>
<td>7 to 10 days or at discharge</td>
<td>(Adrie et al, 2001)</td>
</tr>
<tr>
<td>Bcl2</td>
<td>Increased</td>
<td>Survivors&gt; nonsurvivors</td>
<td>72 hours</td>
<td>(Yasuda et al, 2006)</td>
</tr>
<tr>
<td>Creatinine</td>
<td>Twice upper limit of normal</td>
<td>ARF</td>
<td></td>
<td>(Yasuda et al, 2006)</td>
</tr>
<tr>
<td>Plasma arginine</td>
<td>Initially increased to 200 to 300 pg/ml</td>
<td>Septic Shock</td>
<td>Initially</td>
<td>(Cunningham et al, 2002)</td>
</tr>
<tr>
<td>Plasma arginine</td>
<td>Neurohypophyseal stores of vasopressin deplete and plasma concentration may fall to 30 pg/ml</td>
<td>Septic Shock</td>
<td>After an hour</td>
<td>(Cunningham et al, 2002)</td>
</tr>
<tr>
<td>Serum IL-10</td>
<td>Higher</td>
<td>Develop sepsis</td>
<td>First 72 hours of trauma during hospitalization</td>
<td>(Cunningham et al, 2002)</td>
</tr>
<tr>
<td>Procalcitonin(pre cursor of the)</td>
<td>Higher level</td>
<td>Identifies those septic patients who will</td>
<td></td>
<td>(Claessens &amp;Dhainaut,</td>
</tr>
<tr>
<td>Hormone</td>
<td>Development</td>
<td>Description</td>
<td>Unit</td>
<td>Reference</td>
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</tr>
<tr>
<td>Calcitonin</td>
<td>Involved in</td>
<td>Calcium homeostasis</td>
<td></td>
<td>2007</td>
</tr>
<tr>
<td>atrial natriuretic peptide (ANP), atrial natriuretic factor (ANF)</td>
<td>Significantly higher</td>
<td>As early as the day of admission in surviving sepsis Patients</td>
<td>Survivors: median= 194pmol/l (range 20-2000pmole/l) Non survivors: Median= 853.0pmol/l (range 100-2000pmol/l)</td>
<td>(Morgenthaler, et al, 2004)</td>
</tr>
<tr>
<td>IL-1beta</td>
<td>High</td>
<td>Severe sepsis</td>
<td>0.17(0-0.79) Values are in pg/ml, and a expressed as mean (range).</td>
<td>(Bozza et al, 2007) Day 1. Concentration in blood</td>
</tr>
<tr>
<td>IL-1bata</td>
<td>Higher</td>
<td>Septic shock</td>
<td>1.22(0.01-7.33) As above</td>
<td></td>
</tr>
<tr>
<td>IL-7</td>
<td>Not produced</td>
<td>Severe sepsis</td>
<td>0</td>
<td>As above</td>
</tr>
<tr>
<td>IL-13</td>
<td>Not significant</td>
<td>Severe sepsis</td>
<td>0.27(0.00-4.61) As above</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>Not significant</td>
<td>Severe sepsis</td>
<td>0</td>
<td>As above</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Increased</td>
<td>Septic shock</td>
<td>14.46(2.68-47) As above</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>Not significant</td>
<td>Survivors</td>
<td>8(0-25.72) As above</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>Slightly increased</td>
<td>Non-survivors</td>
<td>9.04(2.31-43.28) As above</td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Not significant</td>
<td>Severe sepsis</td>
<td>0(0-22.77) As above</td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Increased</td>
<td>Septic shock</td>
<td>33.10(0.00-116.7) As above</td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Not significant</td>
<td>Survivors</td>
<td>0.39(0-3.04) As above</td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Slightly increased</td>
<td>Non-survivors</td>
<td>1.30(22-7.21) As above</td>
<td></td>
</tr>
<tr>
<td>G-</td>
<td>No change</td>
<td>Survivor/non-survivor</td>
<td>Mean = 0(range varies) As above</td>
<td></td>
</tr>
</tbody>
</table>
### Table 4: Timeline of different proteins as they appear.

<table>
<thead>
<tr>
<th>Protein Description</th>
<th>Comparison</th>
<th>Group</th>
<th>Value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF (Granulocyte-macrophage colony stimulating factor)</td>
<td>High</td>
<td>Survivors</td>
<td>116 (12-367)</td>
<td>As above</td>
</tr>
<tr>
<td>G-CSF (Granulocyte colony-stimulating factor)</td>
<td>Higher</td>
<td>Non-survivors</td>
<td>423.61 (0-2488.50)</td>
<td>As above</td>
</tr>
<tr>
<td>MT isoform MT-1E: MTs are cystein-rich, low molecular</td>
<td>Two fold</td>
<td>Non-survivors/</td>
<td></td>
<td>(Wong et al, 2007)</td>
</tr>
<tr>
<td>molecular weight, intracellular metal-binding protein</td>
<td>higher</td>
<td>survivors and control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MT isoform MT-1M</td>
<td>2.5 fold</td>
<td>Non-survivor / Survivors</td>
<td></td>
<td>As above</td>
</tr>
</tbody>
</table>
6. Economic Justification

Economic justification of this project will provide an overview of the need for the deliverable report, benefits, who benefits, cost analysis, market of such research, competitors, marketing strategy, potential customers and further funding opportunities.

6.1. Executive Summary

Sepsis is one of the top ten causes of death in the United States. Sepsis-related incidences of deaths are more common than other common and well-known devastating diseases. The key to successfully combating this horrible condition is early diagnosis and swift intervention. There are more possibilities to intervene appropriately for treatment, the sooner the diagnosis and identification of the targeted organ of sepsis is known. Once the sepsis reaches its final stage of septic shock the patient only has a few days to get proper treatment.

