Profiling Cytokine Expression in Trauma Patients Before and After TLR Stimulation

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Abstract

Trauma is the leading cause of morbidity and mortality worldwide for individuals under the age of 45. Despite advances in emergency care, infection continues to be a major complication contributing to significantly high death rates. This is prevalent because traumatic injuries induce a complex host response mediated by pattern recognition receptors that disrupt immune homeostasis and predispose trauma patients to opportunistic infections. Recent studies have demonstrated the immunotherapeutic benefits of using a TLR9 agonist, CpG-ODN in mouse models of trauma. Therefore, it is imperative to continue to investigate the biological effects of CpG-ODN as well as other TLR agonists in order to develop an approach that can protect injured people from infections, restore immune system homeostasis and reduce mortality rates among trauma patients.

We hypothesize that human immune cells will react to TLR9 stimulation to suggest translation capability of TLR9 agonist immunotherapy. In addition, we predict that an A-Class CpG-ODN will stimulate beneficial self-regulating immune stimulatory responses.

We looked at human peripheral blood immune cells. These cells were stimulated with either CpG-ODN 2336 or LPS. After stimulation, we used Luminex technology as a systems immunology research tool to identify specific cytokine responses to CpG-ODN 2336 and LPS activation.

We report that human immune cells have significant cytokine production when stimulated with CpG-ODN 2336 and LPS. Some of these signature cytokines include TNFα, IL-

1α, IL-1α, IL-6, IL-10, MCP-1, IL-1RA, MIP-1β, RANTES and GM-CSF. Stimulation with CpG-ODN 2336 produced a much less potent inflammatory response than LPS, suggesting its non-toxic properties. However, it still induced a sufficient response to potentially activate cells to fight infection.

These results indicate that signature cytokine concentrations are induced or sustained when human immune cells are activated with TLR9 agonists. In addition, TLR9 agonists have implications for fighting infection and restoring immune system homeostasis. Further research is necessary to understand the immunotherapeutic potential of using CpG-ODNs to improve outcomes for trauma and infection.

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Chapter 1: Background

According to the Centers for Disease Control and Prevention, trauma is the leading cause of morbidity and mortality worldwide for individuals under the age of 45 years old. It is also the third leading cause of death in all age populations (1). Severe traumatic injuries can be organized into several groups to include radiation injury, blunt-force trauma, bone fracture and much more. Some of the unfortunate, yet common events that can lead to such injuries can range from motor-vehicle accidents to natural disasters such as earthquakes (2). Given the significant amount of people who suffer from traumatic injuries, it is imperative to develop strategies for emergency preparedness and trauma treatment.

Common causes of death in patients who suffer from severe physical trauma can be immediate and include heart rupture and disruption of the cervical spine. Other causes occur after 24 hours and are usually a result of massive blood loss and subsequent hemorrhagic shock. However, rapid blood volume replacement and other medical advancements have been made to control for massive blood loss (3). Despite these critical advancements in emergency care and medical technology, nosocomial (hospital-acquired) infections, sepsis and multiorgan failure (MOF) are still major complications of trauma that contribute to death. Nosocomial infections are the most common cause of late death occurring between 3 days and 3 weeks after injury (4). The incidence of sepsis occurs in approximately 10% of these trauma patients and is associated with severity of injury and an increase in mortality compared with non-septic patients (3). Patients who initially survive traumatic injuries undergo invasive procedures such as emergent intubation and high-volume blood transfusions that disrupts natural innate barriers and puts them at an increased risk for infection. This gives a chance for commensal bacteria or other

microorganisms such as *Pseudomonas aeruginosa, Streptococcus pneumoniae* and *Staphylococcus aureus* to spread throughout the lower respiratory tract or through skin wounds, contributing to early or late-onset pneumonia (5). In addition, patients who suffer massive blood loss experience a significant decrease in the amount of immune cells, further increasing the risk for infection. Necessary multiple surgeries and administration of sedatives also puts trauma patients at increased risk for infection (6).

Infection is prevalent in trauma patients because traumatic injuries induce a complex physiological immune response mediated by pattern recognition receptors (PRRs) that disrupts immune system homeostasis. PRRs are proteins expressed on innate immune cells that are able to recognize pathogen associated molecular patterns (PAMPs) from microbes. They are also able to detect alarmins or damage associated molecular patterns (DAMPs) from necrotic cells. With the disruption of the immune system homeostasis and the activation of PRRs, opportunistic infections and other injury-associated inflammatory complications become more prevalent (1). While injuries occur in different ways and the level and type of injury may vary, they all share a common component which is severe tissue damage. And this damaged tissue is what usually initiates the early immune system host response.

After severe tissue damage, alarmins and DAMPs released from damaged tissue, such as heat shock proteins (HSPs), Adenosine Triphosphate (ATP), uric acid, mitochondrial DNA (mtDNA) and high-mobility group box nuclear protein 1 (HMGB1) alert the immune system that damage is present (6). Innate immune cells such as neutrophils, dendritic cells and macrophages contain these PRRs that detect these alarmins and DAMPS. This interaction is driven by a certain class of PRRs called toll-like receptors (TLRs) which are prominent in traumatic injury and alarmin detection (7). The figure below highlights how these alarmins initiate the immune

response to traumatic injuries. It also illustrates the different responses that can occur after a system has been primed by alarmins from a physical injury. The response to traumatic injury can be moderate leading to proper resolution and recovery. However, the response can also be excessive or inadequate, which can then lead to further complications.

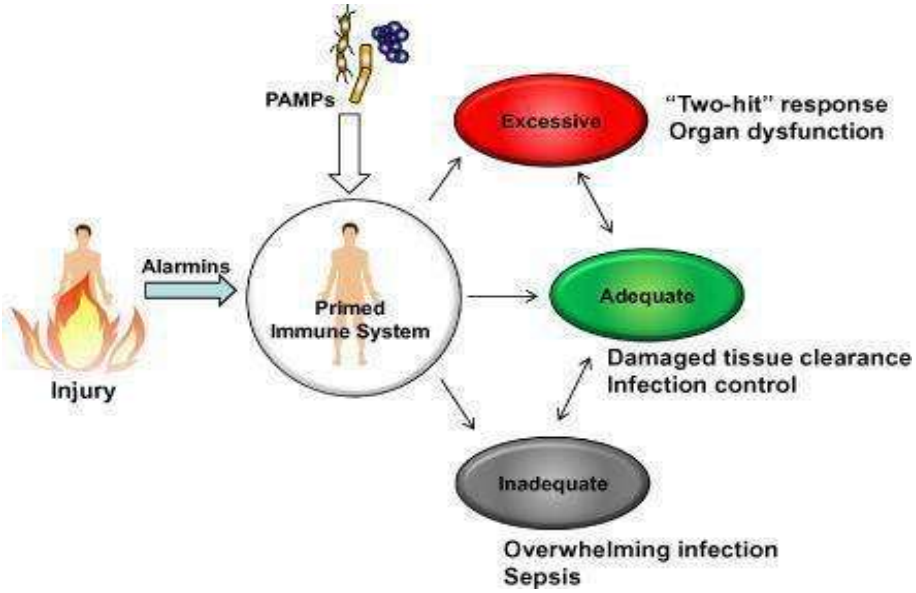
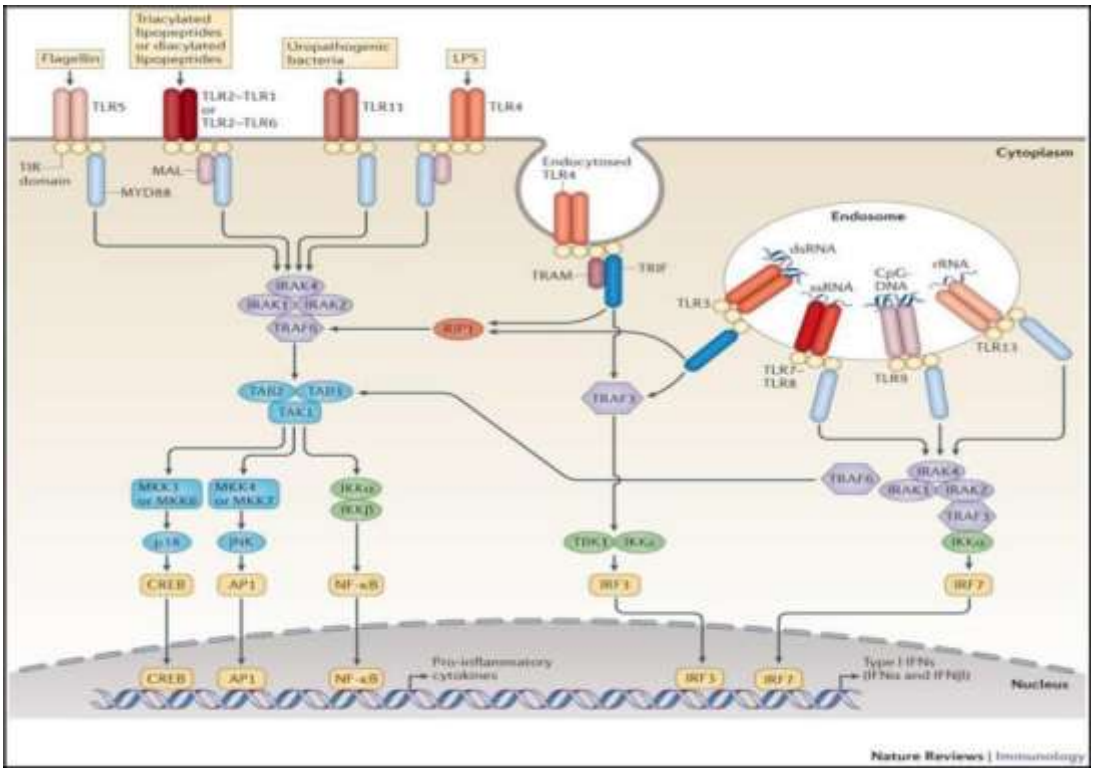


Figure 1.1 Traumatic injury primes the immune system. This schematic depicts the potential responses that can occur after the immune system has been primed by alarmins as a result of physical traumatic injury. The immune response is usually mild or moderate and resolves the tissue damage. However, the primed immune system can trigger a severe response which can lead to the two-hit response or organ dysfunction. It can also trigger an inadequate response which can lead to overwhelming infection or sepsis.