In this project an extensive literature survey has been conducted to draw out recent research related to major proteins and their respective genes that are involved in fever spikes and acute renal failure in response to the infectious agents. All the information is organized in the form of three tables. Table 2 and Table 3 consist of proteins and their respective genes, involved known pathways, targeted organ or clinical outcome, suggested treatment and the references from where the information is cited. Table 4, has the list of proteins and their level of appearance in the blood or serum, level of sepsis or targeted organ, timeline of that event and the list of references. The purpose of such a report is to get a sense of what organ is going to be the victim of sepsis before it actually damages the organ and can be in advanced determined by the physical symptoms. Once
the patient comes to the emergency room, a simple blood test or serum test can identify
the elevated or decreased level of the proteins, then the generated table can be used to
identify the genes and their regulation and their corresponding pathways. Patterns of
pathways that are organized in the table then can identify the targeted organ. At this time
preventive actions can be taken to stop the progression of sepsis towards the organ at risk.
Recent diagnostic tests involve blood cultures and the identification of the infective
pathogen to diagnose the presence of sepsis, and by the time infection is confirmed it is
already too late to stop the progression of sepsis. To avoid this delay recently broad
spectrum antibiotics are being used to cover many kinds of infections, and some times
many kinds of antibiotics have to be tried before the right one is found. Unfortunately, in
patients having sepsis, and especially those with severe sepsis, the progression is very
fast and the medical staff does not have enough time try different antibiotics. Also there
can be very severe and damaging side effects to using broad-spectrum antibiotics.
The intensions are to form a company that will provide genetic and proteometric pathway
analysis. Company’s services will include reports, tables and charts that identify the
progression of sepsis, clinical outcomes and treatments. These services are estimated to
result in 40% reduction of ICU costs, which will save 6.8 billion dollars annually in
United Stares. Revenue model is based on initial license cost of the report at $100K,
annual services for updates is $10K per hospital, insurance companies or other
organizations. It is initially a low-budget project, but due to the huge market size will be
able to breakeven the first year of its operations.
6.2. Problem Statement

Sepsis one of the most challenging conditions for the doctors to diagnose today yet it is the most common cause of death in the critical care units of the hospitals. Early diagnosis is the key for early treatment, but the available blood culture tests take 48 hours to confirm the infection. There is a desperate need for early diagnostic methods to start sepsis treatment sooner so the progression of sepsis can be stopped before it reaches the fatal stages of severe sepsis and septic shock.

6.3. Solution and Value Proposition

The company provides genetic and proteomics pathway analysis to the healthcare providers to diagnose sepsis quickly and accurately. Company’s services, includes providing reports and tables and charts that identify the genes, proteins, pathways and time-course of sepsis. The analysis patterns of genetic pathways associated with the expressed proteins and the level of expressed proteins will not only perform the diagnosis, but also indicate the stage of sepsis, expected clinical outcome and the suggested treatment in a timely manner.

6.4. Market Size

All hospital patients are at risk of getting sepsis:

- 1-2% of all patients in a hospital get sepsis
- 25% of all patients in intensive care get sepsis
- 30-50% result in deaths out of 25% of sepsis patients
- Children under 3 months of age are at higher risk
• According to one study 20% of the patients were under one year of age (Hilmar, 2004)

According to a study conducted in 1995:

Total incidences of sepsis in America: 751,000
Deaths: 215,000

Approximate per patient expenses for the treatment: 22,100 United State Dollars (USD)
Total annual cost nationwide: 16.7 billion USD (Daniel, 2007)

Moreover, according to subsequent studies and surveys, the incidence of sepsis and related deaths has shown an increasing trend. (Daniel, 2007)

Sepsis is one of the most complicated medical conditions and is a real challenge to diagnose and treat for the medical community. Just in California alone there are almost 400 hospitals that can benefit from the diagnostic services provided by our company.

These findings from the literature lead to the conclusion that failing to treat sepsis is very expensive. The average total cost per Intensive Care Unit (ICU) day can start at approximately $1200 dollars per day to about $2500 per day. For example, just the ventilator use for sepsis patients is 15 times more than other patients. On average, patients need to stay in the ICU for as long as 14 days. (Hilmar, 2004)

The incidences of sepsis are increasing across all demographic groups at an average rate of by 8.7% per year (Martin et al., 2003). This increase is mainly due to the growing use of interventional procedures and growing numbers of elderly and vulnerable people. Older people are more susceptible to infections due to aging, co-morbidities, use of invasive surgical techniques and problems associated with institutionalization. (Barcelona declaration, 2007)
6.5. Competitors

As indicated above, there is presently no preferred sepsis marker available in the market. Company’s idea is very unique and there is not a single company that is working in the same area of development. Although it is important to mention here some companies that are providing services that will help in early diagnosis and treatment of the sepsis and other diseases.

6.5.1. Elashoff Consulting:

The company is based in Redwood City, California and might be a possible future competitor due to the services offer, but still they don’t have a specific package that can help hospitals and health care insurers to reduce the cost of sepsis treatment by providing solutions for early sepsis diagnosis. Their services include:

- Disease analysis
- Biomarker identification
- Pathway analysis
6.5.2. Nanogen

Nanogen was awarded $2.5 million grant for development of diagnosis for sepsis and pneumonia for over the next 5 years by the National Institute of Allergy and Infectious Diseases (NIAID), which is a division of NIH. (PRNewswire, 2005)

“Nanogen's advanced diagnostics provide researchers, clinicians, physicians and patients worldwide with improved methods and tests that can predict, diagnose and ultimately help treat disease. Nanogen's products include real-time PCR reagents, the NanoChip(R) Molecular Biology Workstation platform for molecular diagnostic applications and its line of rapid point-of-care diagnostic tests. Nanogen's ten years of pioneering research involving nanotechnology may also have future applications in medical diagnostics, biowarfare and other industries.” (PRNewswire, 2005)

There is an increased interest in sepsis research from other companies as well; some working in this field include:

- Biosite Incorporated is a leading bio-medical company commercializing proteomics discoveries for the advancement of medical diagnosis.
- Eli Lilly
- SIRS-Lab GmbH
- Rules-Based Medicine, Inc. Medicine awarded 849,000 grant by the NIH in 2007
- GATC Biotech: DNA based sepsis diagnostic
- Bioveris Corporation (BIOV)
- Prostherics PLC received 16.3 million pounds in 2006
There are great opportunities to seek and apply for a grant on the basis of the research in this report for further investigation of pathway analysis.

6.6. Customers

6.6.1. Healthcare Service Providers

Sepsis is an extremely complicated condition for a doctor to diagnose and treat. Individual hospitals, clinics and doctors can also be significant customers. Once the diagnosis is made preventive care and treatment can stop sepsis from progressing. The company will provide the immediate information, such as diagnostic tables and reports to the healthcare providers so the diagnosis can be made in a timely manner. The Company will also provide services to update the tables for an annual subscription fee.

6.6.2. Insurance companies

Sepsis is a significant burden on healthcare service providers and they are constantly seeking ways to lower the sepsis cost and better treatment by requiring health care providers to use company’s services.

Below are just a few names of potential healthcare provider customers.

- Aetna
- Health net
- Blue Cross and Blue Shield
- Medicaid
6.6.3. Clinical diagnostic equipment companies

Other biotech companies that are developing products in the field of diagnostic test kits and assay will be greatly interested in this project. Pharmaceutical companies and biotech companies are anxious to learn about potential targets in the body for drug development. This is a great opportunity for them to invest their money in a company like this so they do not have to their own research. Being our customer, investors or clients will save them millions of dollars.

6.6.4. Other Research organizations

Other organization and research institutes, who already have funding and resources to conduct their study about the diagnosis and investigation of sepsis, will be interested in the report. This report will provide them all the initial information that they need to acquire to further investigate. This way they can utilize their funding and time in gathering their own data, clinical trials and other necessary steps for their research.

6.7. Cost Estimation

This report and pathway analysis related services will be provided to the health care providers. This project is initially a very low-budget project that mostly involves literature search, database search and then inferring meaningful information and results from the data and information that is already available. To conduct a clinical study to obtain our own new data and the associated equipment and resources for it can cost up to millions of dollars. This project is using data and information that have already been done by these other higher budget projects. There is wealth of data and findings publicly

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available in published journal and conference papers. Most of all these sources are freely available online or in libraries. There is no manufacturing cost involved so the estimated fixed cost and the variable cost will be responsible for the report and services to the company.

6.7.1. Fixed Costs

“Fixed costs are business expenses that are not dependent on the activities of the business. They tend to be time related such as salaries or rent being paid per month”

(Fixed cost, 2009)

<table>
<thead>
<tr>
<th>Expenses</th>
<th>2009</th>
<th>2010</th>
<th>2011</th>
<th>2012</th>
<th>2013</th>
</tr>
</thead>
<tbody>
<tr>
<td>License and patent</td>
<td>0</td>
<td>$10,000</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Software tools</td>
<td>0</td>
<td>$2000</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Stationary</td>
<td>100</td>
<td>$2000</td>
<td>$2500</td>
<td>$2500</td>
<td>$2500</td>
</tr>
<tr>
<td>Telephone and internet services</td>
<td>$500</td>
<td>$10,000</td>
<td>$10,000</td>
<td>$10,000</td>
<td>$10,000</td>
</tr>
<tr>
<td>Utilities</td>
<td>$500</td>
<td>$1500</td>
<td>$1500</td>
<td>$1500</td>
<td>$1500</td>
</tr>
<tr>
<td>Maintenance</td>
<td>0</td>
<td>$15000</td>
<td>$15000</td>
<td>$15000</td>
<td>$15000</td>
</tr>
<tr>
<td>Furniture lease</td>
<td>0</td>
<td>$1000</td>
<td>$1000</td>
<td>$1000</td>
<td>$1000</td>
</tr>
<tr>
<td>Employee salaries</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chief executive officer (CEO)</td>
<td>1</td>
<td>$90000</td>
<td>$90000</td>
<td>$90000</td>
<td>$90000</td>
</tr>
<tr>
<td>VP of Engineering</td>
<td>0</td>
<td>$70000</td>
<td>$70000</td>
<td>$70000</td>
<td>$70000</td>
</tr>
<tr>
<td>VP of marketing</td>
<td>0</td>
<td>$70000</td>
<td>$70000</td>
<td>$70000</td>
<td>$70000</td>
</tr>
<tr>
<td>Senior Scientist/Engineers</td>
<td>0</td>
<td>$110000</td>
<td>$110000</td>
<td>$110000</td>
<td>$110000</td>
</tr>
<tr>
<td>Junior scientist</td>
<td>0</td>
<td>$70000</td>
<td>$70000</td>
<td>$70000</td>
<td>$70000</td>
</tr>
<tr>
<td>Marketing</td>
<td>0</td>
<td>$50000</td>
<td>$50000</td>
<td>$50000</td>
<td>$50000</td>
</tr>
<tr>
<td>Administration staff</td>
<td>0</td>
<td>$48000</td>
<td>$48000</td>
<td>$48000</td>
<td>$48000</td>
</tr>
<tr>
<td>Accounts</td>
<td>0</td>
<td>$48000</td>
<td>$48000</td>
<td>$48000</td>
<td>$48000</td>
</tr>
<tr>
<td>Employee benefits</td>
<td>0</td>
<td>$50,000</td>
<td>$50,000</td>
<td>$50,000</td>
<td>$50,000</td>
</tr>
<tr>
<td>Insurance</td>
<td>0</td>
<td>$20,000</td>
<td>$20,000</td>
<td>$20,000</td>
<td>$20,000</td>
</tr>
<tr>
<td>Overhead cost</td>
<td>$100</td>
<td>$5000</td>
<td>$5000</td>
<td>$5000</td>
<td>$5000</td>
</tr>
<tr>
<td>Total amount per year</td>
<td>$1200</td>
<td>$557500</td>
<td>$557500</td>
<td>$557500</td>
<td>$557500</td>
</tr>
</tbody>
</table>

Table 5: Estimated fixed cost
6.7.2. Variable cost

“Variable costs are expenses that change in proportion to the activity of the project.”

(Variable cost, 2009) The expected variable cost of the company is shown in Table.

<table>
<thead>
<tr>
<th>Expenses</th>
<th>2009</th>
<th>2010</th>
<th>2011</th>
<th>2012</th>
<th>2013</th>
</tr>
</thead>
<tbody>
<tr>
<td>Advertisement cost</td>
<td>0</td>
<td>$3000</td>
<td>$3000</td>
<td>$5000</td>
<td>$5000</td>
</tr>
<tr>
<td>Website</td>
<td>1000</td>
<td>$500</td>
<td>$500</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>Customer services</td>
<td>0</td>
<td>$2000</td>
<td>$5000</td>
<td>$5000</td>
<td>$5000</td>
</tr>
<tr>
<td>Business expenses</td>
<td>0</td>
<td>5000</td>
<td>7000</td>
<td>7000</td>
<td>10000</td>
</tr>
<tr>
<td>Legal and accounting fees</td>
<td>0</td>
<td>$150,000</td>
<td>$165,000</td>
<td>$165,000</td>
<td>$185,000</td>
</tr>
<tr>
<td>Total</td>
<td>1000</td>
<td>160500</td>
<td>180500</td>
<td>180500</td>
<td>210000</td>
</tr>
</tbody>
</table>

Table 6: Estimated variable cost

6.7.3. Price point

The report price and the services charges have been calculated by literature search and market-analysis.