Many studies have already demonstrated that toll-like receptors on innate immune cells are critical mediators of the early response to bacterial infection. And the lack of increased TLR expression in some individuals is associated with severe infection (8). Other studies have shown that TLR4, a specific type of TLR located in the plasma membrane is a primary receptor for danger and is often highly activated in cases of trauma (9). Additional TLRs such as TLR5 have also been proven to react to alarmins and other danger signals (10). Once alarmins engage these TLRs, adaptor proteins such as myeloid differentiation primary response gene 88 (MyD88) and

TIR-domain-containing adapter-inducing interferon-β (TRIF) control the initiation of TLR responses. Activation of MyD88 initiates a signaling cascade that leads to immune cell activation and cytokine production through the nuclear factor kappa light chain enhancer of activated B cells (NFκB) pathway. The TRIF-dependent pathway also induces a signaling cascade leading to Type I IFN production through the transcription factor, IFN regulatory factor 3 (11). A schematic demonstrating the TLR activation pathways and subsequent signaling is indicated in **Figure 1.2** (12). Other studies have identified the p38 (mitogen-activated protein kinase (MAPK) signaling pathway in mediating amplified TLR responses within traumatic injuries (13).



Source: Nature

Figure 1.2 Mammalian TLR signaling pathways. Toll-like receptors recognize various pathogen associated molecular patterns and play a significant role in the immune response. TLRs follow various signaling pathways as indicated.

Proinflammatory and anti-inflammatory immune responses follow after this initial reaction to injury takes place. Proinflammatory responses are characterized by immune cells and molecular mediators that initiate inflammation to protect the body from harmful stimuli. Alternatively, anti-inflammatory responses are characterized by immune cells and molecular mediators that suppress inflammation and protect the body from tissue damage. In trauma patients with no specific complications, the responses are temporary and well balanced between proinflammatory and anti-inflammatory responses. Damaged tissue is cleared and infection is controlled. However, after severe injury, there is an increased production of inflammatory mediators and the immune system usually tends toward a proinflammatory phenotype (3). This protects the host from secondary infections and heightens anti-microbial immunity. This proinflammatory response can be severe and can lead to what is called a "two-hit" response, defined by Moore (1). The "two-hit" response forms when a traumatic injury activates or primes the host such that the innate immune system, primarily neutrophils and macrophages become hyper-reactive to bacteria and bacterial toxins. These activated neutrophils and macrophages produce amplified levels of proinflammatory cytokines leading to systemic inflammation and detrimental cell-mediated tissue destruction. The clinical term for systemic inflammation is systemic inflammatory response syndrome (SIRS) (14). SIRS is a serious condition affecting the entire body and is characterized by an abnormal production and regulation of cytokines, a body temperature less than 36°C or greater than 38°C, a high respiratory rate and a heart rate greater than 90 beats per minute (15). These symptoms usually indicate that a severe infection is present or developing. SIRS can begin rather quickly, within 30 minutes of severe injury and is accompanied by increased levels of proinflammatory cytokines such as tumor necrosis factor

alpha (TNFα) and interleukin 6 (IL-6) (16). While this phenomenon can be helpful in protecting the host from infection, it can also be detrimental. The uncontrolled immunological response to trauma can lead to the self-destruction of tissues, organ dysfunction and subsequent MOF (17). MOF or multiple organ dysfunction syndrome (MODS) is a clinical disorder in which several organs (i.e. liver, kidneys, and lungs) fail simultaneously. In one study, patients involved in road accidents were assessed for the incidence of MOF and infection. It was found that 56% of patients died due to MOF and 42% of these patients died of MOF after they developed an infection (18). The increased production of cytokines and inflammatory mediators in SIRS ultimately leads to the disruption of immune homeostasis. In response to SIRS, a counter reaction that is believed to act as a natural compensation to trauma-induced inflammation called compensatory anti-inflammatory response syndrome (CARS) develops. CARS is characterized by immunosuppressive activity, an increase in the production of anti-inflammatory cytokines such as interleukin 10 (IL-10) and interleukin 4 (IL-4), an increase in regulatory T cell (Treg) production, suppressed T helper 1 (Th1) activity, increased Th2 activity and an increase in myeloid suppressor cells (19). While this immunosuppressive response protects the host from inflammation and from the two-hit response, its persistency decreases antimicrobial immunity, exposing the patient to overwhelming infection and sepsis. This counter-inflammatory phenotype and immune response also contributes to the development of infection by disrupting immune homeostasis. It was believed that solely SIRS or CARS was dominant in an individual after traumatic injury. However, it has recently been proven that both of these opposing immune response syndromes can occur simultaneously as specified in the term, Mixed Antagonist Response Syndrome (MARS) (20). **Figure 1.3** summarizes the general effects of injury on immune system homeostasis and the subsequent developments of SIRS and CARS (21). This

figure also depicts that SIRS and proinflammatory responses are driven by the innate immune system and are characterized by inflammasome and TLR4 activation in macrophages. Conversely, CARS and anti-inflammatory responses are driven by the adaptive immune system which is dominated by the activation of immunosuppressive regulatory T cells.

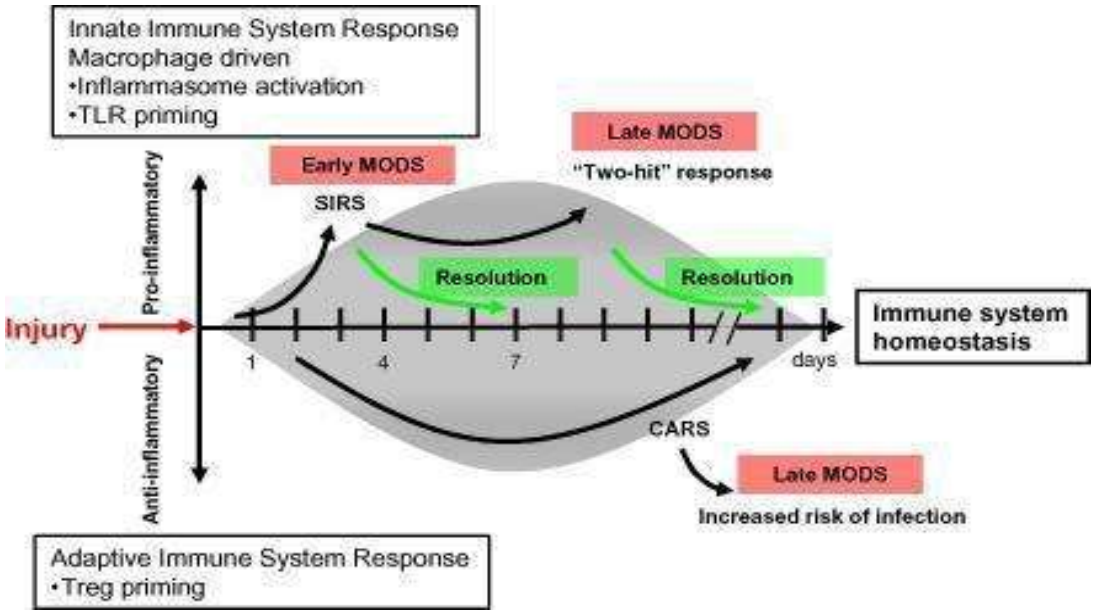


Figure 1.3 Tissue damage disrupts immune system homeostasis. This diagram illustrates the production of proinflammatory (SIRS) and anti-inflammatory (CARS) responses after traumatic injury. The incidence of SIRS and CARS usually disrupts the immune system homeostasis. While the proinflammatory response is driven by the innate immune system, the anti-inflammatory response is driven by the adaptive immune system. The proinflammatory response is characterized by inflammasome activation and TLR priming. The anti-inflammatory response is characterized by regulatory T cell priming. If these syndromes are not resolved in trauma patients, multiple organ dysfunction syndrome can occur as a result of the two hit response or the increased risk of infection.

Research has been conducted by various groups that support the idea that trauma disrupts immune system homeostasis and predisposes patients to opportunistic infections. Interventional approaches to reduce this imbalance can be very beneficial in decreasing the incidences of morbidity and mortality. Nonetheless, it is difficult to develop approaches that are efficient. Diagnosis of severe infection or sepsis in its early stages is extremely challenging. Clinical signs

of MOF usually occur very late and the routine laboratory diagnostic markers such as C-Reactive protein (CRP) and lactate levels are not very helpful in diagnosing these life-threatening complications (18).

Still, there have been quite a few approaches to help attenuate SIRS and modulate the immune response to trauma, using immune response modifiers (IRMs). Immune response modifiers are molecules or substances that are able to induce functional changes to the immune system (22). Some of these IRMs include prostaglandin E1, hydrocortisone and monoclonal antibodies against CD18 (23). Nonetheless, these interventions have not had significant effects on decreasing the incidences of infection and mortality in trauma patients. The reason for this may be due to the heterogeneity in the injury response among trauma patients. There are a number of factors that contribute to the different responses that trauma patients have to different interventional approaches. Some of these prognostic factors include age, severity of injury, comorbidities, chronic conditions, gender, and genetic predisposition. These factors make studying the impact of trauma on the immune system quite challenging because individuals response vastly different to physical injury. However, advances have been made in understanding how injuries influence the immune system and the molecular pathways involved. Particular focus has been made on trying to decipher the development of infections after sustaining traumatic injuries. In fact, a classification system for predicting sepsis was developed by a group of clinicians. This classification system is known as the PIRO scoring system whose acronym stands for – Predisposition, Infection, the Response of the host system and Organ dysfunction (24, 25). A table depicting this important classification tool is indicated in **Table 1.1.** There have been many studies within Netherlands, Portugal and the United States that have

utilized this classification system for sepsis on very large cohorts of patients. And it was found that when compared to the routine scale that is used called, APACHE - (Acute Physiology and Chronic Health Evaluation), PIRO had a significantly higher prognostic value when assessing the risk for infection, MOF and risk of mortality after traumatic injury (26, 27).