These calculations are based on savings accrued by reducing number of days in the ICU for the patient and to the cost associated to medical professionals’ costs, and cost related to treatment of the diagnosis.

Estimation of cost to stay in ICU:

- Average increase in stay in the ICU due to sepsis = 8 days
- Average number of days for sepsis treatment = 19 depending on progression of sepsis, the days do vary so does the cost.
- Mean cost of the ICU stay for a sepsis patient = $39,399 (Teres et al, 2001)

Mean cost of the ICU stay utilizes several hospital resources as listed in the Table 2 and their respective cost all in US dollars.
ICU resources | Sepsis patients | Respiratory patients | All other patients
--- | --- | --- | ---
Pharmacy | 2625 +/- 3555 | 1443 +/- 1767 | 1436 +/- 2495
Lab | 2463 +/- 3109 | 1127 +/- 1220 | 1224 +/- 2009
Imaging | 1217 +/- 1389 | 683 +/- 653 | 784 +/- 1100
Respiratory therapy | 2387 +/- 3245 | 1791 +/- 1809 | 1213 +/- 2117

Table 7: Mean cost for ICU stay. (Teres et al, 2001)

Early diagnosis will help reduce cost of ICU stay by 40%

![Comparison of ICU cost of sepsis patient and all other patients](image)

Figure 19: Comparison of ICU cost of sepsis patient and all other patients

Earlier discharge will lead the hospitals and health care industries to reduce the reduction in cost of the ICU by at least 40%.

Average cost of treating sepsis annually in United States = 17 billion dollars annually

As indicated before that the number of sepsis incidences have been only increasing so does the annual cost associated with its treatment.

So the reduction in 40 percent of the annual cost = 6.8 billion dollars annually reduced.

The actual number of patients that will benefit from this project and the number of days of their stay in ICU likely depend on the type of hospital and standards of care.
Assuming that 10% of all 751,000 incidences of sepsis happen in California then the estimated cost of sepsis treatment is 1.7 billion dollars. The services provided by the company will reduce the cost by 40% will result in 680 million dollars annual savings just in California.

The number of hospitals and clinics in California is approximately 400, so the total savings per hospital is estimated to be 400 million dollars. So based on above savings Company will license the report to the hospitals at initial cost of $100 K and annually for update and services $10K annually per hospital.

6.8. Strength, Weakness, Opportunity and Threats (SWOT) Analysis

To continue the research and generate the income at the same time one need to assess the strengths and weaknesses of the project so appropriate steps can be taken to avoid any future threats.

<table>
<thead>
<tr>
<th>Strengths</th>
<th>Weaknesses</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. One report contains useful and important information from several hundred research papers.</td>
<td>1. Information gathered from only free text papers.</td>
</tr>
<tr>
<td>2. Build a bridge between proteomics and genetics. Get the expression of genes by looking at the present proteins via simple blood test.</td>
<td>2. Only covers Pyrogen and ARF so far.</td>
</tr>
<tr>
<td>3. Getting the idea of gene expression without actually doing gene expression profiling, as it is not possible in a clinical setting to do gene expression profiling on patient.</td>
<td>3. Lack of own experimental data</td>
</tr>
<tr>
<td></td>
<td>4. No wet lab is involved, so further testing needs be done to confirm the result of this literature survey.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Opportunities</th>
<th>Threats</th>
</tr>
</thead>
</table>
1. Apply for grants to continue research at higher level.
2. Sell the report to a customer.

1. Someone else comes up with similar kind of research with his or her own data set and analysis.
2. Due to economics crisis unable to find customers or funding.

Table 8: SWAT analysis

6.9. Investment capital requirements

The company requires $2 million dollars to start operating. Increasing number of sepsis incidences and Health care providers and insurers desperate need of early diagnosis of sepsis and initial funding will be sufficient achieve the breakeven point significantly early.

6.9.1. Estimation of funds or investment

Cash flow analysis (all figures in US dollars, i.e. $):

<table>
<thead>
<tr>
<th>Year</th>
<th>2009</th>
<th>2010</th>
<th>2011</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of sponsors</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total cost</td>
<td>2200</td>
<td>718000</td>
<td>738000</td>
</tr>
<tr>
<td>Available funds</td>
<td>0</td>
<td>200000</td>
<td>1,282000</td>
</tr>
</tbody>
</table>

Table 9: Estimated cash flow analysis

Figure 20: Cash flow analysis graphical representation
6.9.2. Calculations of total income

<table>
<thead>
<tr>
<th>Year</th>
<th>2009</th>
<th>2010</th>
<th>2011</th>
<th>2012</th>
<th>2013</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of customers</td>
<td>0</td>
<td>10</td>
<td>40</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>License fees charged</td>
<td>0</td>
<td>$1000000</td>
<td>$4000000</td>
<td>$8000000</td>
<td>$10000000</td>
</tr>
<tr>
<td>Annual subscription</td>
<td>0</td>
<td>0</td>
<td>200000</td>
<td>500000</td>
<td>800000</td>
</tr>
<tr>
<td>Total revenue</td>
<td>0</td>
<td>$2000000</td>
<td>$5200000</td>
<td>$8500000</td>
<td>$10800000</td>
</tr>
</tbody>
</table>

Table 9: Revenue estimation

6.10. Break-even analysis

Break-even point is the time when the company starts generating profits.

<table>
<thead>
<tr>
<th>Year</th>
<th>No of customer</th>
<th>Fixed Cost (in thousands)</th>
<th>Expense (Fixed cost+variable cost)</th>
<th>Revenue (in thousands)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2009</td>
<td>0</td>
<td>1.2</td>
<td>2.2</td>
<td>0</td>
</tr>
<tr>
<td>2010</td>
<td>10</td>
<td>557</td>
<td>718</td>
<td>1000</td>
</tr>
<tr>
<td>2011</td>
<td>40</td>
<td>557</td>
<td>738</td>
<td>4000</td>
</tr>
<tr>
<td>2012</td>
<td>80</td>
<td>557</td>
<td>738</td>
<td>8000</td>
</tr>
<tr>
<td>2013</td>
<td>100</td>
<td>557</td>
<td>768</td>
<td>10000</td>
</tr>
</tbody>
</table>

Table 10: Breakeven analysis

Figure 21: Breakeven analysis

Breakeven analysis represented by the Table 11 and Figure 21 above indicates that, the company will be successes fully able to reach not only reach the breakeven point, but also will generate profit the first year of its operation.
6.11. Business and revenue model

To gain successes in any business sales and marketing departments plays critical role.