Chart 1 - Currently available and future perspectives for a PIRO based approach in sepsis

	P Predisposition	I Infection	R Response	O Organ dysfunction
Available	Age Comorbidities Chronic conditions Baseline severity Source of admission	Pathogen Susceptibility Bacteremia Bacterial load Site of infection Nosocomial or community-acquired infection	Clinical Resolution Hypoxemia Hypotension Immune Response	ARDS Shock Acute renal failure MODS SOFA
Future	Genetics Polymorphisms of toll-like receptor, tumor necrosis factor, IL-1 and CD14	Genotyping Assay of microbial products (LPS), mannan and bacterial DNA Detection of virulence factors	Biomarkers Nonspecific markers of activated inflammation (PCT or IL-6) or impaired host responsiveness (HLA-DR)	Mithocondrial dysfunction Endothelial damage and activation

ARDS - Acute Respiratory Distress Syndrome; MODS - Multiple Organ Dysfunction Syndrome; SOFA - Sequential Organ Failure Assessment; PCT – procalcitonin; IL-1 – interleukin 1, IL-6 – interleukin 6; LPS – lipopolissacaride; DNA – dexoxiribonucleic acid; HLA-DR – D related human leukocyte antigens.

Table 1.1 PIRO Scoring System. This table depicts the PIRO scoring system used for predicting the incidence of sepsis. Factors taken into consideration include age, comorbidities, and site of infection.

Other studies have investigated the usage of TLR agonists as immune response modifiers after traumatic injury. One study explored the potential beneficial effects of using a TLR5 agonist in mice and non-human primates that were exposed to radiation injuries (10). It was found that the TLR5 agonist, entolimod (CBLB502), which is a polypeptide drug derived from *Salmonella Flagellin* protected mice and non-human primates from gastrointestinal and hematopoietic acute radiation syndromes that are common in individuals who are exposed to dangerous amounts of radiation. CLB502 injection in mice led to the induction of multiple cytokines in mouse plasma including radioprotective cytokines such as granulocyte colony-stimulating factor (G-CSF), IL-6 and TNFα. Survival in mice and non-human primates were also enhanced. These results suggest the potential immunotherapeutic benefits of the TLR5 agonist for patients who have suffered radiation injuries.

Another study conducted within my laboratory investigated the beneficial effects of using a TLR9 agonist on trauma and secondary lung infection in mice (6). The authors used CpG oligodeoxynuncleotide (ODN) which are recognized by the endosomal TLR9. CpG-ODNs are short single-stranded synthetic DNA molecules that contain cytosine and guanine triphosphate deoxynucleotides in particular sequence contexts called CpG motifs. CpG motifs are considered PAMPs because they are abundant in genomic microbial bacterial DNA but are infrequent in vertebrate genomes. CpG-ODNs were previously shown to have different immunostimulatory effects on innate cell subsets (28). Further investigation identified several structurally different classes of CpG-ODNs, which included Classes A, B, C, P and S. These different CpG-ODNs and their characteristics are indicated in **Table 1.2**.

Classifications	Sequence Features	Biological Activities	CpG ODNs
A	CpG phosphodiester palindrome, 5′ and 3′ phosphorothioate Gs	Activates dendritic cells and NK cells, IFN induction	CpG2336 CpG1585 CpGMuMito
B	CpG phosphorothioate backbone	Activates B cells, High IL-6 and IL-10 production	CpG1826
C	CpG phosphorothioate backbone and 3′ duplex formation	Combines A- and B-like activities	CpG2395
P	CpG phosphorothioate backbone and forms multimers and pla	Strong cytokine induction similar to Class A CpG ODNS	CpG21889
S	CpG phophorothioate backbone with 3′ G substitutions	Inhibitory CpG ODNs, TLR9 antagonists	CpG2088

Table 1.2 CpG-ODN characteristics. There are a variety of CpG-ODNs with different sequences and biological activities.

Several CpG-ODNs have already been used as systemic or local adjuvant therapy for cancer, allergies and vaccinations (29, 30). Specifically, an A-Class CpG-ODN, CpG ODN 2336 was shown to elicit strong Th1 responses as well as IFN-α production by plasmacytoid dendritic cells (31). Therefore, the authors of this study took particular interest in this A-class CpG ODN

and its potential immunotherapeutic effects on a mouse model of trauma. A two-hit mouse model was developed where mice were exposed to burn-injury followed by a secondary *S. pneumoniae* lung infection 1 day after injury. Some mice were subcutaneously injected with a single treatment of an A-class CpG ODN 2336 2 hours after injury. It was found that the CpG ODN treatment significantly improved mouse survival rate, increased pathogen clearance and enhanced immune responses when compared to mice who did not receive CpG ODN treatment. Ultimately, CpG-ODN 2336 treatment given after traumatic injury appears to induce heightened acute antimicrobial immune function, but can also make the host tolerant to infection by reducing immune-mediated tissue damage and systemic inflammation. This data also implicated the efficacy of immune response modifying drugs on survival after trauma and early-onset pneumonia. A summary of the procedure and results are indicated in **Figure 1.4**.

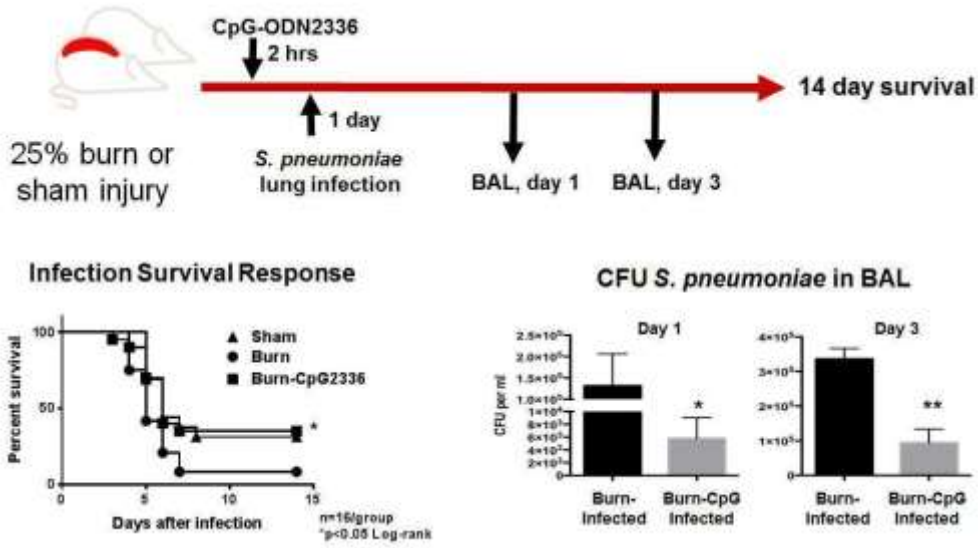


Figure 1.4 CpG-DNA treatment restores anti-microbial immune function in mice treated at 2 hours after burn injury. A two-hit mouse model was developed where mice were exposed to burn injury followed by *S. pneumoniae* lung infection. A portion of the mice also received CpG-ODN treatment. Bronchial alveolar lavage (BAL) fluid was prepared. It was found that there was a significant increase in mouse survival rate and pathogen clearance. Bars represent mean ± SEM. *$p < 0.05$.

The study also performed lung-washouts on mice 3 days after *S. pneumoniae* infection to assess their cytokine levels. After performing, Luminex multiplex assays on lung washout samples, it was found that for those mice treated with CpG-ODN, there were significantly lower levels of proinflammatory cytokines, IL-6 and IFNγ **(Figure 1.5).**

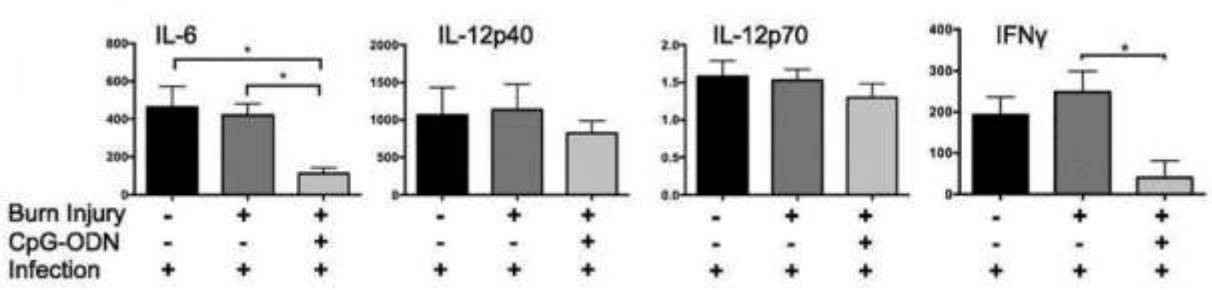


Figure 1.5 Luminex cytokine analysis of lung-washout samples. After burn injury, CpG 2336 administration and *S. pneumoniae* lung infection, pro-inflammatory cytokines were assessed. There were significantly lower levels of proinflammatory cytokines, IL-6 and IFNγ. Bars represent mean ± SEM. *$p < 0.05$.

My study further explores the immunomodulatory behavior of TLR9 agonists in patients who have suffered from traumatic injuries. It is important to point out the translatability of a mouse model of trauma into human models of trauma. While some reports have described mouse models as poor models of trauma, other analyses have indicated that genomic responses to inflammation in mice are similar to genomic responses to inflammation in humans (32, 33). In particular, I focus on profiling the cytokine expression of the total leukocytes in trauma patients before and after CpG-ODN 2336 stimulation. Cytokines are made by immune and non-immune cell types and can transmit local and systemic inflammatory signals to regulate immune responses. They are crucial in governing the immune response to trauma. There has been a growing understanding of the role of cytokines in coordinating the immune response to traumatic injuries. Many of the symptoms observed after trauma or during infection are evoked by exaggerated cytokine production (34, 35). Specific cytokines are more pronounced after

traumatic injuries and provide important information regarding prognosis and mechanism of immune responses (36, 37). Therefore, cytokine profiles may be useful in predicting outcomes of traumatic injuries even early post-trauma. One study assessed the relationship between the cytokine levels of IL-6 and IL-10 and the injury severity and mortality rates within 265 trauma patients over an 18-month period of time (38). It was found that there was a significant correlation between the severity of injury and the serum levels of IL-6 and IL-10. This study confirms the findings from many other individual studies reporting the increased levels of IL-6 and IL-10 contributing to immune dysfunction (39, 40).