The first step that will be taken after generating the report is to meet with few key people from health insurance companies, physicians and hospital administrators and get their feedback.

Another strategy is to reach out to specific media channels, have an article written and publish a paper in leading medicine journals. Another option is to contact the healthcare services, hospitals and marketing agencies directly and showing them the potential benefits and interested companies.

Company will launch their website explaining all the services and reports, tables and their brief description. Link the website to other related website so the message can reach the customers.

6.12. Profit and Lost

Formula used for the profit and lost calculations is as follows:

\[
\text{Profit/loss} = \text{Revenue} - \text{Expense}
\]

![Profit and loss graph](image)

Figure 22: Profit and loss graph
Initially it is a very low budget project and will get profits in the first year. According to the breakeven analysis and revenue details of the company will gain profits in 2010. It is clear from both of the above graphs that breakeven will occur the year company will start its business. Strong marketing and business strategy will expand the company to make customers throughout the California. As mentioned before there are approximately 400 hospitals in California so the goal of the company to reach out to all the hospital and show them the millions of dollars of savings and protection of lives by using the report and services that the company offers.

Norden-Rayleigh Curves:

\[
v(t) = 2adte^{-at^2}, \ a=.01, \ d=2\text{MM}
\]

Figure 23: Funding profile over time; \(v(t) = 2adte^{-at^2}, \ a=.01, \ d=2\text{MM}\)
The initial project budget is estimated at 2 million dollars. Based on other similar research work, this is comparable to other like projects, and, based on the detailed resource requirements for a 2-year program, this fits very well. The value of “a” in the Norden-Rayleigh curve has been chosen to be “0.01”. Although there are uncertainties and risks described in the SWOT section our assessment is that with the cost expenditure outlay given we have a high level of confidence that we can get the required results and accomplish the goals of the 2-year research program within the estimated budget.

6.13. Return on investment (ROI)

This project definitely will provide positive return on investment for the company. If the investors are able to manufacture a drug or diagnostic test based on the research provided by the company there is obvious reasons of getting huge return on investment. Formula for calculating ROI is as follows:
ROI = (Total revenue – Total cost) * 100 / Total cost

<table>
<thead>
<tr>
<th>Year</th>
<th>Total Cost (Millions)</th>
<th>Total Revenue (Millions)</th>
<th>ROI (Percentage)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2009</td>
<td>2200</td>
<td>0</td>
<td>-100</td>
</tr>
<tr>
<td>2010</td>
<td>718000</td>
<td>1</td>
<td>39.27</td>
</tr>
<tr>
<td>2011</td>
<td>738000</td>
<td>4</td>
<td>442</td>
</tr>
<tr>
<td>2012</td>
<td>738000</td>
<td>8</td>
<td>984</td>
</tr>
<tr>
<td>2013</td>
<td>767500</td>
<td>10</td>
<td>1202</td>
</tr>
</tbody>
</table>

Table 11: Return on investment estimation

The ROI graph shows that company will have to face a not significant loss in the first year, but second year in 2010 company will get significant return on investment.

6.14. Exit Strategy - Acquisition by an established company

The company’s future is very bright and there are many opportunities for the company to expand. After California other states and then foreign countries, but one should always be prepared with some alternative exit plan. The company exit strategy is to get acquired by some biopharma company or insurance company. According to the revenue calculations company’s revenues are increasing constantly, but annual gross revenue is 4 million dollars by the year 2011. The desired acquisition price is 4 to 5 times the annual gross revenues, which will be more or less 16 million dollars.
7. Project Schedule

Many of the activities were done concurrently and hence the schedule shows overlapping timelines.

The following table summaries the schedule of the project, activities and milestones.

<table>
<thead>
<tr>
<th>Tasks</th>
<th>Task Name</th>
<th>Start</th>
<th>Finish</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Study the Tang et al paper</td>
<td>9/1/08</td>
<td>9/23/08</td>
</tr>
<tr>
<td>2</td>
<td>Study research papers</td>
<td>9/23/08</td>
<td>3/30/09</td>
</tr>
<tr>
<td>3</td>
<td>Search and study different databases</td>
<td>10/3/08</td>
<td>3/30/09</td>
</tr>
<tr>
<td>4</td>
<td>Collect data on the current success and failure rate for the correct antibiotics</td>
<td>10/3/08</td>
<td>10/15/08</td>
</tr>
<tr>
<td>5</td>
<td>Document and reference literature search information</td>
<td>11/3/08</td>
<td>4/5/09</td>
</tr>
<tr>
<td>6</td>
<td>Explore different protein databases</td>
<td>10/15/08</td>
<td>12/3/08</td>
</tr>
<tr>
<td>7</td>
<td>Explore different databases and tools to analyze the pathway</td>
<td>11/19/08</td>
<td>2/14/09</td>
</tr>
<tr>
<td>8</td>
<td>Develop economic justification</td>
<td>2/14/09</td>
<td>3/10/09</td>
</tr>
<tr>
<td>9</td>
<td>Compile results</td>
<td>2/14/09</td>
<td>4/10/09</td>
</tr>
<tr>
<td>10</td>
<td>Review, edit and complete project</td>
<td>4/10/09</td>
<td>4/17/09</td>
</tr>
</tbody>
</table>

Table 10: Project Schedule for fall 2008/spring 2009
8. Results

- Number of proteins found in sepsis-induced febrile response = 29
- Number of known pathways of associated genes from KEGG, Biocarta and Millipore pathways = 41
- Number of proteins found in sepsis-induced ARF = 30
- Number of known pathways of associated genes from KEGG, Biocarta and Millipore pathways = 51
- Number of additional pathways in ARF = 42

This confirms our hypothesis that a genetic-based pathway analysis can improve sepsis diagnosis and treatment. However further investigation and analysis is needed to confirm the conclusions.