Other studies have discovered the relationship between proinflammatory and anti-inflammatory cytokines and the development of SIRS and CARS as it related to trauma. Proinflammatory cytokines such as TNFα, GM-CSF and interleukin 1β (IL-1β), IL-6 and IL-8 have been known to be prominent in the early cytokine induction after severe tissue damage (41, 42). Cytokines such as IL-1β and TNFα which are usually in excess at the beginning of the proinflammatory cascade increases the production of neutrophils, decreases apoptosis, amplifies phagocytosis and causes an increase in the permeability of the endothelium (43, 44). In one study, it was even found that when TNFα was administered to a sepsis model of rats, mortality was significantly reduced (45). While TNFα and IL-1β can act locally, they also have systemic functions. Both IL-1β and TNFα stimulate the production of chemokines such as IL-8. IL-8 is an important chemokine because it acts as a chemoattractant and activates neutrophils and subsequently induces an influx of neutrophils towards the site of tissue damage (46).

The influx of anti-inflammatory cytokines is usually shown to become more prominent later on in the immune response to traumatic injury. This is because the role of anti-inflammatory

inflammatory plays a vital role in the counter-inflammatory response. It functions to inhibit the production of proinflammatory cytokines such as TNF-α and IL-8. Some studies have noted that IL-10 also inhibits the inflammatory activity of cellular responses such as Th1 cells, natural killer cells and macrophages (47).

My research attempts to understand the cytokine profile after the leukocytes of severely injured patients have been stimulated with the TLR9 agonist, CpG-ODN 2336. I will explore the proinflammatory and anti-inflammatory responses that occur after stimulation has occurred. Specifically, I will utilize systems immunology approaches such as Luminex technology to measure concentration levels of various cytokines. Luminex immunoassays will generate phenotype data that will help identify the role of TLR9 as a regulatory pathway for traumatic injury. This will help us better understand cytokine expression after injury and the potential immunotherapeutic potential of CpG-ODN 2336 in patients who have suffered from severe traumatic injuries.

Chapter 2: Methods and Data

2.1 Materials and Methods

Patient Enrollment and Blood Collection

Blood samples were collected from various trauma patients in Brigham and Women's Hospital on Days 1, 3 and 5 after injury. Patients were enrolled in the study from May 2015 to October 2015. All patients had an injury severity score (ISS) over 20 and were over 18 years old. The injury severity score was calculated according to the criteria set by Baker (48). The average ISS was 34 and most patients suffered from multiple injuries or polytrauma. Exclusion criteria included being pregnant, having a prior medical history or taking medications that predisposed patients to immune dysregulation (for example, chemotherapy or steroid use). Data taken from patients included demographics such as age and gender, mechanisms of injury and culture-proven infection. The average age was 44 years old and most patients were males. Blood from age- and gender-matched, uninjured volunteers at Brigham and Women's Biobank Center were also collected to provide a comparison group. There were 10 control samples taken from the BWH Biobank and 10 samples from trauma patients on three different time points, Day 1, Day 3 and Day 5. Blood samples were drawn from patients if major trauma was suspected. Approximately 8 mL of blood was typically drawn from patients into tubes containing ethylenediaminetetraacetic acid (EDTA) anticoagulants. Before further sampling of blood, written informed consent was obtained from the patients or designated health care proxy. The study protocol was approved by Brigham and Women's Hospital Institutional Review Board (IRB).

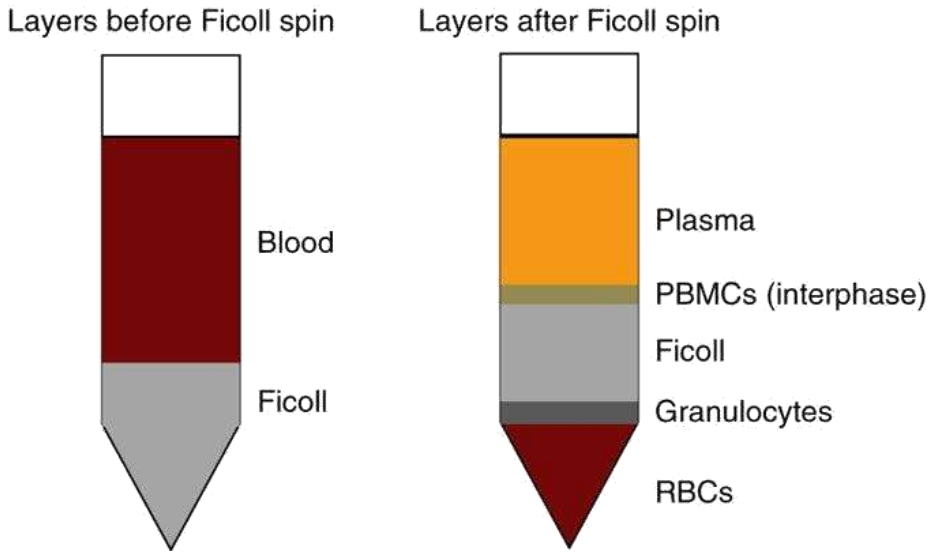
Total Leukocyte Blood Cell Preparation

After blood was collected on days 1, 3 and 5 after injury, blood samples were kept on ice for approximately one hour. Total leukocytes were then prepared from blood samples. Approximately 3mL of blood was transferred from the collection tube into a 50 mL centrifuge tube. 30 mL of an ammonium chloride-based red blood cell (RBC) lysis buffer was then added to a 50 mL centrifuge tube. The RBC lysis buffer was prepared by diluting the stock solution in sterile tissue culture grade deionized water (ddH_2O). The centrifuge tube containing the blood suspension was incubated for five to eight minutes at room temperature with occasional gentle mixing by inverting the tube. It was important to ensure and confirm lysis by examining the clearing of the blood suspension. For some blood samples that had a greater quantity of red blood cells, it was necessary to add an additional 5 mL of lysis buffer if the blood suspension was not clear within the expected time. Adding the additional lysis buffer usually cleared the blood cell suspension within one to two minutes after addition. In order to stop the RBC lysis reaction, 15-20 mL of culture medium was added to the centrifuge tube and mixed gently by inverting tube. The culture medium used in our laboratory consists of various nutrients and antibiotics. This includes 1L of RPMI 1640 supplemented with 45-50 mL of 5% heat inactivated fetal bovine serum (FBS), 10 mL of Antibiotic-Antimycotic, 10 mL of Glutamine, 10mL of hydroxyethyl piperazineethanesulfonic acid (HEPES), 10 mL of non-essential amino acids (NEAA), 25 units/mL of benzonase nuclease (Sigma-Aldrich), 20 units/mL of heparin sodium salt, and 10 mL of beta mercaptoethanol (β-ME). The tube was centrifuged for 10 minutes at a speed of 600 rpm (100g) at room temperature. The supernatant was decanted and 10 mL of culture medium was added to the centrifuge tube in order to resuspend the pellet. The tube was centrifuged for a second time for 10 minutes at 600 rpm (100g) at room temperature. The

supernatant was decanted and the cell pellet was resuspended in 2mL of CryoStor CS10 freezing medium (BioLife Solutions), which contains dimethyl sulfoxide (DMSO). Freezing media is used to protect biological tissues from freezing damage. The suspension was then divided into 0.5 mL aliquots to be placed into four labeled cryogenic vials. The cryogenic vials were then placed into a Nalgene freezing container and stored in the cold room at 4°C. After 20 minutes, the freezing container was placed in the -80°C freezer overnight. Cryogenic vials were then transferred into the liquid nitrogen freezer at -138°C for storage until ready for experimentation.

Plasma Preparation

The plasmas of trauma patients were collected using Ficoll centrifugation. Ficoll centrifugation separates the components of blood based on their unique densities. Approximately 4 mL of blood from the collection tube was transferred into a 15 mL centrifugation tube using a serological pipette. Phosphate-Buffered Saline (PBS) was then added to the blood samples in 1:1 ratio (for example, 4 mL of PBS with 4 mL of blood for a total of 8 mL), in order to dilute the blood and maintain a constant pH. 5 mL of GE Healthcare Ficoll-Plaque PLUS density gradient was then added to a SepMate Tube. Ficoll-Plaque PLUS density gradient is a liquid substance used to separate blood into their different components. The diluted blood was then overlaid into a SepMate tube very carefully using a serological pipette. Once the overlay was complete, the SepMate tube was centrifuged at 1200 rpm (400g) at room temperature for twenty minutes. The SepMate tube was then removed from the centrifuge carefully. There were five layers in the tube following centrifugation. **Figure 2.1** depicts the separation of layers in a blood sample using Ficoll centrifugation. The top layer contains the plasma while the second layer contains the peripheral blood mononuclear cells (PBMCs). The third layer contains the



Source: Nature

Figure 2.1 Ficoll Centrifugation. Blood is overlaid into the Sepmate Tube directly onto the Ficoll-Plaque solution. Ficoll centrifugation results in the separation of blood into the layers indicated. These layers include plasma, PBMCs, Ficoll solution, Granulocytes, and red blood cells.

Ficoll-Plaque solution. The fourth and fifth layer contains the granulocytes and erythrocytes consecutively. The plasma in the top layer was carefully removed with a transfer pipette and placed into labeled cluster tubes. The cluster tubes containing the plasma were then placed into a -30°C freezer until they were ready for experimentation.

Thawing Method and TLR Agonist Stimulation

Prior to thawing total leukocytes, 10 mL of culture medium and 1µl of benzonase nuclease was added to a 15 mL centrifuge tube and warmed within the incubator at 37°C for approximately ten minutes. Cryogenic vials containing total leukocyte samples were removed from the liquid nitrogen freezer and thawed at 37°C in a warm bath without agitation for exactly three minutes. Total leukocytes were slowly mixed with the pre-warmed culture medium by slow sequential volume mixing of 0.25 mL of warm culture medium using a transfer pipette. This was

done in order to slowly introduce cells to the new environment. Cells were then slowly mixed into the 10 mL of culture medium 0.5 mL at a time using the transfer pipette. The tube containing total leukocytes and culture medium were then centrifuged for 10 minutes at 600 rpm (100g) at room temperature. Supernatants were decanted and cells were distributed in 1 mL of the culture medium. Cell numbers for each sample were then counted using the MACSQuant Analyzer 10 flow cytometer and adjusted for 500,000 cells per well.

TLR Agonist Stimulation

TLR agonist stimulation of total leukocytes was done in a sterile 96-well round-bottom culture plate (Fisherbrand). 100 µl of cells adjusted for 500,000 cells per well were distributed in each well of the culture plate. Immune-modulating TLR agonists were also prepared. The conditions included no TLR agonist (unstimulated), 0.1 µg/mL of ultra-pure LPS (lipopolysaccharide), which served as a positive control and 3µg/mL of an A-class CpG-ODN 2336. LPS is a very strong stimulant this is expected to induce high production of cytokines. These particular concentrations of LPS and CpG were chosen after optimization. Various concentration of CpG-ODN 2336 and LPS were used to stimulate leukocytes to find the optimal concentration needed for induction of cytokines. Ultra-Pure LPS B5 (LPS from E.coli 055:B5) and CpG-ODN 2336 were obtained from InvivoGen, a manufacturer of Toll-like receptor related products. Once obtained, stock solutions were prepared as indicated in the instructions provided by InvivoGen. One mL of endotoxin-free or tissue-culture grade Sigma water was added to the TLR agonists and then homogenized. This mixture was then divided into 10µl or 20µl aliquots, placed in cluster tubes and stored at -30ºC. TLR agonists were then mixed with approximately 1 mL of culture medium to prepare accurate concentrations. 100 µl of each prepared TLR agonist

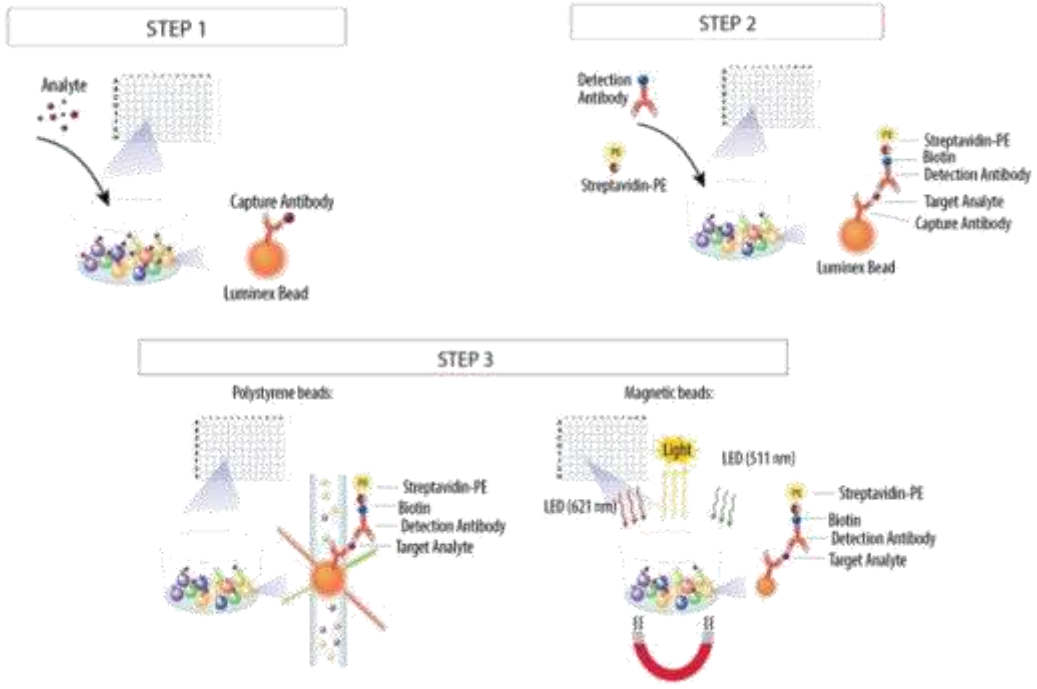
was plated in each well of the 96-well culture plate that contained 100 µl of total leukocyte cells. The culture plate was then placed in the incubator overnight at 37°C. The following day, 180µl of supernatants from each sample were then harvested and transferred into labeled cluster tubes using a multichannel pipette. The supernatants were then frozen at -30°C until they were used in the Luminex cytokine detection assay to screen for changes in cytokine and chemokine production patterns.

Multiplex Cytokine Analysis

Frozen plasma and supernatants were removed from the -30°C freezer and placed in the incubator to thaw. The plasma and total leukocyte concentrations of TNF-α, IL-1α, IL-1□, IL-1RA, IL-10, IL-6, IFNγ, MCP-1 (CCL2), IL-8, MIP-1β (CCL4), IL-12/23, RANTES (CCL5), TNFα, GM-CSF, and TREM-1 were assessed using the cytokine multiplex assay technology by Luminex. This cytokine panel was chosen based on previous literature that has identified these cytokines as significantly reduced or induced in trauma patients (49, 50). A 96-well V-bottom plate (Fisherbrand) was obtained and the plate cover was labeled for easy detection of the sample number and TLR agonist used. Luminex Incubation Buffer was poured into a separate container for usage. The Luminex incubation buffer consists of 1 L DPBS, 0.5 mL Tween (Sigma P1379), 0.25 mL Igepal CA (Sigma 18896), and 0.5 g Sodium Azide and 0.5% BSA (5g/L of DPBS). Primary and secondary antibodies are diluted in incubation buffer in order to maintain their structure. 20 µl of this Luminex incubation buffer was added into each well of the first column. A reference cytokine standard was prepared and then added to each well of the first column using a 1:3 dilution. Frozen supernatants and plasma in cluster tubes were then removed from the -30°C freezer and allowed to thaw at room temperature. 40 µl of each supernatant or plasma was

added to its given well. It critical to note that when conducting Luminex cytokine detection assays on plasma, horse serum was diluted into incubation buffer using a 1:4 dilution. 20 µl of the incubation buffer/horse serum mixture was then added to each well that contains the plasma. Plasma is very rich in proteins and horse serum dilutes and prevents the blocking of signaling. Next, a mixture of human primary antibodies stored in a darkened cryovial tube was obtained. The antibody mix was then vortexed and sonicated for at least 10 to 20 seconds total. An incubation buffer and primary antibody mixture was created depending on the amount of samples and cytokines using a 1:100 dilution. The primary antibody is also known as a capture antibody with a magnetic Luminex bead attached to it. It is added to the sample and the antibody captures the specific cytokine of interest. 40 µl of the antibody mixture was added to each well containing the sample. The antibody mixture was also added to the column of wells that contained the standards. The 96 well V bottom plate was then placed on a lab shaker and covered with aluminum foil for one hour in order to properly mix supernatants or plasma with antibodies. The plate was then removed from the lab shaker, placed on a magnetic bead washer and a Luminex wash buffer was obtained. The Luminex wash buffer consisted of 1 L DPBS, 0.5 mL Tween 20 (Sigma P1379) and 0.5 g sodium azide. The wash buffer serves to remove any excess antibody that has not captured the cytokines of interest. It also serves to decant any supernatant or plasma left. The magnetic washer is used to securely hold the magnetic beads in place. Using a multichannel pipette, 150 µl of Luminex wash buffer was added to each well and was incubated on the magnetic bead washer for 1 minute. This step was done twice. Human secondary biotinylated detector antibodies were then obtained. A biotinylated secondary antibody is also known as a detection antibody and binds the primary antibody. The biotin on the secondary antibody has a high affinity for Streptavidin which is added afterwards. An incubation

buffer and secondary antibody mixture was created according to the amount of samples and cytokines being measured, using a 1:1000 dilution. 40 µl of this mixture was then added to given wells. The plate was then placed on the lab shaker and covered with aluminum foil for one hour. Once the plate was removed from the lab shaker and placed on a magnetic bead washer, 150 µl of Luminex wash buffer was added to each well and incubated for one minute. This wash was also performed for a second time. Streptavidin Phycoerythrin (PE) was obtained. Streptavidin is a protein purified from a bacterium and is known to have a high affinity for biotin. Phycoerythrin is a fluorescence based indicator that is conjugated to Streptavidin in order to detect the biotinylated antibody. An incubation buffer and Streptavidin PE mixture was created using a 1:500 dilution (for example, 1000 µl of incubation buffer and 2µl of Streptavidin PE). 40 µl of the Streptavidin/PE mixture was added to each well. Again, the plate was placed on the lab shaker and covered for 30 minutes. After the 30 minute incubation with Streptavidin PE, the plate was removed from the shaker and washed twice on the magnetic bead washer in 150 µl of Luminex wash buffer. Finally, the samples were resuspended in 150 µl of Luminex Wash Buffer. The plate was then placed in the MagPix instrument for cytokine level determination. The MagPix instrument contains two different LED lights that illuminate the Streptavidin PE and the magnetic Luminex bead separately **(Figure 2.2).** The illumination of PE allows for the quantification of the amount of cytokine present in the sample while the illumination of the magnetic bead is used for detecting what cytokine is actually being measured. A computer is attached to the MagPix instrument for simple setup of the protocol and viewing the quantification of cytokine production.



Source: R&D Systems

Figure 2.2 Luminex Immunoassay. The Luminex assay uses a mixture of antibodies and magnetic beads to form an antibody-antigen sandwich that is then read on a MagPix instrument for cytokine detection.

Statistical Analysis

GraphPad Prism 7.02 (La Jolla, CA), a statistical analysis software was used for organizing the concentrations of cytokines and measuring statistical significance. A one-way ANOVA test with Tukey multiple comparisons was used to analyze these data. For all data, $p < 0.05$ was considered statistically significant with a 95% confidence interval.