9. Future work

This area of study is an on going process and requires further investigation of the pathways of different sepsis-induced clinical outcomes. So the next step will be to continue analyzing other organ failure and complications produced by sepsis. Below is the list of other areas of investigation.

9.1. Disseminated intravascular coagulation (DIC)

“DIC is a life threatening condition that prevents a person’s blood from clotting normally. It may cause excessive clotting (thrombosis) or bleeding (hemorrhage) throughout the
body and lead to shock, organ failure, and death.” (Disseminated intravascular coagulation, 2007)

Figure 26: Extreme Case Of DIC

Above is a picture of an extreme case of DIC and its treatment in the hospital.

9.2. Acute respiratory distress syndrome (ARDS)

“ARD is characterized by inflammation of the lung parenchyma leading to impaired gas exchange with concomitant systemic release of inflammatory mediators causing inflammation, hypoxemia and frequently resulting in multiple organ failure. This condition is often fatal, usually requiring mechanical ventilation and admission to an intensive care unit.” (Acute respiratory distress syndrome, 2009)

9.3. Acute Liver Failure

“In the majority of acute liver failure (ALF) there is widespread hepatocellular necrosis beginning in the centri-zonal distribution and progressing toward portal tract. The degree of parenchymal inflammation is variable and is proportional to duration of disease.” (Acute liver failure, 2009)
9.4. Cardiac Arrest

“A cardiac arrest also known as cardiopulmonary arrest or circulatory arrest, is the abrupt cessation of normal circulation of the blood due to failure of the heart to contract effectively during systole.” (Cardiac arrest, 2009)

9.5. Continuation of time line table (Table 4)

There are numerous studies that have been done regarding different proteins involved in the consequences of sepsis. The literature survey should continue to find more articles and added to the results in table 4 of this report so it will be more useful in the diagnosis and treatment of sepsis.
10. Conclusion

Presently, even after amazing advancements in medical technologies and research, sepsis diagnosis lacks selectivity and specificity. Any effort that can improve the diagnostic process of this horrible medical condition will make a significant contribution to saving thousands of life. Treating sepsis involves billions of dollars in just the United States of America. This toll is a huge burden on the health care infrastructure. A table consisting of proteins and their associated genes and pathways will aid diagnosing of sepsis and its progress and also in understanding the stage of organ failure in sepsis in a clinical setting without performing any gene expression profiling. This is an on going project and further investigation is required related to other organ failure and clinical outcomes of sepsis to see the different patterns of pathways involved in different sepsis induced outcomes. This will make it possible to make the diagnosis more accurate, specific and selective.
References


Miller, T. A., Bass, B.L., Fabri, P.J., Haisch, C. E., Mercer, D. W., Merrell, R.C. and Myers, S.I. Physiologic Foundations and clinical applications. Modern surgical care. Retrieved on March, 2009 from http://books.google.com/books?id=8SuNnLearBgC&pg=PA232&lpg=PA232&dq=Macrophages+or+dendritic+cells+that+have+previously+ingested+necrotic+cells+will+induce+an+inflammatory+cytokine+profile+(Th1).&source=bl&ots=uEWTtVc36R&sig=SvP5p8u8Qd8wbozQ3B1FLUHwSeRQ&hl=en&ei=vOPoSaTGG4TktQPrG


Appendix A

Macrophage: are white blood cells within tissues, produced by the division of the Monocyte. (Macrophage, 2009)

Premotor cortex: is an area of motor cortex in the frontal lobe of the brain. (Premotor cortex, 2009)

POA: preoptic area is a region of the hypothalamus. The POA is responsible for thermoregulation and receives nervous stimulation from thermoreceptor in the skin, mucous membrane and hypothalamus itself. (preoptic area, 2009)

OVLT: Organum vasculosum of the lamina terminalis.

Interesting links to get more information about sepsis syndrome:

1. Sepsis.com
2. International sepsis forum: http://www.sepsisforum.org
5. Surviving sepsis Campaign: http://www.survivingsepsis.org
Appendix B

List of all the pathways of pyrogen for fever spikes:

1. Toll-like receptor signaling pathway
2. Cytokines and inflammatory response
3. Cytokine network
4. IL2 signaling pathway
5. Cheperon modulate interferon signaling
6. IFN gamma signaling
7. Th1/Th2 differentiation pathway
8. Cytokine-cytokine receptor attraction
9. TNF/stress related signaling
10. TNFR1 signaling
11. IL10 anti-inflammatory signaling
12. JAK-STAT signaling pathway
13. TNFR2 signaling
14. Apoptosis pathway
15. Signal transduction through IL1R
16. Mitogen - activated protein signaling
17. Aspirin blocks signaling pathway involved in platelet activation
18. P38 MAPK signaling
19. NF-KB signaling
20. WNT signaling pathway
21. ALK in cardiac myocytes
22. Rho cell motility signaling
23. Role of P13 K subunit P85 in regulation of actin organization and cell migration
24. Y branching of actin filament
25. T cell receptor signaling
26. Signaling pathways from G protein families
27. Ras signaling pathway
28. Rac 1 cell motility signaling pathway
29. Phospholipidase as signaling
30. Intigrin signaling
31. BCR signaling
32. Immune response during T-cell polarization
33. Pertussis toxin-intensive CCR5 signaling in macrophage
34. Selective expression of chemokines receptors during polarization
35. Inactivation of GsK3 by AKT causes accumulation of β-catenin in aveolar macrophages
36. Mechanism of genes regulation by peroxisome proliferators via PPARα (alpha)