2.2 Results

Plasma Cytokine Expression

The average cytokine expression in the plasma of trauma patients before any TLR stimulation is indicated in **Figure 2.3**. The average cytokine expression in the plasma of control samples taken from the Biobank is also indicated. While the cytokine panel consisted of a variety of cytokines, IL-6, IL-10 and IL-1RA (Interleukin-1 Receptor Antagonist) specifically trended towards elevation at Day 1 and then reduction on subsequent days. IL-6, a pro-inflammatory cytokine has already been designated as an early indication of injury severity in trauma patients by numerous authors (29-33). IL-6 is secreted by macrophages and plays a significant role in stimulating the immune response to tissue damage. IL-6 secretion peaks 1-4 hours after physical injury and then declines afterwards (51, 52). Another cytokine that peaked at Day 1 in the plasma of trauma patients was IL-10. Research has also implicated IL-10 as an early indication of injury severity in trauma patients (31-33). IL-10 is an anti-inflammatory cytokine that is responsible for downregulating Th1 type responses and suppressing cytokine secretion. Therefore, it is also released right after traumatic injury in order to cope with pro-inflammatory responses and restore homeostasis. IL-1RA is an additional anti-inflammatory cytokine that followed the same trend as IL-6 and IL-10. IL-1RA is an inhibitor of the pro-inflammatory cytokine, IL-1β. Research has also shown that IL-1RA is an early-response cytokine in the serum plasma of patients who suffer traumatic injury (53). Ultimately, the results obtained from the plasma of trauma patients coincided with data from the literature.

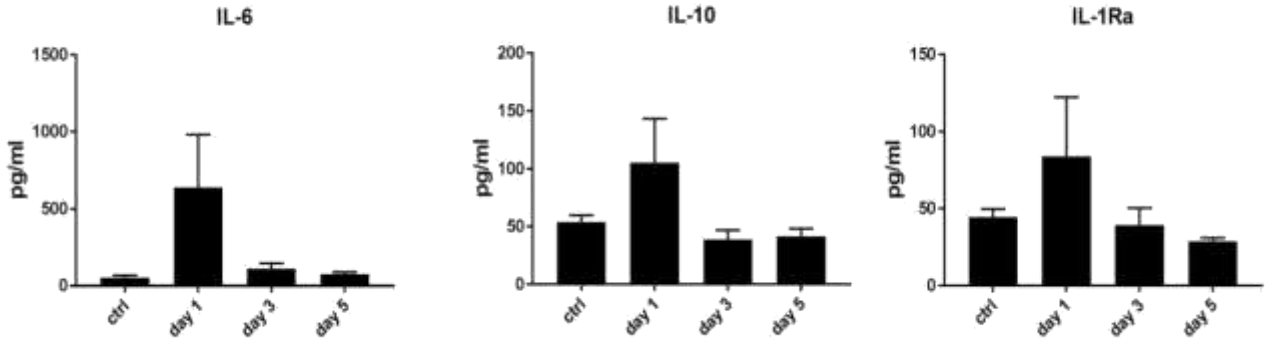


Figure 2.3 Plasma Cytokine Concentrations. The concentrations of these cytokines measured in pg/ml tended to trend towards elevation on Day 1 and then reduction on Days 3 and 5. Bars represent ± SEM. n = 10 per group.

Cytokine Expression in Total Leukocytes

The cytokine expression of unstimulated and stimulated total leukocytes from trauma patients on Days 1, 3 and 5 is displayed in the following figures. The results from the Luminex immunoassay provides information about released cytokines during the immune response to trauma as well as the immune response to TLR stimulation. The cytokines that showed significant induction after TLR stimulation included TNFα, IL-1α, IL-1β, IL-6, RANTES, MIP-1β, IL-10 and GM-CSF.

TNFα

TNFα, also known as tumor necrosis factor alpha is a pro-inflammatory cytokine primarily produced by macrophages. It is induced in response to pathogens, especially gram negative bacteria. It also recruits neutrophils and macrophages to sites of infection (54). High concentrations of TNFα are implicated in a variety of diseases such as cancer, rheumatoid arthritis, inflammatory bowel disease (IBD), and psoriasis. While TNFα is important for combatting infection, increased concentrations of TNFα is correlated with the development of

endotoxemia or septic shock in patients and can be quite detrimental (55). In **Figure 2.4**, TNFα is strongly induced when the total leukocytes are stimulated with LPS. However, CpG 2336 stimulation does not induce TNFα, suggesting its non-toxic properties. It is also important to note the decrease of TNFα in LPS stimulated total leukocytes of trauma patients compared to healthy volunteers. This suggests the ability of injury to prime the immune system and prevent extensive induction of proinflammatory cytokines when bacterial infection arises.

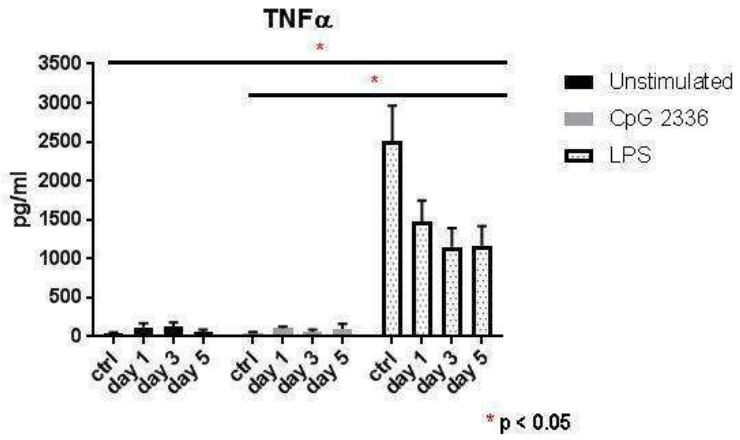


Figure 2.4 TNFα Production in Total Leukocytes of Trauma Patients and Healthy Volunteers. TNFα is strongly induced by LPS stimulation. However, it is not induced by CpG 2336 when compared to unstimulated total leukocytes. There is a reduction of TNFα in LPS stimulated total leukocytes of trauma patients compared to healthy volunteers. Bars represent ± SEM. n = 10 per group. * $p < 0.05$.

IL-1α

IL-1α or Interleukin 1 alpha is another proinflammatory cytokine mainly produced by macrophages. It has similar effects as TNFα, responding to pathogens by promoting fever and sepsis. It also plays a role in activating CD4+ and CD8+ T cells (56). In **Figure 2.5**, the production of IL-1α is significantly higher after LPS stimulation compared to CpG 2336

stimulation. This data also indicates the non-toxic properties of CpG 2336. Like TNFα, there is also a reduction in IL-1α in LPS stimulated total leukocytes of trauma patients compared to healthy volunteers, indicating the injury-primed immune system's resistance to inflammation.

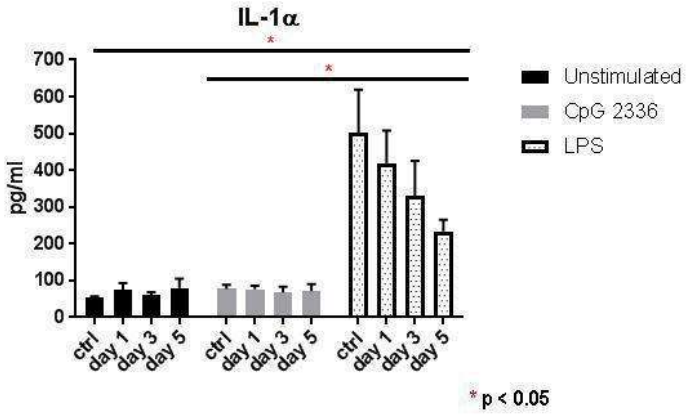


Figure 2.5 IL-1α Production in Total Leukocytes of Trauma Patients and Healthy Volunteers. IL-1α is strongly induced by LPS stimulation. However, it is not induced by CpG 2336 when compared to unstimulated total leukocytes. There is a sequential decrease of IL-1α in LPS stimulated total leukocytes of trauma patients. Bars represent ± SEM. n = 10 per group. * $p < 0.05$.

IL-1β

IL-1β or Interleukin 1 beta is an additional proinflammatory cytokine that has very similar functions to IL-1α. Although, it is known for its inflammatory response to pathogens, it also plays an active role in inflammasome activation and Th17 differentiation (56). **Figure 2.6** also indicates that while LPS is able to stimulate IL-1β induction, CpG 2336 does not significantly induce IL-1β, indicating its non-toxicity. The reduction of IL-1β production in trauma patients after LPS stimulation is also noted.

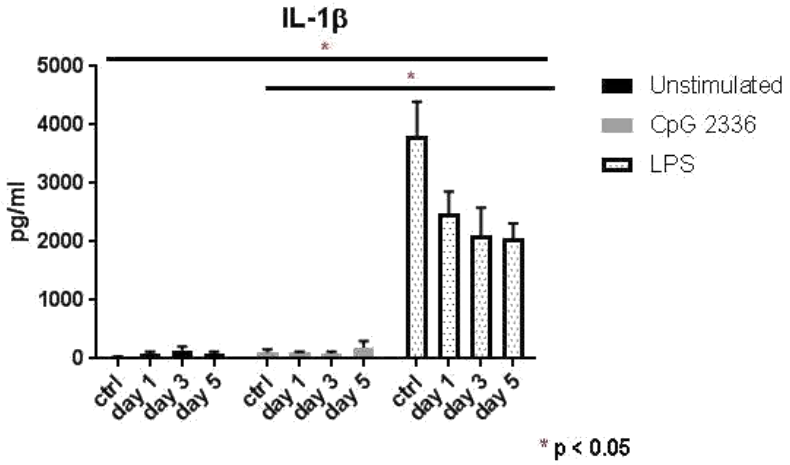


Figure 2.6 IL-1β Production in Total Leukocytes of Trauma Patients and Healthy Volunteers. IL-1β is strongly induced by LPS stimulation. However, it is not induced by CpG 2336 when compared to unstimulated total leukocytes. There is a decrease of IL-1β in LPS stimulated total leukocytes of trauma patients. Bars represent ± SEM. n = 10 per group. * $p < 0.05$.

IL-6

Interleukin 6 or IL-6 is known to have both proinflammatory and anti-inflammatory properties. It is secreted by macrophages and T cells during infection to promote inflammatory responses. In the context of trauma, increased IL-6 concentration has been shown to be correlated with severity of injury and mortality (38-40). **Figure 2.7** shows that IL-6 is significantly induced by LPS total leukocyte stimulation. However, it is not significantly induced by CpG 2336 total leukocyte stimulation. Still, the IL-6 production is increasing over Days 1, 3 and 5. This is consistent with data from the literature regarding the induction of IL-6 over time due to injury.

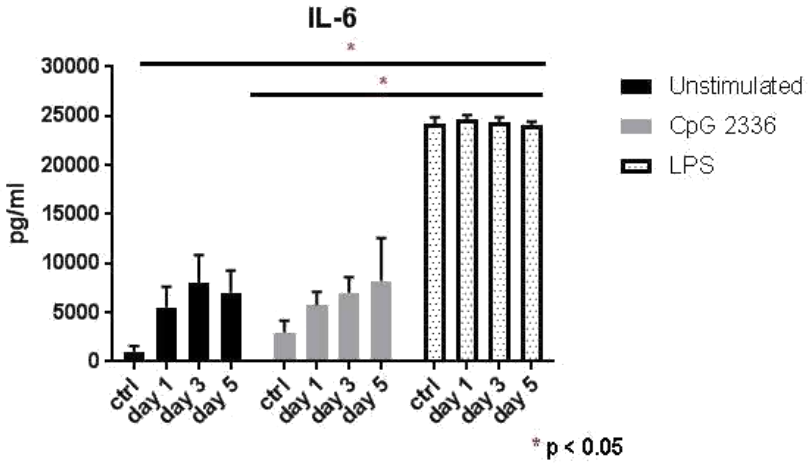


Figure 2.7 IL-6 Production in Total Leukocytes of Trauma Patients and Healthy Volunteers. IL-6 is strongly induced by LPS stimulation. However, it is not induced by CpG 2336 when compared to unstimulated total leukocytes. Still, the IL-6 response is increasing over time in total leukocytes stimulated with CpG 2336. Bars represent ± SEM. n = 10 per group. * $p < 0.05$.

RANTES

RANTES (regulated on activation, normal T cell expressed and secreted), also known as chemokine ligand 5 (CCL5) is a chemotactic cytokine that plays an important role in recruiting several leukocytes (for example, T cells, eosinophils and basophils) into inflammatory sites. RANTES also induces the proliferation and activation of natural killer (NK) cells (57). In **Figure 2.8**, RANTES is strongly induced by both LPS and CpG 2336. While previous figures have indicated CpG 2336's low induction of cytokine and therefore non-toxic properties, it is also important that CpG 2336 can induce an inflammatory response to fight infection and reduce tissue damage in trauma patients. RANTES production also increases over time in LPS and CpG 2336 treated trauma patients. It is unclear why the concentration of RANTES is high in healthy patients and low in trauma patients. Further investigation is necessary.

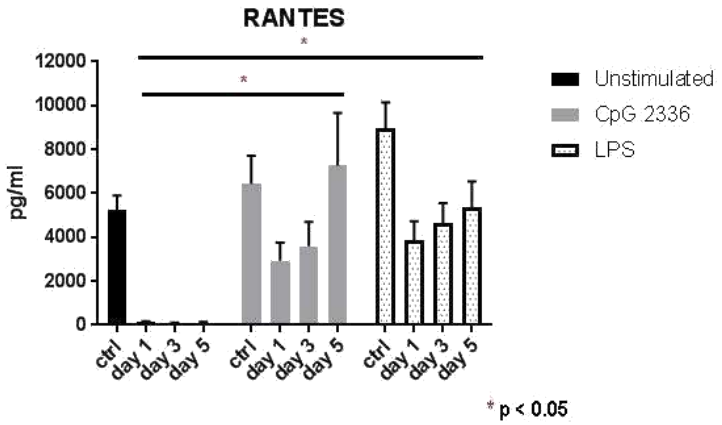


Figure 2.8 RANTES Production in Total Leukocytes of Trauma Patients and Healthy Volunteers. RANTES is significantly induced by LPS stimulation and CpG 2336 stimulation. There is a sequential increase of RANTES in trauma patients stimulated with either LPS or CpG 2336. Bars represent ± SEM. n = 10 per group. * $p < 0.05$.

MIP-1β

MIP-1β, also known as macrophage inflammatory protein 1 beta or CCL4 is a chemokine that helps in recruiting monocytes and NK cells towards the site of infection (58). MIP-1β is produced by macrophages and is crucial in inducing the release of other pro-inflammatory cytokines such as IL-1, IL-6 and TNFα (59). In **Figure 2.9**, unlike RANTES, there is no significant induction of MIP-1β when total leukocytes are stimulated with CpG 2336, also indicating its non-toxic properties.

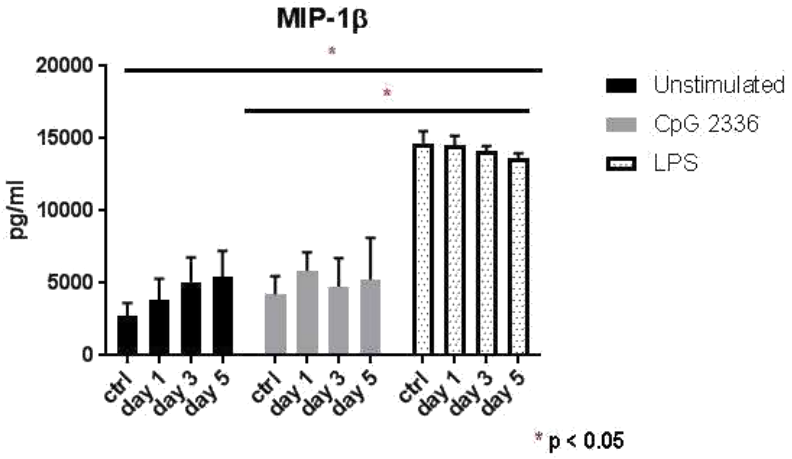


Figure 2.9 MIP-1β Production in Total Leukocytes of Trauma Patients and Healthy Volunteers. MIP-1β is strongly induced by LPS stimulation. However, it is not significantly induced by CpG 2336 when compared to unstimulated total leukocytes. Bars represent ± SEM. n = 10 per group. * $p < 0.05$.

MCP-1

MCP-1, also known as monocyte chemoattractant protein 1 or CCL2 is another chemokine that recruits monocytes, T cells and dendritic cells to sites of inflammation especially in injury or infection (60). In **Figure 2.10**, while there is no significant difference among stimulated and unstimulated groups, it is clear that injury induces MCP-1 when compared to healthy and uninjured volunteers. In addition, LPS stimulation tends towards lower MCP-1 production compared to unstimulated and CpG 2336 stimulated groups.

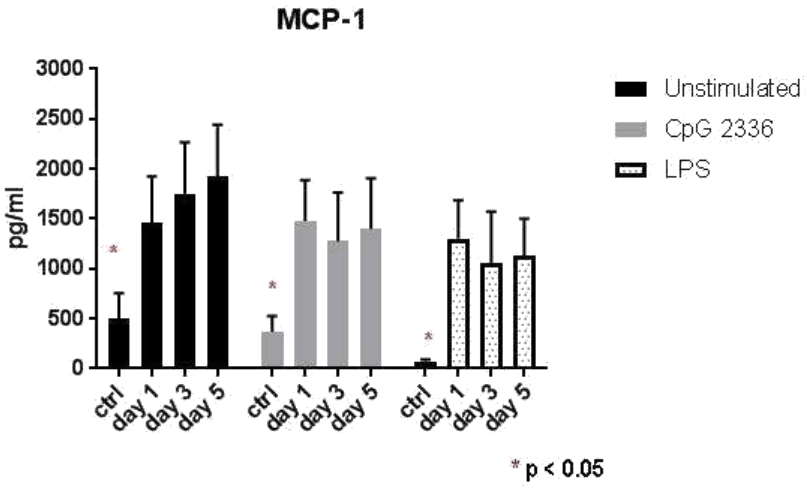


Figure 2.10 MCP-1 Production in Total Leukocytes of Trauma Patients and Healthy Volunteers. Injury significantly induces MCP-1 in both unstimulated and stimulated groups. Bars represent ± SEM. n = 10 per group. * $p < 0.05$.

IL-10

IL-10 or Interleukin 10 is an anti-inflammatory cytokine that is also highly implicated in patients who suffer from severe traumatic injury. It is primarily produced by monocytes and T helper cells. IL-10 serves to downregulate Th1 cells, NK cells and macrophages. Consequently, it suppresses excessive inflammation in cases of severe injury and infections (61). **Figure 2.11** displays its significant induction when stimulated with LPS. However, when total leukocytes are stimulated with CpG 2336, there does not seem to be any significant induction when compared with unstimulated total leukocytes. This also demonstrates the non-toxicity of CpG 2336. Furthermore, there is a sequential decrease of IL-10 in LPS stimulated total leukocytes of trauma patients, suggesting injured patients' resistance to counter inflammatory responses.

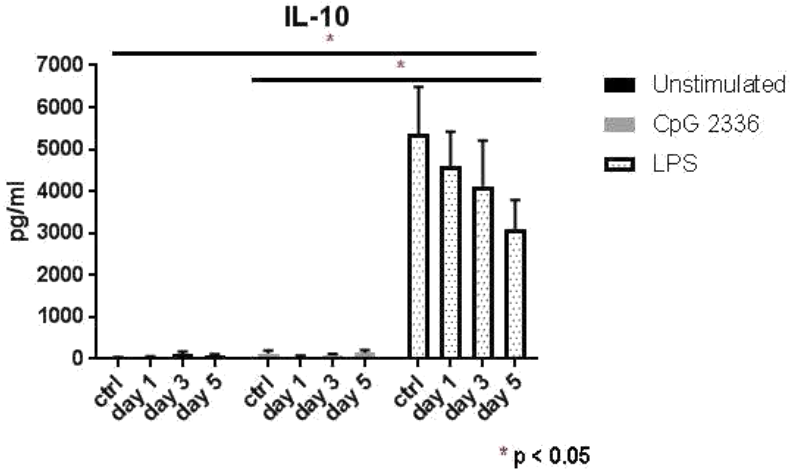


Figure 2.11 IL-10 Production in Total Leukocytes of Trauma Patients and Healthy Volunteers. IL-10 is strongly induced by LPS stimulation. However, it is not significantly induced by CpG 2336 when compared to unstimulated total leukocytes. There is a sequential decrease of IL-10 in LPS stimulated total leukocytes of trauma patients. Bars represent ± SEM. n = 10 per group. * $p < 0.05$.