List Acute Renal Failure Pathways:
1. Dendritic cells in regulation TH1 and TH2 development
2. Hypoxia-inducible factor in the cardiovascular system
3. Corticosteroid and cardio protection
4. Ion Channel and their functional role in vascular endothelium
5. VEGF, hypoxia, and angiogenesis
6. Inhibition of matrix metalloprotein
7. CD40L signaling
8. Stress induction of HSP regulation
9. NO independent IL 12 pathway in NK cell
10. Caspase cascade in apoptosis
11. D4-GD1 signaling
12. FAS signaling
13. IL 8 Signaling
14. Induction of apoptosis through DR3 and Dr45 death receptor
15. IL 2 receptor beta chain in T cell activation
16. CCR3 signaling in eosinophils
17. CXCR4 signaling pathway
18. Growth hormone signaling pathway
19. IL-5 signaling pathway
20. IL12 and Tat4 dependent signaling pathway in Th1 development
21. IL 22 soluble receptor signaling pathway
22. Msp/Ron receptor signaling pathway
23. Signaling of hepatocyte growth factor receptor
24. Phospholipase C- epsilon pathway
25. T helper cell surface
26. Wnt-β-catenin pathways
27. Cell to cell adhesion signaling
28. Presenilin action and signaling pathway of notch
29. Trefoil factors initiate mucosal healing
30. ETS-1 pathway
31. Regulate the transcription of cyclin D1 and control the regeneration of renal tubules in ARF
32. Adhesion and diapedesis of lymphocytes
33. Cells and molecule
34. Involved in local acute inflammatory response
35. Cyclo-oxygenase pathways
36. Neuropeptides signaling through G-protein alpha-I & G-protein alpha-q
37. Development glucocorticoids receptor signaling
38. Control of skeletal myogenesis by HDAC & calcium/calmodulin-dependent kinase (CaMK)
39. ErK1/Erk2 MAPK signaling pathway
40. IGF-1 signaling pathway
41. Multiple antiapoptotic pathways from IGF-1R signaling lead to BAD phosphorylation
42. Regulation of BAD phosphorylation
43. Skeletal muscle hypertrophy is regulated via AKT/mTOR pathway
Appendix C

NF-KB signaling:

Figure 27: NF-KB pathway (Retrieved from Biocarta pathways, 2009)

Nuclear factor kB (NF-kB) is a nuclear transcription factor that regulates expression of a large number of genes that are critical for the regulation of apoptosis, viral replication, tumorigenesis, inflammation, and various autoimmune diseases. The activation of NF-kB is thought to be part of a stress response as it is activated by a variety of stimuli that include growth factors, cytokines, lymphokines, UV, pharmacological agents, and stress. In its inactive form, NF-kB is sequestered in the cytoplasm, bound by members of the IκB family of inhibitor proteins, which include IκBa, IκBb, IκBg, and IκBe. The various stimuli that activate NF-kB cause phosphorylation of IκB, which is followed by its ubiquitination and subsequent degradation. This results in the exposure of the nuclear localization signals (NLS) on NF-kB subunits and the subsequent translocation of the
molecule to the nucleus. In the nucleus, NF-kB binds with a consensus sequence (5’GGGACTTTCC-3’) of various genes and thus activates their transcription. IkB proteins are phosphorylated by IkB kinase complex consisting of at least three proteins; IKK1/IKKa, IKK2/IKKb, and IKK3/IKKg. These enzymes phosphorylate IkB leading to its ubiquitination and degradation. Tumor necrosis factor (TNF) which is the best-studied activator binds to its receptor and recruits a protein called TNF receptor death domain (TRADD). TRADD binds to the TNF receptor-associated factor 2 (TRAF-2) that recruits NF-kB-inducible kinase (NIK). Both IKK1 and IKK2 have canonical sequences that can be phosphorylated by the MAP kinase NIK/MEKK1 and both kinases can independently phosphorylate IkBa or IkBb. TRAF-2 also interacts with A20, a zinc finger protein whose expression is induced by agents that activate NF-kB. A20 functions to block TRAF2-mediated NF-kB activation. A20 also inhibits TNF and IL-1 induced activation of NF-kB suggesting that it may act as a general inhibitor of NF-kB activation. (Biocarta.com)
Inflammation is a protective response to infection by the immune system that requires communication between different classes of immune cells to coordinate their actions. Acute inflammation is an important part of the immune response, but chronic inappropriate inflammation can lead to destruction of tissues in autoimmune disorders and perhaps neurodegenerative or cardiovascular disease. Secreted cytokine proteins provide signals between immune cells to coordinate the inflammatory response. Some cytokines such as IL-1, IL-6 and TNF act to broadly provoke the inflammatory response while others act on specific types of immune cells. Macrophages and other phagocytic cells provide a front-line defense against bacterial infection. Macrophages stimulate the inflammatory responses of neutrophils, fibroblasts, and endothelial cells in response infection by secreting IL-1 and TNF. IL-1 and TNF cause fever through
alteration of the body temperature set-point in the hypothalamus. Fibroblasts and endothelial cells respond to IL-1 and TNF by recruiting more immune cells to the site of inflammation. Secreted IL-8 is a chemokine that attracts neutrophils to sites of infection.

Macrophages also present antigen to T helper cells that play a central role in coordinating immune responses. T helper cells induce clonal expansion of T cells that respond to antigen, with IL-2 as a key mediator of T cell proliferation and activation. TGF-beta is a negative regulator of proliferation in many cells, have anti-inflammatory actions in some settings (see TGF-beta pathway). The cytotoxic activity of Natural Killer cells (NK cells) and lymphokine activated killer cells (LAK cells) toward viral infected or tumor cells is stimulated by IL-2 and other cytokines. T helpers secrete IL-3 and IL-5 to stimulate eosinophil proliferation and activation. Eosinophils are involved in the immune response to parasitic infection. T helper cells are required to stimulate B cell responses as well, with the cytokines IL-10, IL-4 and other cytokines regulating the clonal selection and differentiation of antigen-specific B cells to form antibody-secreting plasma B cells and memory cells. In addition to inducing activation and proliferation of specific differentiated immune cells, cytokines act on hematopoietic stem cells, causing their proliferation and differentiation into the full range of immune cells.
Th1/Th2 differentiation:

Helper T cells are found in two distinct cell types, Th1 and Th2, distinguished by the cytokines they produce and respond to and the immune responses they are involved in. Th1 cells produce pro-inflammatory cytokines like IFN-γ, TNF-β and IL-2, while Th2 cells produce the cytokines IL-4, IL-5, IL-6 and IL-13. The cytokines produced by Th1 cells stimulate the phagocytosis and destruction of microbial pathogens while Th2 cytokines like IL-4 generally stimulate the production of antibodies directed toward large extracellular parasites. IL-5 stimulates eosinophil responses, also part of the immune response.
response toward large extracellular parasites Th1 and Th2 are produced by differentiation from a non-antigen exposed precursor cell type, Thp. Exposure of Thp cells to antigen by antigen-presenting cells may result in their differentiation to Th0 cells, not yet committed to become either Th1 or Th2 cells, although the existence of Th0 cells is controversial. Cells committed as either Th1 and Th2 cells are called polarized, whether they are effector cells actively secreting cytokines or are memory cells. The stimulation of Thp cells by exposure to antigen-presenting cells induces the proliferation of undifferentiated cells, and their expression of IL-2 and IL-2 receptor. The differentiation of Th1 cells and Th2 cells depends on the cytokines they are exposed to. IL-12 causes Th1 differentiation and blocks Th2 cell production, while IL-4 causes Th2 differentiation and antagonizes Th1 development. IL-18 also induces Th1 differentiation Polarized Th1 and Th2 cells also express distinct sets of chemokine receptors that further modify their homing and other cellular responses improved understanding of Th1 and Th2 differentiation will improve our overall understanding of the immune system, its response to infection and aberrant responses that lead to disease. (Retrieved from Biocarta pathways)
Cell to Cell Adhesion Signaling:

Interactions between cells responsible for cell to cell adhesion also can communicate signals into the cellular interior, often involving interactions with cytoskeletal elements to produce changes in cell motility, migration, proliferation and shape. The cadherins are cell surface adhesion molecules that help form tight junctions between cells such as formation of epithelial cell layers. E-cadherin inactivation has been implicated in cancer development. In addition to mediating adhesion with other cells, cadherins transduce signals into cells through interactions with the catenins. Catenins probably affect actin cytoskeletal function through interactions with proteins like actinin and vinculin. Catenins also probably trigger changes in cell shape and motility with signals through the Rho small GTPases. Another important cell adhesion molecule is CD-31, or PECAM-1,
involved in the formation of junctions between endothelial cells, cell migration, migration of lymphocytes, and regulation of lymphocyte activation. Src phosphorylates PECAM-1 on tyrosine residues causing SHP-2 association with PECAM-1. Paxillin acts as an adaptor protein between proteins involved in adhesion signaling like FAK and src and cytoskeletal elements. In addition to signals created by adhesion molecules to alter cellular responses, other signaling pathways can alter adhesion through components of the focal adhesion complex.

**D4-GDI signaling pathway:**

![D4-GDI signaling pathway](image)

Figure 31: D4-GDI signaling pathway (Biocarta pathway, 2009)

D4-GDI (GDP dissociation inhibitor) is a negative regulator of the ras related Rho Family of GTPases. Since the rho GTPases promote cytoskeletal and membrane changes associated with apoptotic cell death, the removal of the D4-GDI block through its
cleavage is important for inducing apoptosis. Caspase-3 cleaves the 28 kDa mature form of D4-GDI to give a 5 kDa and 23 kDa Size fragment. The 23 kDa fragment then translocates to the nucleus. The mechanisms involving cleavage of D4-GDI with apoptosis are not presently known. Activation of the Jun N-Terminal kinase, a regulator of apoptosis, may be one of the mechanisms. (Biocarta.com, 2009)

VEGF, Hypoxia, and Angiogenesis:

![VEGF, Hypoxia, and Angiogenesis](image)

Figure 32: VEGF, Hypoxia, and Angiogenesis (Biocarta pathways, 2009)

Vascular endothelial growth factor (VEGF) plays a key role in physiological blood vessel formation and pathological angiogenesis such as tumor growth and ischemic diseases.
Hypoxia is a potent inducer of VEGF in vitro. The increase in secreted biologically active VEGF protein from cells exposed to hypoxia is partly because of an increased transcription rate, mediated by binding of hypoxia-inducible factor-1 (HIF1) to a hypoxia responsive element in the 5'-flanking region of the VEGF gene. bHLH-PAS transcription factor that interacts with the Ah receptor nuclear translocator (Arnt), and its predicted amino acid sequence exhibits significant similarity to the hypoxia-inducible factor 1 alpha (HIF1a) product. HLF mRNA expression is closely correlated with that of VEGF mRNA. The high expression level of HLF mRNA in the O2 delivery system of developing embryos and adult organs suggests that in a normoxic state, HLF regulates gene expression of VEGF, various glycolytic enzymes, and others driven by the HRE sequence, and may be involved in development of blood vessels and the tubular system of lung. VEGF expression is dramatically induced by hypoxia due in large part to an increase in the stability of its mRNA. HuR binds with high affinity and specificity to the VRS element that regulates VEGF mRNA stability by hypoxia. In addition, an internal ribosome entry site (IRES) ensures efficient translation of VEGF mRNA even under hypoxia. The VHL tumor suppressor (von Hippel-Lindau) regulates also VEGF expression at a post-transcriptional level. The secreted VEGF is a major angiogenic factor that regulates multiple endothelial cell functions, including mitogenesis. Cellular and circulating levels of VEGF are elevated in hematologic malignancies and are adversely associated with prognosis. Angiogenesis is a very complex, tightly regulated, multistep process, the targeting of which may well prove useful in the creation of novel therapeutic agents. Current approaches being investigated include the inhibition of angiogenesis stimulants (e.g., VEGF), or their receptors, blockade of endothelial cell
activation, inhibition of matrix metalloproteinases, and inhibition of tumor vasculature. Preclinical, phase I, and phase II studies of both monoclonal antibodies to VEGF and blockers of the VEGF receptor tyrosine kinase pathway indicate that these agents are safe and offer potential clinical utility in patients with hematologic malignancies.

(Biocarta.com, 2009)

**Immune response CCR3 signaling in eosinophils:**

![Immune response CCR3 signaling in eosinophils](image.png)

Figure 33: Immune response CCR3 signaling in eosinophils (Millipore pathways, 2009)

Human eosinophils are key effector cells implicated in a number of chronic inflammatory reactions, associated with bronchial asthma, allergic inflammatory disease and parasitic infections. Chemoattractants/chemokines, generated at the affected sites, promote migration of eosinophils from vasculature into tissues. Chemotactic response of
eosinophils is mostly mediated by CC Chemokine Receptor-3 (CCR3), a member of G-protein-coupled receptor family, which activates G-protein alpha-i-family. Chemokines of the eotaxin group (Eotaxin, Eotaxin-2 and Eotaxin-3), acting exclusively via CCr3, induce recruitment of eosinophils to the sites of inflammation. Other eosinophil-activating chemokines (such as CCL5, CCL7, CCL8 and CCL13) can signal via CCr3 and play a crucial role in eosinophil migration tissues. These chemokines are not selective and can signal via additional receptors. (Millipore pathways, 2009)