GM-CSF

GM-CSF, also known as Granulocyte-Macrophage Colony-Stimulating Factor is a glycoprotein that functions as a proinflammatory cytokine. GM-CSF stimulates stem cells to produce granulocytes such as macrophages, eosinophil, basophils and monocytes when infection occurs. These granulocytes then aid in inflammation and fighting infection (62). In **Figure 2.12,** LPS stimulation significantly induces GM-CSF. However, CpG 2336 stimulation does not significantly induce GM-CSF. Again, this indicates CpG 2336's non-toxicity. Unlike other cytokines, there is an increase in GM-CSF in LPS stimulated total leukocytes of trauma patients compared to healthy volunteers. Further research is also necessary to describe this finding.

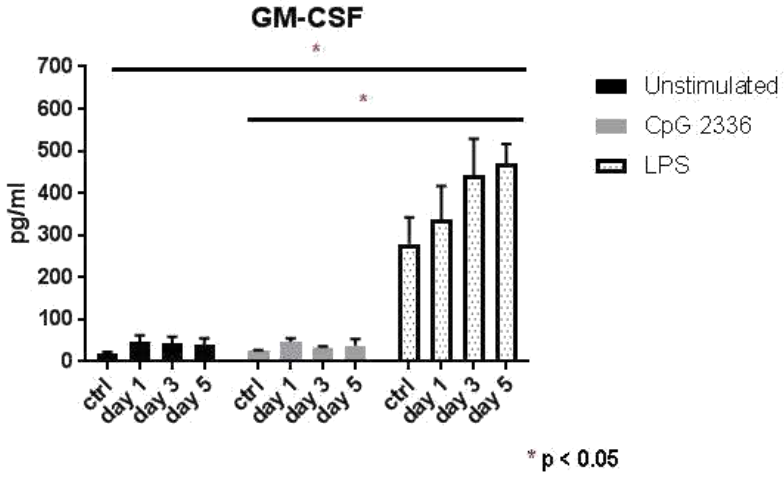


Figure 2.12 GM-CSF Production in Total Leukocytes of Trauma Patients and Healthy Volunteers. GM-CSF is strongly induced by LPS stimulation. However, it is not significantly induced by CpG2336 when compared to unstimulated total leukocytes. There is a sequential increase in GM-CSF in LPS stimulated total leukocytes of trauma patients. Bars represent ± SEM. n = 10 per group. * $p < 0.05$.

2.3 Brief Discussion

Although there have been major advances in emergency care medicine, traumatic injuries still remain to be a significant source of morbidity and mortality throughout the world. Patients who survive the initial causes of mortality such as massive blood loss and cervical spine disruption are still at risk of death due to the prevalence of opportunistic infection (3). Bacterial pneumonia is the most common infection after injury and it is often caused by either emergent intubation or aspiration (3). Subsequent complications of infection include sepsis and multiple organ failure, often leading to death. And for those patients who develop sepsis and multiorgan failure following traumatic injury, it is probable that the disruption of immune system homeostasis occurs. This disruption places individuals at an increased vulnerability to infections and inflammatory-mediated disorders. Therefore it is imperative to understand the complex

immune responses that occur after trauma. This increased knowledge will help clinicians identify prognostic markers that may predict which patients are more susceptible to infection post-trauma.

While there have been several studies that have shown the relationship between severity of trauma and the resulting immune response there is still relatively little known about how immune response modifiers influence the anti-microbial response in patients who suffer from traumatic injury. In this project, I assessed the cytokine concentrations in the blood plasma of healthy individuals and trauma patients. I also used an immune response modifier, CpG-ODN to stimulate the total leukocytes of severely injured patients. This was done in order to better understand the immune response phenotype that would result after immunotherapeutic treatments. Specifically, CpG 2336 was used as it already has been implicated as beneficial in mice (6). Lipopolysaccharide (LPS) was used as a comparative positive control. We expected LPS's high toxicity and induction of cytokines. Luminex-based immunoassays were performed to identify the cytokine induction after TLR stimulation.

In the trauma patients, it was found that changes in cytokine production occurred after injury. The blood plasma of patients revealed that proinflammatory cytokines IL-6 and IL-1RA are induced right after injury and then subsides on the following days. The same trend occurred in the anti-inflammatory cytokine, IL-10. This suggests that injury does in fact induce cytokine production but not the severe cytokine storm that is suggested by other studies.

After stimulation, the results demonstrated that LPS strongly induced a variety of proinflammatory and anti-inflammatory cytokines. In contrast, CpG 2336 stimulation did not significantly induce proinflammatory and anti-inflammatory cytokines except RANTES. This implies that CpG 2336 is low in toxicity and safe to use in patients as an immunotherapeutic treatment. However, it still may be able to produce an anti-microbial immune response and

promote inflammation as seen in RANTES. Expanding the Luminex antibody panel may suggest CpG 2336's ability to induce other cytokines.

Although it may appear from these results that CpG 2336 is not biologically active, companion studies using CyTOF mass cytometry to profile immune cell subset activation by CpG 2336 clearly shows that CpG 2336 can activate B cells, monocytes and natural killer (NK) cells. This CpG 2336 has immunostimulatory properties that are independent of cytokine inducing activity.

In closing, Luminex technology proved to be an efficient and powerful systems immunology tool that can be used for profiling cytokine expression in immune cells at different time points after traumatic injury. Ultimately, this project along with accompanying projects will help expand the potential of using CpG 2336 as a clinical treatment strategy to stabilize immune function after traumatic injury or opportunistic bacterial infection.

Chapter 3: Discussion and Perspectives

3.1 Limitations

While my project provides valuable data regarding the potential of using CpG 2336 as a clinical treatment strategy for traumatic injury, there still are a number of limitations to be noted. I was able to profile the cytokine expression of TLR stimulated total leukocytes using a limited cytokine panel. However, there may be other proinflammatory and anti-inflammatory cytokines that are reduced or induced after TLR stimulation. These may include cytokines such as IL-2, IL-12, TGFβ, IL-4, IL-5, IL-13, IFNβ and IL-17 amongst others. Nonetheless, the cytokine panel utilized in this study is an introduction to understanding the potential beneficial effects that CpG-ODN has on trauma in humans. Another limitation of this project was that I was only able to assess trauma-induced phenotypic changes in blood immune cells which only allows for evaluating data on immune cells in the blood or immune cells released from tissues. There are also immune cells within the skin, lymph nodes, bone marrow, spleen and thymus that are incapable of being measured in this study of trauma patients. Immune cells within these organs may also be influential in governing the immune response to trauma. Nevertheless, my laboratory is actively conducting research using mouse models of trauma to investigate cellular and cytokine changes in the immune cells of the lymph nodes, the bone marrow and the spleen. In addition, trauma patients' immune cells were stimulated with TLR agonists whether or not they developed infections. Although it is ideal to provide patients with immunotherapeutic treatment before they acquire infections, it would be useful to gain knowledge on how CpG-ODN treatment directly affects individuals who have already acquired infections. It can be useful to stimulate the total leukocytes of patients who have infections and profile the cellular and cytokine response afterwards. In addition, total leukocytes of trauma patients can be stimulated

with LPS and CpG 2336, respectively. The cellular and cytokine response after simultaneous stimulations can also be assessed. This may provide new knowledge about the effects of TLR9 agonists on infection and inflammation-related disorders. Finally, my study included ten different injured patients at varied time points from a single trauma center at Brigham and Women's Hospital. This is a relatively small sample size. Many studies that have investigated immune responses to traumatic injuries have large sample sizes ranging from hundreds to thousands of patients. Blood samples from trauma patients are difficult to acquire and are limited. However, further sampling of many more patients from different trauma centers is necessary to confirm these findings. Additionally, the patients enrolled in this study are severely injured with an average injury severity score of 34. These findings may not be pertinent to less severely injured patients with different demographics or injury characteristics.

3.2 Future Perspectives

In summary, by using Luminex technology, time-dependent phenotypic changes in the cytokine profile were identified following major trauma and TLR stimulation. CpG-ODN 2336 treatment proved to be low in toxicity, as it did not particularly produce high levels of cytokines. Its induction of RANTES does provide insight concerning its ability to produce an inflammatory response. However, further investigation regarding RANTES and its relation to injury is necessary. Future directions will also include more thorough analysis of additional cytokines, specific immune cell subsets and its influences on injury. It is important to note that my project accompanies what is being done by other members of our lab. Our lab uses a novel technology, mass-cytometry of-flight (CyTOF) to detect and quantify multi-cellular responses to trauma in mouse models and in peripheral blood mononuclear cells (PBMCs) from trauma patients. CyTOF is similar to flow cytometry but this technique uses rare-earth metal isotope labeled

antibodies for detection. As a result, there is no background or fluorescence overlap. This allows for simultaneous detection of multiple cell markers (up to 45). Our lab has already identified the induction of Th17-type $CD4^+$ T cells, NK cells, and blood monocytes after severe injury in trauma patients. In mouse models of trauma treated with CpG 2336, our lab identified coexpression of CD11c and F4/80 on macrophages and a unique population of CD172a+ cells. Therefore, using both CyTOF and Luminex technologies to phenotype cellular and cytokine responses after CpG 2336 stimulation will be important for delineating the mechanism-of-action for CpG-ODN treatments.

Additionally, it would be useful to profile patients based on demographics such as age, gender, comorbidities, and type of injury to gain understanding on other factors that may contribute to different cellular and cytokine response after trauma. The data provided in this study contributed to new information regarding immune responses to CpG-ODN 2336 treatment. Future studies are necessary for understanding its immunotherapeutic benefits on trauma patients. Other studies identifying the cytokine and cellular profile after using different classes or sequences of CpG-ODN may also be useful for gaining insight into their beneficial effects. Different TLR agonists such as flagellin (TLR5) and R848 (TLR7/8) can also be used to stimulate human total leukocytes and investigate their resulting immune responses.

In conclusion, the results from this Luminex-based experiment not only contributes to understanding the beneficial effects of CpG-ODN treatment but has also helped identify the role of TLR9 as a regulatory pathway for injury and infection. It has also provided additional information about the induction of cytokines after injury. Further investigation using systems immunology tools that build upon the data presented in the project will provide us with useful

information about utilizing CpG-ODNs as a treatment tool for mass casualty or trauma scenarios. This can then help restore or redirect immune system homeostasis in patients and prevent the development of life-threatening complications caused by opportunistic infections.

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